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Formulations in situ de donneurs de monoxyde d'azote

Marianne Parent

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Ecole Doctorale BioSE (Biologie-Santé-Environnement)**Thèse**

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Mention : « Sciences de la Vie et de la Santé »

Par Marianne PARENT

**FORMULATIONS *IN SITU* DE
DONNEURS DE MONOXYDE D'AZOTE**

Le 28 octobre 2013

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TRAVAUX SCIENTIFIQUES

PUBLICATIONS

- Article n°1 : « Quel avenir en thérapeutique cardiovasculaire pour le monoxyde d'azote et ses dérivés ? » ; **M. Parent**, F. Dupuis, P. Maincent, C. Vigneron, P. Leroy, A. Boudier. *Annales Pharmaceutiques Françaises* 2013 ; 71 : 84-94.
- Article n°2 : « S-nitrosation/Denitrosation in cardiovascular pathologies: facts and concepts for the rational design of S-nitrosothiols » ; C. Gaucher, A. Boudier, F. Dahboul, **M. Parent**, P. Leroy. *Current Pharmaceutical Design* 2013 ; 19 : 458-472 (IF : 3,311).
- Article n°3 : « PLGA *in situ* implants formed by phase inversion : critical physicochemical parameters to modulate drug release » ; **M. Parent**, C. Nouvel, M. Koerber, A. Sapin, P. Maincent, A. Boudier. *Journal of Controlled Release* 2013 ; 172 : 292-304 (IF : 7,633).
- Article n°4 : « A complete physicochemical identity card of S-nitrosoglutathione » ; **M. Parent**, F. Dahboul, R. Schneider, I. Clarot, P. Maincent, P. Leroy, A. Boudier. *Current Pharmaceutical Analysis* 2013 ; 9 : 31-42 (IF : 1,333).
- Article n°5 : « Are *in situ* formulations the keys for the therapeutic future of S-nitrosothiols? » ; **M. Parent**, A. Boudier, F. Dupuis, C. Nouvel, A. Sapin, I. Lartaud, J-L. Six, P. Leroy, P. Maincent. *European Journal of Pharmaceutics and Biopharmaceutics* (IF : 3,826) : sous presse.

COMMUNICATIONS ORALES

- Groupe de recherche GRIMIT, 25-27 septembre 2013, Paris (France) : « Implants formés *in situ* pour la médecine régénérative de l'infarctus du myocarde ».
- 23rd European Meeting on Hypertension and Cardiovascular protection, 14-17 juin 2013, Milan (Italie) : « A sustained release of S-nitrosoglutathione has a longer impact on pulse than on mean arterial pressure ».
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- Congrès Data Sciences International, 21-22 mars 2013, Berlin (Allemagne) : « Telemetry allows to predict a change in stroke volume via a longer decrease in pulse than in mean arterial pressure in response to NO-donors ». Présenté par I. Lartaud.
- GTRV 2012, 3-5 décembre 2012, Chilly-Mazarin (France) : « Sustained hypotension in rats with *in situ* formulations loaded with S-nitrosothiols ».
- Première rencontre « NutriOx », 6-9 septembre 2012, Särrebruck (Allemagne) : « Development, characterisation and evaluation of S-nitrosothiols ».
- Colloque « Redox mechanism and drug development », 5 juillet 2012, Nancy (France) : « Implication of redox imbalance in tolerance phenomenon of organic nitrates used as nitric oxide donors ».

COMMUNICATIONS AFFICHÉES

- Rencontre annuelle 2013 de l'AAPS (American Association of Pharmaceutical Scientists), 10-14 novembre 2013, San Antonio (USA) : « *In situ* forming microparticles : a promising formulation to bring *S*-nitrosothiols from bench to bedside »; **M. Parent**, A. Boudier, F. Dupuis, A. Sapin, C. Nouvel, J-L. Six, I. Lartaud, P. Maincent.
- 19th International symposium on microencapsulation, 9-11 septembre 2013, Pampelone (Espagne) : « *In situ* forming microparticles : a promising formulation for *S*-nitrosothiols sustained release »; **M. Parent**, A. Boudier, F. Dupuis, A. Sapin, C. Nouvel, J-L. Six, I. Lartaud, P. Maincent.
- GTRV 2011 (Bruxelles, Belgique) : « Sustained release of nitric oxide donors by *in situ* forming implants and microparticles »; **M. Parent**, A. Boudier, R. Schneider, C. Nouvel, J-L. Six, P. Leroy, P. Maincent.
- GTRV 2010 (Toulouse, France) : « W/O/W multiple self-emulsifying nanoemulsion (MSEN) for oral delivery of insulin »; **M. Parent**, M. Socha, S. Gibaud, P. Maincent.

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Téléométrie et administration de donneurs de monoxyde d'azote :

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LISTE DES ABREVIATIONS

ADP : adénosine diphosphate
Ang-1 : angiotensine-1
Ang-2 : angiotensine-2
DMEM : *Dulbecco's modified eagle medium*
ECA : enzyme de conversion de l'angiotensine
EDHF : facteur hyperpolarisant dérivé de l'endothélium
EDRF : facteur relaxant dérivé de l'endothélium
End-1 : endothéline-1
eNOS : *endothelial nitric oxide synthase*
ERO : espèces réactives de l'oxygène
GCs : guanylate cyclase soluble
GSNO : *S*-nitrosoglutathion
HPLC/CLHP : *high performance liquid chromatography*/ chromatographie liquide haute performance
HR : fréquence cardiaque (*heart rate*)
IDM: infarctus du myocarde
IM : administration intramusculaire
iNOS : *inducible nitric oxide synthase*
ISDN : dinitrate d'isosorbide
ISMN : mononitrate d'isosorbide
ISI : implants *in situ* (*in situ implants*)
ISM : microparticules *in situ* (*in situ microparticles*)
ISPI : implants *in situ* poreux (*in situ porous implants*)
MAP : pression artérielle moyenne (*mean arterial pressure*)
MetHb : méthémoglobinémie
MM : masse molaire
Mn : masse molaire moyenne en nombre
MTT : 3-(4,5-diméthylthiazol-2-yl)-2,5-diphényltetrazolium bromide
NADPH_{ox} : NADPH oxydases
NMP : *N*-méthyl-2-pyrrolidone
NONOates : diazéniumdiolates
PAP : pression artérielle pulsée (*pulse arterial pressure*)
PBS : *phosphate buffer solution*
PCL : poly-(ϵ -caprolactone)
PDI : indice de polymolécularité
P_{dia} : pression artérielle diastolique

TEP-SCAN : tomographie par émission de positrons couplée à l'imagerie

PGA : poly(glycolide)

PLA : poly(lactide)

PLGA : poly(lactide-*co*-glycolide)

PGI₂ : prostacycline

PRP : plasma riche en plaquettes

PPP : plasma pauvre en plaquettes

P_{sys} : pression artérielle systolique

RSNO : *S*-nitrosothiol(s)

SC : administration sous-cutanée

SEM : *scanning electron microscopy*

SNAP : *S*-nitroso-*N*-acétylpénicillamine

TA : triacétine

T_g : température de transition vitreuse

TRAP-6 : *Thrombin-receptor agonist peptide-6*

TxA₂ : thromboxane A₂

VEGF : facteur de croissance de l'endothélium vasculaire

INTRODUCTION

Depuis sa découverte dans les années 80 [1], le monoxyde d'azote (ou oxyde nitreux, NO) suscite un vif intérêt au sein de la communauté scientifique. Ce messenger gazeux, radicalaire et ubiquitaire intervient en effet dans de nombreux processus physiologiques comme la vasodilatation, la migration cellulaire, la neurotransmission ou la réponse immunitaire [2]. Son homéostasie est cependant perturbée dans de nombreuses pathologies, notamment cardiovasculaires : athérosclérose, insuffisance cardiaque, infarctus... [3–5]. Ces pathologies touchent un nombre croissant de personnes, en particulier en Europe du fait du vieillissement de la population et des habitudes de vie. Elles ont des conséquences lourdes en termes de mortalité, mais également de morbidité et de coûts associés à la prise en charge [6].

A l'heure actuelle, NO gazeux est utilisé en inhalation dans le traitement de l'hypertension artérielle pulmonaire [7]. Sa demi-vie est très courte (de l'ordre de la seconde [8]) et par conséquent il ne peut exercer qu'une action vasodilatatrice locale au niveau des vaisseaux pulmonaires. Il n'existe sur le marché qu'une poignée de molécules précurseurs de NO ayant une demi-vie suffisante pour permettre une action systémique. Elles appartiennent à la famille des nitrates organiques ou des sydnominines et leur usage est limité à l'angine de poitrine (ou angor) et à l'insuffisance cardiaque. Leurs principales limitations sont un phénomène d'échappement thérapeutique (tolérance) et des problèmes de toxicité par induction d'un stress oxydant/nitrosant [9,10].

De nombreuses équipes de recherche s'intéressent à de nouvelles familles de composés, aux structures chimiques différentes, susceptibles d'activer les voies de signalisation NO-dépendantes sans présenter les inconvénients des médicaments existants. Les diazéniumdiolates (NONOates) et les *S*-nitrosothiols (RSNO) sont les principales familles étudiées [11,12]. Néanmoins, les RSNO sont susceptibles d'être moins toxiques, d'une part car certains RSNO existent de façon endogène et constituent les réservoirs physiologiques de NO [13] et d'autre part car l'administration de NONOates pourrait entraîner la production de nitrosamines cancérigènes [14]. Le potentiel thérapeutique de ces nouveaux précurseurs de NO est cependant restreint par leur manque de stabilité. Deux approches complémentaires sont possibles pour pallier ce problème : soit une modification chimique de la structure du composé, soit une « protection » du composé par une forme galénique appropriée.

Ce travail s'inscrit en marge du projet NanoSNO « Formulation nanoparticulaire de *S*-nitrosothiols en vue d'améliorer la biodisponibilité de l'oxyde nitrique. Evaluation toxicologique et pharmacologique » soutenu par l'Agence Nationale de la Recherche de 2010 à 2013. L'un des sous-objectifs de ce projet repose sur la synthèse de *S*-nitrosothiols dont la structure chimique comporte des modifications permettant d'augmenter leur stabilité et leur hydrophobie, les rendant ainsi plus adaptés à une potentielle utilisation clinique. Cependant, suite à des difficultés expérimentales importantes, jusqu'à présent seules deux molécules, ne présentant pas d'originalité, ont pu être synthétisées, purifiées et isolées : le *S*-nitrosogluthation (GSNO) et la *S*-nitroso-*N*-acétylpénicillamine (SNAP). Leur encapsulation dans des formes galéniques micro- ou nano-particulaires (autre sous-objectif du

projet NanoSNO) s'est révélée délicate, de par leur faible stabilité et les conditions expérimentales parfois drastiques de leur préparation.

Dans ce contexte, nous avons cherché à développer une formulation originale de ces RSNO et nous avons travaillé sur leur intégration à des implants et microparticules *in situ* [15,16]. Ces systèmes sont préparés simplement et injectés dans le tissu d'intérêt (en général en sous-cutané) sous forme de solution, de suspension ou d'émulsion. Leur élément principal est un copolymère d'acide lactique et glycolique (PLGA), qui est biocompatible et bioassimilable, insoluble dans l'eau. Une fois en contact avec les fluides aqueux de l'organisme, le polymère précipite au site d'injection, ce qui conduit à la formation d'une matrice solide incorporant le principe actif. Cette matrice sera progressivement dégradée dans l'organisme en libérant le principe actif.

Le but principal de cette étude repose sur la démonstration de l'encapsulation de ces molécules dans les formulations *in situ* et l'apport de la preuve de concept d'une libération prolongée, tant *in vitro*, qu'*in vivo* après administration parentérale.

La première partie de ce manuscrit est consacrée à l'état de l'art. Il s'agit dans un premier temps de revenir sur les avantages et les inconvénients des médicaments donneurs de NO existants et sur le potentiel thérapeutique de nouvelles molécules comme les RSNO (Article 1). Dans un deuxième temps, les mécanismes de libération physiologiques de NO à partir des RSNO sont exposés. A partir de cela, les modifications structurales susceptibles d'améliorer leurs propriétés physicochimiques (stabilité et demi-vie) tout en maintenant leur activité, ainsi que les formulations déjà présentées dans la littérature sont décrites (Article 2). Enfin, le dernier chapitre est consacré aux formes *in situ* étudiées dans ce travail : les mécanismes de formation, de dégradation des matrices et de libération de principes actifs à partir de telles formulations sont présentés, ainsi que les diverses optimisations possibles (Article 3).

La deuxième partie correspond au travail expérimental. Le premier chapitre est consacré à l'étude physicochimique de GSNO (chef de file des RSNO) synthétisé, isolé et purifié grâce à notre collaboration avec le Pr R. Schneider (UMR 7274, ENSIC). Cette étude a permis de valider le protocole de préparation de ce RSNO ainsi que les méthodes d'analyses colorimétriques et chromatographiques (Article 4). Ceci permet de garantir l'utilisation d'un produit isolé et pur, de qualité pharmaceutique, pour les expériences suivantes. Cette démarche a été étendue aux autres RSNO étudiés au laboratoire et plus particulièrement au SNAP dans le cadre de cette thèse.

Le deuxième chapitre porte sur l'incorporation de GSNO et SNAP dans des formes *in situ* de type implants et microparticules. Les résultats démontrent l'obtention d'une libération prolongée *in vitro*, influencée notamment par l'hydrophobie du principe actif (Article 5). Finalement, un effet systémique et prolongé de SNAP (Article 5) et de GSNO avec les formulations a été mis en évidence *in vivo*.

Le troisième chapitre explore une application future de ces systèmes *in situ* dans le traitement de l'infarctus du myocarde. Ainsi, leur injection directement au sein de la zone infarctée permettrait à la fois de libérer NO et d'accueillir des cellules au fur et à mesure de la dégradation de la matrice (par augmentation progressive de la porosité). Ceci serait favorable à la reconstruction post-infarctus. En premier lieu, une étude a été menée *in vitro* avec des implants *in situ* dont la porosité a été modulée afin de simuler des implants *in situ* tels qu'ils se structurent *in vivo* et également tels qu'ils deviennent au cours de leur dégradation. Ils ont été utilisés comme un modèle pour étudier la capacité à libérer des donneurs de NO et à être colonisés par des cellules. En parallèle, des expériences d'injections intramyocardiques ont été menées chez des rats sains ou souffrant d'un infarctus du myocarde. Les résultats présentés dans ce chapitre sont préliminaires mais semblent prometteurs.

Ce manuscrit se termine par une discussion, présentant la justification du choix du sujet et de la méthodologie, avec la mise en évidence des biais potentiels. De plus, les résultats obtenus sont mis en parallèle de ceux de la littérature et les perspectives du travail sont évoquées. Enfin, une conclusion générale rappelle la problématique du travail et les réponses apportées.

ÉTUDE BIBLIOGRAPHIQUE

1. GENERALITES SUR LES DONNEURS DE MONOXYDE D'AZOTE

1.1. Introduction

1.1.1. Les maladies cardiovasculaires

D'après l'Organisation Mondiale de la Santé, les maladies cardiovasculaires représentent la première cause de mortalité dans le monde, quel que soit le sexe [17]. En France, elles constituaient en 2008 la seconde cause de décès, légèrement devancées par les tumeurs (Figure 1).

Les maladies cardiovasculaires forment un groupe hétérogène de pathologies, aiguës ou chroniques, touchant les vaisseaux sanguins, le muscle ou les valves cardiaques. Les plus connues sont l'angine de poitrine, l'infarctus du myocarde, l'insuffisance cardiaque, les thromboses veineuses et les accidents vasculaires cérébraux. Elles représentent un coût humain et financier important. Rien que pour les Etats-Unis, les estimations de l'American Heart Association prévoient d'ici 2030 une augmentation de 10 % de leur prévalence (40,5 % de la population atteinte), de 61 % des coûts indirects (par perte de productivité, soit une facture de 276 milliards de dollars) et de 200 % des coûts directs (818 milliards de dollars) [18].

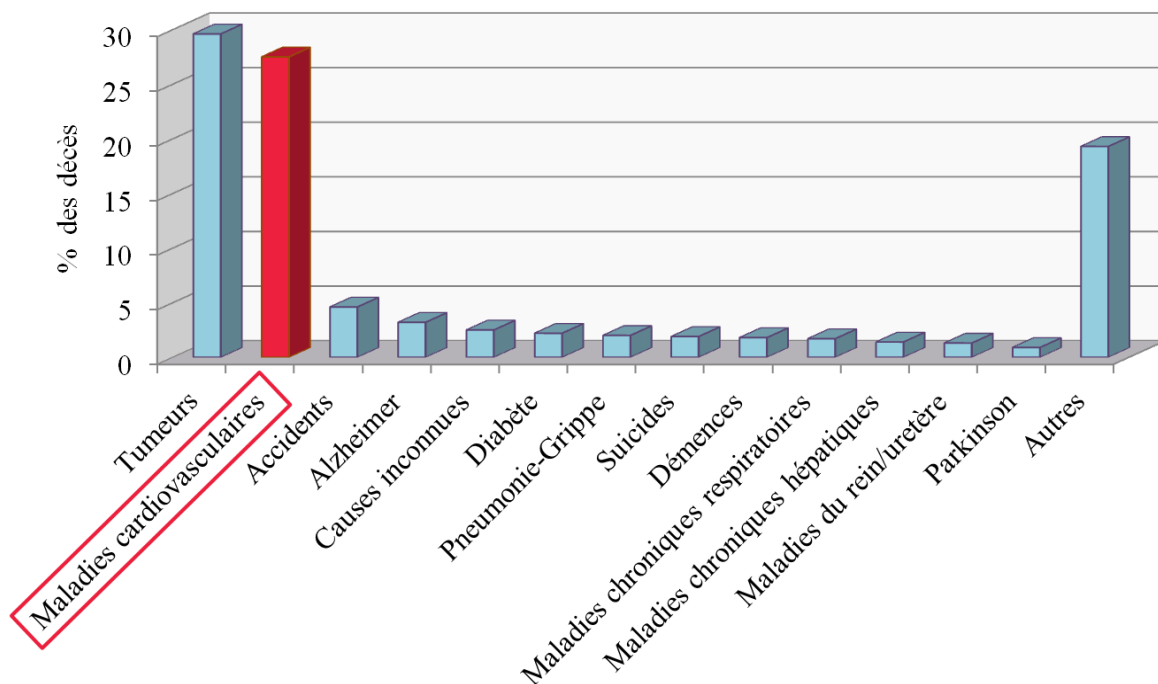


Figure 1 : Principales causes de mortalité en France en 2008, d'après les données de l'Institut National de Veille Sanitaire [19].

Les facteurs de risque des maladies cardiovasculaires sont bien connus. Il s'agit d'une part de facteurs non contrôlables tels que l'âge, le sexe, l'ethnie et les antécédents familiaux ou personnels et d'autre part de facteurs modulables (hypertension artérielle, hypercholestérolémie, sédentarité, surpoids, consommation excessive d'alcool, tabagisme et stress). Ces derniers possèdent un dénominateur commun : ils provoquent une dysfonction endothéliale et participent ainsi à l'athérosclérose [20] (Figure 2).

L'athérosclérose est une maladie vasculaire chronique de la paroi des artères, qui conduit à leur obstruction progressive. C'est un processus séquentiel complexe, initié dès l'enfance, mettant en jeu des réactions inflammatoires et prolifératives exacerbées en réponse à divers stimuli [21]. Parmi les différents acteurs impliqués, les cellules endothéliales jouent un rôle clé, et ce dès les premières étapes de l'athérosclérose.

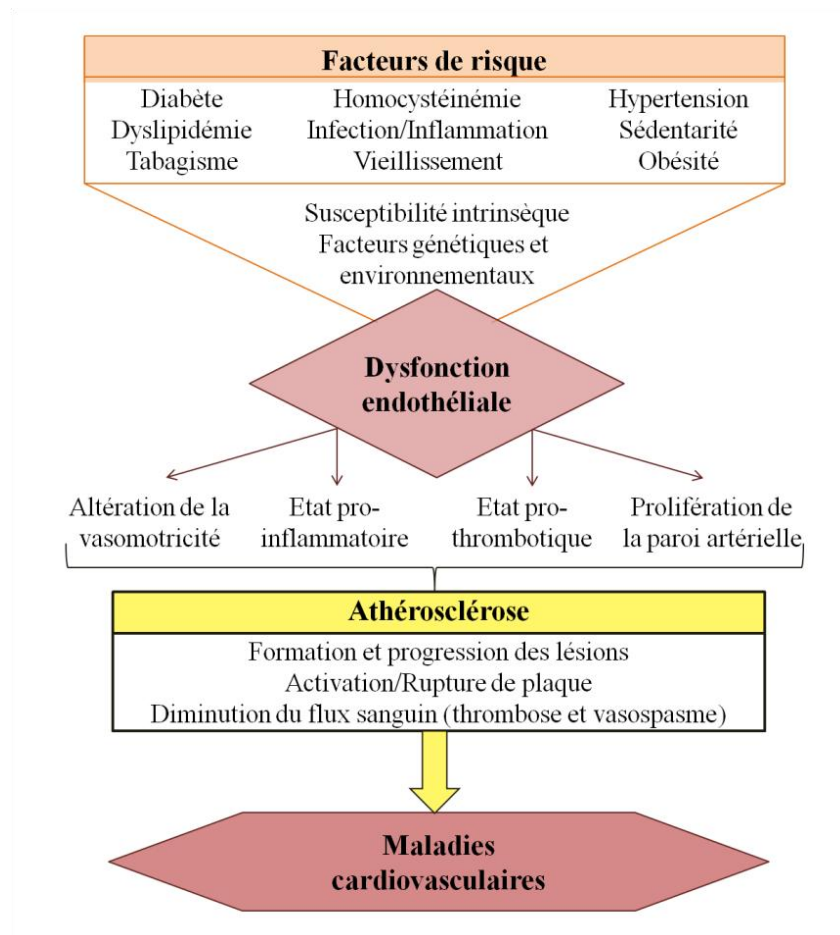


Figure 2 : Rôle de la dysfonction endothéliale dans la genèse des maladies cardiovasculaires, modifié d'après [22].

1.1.2. L'endothélium vasculaire

La monocouche de cellules endothéliales constituant l'endothélium vasculaire occupe une position stratégique à l'interface entre le sang (pôle apical) et les tissus environnants (pôle basal) (Figure 3). Longtemps considéré comme une simple barrière physique, l'endothélium vasculaire est en réalité un élément majeur de l'homéostasie vasculaire.

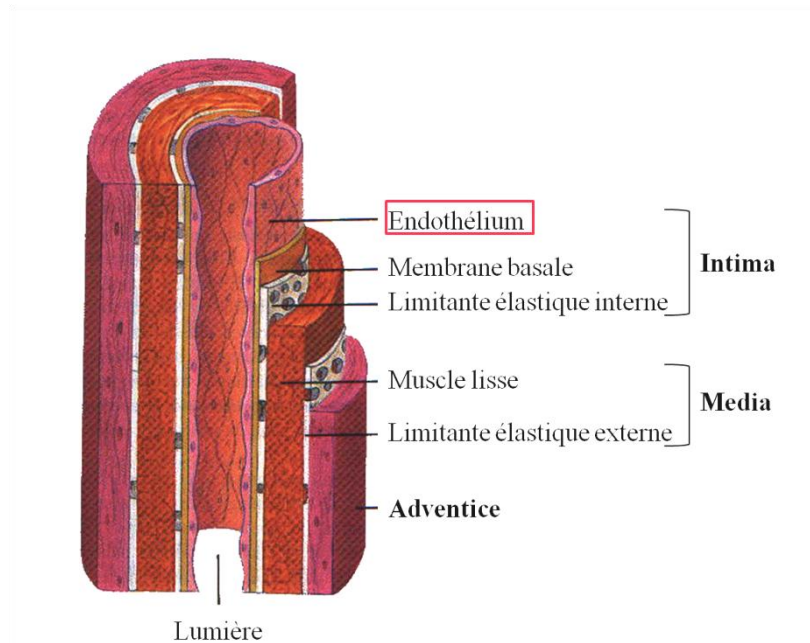


Figure 3 : L'endothélium, une structure clé des vaisseaux sanguins (ici une artère), adapté d'après une image de la Fédération Française de Cardiologie [23].

Les cellules endothéliales vasculaires sont exposées en permanence à des stimuli chimiques (hormones, neurotransmetteurs) et mécaniques (contraintes de cisaillement). En réponse, elles sécrètent différentes substances modulant la vasomotricité, l'angiogenèse, la perméabilité vasculaire, l'hémostase et l'inflammation. La Figure 4 présente les différentes substances produites au niveau de l'endothélium modulant le tonus des cellules musculaires lisses avoisinantes (et donc la vasomotricité). En condition physiologique, la libération des facteurs de relaxation des cellules musculaires lisses (en particulier du monoxyde d'azote, NO) prédomine sur celle des facteurs de contraction.

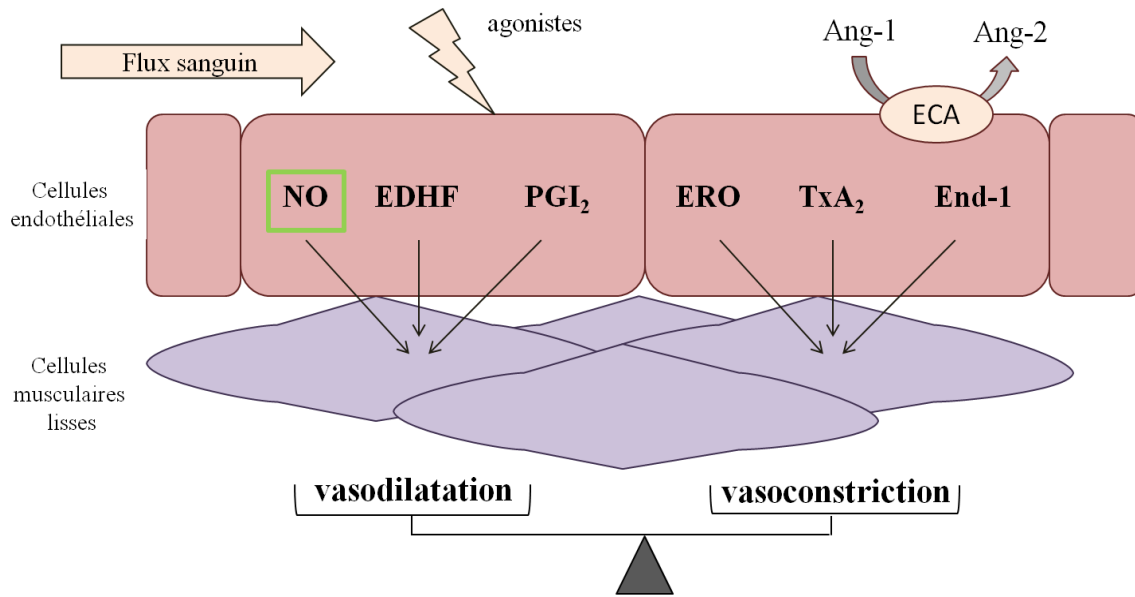


Figure 4 : La modulation du tonus vasculaire par l'endothélium résulte de la synthèse et de la libération de vasodilatateurs (monoxyde d'azote NO, facteur hyperpolarisant dérivé de l'endothélium EDHF, prostacycline PGI₂) et de vasoconstricteurs (espèces réactives de l'oxygène ERO, thromboxane A2 TxA₂, endothéline-1 End-1, et conversion à la surface endothéliale de l'angiotensine-1 (Ang-1) en angiotensine-2 (Ang-2) par l'enzyme de conversion de l'angiotensine (ECA)).

1.1.3. Le monoxyde d'azote

Le monoxyde d'azote est un radical libre, gazeux et soluble, à très faible demi-vie [8]. Il est produit en continu au niveau des cellules endothéliales par une enzyme, l'*endothelial nitric oxide synthase* (eNOS), à partir de la *L*-arginine. Cette enzyme est active de façon constitutive mais peut être stimulée par différents agonistes comme par exemple la thrombine [24], l'adénosine 5'-diphosphate [25] ou les kinines [26]. Néanmoins, le principal stimulus de cette production basale de NO semble être la contrainte de cisaillement exercée par le flux sanguin [27–29]. Hecker *et coll.* ont par exemple observé une diminution du taux basal de NO lorsque des cellules endothéliales sont cultivées *ex vivo* sans contrainte mécanique [30].

Le monoxyde d'azote exerce de multiples effets au niveau vasculaire (Figure 5). Comme évoqué précédemment, il intervient dans le maintien du tonus basal en relaxant les cellules musculaires lisses vasculaires [31]. Il possède de plus des propriétés anti-athérogéniques : il agit par exemple sur les plaquettes, non seulement en inhibant l'adhésion, l'activation, la sécrétion du contenu des granules et l'agrégation des plaquettes, mais encore en facilitant leur désagrégation [32]. NO prévient également le chimiotactisme des monocytes [33], inhibe l'adhésion des leucocytes à l'endothélium [34] ainsi que la migration [35] et la prolifération [36] des cellules musculaires lisses. Ceci limite la prolifération

néointimale survenant après un traumatisme vasculaire. Combiné à son effet stimulant sur la migration et la prolifération des cellules endothéliales [37], ceci suggère un rôle de NO dans la réparation des vaisseaux. Par ailleurs, ce radical, à concentration physiologique, présente des propriétés anti-inflammatoires, notamment en diminuant la perméabilité vasculaire [38], en inhibant la dégranulation des neutrophiles [39] et des mastocytes [40], ou encore en inhibant la production de cytokines immuno-modulatrices (interleukines IL-12 et IL-1) par les macrophages [41]. Enfin, il exerce un effet antioxydant de par sa capacité à capter les radicaux libres [42] comme l'anion superoxyde ($O_2^{\cdot-}$) [43] ou à inhiber leur production [44]. NO agit *via* différentes voies de signalisation : ce point sera évoqué en détail par la suite.

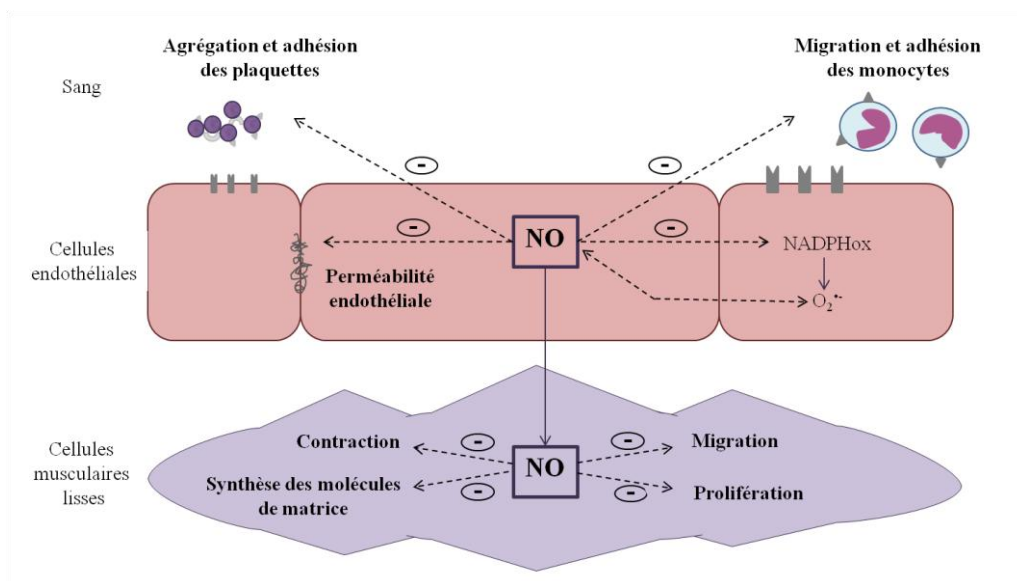


Figure 5 : Représentation schématique des différents effets du monoxyde d'azote sur les cellules sanguines et sur celles de la paroi vasculaire, modifiée d'après [45]. NADPHox : NADPH oxydases, $O_2^{\cdot-}$: anion superoxyde.

Les concentrations physiologiques de NO sont généralement considérées comme étant de l'ordre du pico- ou nano-molaire [46]. Au-delà (micro-molaire), par exemple suite à l'induction inappropriée de l'isoforme enzymatique iNOS ou dans un environnement soumis à un stress oxydant, NO peut favoriser l'inflammation et amplifier le stress [47].

1.2. Article 1 : « Quel avenir en thérapeutique cardiovasculaire pour le monoxyde d'azote et ses dérivés ? »

Les maladies cardiovasculaires représentent un challenge thérapeutique majeur, tant d'un point de vue humain que financier. La dysfonction endothéliale constitue l'élément pivot de ces pathologies. Elle est essentiellement associée à une diminution de la production et/ou de la disponibilité de NO [20]. Sa très faible demi-vie restreint son utilisation directe en thérapeutique. Cependant, toute molécule susceptible de délivrer NO ou d'agir au niveau de ses cibles représente donc une avancée thérapeutique importante. Néanmoins, cette stratégie thérapeutique doit être appliquée avec prudence, car des concentrations de NO trop importantes peuvent être responsables d'effets délétères [47].

L'Article 1 constitue une revue bibliographique parue dans les *Annales Pharmaceutiques Françaises*. Après un bref rappel sur NO et sa physiopathologie, cette revue s'intéresse aux rares médicaments donneurs de NO sur le marché et plus particulièrement aux nitrates organiques. Ces molécules sont utilisées depuis un siècle dans le traitement symptomatique de l'angine de poitrine et souffrent, malgré leur efficacité, d'inconvénients majeurs comme un phénomène d'échappement thérapeutique ou l'instauration d'un stress oxydant et nitrosant. Cet article se termine par une ouverture vers de nouvelles pistes de composés prodrogues de NO, tels que les *S*-nitrosothiols (RSNO).



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REVUE GÉNÉRALE

Quel avenir en thérapeutique cardiovasculaire pour le monoxyde d'azote et ses dérivés ?

Which future in cardiovascular therapy for nitric oxide and its derivatives?

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MOTS CLÉS

Monoxyde d'azote ;
Dérivés nitrés ;
Échappement
thérapeutique ;
Stress oxydant ;
Préconditionnement ;
S-nitrosothiols

Résumé Le monoxyde d'azote (NO) est impliqué dans la régulation de nombreux phénomènes physiologiques comme l'homéostasie vasculaire. L'administration exogène de NO présente un intérêt thérapeutique majeur, notamment dans le traitement de la maladie artérielle coronaire, les syndromes ischémiques et autres pathologies cardiovasculaires. Néanmoins, l'utilisation directe de NO est limitée par sa faible demi-vie. Des prodrugs de NO sont commercialisées depuis plusieurs décades, comme les dérivés nitrés pour le traitement de l'angine de poitrine. Ces derniers présentent des avantages certains comme le soulagement de la crise d'angor ou l'effet de preconditionnement. En revanche, ils souffrent de différents inconvénients : toxicité, échappement thérapeutique, exacerbation de la dysfonction endothéliale. Ces effets négatifs sont provoqués par la production massive d'espèces réactives de l'oxygène et de l'azote, responsables d'un stress oxydant et nitrosant. De nouveaux donneurs de NO sont à l'étude pour pallier ces désavantages, parmi lesquels la famille des S-nitrosothiols, particulièrement prometteurs.

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KEYWORDS

Nitric oxide;
Organic nitrates;
Tolerance;
Oxidative stress;
Preconditioning;

Summary Nitric oxide (NO) is involved in the regulation of several physiological processes such as vascular homeostasis. Exogenous NO supply offers major therapeutic interest, especially in the treatment of coronary artery disease, ischemic syndromes and other cardiovascular pathologies. Nevertheless, the administration of NO itself is limited by its short half-life. NO prodrugs have been marketed for decades, e.g. organic nitrates for angina pectoris. These prodrugs display undeniable advantages such as angina crisis relief and preconditioning effect. Nevertheless, they suffer from several drawbacks: toxicity, tolerance, endothelial dysfunction

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S-nitrosothiols

exacerbation. These negative effects are related to massive production of reactive species derived from oxygen or nitrogen, which trigger oxidative and nitrosative stress. New NO donors are under development to overcome those disadvantages, among which the S-nitrosothiols family seems especially promising.

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Introduction

La découverte de l'oxyde nitreux ou monoxyde d'azote (NO) en tant que messager physiologique chez les mammifères a valu le prix Nobel (Physiologie ou Médecine, 1998) à R. Furchgott, L. Ignarro et F. Murad. Le NO est produit à partir de la L-arginine et du dioxygène par une réaction enzymatique catalysée par la famille des NOS (« nitric oxide synthase », EC 1.14.13.39) [1], dont trois isoformes ont été décrites (Tableau 1). La NOS endothéliale (eNOS, NOS III) et la NOS neuronale (nNOS, NOS I) sont constitutionnelles et sont activées par une élévation de la concentration en calcium au sein de la cellule. Elles produisent de faibles concentrations de NO de façon transitoire : il agit alors comme médiateur physiologique, par exemple dans l'homéostasie vasculaire ou comme neurotransmetteur. La troisième isoforme est la NOS inducible (iNOS, NOS II), généralement exprimée par les cellules du système immunitaire après induction par un stimulus pathogène ou par des cytokines pro-inflammatoires. Les iNOS fonctionnent indépendamment de la concentration de calcium : après induction, elles produisent de fortes concentrations de NO de façon prolongée. Au niveau des macrophages activés, cette production massive de NO associée à celle d'anion superoxyde ($O_2^{\cdot-}$) par les NADPH oxydases entraîne la formation d'espèces réactives de l'oxygène et de l'azote qui permettent la destruction des pathogènes (bactéries, virus et parasites). Outre ce rôle dans la défense immunitaire non spécifique, les iNOS peuvent aussi être exprimées par d'autres types cellulaires de façon quasi ubiquitaire.

Une augmentation de la production de NO par les nNOS ou les iNOS est retrouvée dans de nombreuses situations pathologiques, par exemple les maladies neurodégénératives ou les maladies inflammatoires chroniques. Par conséquent, des inhibiteurs de NOS sont en développement, idéalement avec la plus grande sélectivité possible pour la iNOS et/ou la nNOS [12]. En effet, en ce qui concerne l'eNOS, c'est son inhibition et non son activation qui conduit à des situations pathologiques.

Cette revue s'intéressera uniquement aux applications thérapeutiques cardiovasculaires de molécules donneuses de NO. Dans un premier temps, un bref aperçu des donneurs de NO déjà commercialisés sera donné. Dans un deuxième temps, les dérivés nitrés seront décrits de façon plus approfondie, car des avancées récentes ont permis de mieux comprendre les mécanismes de leurs effets bénéfiques et de leurs effets indésirables, comme le phénomène d'échappement thérapeutique caractéristique de ces composés. Enfin, des donneurs de NO en développement seront évoqués et plus particulièrement les S-nitrosothiols.

Le monoxyde d'azote au niveau cardiovasculaire

Un médiateur physiologique déterminant via les phénomènes de nitrosylation et nitrosation

Le NO est un gaz de nature radicalaire, susceptible de réagir avec de nombreuses protéines et enzymes, notamment via leurs cofacteurs métalliques (nitrosylation) ou leurs résidus cystéine (nitrosation) [13]. Une de ses cibles particulièrement importante est la guanylate cyclase soluble (GCs, EC 4.6.1.2). Cette enzyme est présente dans le cytoplasme de la plupart des cellules des mammifères et transforme la guanosine triphosphate (GTP) en guanosine monophosphate cyclique (GMPc) [14]. NO est capable de se fixer sur le groupement prosthétique hème de la GCs, ce qui augmente l'activité de l'enzyme d'un facteur 100 à 200 [15]. Le GMPc produit active les protéines kinases G (PKG), ce qui déclenche une signalisation complexe et donc de nombreux changements physiologiques.

Bien que la nitrosylation de la GCs et la signalisation GMPc qui en découle soient les effets de NO les mieux connus et les plus détaillés, la S-nitrosation a émergé récemment comme étant une autre voie de signalisation extrêmement importante. La fonction mitochondriale, en particulier, est régulée par la S-nitrosation transitoire de protéines impliquées dans la chaîne respiratoire, la morphologie et la perméabilité mitochondriale ou le métabolisme des acides gras [16,17].

NO et système cardiovasculaire

Le NO joue un rôle particulièrement important au niveau vasculaire, où il est produit principalement par les eNOS des cellules endothéliales. Sa diffusion vers les cellules musculaires lisses vasculaires a longtemps été considérée comme un phénomène uniquement passif, mais des travaux récents ont montré l'existence d'un mécanisme actif au niveau des jonctions myoendothéliales impliquant l'hémoglobine alpha et la cytochrome b5 réductase 3 [18]. NO provoque la relaxation des cellules musculaires lisses et induit ainsi une vasodilatation [19] (Fig. 1). De plus, il inhibe la prolifération des cellules musculaires lisses vasculaires et empêche donc le développement d'une hyperplasie néo-intimale [20]. NO peut également agir sur les éléments figurés du sang, plaquettes et leucocytes, principalement par la signalisation GMPc-dépendante. NO diminue l'activation et l'agrégation plaquettaire [21], ainsi que le roulement des leucocytes et leur adhésion aux parois vasculaires [22]. En résumé, NO est essentiel pour maintenir l'homéostasie vasculaire. Un défaut de NO (par production endogène

Tableau 1 Principales caractéristiques des isoformes *nitric oxide synthase* (NOS).
Main characteristics of NOS isoforms.

	eNOS (NOS III)	nNOS (NOS I)	iNOS (NOS II)
Expression		Constitutive	Inductible
Production de NO		Faible, transitoire	Forte, prolongée
Principaux lieux d'expression	Cellules endothéliales (cardiomyocytes, neurones, plaquettes)	Neurones (CMLV, neutrophiles)	Macrophages activés (neurones et cellules gliales, CMLV, cardiomyocytes)
Rôle physiologique principal	Homéostasie vasculaire Angiogenèse	Neurotransmetteur Plasticité synaptique Mémoire	Défense immunitaire non spécifique
Principales implications pathologiques	Dysfonction endothéliale [2] (↓) Insuffisance cardiaque [3] (↓) Diabète [4] (↓)	Migraine [5] (↑) Ischémie-reperfusion cérébrale [6] (↑) Dépression [7] (↑ ou ↓)	Maladie de Parkinson [8] (↑) Inflammation et allergie chroniques [9] (↑) Carcinogenèse [10] (↑) Diabète [11] (↑)

CMLV : cellules musculaires lisses vasculaires ; ↑ : activité augmentée ; ↓ : activité diminuée.

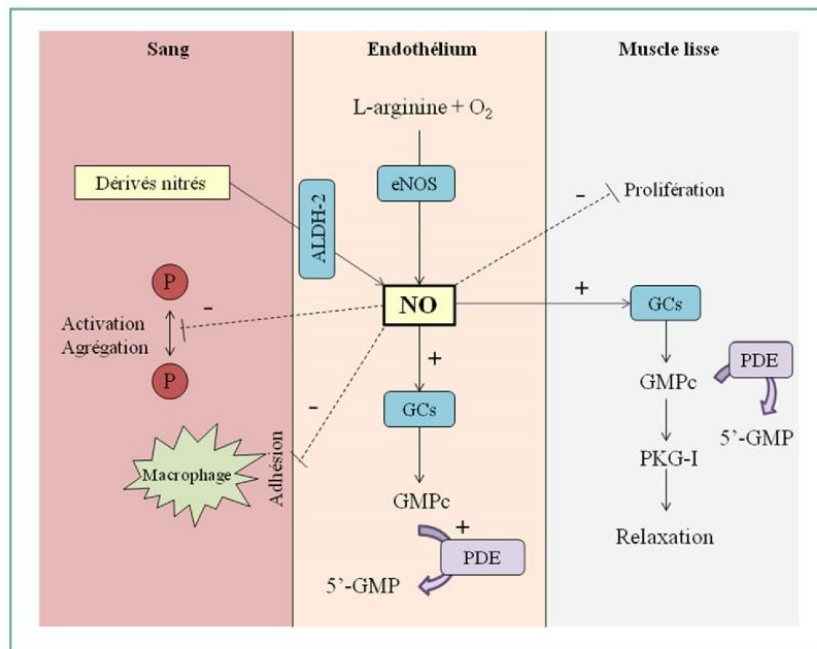


Figure 1. Effets vasculaires du monoxyde d'azote (NO). P: plaquettes; ALDH-2: aldéhyde déshydrogénase mitochondriale; eNOS: *endothelial nitric oxide synthase*; GCs: *guanylate cyclase soluble*; PDE: phosphodiesterase; GMPc: guanosine monophosphate cyclique; PKG-I: protéine kinase GMPc-dépendante de type I. *Vascular effects of nitric oxide (NO).*

diminuée et/ou dégradation augmentée) peut entraîner une vasoconstriction (donc une hypertension), un état de pré-activation des plaquettes (risque de thrombose) et une levée de l'inhibition du recrutement leucocytaire (état pro-inflammatoire).

Par ailleurs, au niveau cardiaque, des liens ont été établis entre la 5-nitrosation de protéines clés de la

mitochondrie et l'effet de préconditionnement ischémique, ou la protection contre les dommages liés au phénomène d'ischémie-reperfusion [23].

En conséquence, ces différents mécanismes d'action rendent un apport exogène de NO particulièrement prometteur en thérapeutique cardiovasculaire.

Les donneurs de NO en thérapeutique

La vasodilatation induite par NO (diminution des résistances vasculaires) entraîne une diminution de la pression sanguine. Cette propriété est exploitée en thérapeutique, en premier lieu avec l'utilisation de NO gaz dans le traitement de l'hypertension artérielle pulmonaire [24]. NO inhalé agit uniquement localement, à cause de sa très faible demi-vie inférieure à 0,1 s [25]. Il n'est donc pas utilisable pour un effet systémique.

En conséquence, des prodrogues ont été développées et commercialisées avec des indications élargies aux pathologies ischémiques et à l'insuffisance cardiaque (pour leur effet sur la pré-charge). Il s'agit notamment des sydnonimines (molsidomine), du nicorandil et des dérivés nitrés. Les sydnonimines ne sont pas utilisées en première intention car elles libèrent à la fois NO et l'anion superoxyde $O_2^{\bullet -}$ [26] et pourraient donc créer ou aggraver une dysfonction endothéliale. En ce qui concerne le nicorandil, il s'agit d'une molécule à double potentialité : à la fois donneur de NO et activateur des canaux potassiques sensibles à l'adénosine triphosphate (K-ATP). Cette dernière propriété pourrait être à l'origine d'un effet de preconditionnement ischémique [27]. Le nicorandil fait partie de la thérapeutique anti-angineuse comme l'une des alternatives en cas d'intolérance aux bêtabloquants [28]. Néanmoins, des cas d'ulcération des muqueuses ont été rapportés [29,30]. Quant aux dérivés nitrés, ils constituent l'une des trois principales familles utilisées dans le traitement de l'angine de poitrine. Ils seront décrits plus en détail, comme un exemple de donneurs de NO de « première génération », afin de mieux comprendre les enjeux d'une thérapie par des prodrogues de NO.

Les dérivés nitrés

Prise en charge de l'angor

Les dérivés nitrés sont utilisés depuis plus d'un siècle dans le traitement de l'angor (angine de poitrine). Ce n'est que récemment que leur propriété de donneur de NO a été découverte. La compréhension progressive de leur mode d'action et des mécanismes de leurs effets indésirables ouvre la voie à de nouveaux donneurs de NO, aussi efficaces mais moins toxiques.

L'angine de poitrine est caractérisée par une douleur thoracique intense, symptomatique d'une coronaropathie, décrite pour la première fois en 1768 [31]. Il s'agit en fait d'une inadéquation entre les besoins du cœur en oxygène et nutriments et leur apport via les artères coronaires. La coronaropathie représente actuellement la première cause de mortalité dans les pays très et moyennement développés [32]. Les buts de la prise en charge de l'angor reposent sur la réduction de la morbidité et des risques de mortalité (infarctus, arythmies, insuffisance cardiaque), mais aussi sur la réduction ou l'élimination des symptômes, afin d'améliorer la qualité de vie des patients et d'augmenter leur capacité à l'exercice [33]. Historiquement, il existe trois classes majeures d'anti-ischémiques : les dérivés nitrés, les bêtabloquants et les inhibiteurs calciques. Les recommandations actuelles privilégient pour le

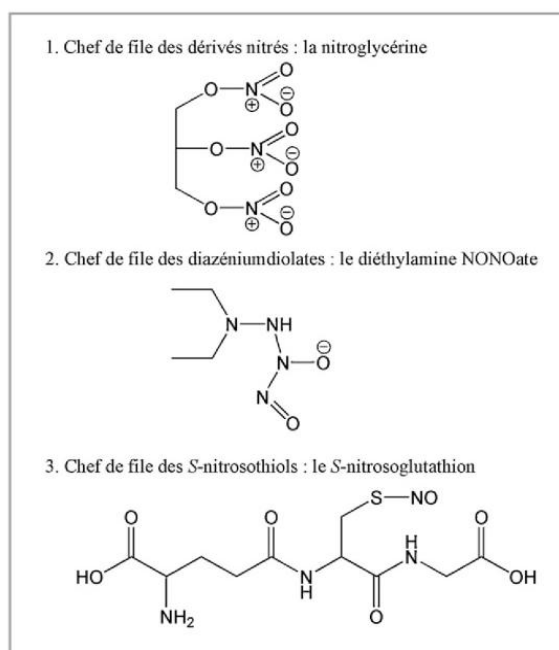


Figure 2. Structure des chefs de file des principales familles de donneurs de NO. Les dérivés nitrés sont commercialisés alors que les diazéniumdiolates et les S-nitrosothiols sont en développement. *Main families of NO donors: structure of the leader drug. Organic nitrates are already marketed while diazeniumdiolates and S-nitrosothiols are under development.*

traitement de fond en première intention une monothérapie par bêtabloquant (en l'absence de contre-indication), car ils ont montré une réduction significative de la morbi-mortalité. Les dérivés nitrés et inhibiteurs calciques peuvent être utilisés à la place des bêtabloquants en cas de contre-indication, ou en complément de ceux-ci s'ils ne suffisent pas à contrôler les symptômes [28,33]. Récemment, de nouveaux médicaments avec d'autres modes d'action ont été introduits, notamment l'ivabradine, un inhibiteur des canaux I_f , qui peut être utilisé en cas d'intolérance aux anti-ischémiques ou en cas d'insuffisance cardiaque systolique [34]. Des traitements chirurgicaux tels que l'angioplastie ou le pontage coronarien permettent également de soulager la symptomatologie angineuse en levant ou en contournant l'obstruction artérielle. Dans tous les cas, le traitement de la crise se fait par administration sublinguale de nitroglycérine (NTG).

Historique

La NTG, chef de file de la famille des nitrates organiques, a été synthétisée en 1846 par un chimiste italien [35] (Fig. 2). Elle a d'abord été considérée comme un explosif très puissant et extrêmement dangereux car instable. Par la suite, Alfred Nobel parviendra à la stabiliser par mélange avec de la terre de diatomée et la commercialisera sous le nom de « dynamite » en 1867.

La manipulation du produit pouvant provoquer d'intenses maux de tête, une action vasodilatatrice de la NTG fut

suspectée. Murrel l'introduisit en 1878 dans la thérapeutique de l'angor et obtint d'excellents résultats à faible dose. Il conclut que les maux de tête observés étaient vraisemblablement liés à un surdosage [36]. Étant donné l'historique « détonant » de la NTG, le composé fut rebaptisé « trinitrine » pour l'usage thérapeutique, afin de ne pas inquiéter les patients.

La NTG est rapidement métabolisée par le foie et a donc une durée d'action assez faible. D'autres dérivés nitrés à plus longue durée d'action ont été développés par la suite. Il s'agit tout d'abord du tétranitrate de pentaérythritol au début du ^{xx} siècle, puis du dinitrate et mononitrate d'isosorbide (ISDN et ISMN, respectivement) dans les années 1930. Aujourd'hui, seuls la NTG, l'ISDN et l'ISMN sont encore autorisés en France (Tableau 2). La NTG est utilisée essentiellement dans la prévention à court terme ou le traitement de la crise du fait de sa rapidité d'action. Elle est administrée par voie sublinguale ou transcutanée afin de s'affranchir de l'effet de premier passage hépatique. En ce qui concerne l'ISDN et l'ISMN, ils sont principalement administrés par voie orale pour la prévention à long terme de la crise d'angor.

Des donneurs de NO

Le mécanisme d'action des dérivés nitrés est longtemps resté inconnu et commence seulement à être élucidé. Il semblerait qu'ils soient des prodrogues de NO, dont la libération nécessiterait une activité enzymatique : aldéhyde déshydrogénase mitochondriale (ALDH-2, EC 1.2.1.3 [37]) pour la NTG et cytochromes P450 pour l'ISMN et l'ISDN [38].

Les dérivés nitrés sont donc des vasodilatateurs avec, à doses thérapeutiques, une action plus marquée sur les veines que sur les artères [39]. Cet effet spécifique est imparfaitement compris, mais pourrait être lié à une expression et/ou une activité plus importante(s) des enzymes transformant ces molécules en NO au niveau veineux [40]. La dilatation veineuse et dans une moindre mesure artérielle permet de réduire à la fois la précharge et la postcharge, et donc de diminuer la consommation en oxygène du myocarde. Par ailleurs, les dérivés nitrés exercent également un effet vasodilatateur sur les coronaires (de manière prépondérante au niveau des troncs épicardiques), permettant ainsi d'améliorer la perfusion du cœur. Leur propriété de donneur de NO leur permet également d'exercer un effet antiagrégant plaquettaire [41] et potentiellement de limiter l'adhésion des macrophages, deux effets particulièrement intéressants dans le cadre de la maladie artérielle coronaire.

Les dérivés nitrés sont généralement considérés comme des molécules ayant peu d'effets indésirables. Néanmoins, lors de la mise en route du traitement, des maux de tête importants peuvent survenir, accompagnés d'hypotension et parfois même de bradycardie. Malgré leur efficacité et leur faible toxicité, les dérivés nitrés souffrent d'un inconvénient majeur : l'apparition rapide d'un échappement thérapeutique (phénomène de tolérance), mettant en jeu une production massive d'espèces réactives délétères.

Échappement thérapeutique (tolérance)

Le phénomène d'échappement thérapeutique (ou tolérance) était connu avant même l'utilisation en

thérapeutique des dérivés nitrés. En effet, chez les ouvriers des usines de production de NTG, il était courant de souffrir de la « migraine du lundi matin ». En fait, ces ouvriers développaient une tolérance pendant leur semaine de travail, qui était réversible durant le week-end. Lors de leur retour le lundi matin, l'exposition à de fortes quantités de NTG entraînait une vasodilatation massive et donc une tachycardie, une somnolence et des maux de tête.

Chez les patients angineux traités par dérivés nitrés, la tolérance se manifeste par une perte de l'effet des médicaments, notamment la diminution de la capacité d'exercice sans déclenchement d'une crise [42]. Pour les dérivés nitrés, les caractéristiques de ce phénomène sont une apparition mais aussi une disparition rapides. Aussi, l'échappement est classiquement prévenu en ménageant une fenêtre thérapeutique, c'est-à-dire une période quotidienne d'au moins huit heures sans traitement par les dérivés nitrés [43]. Malheureusement, un phénomène de rebond peut alors se produire, avec des épisodes ischémiques nocturnes [44]. En conséquence, un traitement concomitant par un autre anti-ischémique est nécessaire pour couvrir cette période. Cet effet de rebond avait déjà été observé chez les ouvriers des usines de production de NTG, quand ils n'étaient plus exposés à de fortes doses. Il était responsable de nombreuses « attaques cardiaques du dimanche » [35].

Différentes hypothèses ont été avancées au fil des ans pour tenter d'expliquer la tolérance : une diminution de la quantité et/ou de l'effet de NO disponible, des altérations dans le métabolisme des dérivés nitrés (avec une augmentation du stress oxydant et/ou la déplétion de groupements thiols [-SH] participant à un effet antioxydant tissulaire), une régulation neuro-hormonale ou une altération de la signalisation GMPc. En définitive, tous ces mécanismes seraient vraisemblablement imbriqués et convergeraient vers la formation d'espèces réactives de l'oxygène et de l'azote (ER), en particulier l'anion peroxy-nitrite ONOO⁻ (Fig. 3).

D'une part, il peut exister une résistance à NO, en particulier lors de l'existence d'une dysfonction endothéliale, ce qui est le cas chez les patients traités par dérivés nitrés [45]. Cette résistance, indépendante de la production de NO, repose principalement sur la consommation de NO par réaction avec l'anion superoxyde (O₂^{•-}) ainsi que sur l'oxydation (réversible ou non) de la GCs [46]. Dans les cellules endothéliales, mais également dans les cellules musculaires lisses, la réaction de NO et O₂^{•-} produit l'anion peroxy-nitrite (ONOO⁻), un oxydant très puissant. Il est capable notamment d'oxyder la tétrahydrobioptérine, un des cofacteurs de l'eNOS. L'enzyme est alors découplée (eNOS_d) et produit O₂^{•-} au lieu de NO, amplifiant ainsi le phénomène [47]. Les ER produites peuvent réagir avec de nombreuses autres cibles protéiques ou enzymatiques, au niveau de groupements thiols (-SH) ou de groupements comportant un cation métallique (hème par exemple). L'oxydation des groupements -SH peut diminuer les défenses antioxydantes : il y a par conséquent augmentation des espèces oxydantes en parallèle de la diminution des espèces antioxydantes et donc génération d'un stress oxydant important. De plus, le site catalytique de l'ALDH-2 comporte plusieurs résidus cystéine adjacents. Lors de la réaction avec les dérivés nitrés, ces groupements -SH sont oxydés et l'enzyme inactivée [37]. Une régénération du site catalytique est normalement

Tableau 2 Caractéristiques des donneurs de monoxyde d'azote commercialisés en France. <i>Characteristics of nitric oxide donors marketed in France.</i>					
Principes actifs	Indications	Voies d'administration	Délai d'action	Durée d'action	Remarques
Monoxyde d'azote	Hypertension artérielle pulmonaire	Inhalation	Aucun	/	
Nitroglycérine Dinitrate d'isosorbide	Insuffisance cardiaque Œdème aigu pulmonaire d'origine cardiogénique	Intraveineuse	2 min 30–60 s	10 min 30–60 min	Réserve hospitalière
Dinitrate d'isosorbide	Levée de spasme artériel Coronarographie	Intracoronaire	Aucun	/	
Nitroglycérine Dinitrate d'isosorbide	Traitement préventif à très court terme ou curatif de la crise d'angor	Sublinguale	2–3 min	30–60 min 1–2 h	/
Nitroglycérine	Traitement préventif de la crise d'angor Insuffisance cardiaque (en association)	Transdermique	/	24 h	Nécessité d'un intervalle libre ≥ 8 h
Mononitrate et dinitrate d'isosorbide		Orale	15–60 min	4–24 h	Nécessité d'un intervalle libre ≥ 12 h
Molsidomine Nicorandil	Traitement préventif de la crise d'angor	Orale	20 min 30–60 min	3 h 12 h	/ /

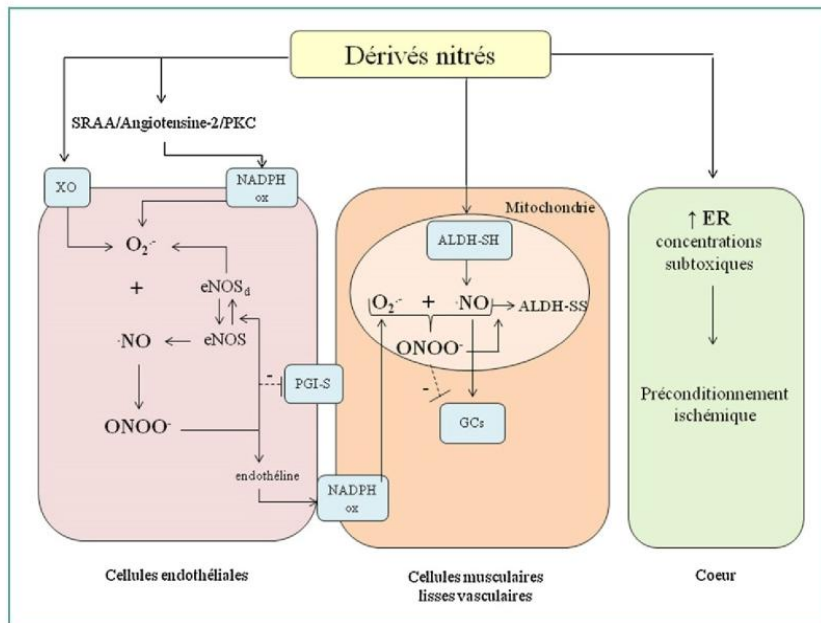


Figure 3. Effets négatifs (tolérance, au niveau des cellules endothéliales et musculaires lisses vasculaires) et positifs (préconditionnement, au niveau des cellules cardiaques) des dérivés nitrés. ER : espèces réactives de l'oxygène et de l'azote ; SRAA : système rénine-angiotensine-aldostérone ; PKC : protéine kinase C ; XO : xanthine oxydase ; NADPHox : NADPH oxydase ; eNOS : endothelial nitric oxide synthase (d : découplée) ; PGI-S : prostaglandine synthase ; GCs : guanylate cyclase soluble ; ALDH : aldéhyde déshydrogénase mitochondriale (SH : forme active, SS : forme oxydée, inactive).
Negative (tolerance, endothelial and vascular smooth muscle cells) and positive (preconditioning, cardiac cells) effects of organic nitrates.

effectuée grâce à d'autres thiols de l'organisme. Les ER sont susceptibles de maintenir l'ALDH-2 en conformation inactive, soit en oxydant directement les thiols du site catalytique [48], soit en oxydant les thiols utilisés pour sa régénération. En conséquence, le métabolisme des dérivés nitrés est ralenti et donc la libération de NO à partir de ces composés est diminuée.

D'autre part, un mécanisme de régulation neuro-hormonale peut se mettre en place, avec l'atténuation progressive des effets des dérivés nitrés, à cause d'une augmentation de sécrétion de substances vasoconstrictrices (comme les catécholamines, l'endothéline-1 ou l'angiotensine-2), qui peut également être associée à une diminution de sécrétion des espèces vasodilatrices (comme la prostaglandine I₂). En effet, les dérivés nitrés activent le système rénine-angiotensine-aldostérone : l'angiotensine-2 active alors la protéine kinase C, qui stimule la production d'O₂^{•-} par les xanthines oxydases et les NADPH oxydases membranaires [49] : l'intensité du stress oxydant est encore augmentée. Ce phénomène a notamment été impliqué dans le rebond ischémique lors du retrait brutal des dérivés nitrés [44].

Enfin, l'échappement apparaît lié à une détérioration progressive de la bioactivation enzymatique des dérivés nitrés (par exemple par l'oxydation du groupement -SH du site catalytique de l'enzyme [48]), couplée à une production importante d'ER dont O₂^{•-}. Ces dernières créent alors un cercle vicieux, en particulier en amplifiant la résistance à NO [50]. Elles peuvent également affecter la signalisation du NO via la S-nitrosation de la GCs qui se traduit par la diminution de la production de GMPc [51].

La compréhension des mécanismes de l'échappement thérapeutique aux dérivés nitrés n'a été obtenue que récemment : ils sont complexes mais apparaissent centrés sur la production massive d'ER. Ces progrès ont également permis de mieux cerner d'autres effets des dérivés nitrés comme leur impact sur la dysfonction endothéliale et le préconditionnement, évoqués ci-après.

NO et dysfonction endothéliale

L'endothélium joue un rôle clé dans le maintien de l'homéostasie vasculaire via la libération de facteurs relaxants ou contractants. Un déséquilibre de cette balance conduit à un état pathologique appelé dysfonction endothéliale. Cette dernière est communément associée à un certain nombre de facteurs de risques de maladies cardiovasculaires comme l'athérosclérose, l'hypercholestérolémie, le tabagisme ou l'hypertension [52]. Différents mécanismes peuvent être impliqués, mais une inactivation accélérée et/ou une production réduite de NO sont couramment observées dans la dysfonction endothéliale et représentent un facteur de risque indépendant de problèmes cardiovasculaires majeurs [53]. Du fait des effets vasculaires du NO, à la fois sur les cellules musculaires lisses, les plaquettes et les cellules inflammatoires, un apport de NO exogène semble représenter une thérapeutique intéressante, notamment dans le traitement de l'athérosclérose.

Néanmoins, l'apport de NO via les dérivés nitrés s'accompagne d'un développement/exacerbation de la dysfonction endothéliale [54]. Celle-ci peut s'expliquer par une augmentation du stress oxydant (cf. précédemment). En

effet, les ER, notamment l'espèce ONOO⁻, sont fortement impliquées dans la dysfonction endothéliale. L'exacerbation de cette situation pathologique peut conduire à un mauvais pronostic. Cependant, les conclusions des essais cliniques sur l'efficacité à long terme des dérivés nitrés sont contradictoires [55]. Une méta-analyse a par exemple suggéré que leur usage pouvait être délétère chez les patients atteints de maladie coronaire [56]. En revanche, d'autres études plus récentes ont conclu qu'ils n'augmentent ni la mortalité ni les événements coronaires [57], voire même qu'ils améliorent significativement les dégâts cardiaques provoqués par un événement coronaire aigu [58]. Ces résultats contradictoires peuvent sans doute s'expliquer par les effets à la fois délétères (échappement, dysfonction endothéliale) et bénéfiques (effet de préconditionnement) des dérivés nitrés.

NO et préconditionnement ischémique

En plus d'être les seuls composés susceptibles de prévenir à court terme/stopper la crise, les dérivés nitrés pourraient également exercer un effet protecteur non hémodynamique, en induisant un phénotype similaire à celui du préconditionnement au niveau des cardiomyocytes (Fig. 3) [59].

Le préconditionnement est un mécanisme endogène de protection contre l'ischémie : une ou plusieurs brèves périodes d'ischémie rendent un tissu plus résistant à une ischémie ultérieure prolongée. Ce phénomène complexe implique les radicaux libres de l'oxygène, le pore de transition de perméabilité et les canaux à ions chlorure mitochondriaux [60]. Le préconditionnement limite la libération massive d'ER lors d'un épisode d'ischémie/reperfusion (en empêchant leur sortie de la mitochondrie) [61] et stimule les défenses antioxydantes [62], permettant ainsi de diminuer les dommages tissulaires. Enfin, le préconditionnement ischémique permettrait aussi de promouvoir l'angiogenèse [63] et donc de sauver une partie des cellules lors d'un infarctus, au moins dans les régions de périphérie de la lésion, en permettant leur approvisionnement en oxygène et nutriments. Ce mécanisme a été mis en évidence dans différents tissus dont le tissu cardiaque [60]. Il peut également être obtenu par l'administration de différents agents pharmacologiques [60], dont le nicorandil [64] et les dérivés nitrés [59,65]. Néanmoins, la faisabilité et l'importance clinique d'un préconditionnement par les donneurs de NO restent encore non déterminées à ce jour.

En résumé, avec les dérivés nitrés, des ER produites en fortes quantités en continu participent au phénomène d'échappement, alors que produites en faible quantité et sur une faible durée, elles participent à la transduction du signal de préconditionnement ischémique [65].

Toutes ces avancées permettent de déterminer une orientation nouvelle pour l'arsenal thérapeutique futur, avec des composés donneurs de NO qui seraient dénués des effets secondaires des dérivés nitrés.

Autres donneurs de NO en développement

Comme nous l'avons décrit précédemment, les dérivés nitrés utilisés depuis plus d'un siècle dans le traitement de l'angine de poitrine sont des donneurs de NO efficaces,

mais qui souffrent du développement rapide d'un échappement thérapeutique. En conséquence, d'autres précurseurs de NO sont à l'étude, en vue d'obtenir des composés actifs, stables, peu ou non toxiques, capables de générer tous les effets positifs de NO sans production massive d'ER.

Certains auteurs proposent d'utiliser les ions nitrite ou nitrate comme précurseurs de NO. En effet, ces espèces peuvent participer dans l'organisme à la production de NO : les ions nitrate sont apportés par l'alimentation et sont transformés en ions nitrite par les réductases des bactéries commensales du tube digestif [66]. Les ions nitrite ainsi produits (ou apportés par l'alimentation) peuvent ensuite être réduits en NO dans certaines conditions, comme par exemple l'ischémie [67]. L'administration de ces composés n'entraîne pas de phénomène d'échappement et il existe de nombreuses applications potentielles, notamment dans les pathologies ischémiques où la production de NO par les eNOS est limitée par l'hypoxie [66]. Néanmoins, des doutes subsistent sur l'éventuelle formation de nitrosamines cancérigènes après administration de ces ions en forte quantité et constituent un obstacle majeur à leur utilisation.

Parmi les donneurs de NO en développement, une autre famille souffre également de ce risque carcinogène : les diazéniumdiolates ou NONOates. Classiquement, les NONOates sont des composés présentant un groupement diolate $[N(O)_2N=O]$ relié par un des azotes à un atome de carbone, d'azote, d'oxygène ou de soufre [68] (Fig. 2). Ils se décomposent spontanément à pH physiologique en libérant deux équivalents de NO par NONOate. Leur décomposition ne dépend pas de composés biologiques : elle est prévisible et peut être modulée par la structure du composé. Cependant, leur utilisation est également limitée par la formation de nitrosamines [69].

Enfin, les S-nitrosothiols ou RSNO (groupement NO lié à un atome de soufre d'un composé organique) présentent un certain nombre d'avantages (Fig. 2). Premièrement, ils ont des demi-vies du même ordre que celles des dérivés nitrés et n'entraînent pas de phénomène de tolérance [70–73]. Cela pourrait s'expliquer par les nombreux facteurs permettant la libération de NO à partir de ces composés (lumière, température, métaux de transition, thiols, enzymes... [26]), donc leur moindre dépendance au métabolisme enzymatique par rapport aux nitrates organiques. Deuxièmement, les RSNO sont des agents vasorelaxants [74] et exercent également un effet antiagrégant plaquettaire à des doses inférieures à celles modifiant le tonus vasculaire [75,76]. Enfin, certains RSNO existent de façon endogène (par exemple la S-nitrosoalbumine, la S-nitrosohémoglobine ou le S-nitrosoglutathion à des concentrations de 250 nM dans certains tissus [77]) et sont donc a priori peu susceptibles de toxicité. Chez l'animal, ils ont montré un effet protecteur contre les dommages dus à l'ischémie-reperfusion au niveau cérébral [78–80] ou cardiaque [81]. Cet effet protecteur pourrait être amplifié par la mise au point de RSNO ciblant spécifiquement la mitochondrie, comme proposé dans les travaux de Chouchani et Prime [17,82].

Le potentiel des RSNO en thérapeutique cardiovasculaire a déjà été en partie exploré chez l'homme, notamment en ce qui concerne leur effet antiagrégant plaquettaire (y compris dans la réduction des embolies cérébrales silencieuses) ou pro-cicatrisant [83,84]. Pour parvenir à un usage clinique, une forme galénique appropriée doit être trouvée.

Idéalement, elle devrait être capable de protéger les RSNO, tout en les libérant de façon progressive et prolongée de préférence aux lieux de production de NO endogène (jonction myoendothéliale...), afin de se rapprocher des conditions physiologiques. Dans ce but, différents matériaux polymériques capables de libérer NO (à partir de NONOates ou de RSNO) ont été créés [85,86] : ils sont destinés à être utilisés comme stent ou comme revêtement anti-thrombotique (par exemple pour les tubulures utilisées en circulation extracorporelle).

Pour conclure, la délivrance de NO exogène est une option thérapeutique attrayante dans le domaine cardiovasculaire. Néanmoins, pour être efficace, la libération de NO ne doit pas s'accompagner d'une augmentation du stress oxydant, comme c'est le cas avec les dérivés nitrés. Ces molécules sont pourtant utilisées depuis une centaine d'années dans le traitement de l'angine de poitrine, mais c'est seulement récemment que les mécanismes de tolérance et de preconditionnement ont été découverts et en partie élucidés. Nul doute que cette meilleure compréhension permettra la mise au point de donneurs de NO moins toxiques et au moins aussi efficaces. Les RSNO semblent représenter une alternative thérapeutique plutôt intéressante puisque certains ont déjà été testés en clinique [83]. L'avenir confirmera peut-être l'arrivée de ces molécules dans l'arsenal thérapeutique, dans le cadre de la maladie artérielle coronaire ou plus largement dans les pathologies ischémiques. D'autres applications thérapeutiques de donneurs de NO sont à l'étude : dans l'ostéoporose [87], l'hémorragie cérébrale [88], en obstétrique [89], en cancérologie [90], dans le sepsis [91] ou le diabète [92].

Déclaration d'intérêts

Les auteurs déclarent ne pas avoir de conflits d'intérêts en relation avec cet article.

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1.3. Conclusion

Dans l'Article 1, les avantages et les inconvénients des dérivés nitrés, donneurs de NO commercialisés depuis 100 ans, ont été exposés.

Le développement de nouvelles familles de donneurs de NO avec des effets bénéfiques égaux ou renforcés et ne provoquant pas de phénomène d'échappement thérapeutique ni de stress oxydant constitue un défi, particulièrement important dans un contexte d'augmentation de la prévalence des maladies cardiovasculaires. Plusieurs pistes (nitrates inorganiques, RSNO, NONOates...) ont été évoquées : la réactivité de ces familles de composés est potentiellement différente, ce qui peut conduire à l'activation de plusieurs voies de signalisation NO-dépendantes. Ces points seront présentés plus en détail dans la section suivante, en particulier en ce qui concerne les *S*-nitrosothiols. Enfin, le problème de la formulation galénique des molécules sera abordé.

2. VOIES DE SIGNALISATION ET DONNEURS DE NO

2.1. Trois grands types de signalisation

Comme abordé dans l'Article 1, étant donné sa très faible demi-vie, NO ne peut pas être stocké tel quel, et doit en conséquence, soit être synthétisé « à la demande », soit former des adduits plus stables afin d'exercer ses effets biologiques spécifiques. Ainsi, trois grands types de modifications des protéines par NO ont été identifiés à l'heure actuelle (Figure 6).

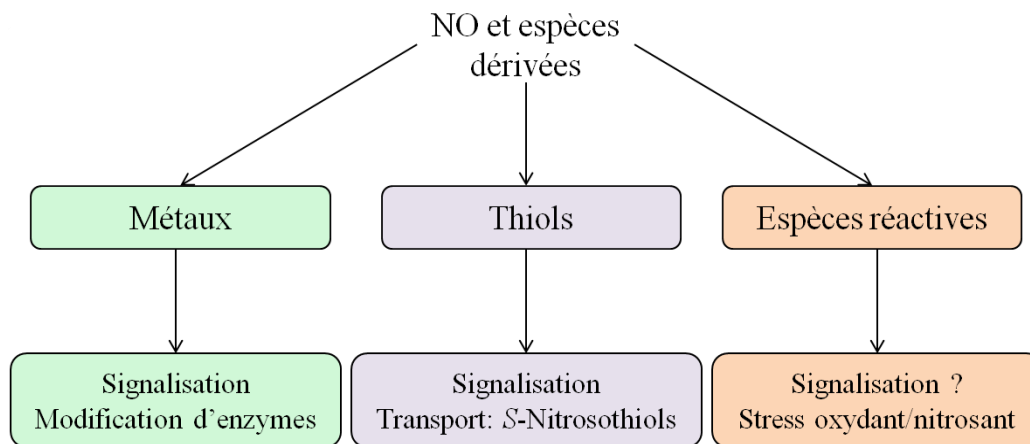


Figure 6 : Impact de NO et ses dérivés sur les protéines de la signalisation cellulaire.

D'une part, la *S*-nitrosylation est la réaction de NO ou de ses dérivés avec les métaux de transition. L'exemple le plus connu est la liaison de NO avec l'atome de fer du groupement hème de l'enzyme guanylate cyclase soluble (GCs), ce qui augmente son activité plus de 200 fois [48]. La GCs catalyse la transformation du triphosphate de guanosine en 3'-5' monophosphate de guanosine cyclique. Ce second messager atteint de nombreuses cibles (protéines kinases G, canaux ioniques liés aux nucléotides cycliques et phosphodiésterases), ce qui explique que cette voie de signalisation soit impliquée dans la majorité des fonctions physiologiques de NO. Un autre exemple de *S*-nitrosylation est la liaison de NO avec le fer hémique de la cytochrome c oxydase. Compétitive avec la liaison de l'oxygène, elle permet la régulation de la consommation en oxygène au niveau de la mitochondrie [49].

D'autre part, la réaction de *S*-nitrosation est la modification covalente de groupements thiols par NO et forme des *S*-nitrosothiols. Elle peut se produire au niveau des groupements thiols de composés de faible masse molaire comme le glutathion, ou avec des fonctions thiols présentes dans des protéines (ex : l'albumine) au niveau des acides aminés de type cystéine. Cette modification redox réversible est désormais reconnue comme une modification post-traductionnelle des protéines aussi importante que la phosphorylation et une voie majeure de signalisation liée au NO [50]. Les protéines susceptibles

d'être *S*-nitrosées peuvent être impliquées dans la fonction cellulaire normale (ex : trafic intracellulaire des protéines [51], contractibilité musculaire [52], apoptose [53], circulation sanguine [54]) et/ou dans des situations pathologiques variées (ex : diabète [55], pathologies neurologiques [56], cancers [57]). Il est important de faire remarquer que dans la littérature anglophone, il est souvent (de façon impropre) fait référence à la *S*-nitrosation par le terme de *S*-nitrosylation.

Enfin, NO appartient au groupe des espèces réactives de l'azote. Il peut subir une interconversion en cation nitrosonium (NO^+) ou anion nitroxyl (NO^-) [58]. Il peut également réagir avec des espèces réactives de l'oxygène. Par exemple, sa réaction avec l'anion superoxyde ($\text{O}_2^{\cdot-}$) conduit à la formation de l'anion peroxydinitrite (ONOO^-), qui peut ensuite générer de l'acide peroxydinitreux (HOONO), espèce instable et fortement oxydante. Le peroxydinitrite peut réagir avec les acides aminés des protéines (ex : oxydation des cystéines, nitration des tyrosines [59]). L'éventuel rôle physiologique de la nitration des protéines n'est pas encore élucidé, même s'il pourrait concerner la régulation du *turn-over* des protéines [60]. Par contre, la présence de protéines aux résidus tyrosines nitrés a été mise en évidence dans de nombreuses pathologies, y compris l'athérosclérose, l'infarctus du myocarde, la myocardite, le diabète et les maladies neurodégénératives ou inflammatoires [61]. Il peut aussi oxyder des lipides, comme les lipoprotéines de faible densité [62] ou initier la réaction en chaîne de peroxydation lipidique au niveau des membranes [63]. A l'inverse, NO permet la terminaison de cette réaction en chaîne [49]. De plus, le peroxydinitrite peut endommager l'ADN, en modifiant les bases ou les sucres [64]. Finalement, l'exposition au peroxydinitrite peut déclencher la mort cellulaire, soit par apoptose, soit par nécrose [65].

Des rôles clés de NO ont été identifiés dans quasiment tous les systèmes physiologiques chez les Mammifères. Cette molécule de signalisation multifonctionnelle est atypique, car son action dépend de modifications des peptides et protéines et non d'une simple interaction de type « clé-serrure ». Par conséquent, l'activité biologique de NO est déterminée par sa réactivité chimique. De plus, de nombreuses réactions de compétition entre NO et les espèces dérivées de NO sont possibles. Elles sont potentiellement influencées par les changements de l'environnement (pH, potentiel redox...). Etant donnée la complexité de la chimie de l'azote, la réactivité exacte des donneurs de NO, et donc la/les voie(s) de signalisation impliquée(s) sont encore mal définies et sujettes à discussion dans la littérature [66–68]. Par conséquent, l'expression « donneur de NO » communément utilisée dans la littérature pour désigner différentes classes comme les nitrates organiques, les diazénium diolates (NONOates), les RSNO, etc., est controversée [69]. Elle représente vraisemblablement un raccourci, car il est peu probable que tous ces composés libèrent la(les) même(s) espèce(s) chimique(s). Ceci est important car cette réactivité chimique peut conduire à des différences dans les voies de signalisation activées ou réprimées et donc dans les effets thérapeutiques/ secondaires de ces composés. L'expression « donneur de NO » sera néanmoins utilisée dans cette thèse car la plus communément admise dans la littérature.

2.2. Article 2 : « *S*-nitrosation/dénitrosation in cardiovascular pathologies: facts and concepts for the rational design of *S*-nitrosothiols »

Au cours des dernières années, l'importance physiopathologique de la *S*-nitrosation a progressivement été reconnue comme égale, voire supérieure, à celle de la *S*-nitrosylation. La revue suivante (Article 2 paru dans *Current Pharmaceutical Design*) fait le point sur ce processus majeur, avec un intérêt particulier pour les pathologies cardiovasculaires. La *S*-nitrosation, c'est-à-dire la formation de *S*-nitrosothiols, constitue une des modifications post-traductionnelles des protéines. Sa régulation dépend en partie de réactions de transnitrosation (déplacement du groupement NO d'un nitrosothiol vers une fonction thiol libre) et de dénitrosation. Par conséquent, dans une première partie de cette revue, deux familles enzymatiques impliquées dans les réactions de transnitrosation/dénitrosation sont étudiées : les thiorédoxines et les protéines disulfures isomérasés. Or, comme il sera décrit, les RSNO présentent un grand potentiel thérapeutique (démonstré chez l'animal et chez l'Homme) avec vraisemblablement peu ou pas de toxicité, puisqu'ils constituent le réservoir endogène de NO. Néanmoins, la faible stabilité de la liaison S-N (en particulier face aux enzymes dénitrosantes) constitue un obstacle majeur à leur utilisation en thérapeutique. La deuxième partie de la revue est donc consacrée aux stratégies pour améliorer la stabilité de ces composés, d'une part par des modifications adéquates de leur structure chimique et d'autre part par leur intégration dans des formes galéniques.

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S-nitrosation/Denitrosation in Cardiovascular Pathologies: Facts and Concepts for the Rational Design of S-nitrosothiols

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Abstract: Nitric oxide (*NO) is a physiological mediator of vasorelaxation constitutively synthesized by endothelial nitric oxide synthase. Because *NO has a short half-life, it is stored by proteins through S-nitrosation reactions. S-nitrosation was recently defined as a post-translational modification of proteins for cellular signalling, as important as glycosylation and phosphorylation. Disulfide forming/isomerizing enzymes like thioredoxin (Trx), protein disulfide isomerase (PDI), which are chaperone proteins, are implicated into transnitrosation reactions, which are the transfer of *NO from one cysteine residue to another one. Furthermore, Trx has been shown to denitrosate S-nitrosothiols depending on its redox status. S-nitrosation of Trx on Cys residues apart from active site, under nitrosative or oxidative stresses, enhances its activity, thereby reducing intracellular reactive oxygen species. Trx and PDI have therefore an essential role for cell signalling control which leads, among other actions, to cardio and vasculo-protection. The diminution of either *NO synthesis or bioavailability is implicated into a large number of cardiovascular pathologies associated to hypoxia or vasoconstriction like, endothelial dysfunction, arterial hypertension and atherosclerosis. In order to mimic the physiological storage of *NO as S-nitrosothiols, the development of *NO donors should be based on the covalent S-NO bond. The chemical stabilisation of the S-NO bond and protection against enzymatically active proteins such as PDI/Trx are major points for the design of stable compounds. S-nitrosothiols entrapment in innovative formulations (films, gels, microparticles, nanoparticles) is an emerging field in order to stabilise and protect them, and to deliver *NO under a sustained release at the targeted site.

Keywords: Thioredoxin, protein disulfide isomerase, nitric oxide, nitrosation, S-nitrosothiol, *NO donors, innovative formulation.

INTRODUCTION

The biochemical actions of nitric oxide (*NO) are not easily compatible with its chemical properties. Indeed, from the chemical point of view, *NO is highly reactive with a short half-life and has selective signalling biochemical actions. An intriguing question is "how *NO can exist and move inside the cell without being instantaneously sequestered by other molecules". Possible answer is to consider the implication of peptides and proteins in *NO transport, storage and propagation of the biochemical signalling of *NO . NO-bonomic is the novel metabonomics approach for the global profiling of *NO metabolism and *in vivo* signalling. It has been defined by Janero *et al.*, as the "global NO metabolic profiling which would encompass the quantitative *in vivo* mapping of NO-related metabolites of either endogenous or exogenous (*i.e.*, extragenomic) origin and how they are affected by disease, drug exposure, or other (patho)physiological influences. The set of NO-related metabolites and any products of their interaction identified under the prevailing conditions of the organism would constitute a NO-bonome" [1]. Moreover, S-nitrosation and nitrosylation of proteins implicated in cellular functional regulation are part of the NO-bonomic. Protein S-nitrosation, which is the addition of *NO to a cysteine residue (RSH), forming S-nitrosothiols (RSNO), constitutes a post-translational modification of proteins for cellular signalling. This modification is as important as glycosylation and phosphorylation. Nitrosylation is the addition of the radical *NO to the metallic center (mostly iron) of metalloproteins like soluble guanylate cyclase (sGC) and cytochrome P450 regulating their enzymatic activities. *NO is synthesized *in vivo* from L-arginine through catalytic action of three NO synthases (NOS) isoenzymes, including neuronal (nNOS), inducible (iNOS), and endothelial NOS (eNOS). eNOS is expressed in endothelial cells (EC) as a constitutive enzyme and produces picomolar to nanomolar concentrations of *NO for short periods in response to receptor stimulation (acetylcholine, bradykinin) or shear stress [2,3]. *NO through its vasodilator activity is

crucial for the maintenance of vascular homeostasis. Indeed, *NO diffuses from EC to smooth muscle cells where it activates sGC through nitrosylation to produce cyclic guanosine monophosphate (cGMP), activating a signalling pathway inducing vasorelaxation (Fig. (1)).

According to a pharmaceutical point of view, *NO donors are prescribed into cardiovascular diseases. Since *NO is characterised by an extremely short half-life *in vivo* (*ca.* 5 s), it is administered as a prodrug. Nonetheless, classical *NO donors have shown some limitations as a fast release of *NO , a relatively poor distribution to the targeted site (the vascular smooth muscle), an oxidative stress induction, and a tolerance phenomenon generation [4]. A way to solve these problems consists in the development of *NO donors based either on physiological S-nitrosothiols (RSNO), such as S-nitrosoglutathione (GSNO) and S-nitrosoalbumin (AlbNO), or exogenous ones, such as S-nitroso-N-acetylcysteine (NACNO) and S-nitroso-N-acetylpenicillamine (SNAP). Endogenous high molecular weight RSNO such as AlbNO, S-nitrosohemoglobin and low molecular weight ones (e.g. GSNO), are a physiological reservoir of *NO , thus they can become potential *NO donors for cardiovascular disease treatments. As albumin is the most abundant protein in plasma (35-50 g/L), it is not surprising that the major circulating plasma S-nitrosothiol is AlbNO [5], which has been proposed to act as a reservoir of *NO within the circulation, transporting and releasing *NO inside the vascular beds. A major subset of S-nitrosated proteins including circulating AlbNO are in equilibrium through spontaneous transnitrosation reactions with reduced glutathione (GSH), leading to a low molecular weight RSNO, *i.e.* GSNO, which acts as a second messenger to transduce *NO bioactivity [6,7]. Indeed, some studies clearly showed that RSNO denitrosation takes place nonenzymatically by rapid transnitrosation reactions in whole blood between GSNO and albumin or in intact cells between albumin and GSH [8,9]. However, some differences in the denitrosation rate of individual RSNO have been noted, suggesting the existence of additional structural factors [9]. The transfer can also be spontaneous implicating the thiols at the cell membrane [10]. However, the relatively unfavorable kinetics of transnitrosation reactions [11] suggest that enzyme-mediated systems are required for the process to happen in a sufficient fast way to medi-

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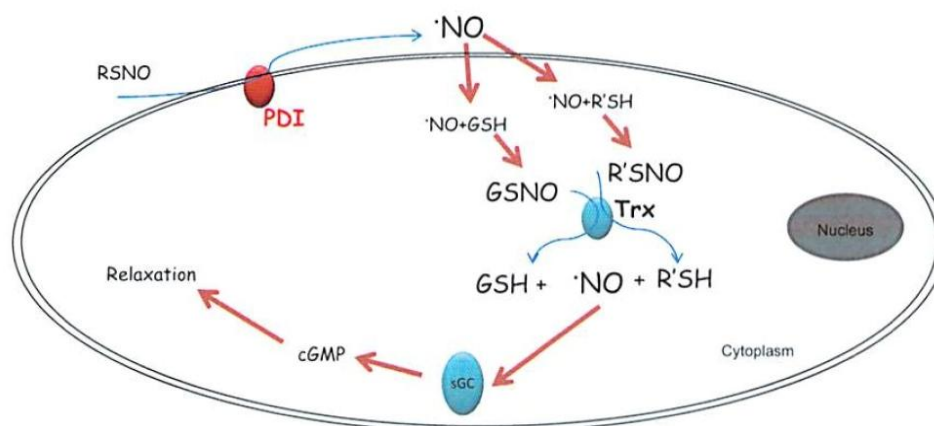


Fig. (1). Transfer and release of $\cdot\text{NO}$ by thiol disulfide oxidoreductase enzymes such as protein disulfide isomerase (PDI) and thioredoxin (Trx). Only the PDI at the plasma membrane of the cell is represented here because it is involved in the RSNO influx. Inside smooth muscle cells, $\cdot\text{NO}$ induces vasorelaxation through the activation of the soluble guanylate cyclase (sGC) pathway. cGMP: cyclic guanosine monophosphate; GSNO: S-nitrosoglutathione; GSH: reduced glutathione; RSNO: S-nitrosothiol.

ate signalling events. These systems include disulfide forming/isomerizing enzymes, which are the main enzymes acting to trans/denitrosate RSNO and finally to deliver $\cdot\text{NO}$. The two major enzyme families forming this class are the thioredoxin (Trx) and the protein disulfide isomerase (PDI), both of which have been shown to be involved into transnitrosation and denitrosation processes.

Consequently, endogenous RSNO that include $\cdot\text{NO}$ -traffic proteins (high molecular weight) or peptides (low molecular weight) can be used as reliable and safe $\cdot\text{NO}$ donors [12]. A $\cdot\text{NO}$ -traffic protein is a protein with high efficiency of S-nitrosation, high stability of the S-nitroso group in the blood stream and high efficiency in transnitrosation inside the cells [13]. They protect $\cdot\text{NO}$ from degradation (mostly oxidation and radical reactions) and can deliver it to the appropriate site of action (tissues and cells). So, this kind of $\cdot\text{NO}$ donors will trigger physiological dynamics to deliver $\cdot\text{NO}$ to its cellular target. Considering the lability of the S-NO bond (under exposure to light, metals, reducing agents, enzymes), a rational drug design of $\cdot\text{NO}$ donors is required to obtain a prolonged and controlled release of $\cdot\text{NO}$. In this way, the chemical stabilisation of the S-NO bond is the key to increase $\cdot\text{NO}$ donors half-life. Indeed, the alpha or beta substitutions on the carbon atom ($\text{C}\beta$ - $\text{C}\alpha$ -S-NO), with an electrodonor group and a positive charge respectively, are of great importance to promote RSNO stability [14,15]. Furthermore, RSNO formulation into nanoparticles (NP), making a physical barrier between RSNO and the environment, will help to increase its half-life in the blood stream. Otherwise, physiological RSNO like GSNO are hydrophilic, thus further modifications of RSNO increasing their lipophilicity will ensure a long time $\cdot\text{NO}$ delivering formulation.

This review will focus on the process of RSNO denitrosation and transnitrosation *via* disulfide forming/isomerizing enzymes such as Trx and PDI. In addition, we will review the relationship between disulfide forming/isomerizing enzymes and pathology. Finally through the overview of the specificity of the sites and motifs of S-nitrosation, we will give some keys to identify potential candidates for new $\cdot\text{NO}$ delivery drugs development and their rational design.

TRANSNITROSATION/DENITROSATION

It is widely assumed that the biological effects of RSNO are attributed to the homolytic cleavage of the S-NO bond with release of $\cdot\text{NO}$ [16,17]. The rate of $\cdot\text{NO}$ release from RSNO is dependent

upon many factors, such as pH, partial pressure of O_2 , light, metal ion concentration, presence of reducing and chelating agents, excess of thiols and enzymes [18,19]. However, Amelle *et al.* demonstrated that NO^- (*via* nitrosyl transfer), $\cdot\text{NO}$, and NO (nitroxyl anion) are released from RSNO at physiological pH [18]. Furthermore, the rate of $\cdot\text{NO}$ group transfer (transnitrosation) from a RSNO to myoglobin is more rapid than rates of $\cdot\text{NO}$ release by a RSNO alone [18]. These results suggest that heterolytic mechanisms of decomposition constitute significant pathways in RSNO metabolism.

S-nitrosothiols can undergo spontaneous [20] or assisted transnitrosation from one thiol to another one. The spontaneous transnitrosation from high to low molecular weight RSNO depends on the pKa of the thiol group. Indeed, transnitrosation occurs as the nitroso-nitrogen atom is attacked by the nucleophilic thiolate anion. So, the reaction rate is dependent on the reactivity of the thiol, which is correlated with the pKa of the sulfhydryl group. Besides, Wang *et al.* showed that transnitrosation rate constant increases in a linear relationship with the pKa of the thiol group [20].

Inside the cell cytoplasm, $\cdot\text{NO}$ can react with GSH to form GSNO, an intracellular pool of $\cdot\text{NO}$. Transnitrosation can be catalyzed by disulfide forming/isomerizing enzymes such as Trx and PDI. This class of proteins commonly known as thiol disulfide oxidoreductases catalyzes thiol disulfide exchange *in vivo* reactions [21]. The activity of these enzymes depends on a common motif with one pair of cysteine (Cys) residues arranged in a Cys-X-X-Cys motif (X is any amino acid). This motif is usually integrated in a domain sharing structural homology with Trx. The Cys-X-X-Cys motif has become the hallmark of proteins that are involved in forming or breaking disulfide bonds.

Thioredoxin

The thioredoxin system consists of Trx, NADPH and thioredoxin reductase [22]. It is essential for the regulation of cellular redox state and plays an important role in reducing the disulfide bonds of proteins through an interaction with the redox-active site center of Trx. Human Trx contains five Cys residues, including two, Cys32 and Cys35 in its evolutionary conserved -Trp-Cys-Gly-Pro-Cys-Lys- catalytic center. In their reduced thiol form (*r*Trx), Cys32 and Cys35 can catalytically reduce specific protein disulfide bonds and can serve as a denitrosase toward specific S-nitrosated proteins [23-25], resulting in the formation of a disulfide bond be-

tween Cys 32 and Cys 35 (Fig. (2)). There are multiple forms of Trx encoded by different genes. Thioredoxin-1 (Trx-1), the most extensively studied, exists primarily in the cytosol, but is also found in the nucleus and blood plasma [22]. The two other isoforms are thioredoxin-2 (Trx-2) also known as a mitochondrial Trx, and SpTrx, an isoform expressed in spermatozoa [26].

In this review, we will focus on cytosolic and circulating Trx-1, which are the most important location for RSNO (as $\cdot\text{NO}$ donor) catabolism. It has been shown that Trx is able to denitrosate low molecular weight peptides (23 to 30 kDa) like GSNO or *S*-nitrosocysteine (CysNO), or high molecular weight proteins like AlbNO, *S*-nitrosometallothionein and *S*-nitrosocaspases 3 and 8 [23,27,28]. Human Trx-1 is a 12 kDa oxidoreductase enzyme containing a dithiol-disulfide active site. It is a ubiquitous protein induced by oxidative stress that is found in the cytosol and in the mitochondria of cells. Some reports also showed that Trx accumulates in the lipid raft fraction of the plasmic membrane of EC that may be involved in the adherence of leukocytes [29]. *In vitro* experiments showed that Trx is able to reduce nitrosated substrates to thiols in a variety of proteins and peptides like the transcription factor AP-1, caspase-3, metallothionein, and GSNO [27,30,31].

Haendeler *et al.* showed that Trx is constitutively *S*-nitrosated in EC at a single allosteric Cys residue (Cys69), which is located outside of the active site (which contains Cys32 and Cys35) [32]. Furthermore, *S*-nitrosation of Cys69 is the main facilitating determinant of the oxidoreductase capacity in EC. Indeed, Trx can denitrosate and thereby activate auto-*S*-nitrosated NOS, which is consistent with the finding that active Trx helps to maintain cellular SNO content. It has also been shown that auto-*S*-nitrosation of eNOS inhibits its activity [33,34].

Trx also binds and inactivates the apoptosis signal-regulating kinase-1 (ASK1). ASK1 acts as a mitogen-activated protein kinase (MAPK), which activates the Jun N-terminal kinase (JNK) and p38 MAPK pathways and is required for TNF α -induced apoptosis. The analysis of Haendeler *et al.* indicated that *S*-nitrosation of Trx at Cys69 is necessary for its basal anti-apoptotic function in EC [32]. However, it has also been reported that treatment with GSNO can induce *S*-nitrosation of Trx in intact cells and dissociate ASK1 [35], possibly by targeting active-site Cys32 and/or Cys35. Accordingly, whereas basal *S*-nitrosation of Cys69 will facilitate oxidoreductase and anti-apoptotic functions, *S*-nitrosation of active-site Cys (which might be induced by nitrosative stress) might exert an apoptotic effect. This would occur through the release of ASK1 and the inhi-

bition of oxidoreductase function (including ROS scavenging), which would result from active-site thiol modification.

Human Trx contains five Cys (Cys32, Cys35, Cys62, Cys69, and Cys73) that can be readily *S*-nitrosated both *in vitro* and *in vivo* [32,35,36,37]. There are inconsistent results pertaining to the specificity of GSNO-dependent nitrosation of Trx. Nitrosation has been reported on all three of the non-active-site Cys, Cys62 [38,39], Cys69 [32,40,41] and Cys73 [37,40,42,43,44]. More recently, Barglow *et al.* showed that the differences in Cys selectivity for nitrosation between the reduced (rTrx) and oxidized (oTrx) forms of Trx may represent a redox-dependent "switch" for signal transduction via cascading transnitrosation reactions (Fig. (3)) [45]. Because Cys73 and Cys62 are on opposite faces of the protein, it is possible that oTrx-SNO and rTrx-SNO have different binding partners and thus different downstream transnitrosation targets such as caspase-3 under oxidative stress. This would provide an alternate mechanism of signal transduction during periods of nitrosative or oxidative stress.

From all of these considerations on nitrosation of Cys belonging to the active site or the three other Cys residues, arose ideas on the distinction between transnitrosation and denitrosation activities of Trx. Indeed, it is known that oTrx can be nitrosated on Cys73 and can still act as a transnitrosating agent [37,44]. When Cys32 and Cys35 are reduced, Trx can act as a denitrosylase or as a reductase. Clear evidences have been provided that Trx can denitrosate some proteins and transnitrosate other ones. Indeed, Benhar *et al.* demonstrated that Trx is unable to denitrosate the key antioxidant enzyme peroxiredoxin 1 [24] whereas Wu *et al.* found that peroxiredoxin 1 is transnitrosated by SNO-Trx [44]. Furthermore, a recent study investigated the distinction between Trx transnitrosation and denitrosation activities and target proteins [25]. For example, both α -tubulin and GAPDH are sensitive to both SNO-Trx transnitrosation and rTrx denitrosation, while peroxiredoxin 1, peptidyl-propyl cis-trans isomerase A and α -enolase are only SNO-Trx transnitrosating targets and not Trx denitrosating targets [25].

Thioredoxin is a key oxidoreductase enzyme that has firstly been shown to be implicated in the denitrosation of *S*-nitroso-proteins. Multiple studies presently cited, have clearly demonstrated that Trx can undergo post-translational modifications through *S*-nitrosation of two Cys not included in its active site. These post-translational modifications lead to the transnitrosating activity of Trx. However, Trx is not the only oxidoreductase enzyme present in eukaryotic cells. PDI must have also its own role in $\cdot\text{NO}$ transfer.

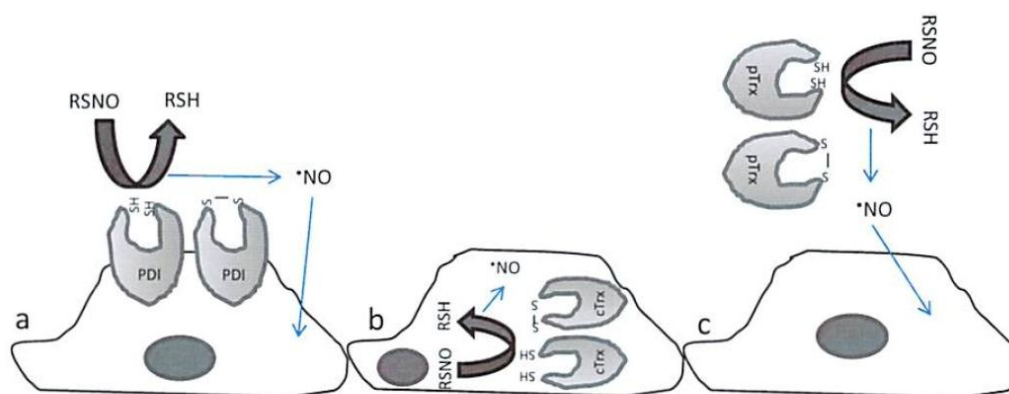


Fig. (2). The different locations of thiol disulfide oxidoreductase enzymes such as protein disulfide isomerase (PDI) and thioredoxin (Trx) implicated into the transfer of $\cdot\text{NO}$ from the blood circulation to the cytoplasm of cells. The *S*-NO bond is catabolized by the reduced Cys of thiol disulfide oxidoreductase enzymes releasing $\cdot\text{NO}$ and oxidizing the enzyme into its disulfide form. a) Protein disulfide isomerase (PDI); b) Cytoplasmic thioredoxin (cTrx); c) Plasmatic Trx (pTrx). RSNO: *S*-nitrosothiol; RSH: thiol.

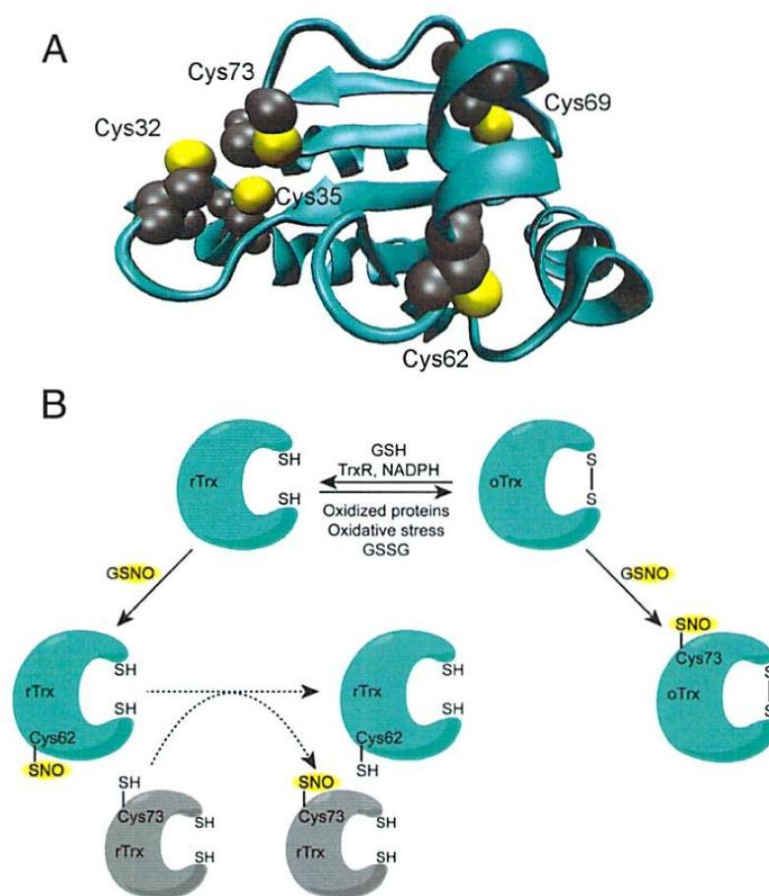


Fig. (3). Model of nitrosation selectivity for oxidized and reduced thioredoxin (oTrx and rTrx). (A) rTrx structure (based on PDB ID code 1ERT) showing the location of the 5 Cys. (B) Proposed model for interconversion and nitrosation of rTrx and oTrx. From [45]; copyright authorized by PNAS.

Protein Disulfide Isomerase

PDI was the first thiol disulfide oxidoreductase identified about 40 years ago [46]. Seventeen human PDI-family members have now been identified in the endoplasmic reticulum (ER), with a wide range of domain architectures and active-site chemistries [47]. PDI is a versatile enzyme. Indeed, depending on the redox environment and the characteristics of the protein substrates, PDI can catalyze the formation, the reduction or the isomerization of disulfide bonds. Although large levels of PDI are found in the ER, it can also be secreted from cells and associates electrostatically with cell surface, for instance in endothelial, smooth muscle cells and platelets [48-50]. This enzyme family shows little specificity among its protein substrates and it acts on a wide variety of proteins ranging from small, single-domain proteins to large oligomers with multiple domains.

An additional novel activity of PDI is its ability to denitrosate RSNO. PDI is a candidate for specific reactivity toward $\cdot\text{NO}$ on the cell surface (Fig. (2)). Indeed, some studies have shown evidence for the involvement of PDI expressed on the cell plasma membrane in the RSNO influx (e.g. $\cdot\text{NO}$ donors based on RSNO) [10,49,51,52]. Furthermore, the active site of PDI includes two Cys residues, one of which has a low pK_a (4.5) [53], allowing the result-

ing thiolate anion available for reaction with the nitrosonium ion (NO^+) or facilitating the nucleophilic attack of the S-NO bond. Finally, PDI is involved in thiol-disulfide exchange reactions, which are mechanistically similar to RSH-RSNO exchange reactions.

PDI has been shown to denitrosate GSNO [54] and it is postulated that the $\cdot\text{NO}$ released through this reaction combines with dioxygen in the hydrophobic environment of either the cell membrane or the PDI protein itself, to form N_2O_3 . In that way, PDI becomes a $\cdot\text{NO}$ carrier via N_2O_3 mediated auto-S-nitrosation of its thiol active sites.

PDI and Trx are two disulfide forming/isomerizing enzymes involved in the transfer of $\cdot\text{NO}$ from a protein to another one. However, this transfer has to satisfy nitrosation specificities in term of Cys environment.

SPECIFICITY OF S-NITROSATION, SNO SITES AND MOTIFS

The identification of SNO proteins was initially an analytical challenge. In fact, the first techniques available to detect SNO proteins were limited to mercury (Hg^{2+}) reaction coupled with photolysis/chemiluminescence or spectrophotometry. However, these techniques were unable to identify neither the SNO proteins nor the

modified Cys within the proteins. The development of the biotin switch technique by Jaffrey *et al.* made the identification of SNO-proteins possible [55]. Furthermore, the biotin switch technique enables the identification of the modified Cys within the protein when coupled to mass spectrometry.

Among the multiple Cys residues in a protein, only specific ones are modified and are responsible for altering protein functions [56-58].

It has been proposed that nitrosable Cys residues are flanked within 6 Å by basic (Lys, Arg, His) and acidic (Asp, Glu) residues. These residues could regulate S-nitrosation and denitrosation by altering thiol nucleophilicity [59]. Recently, Greco *et al.* identified specific proteins and motifs of S-nitrosation in human vascular smooth muscle cells [60]. In smooth muscle cells incubated with Cys-NO, a ^{*}NO donor, up to 18 S-nitrosocysteine-containing peptides belonging to 16 proteins such as cytoskeletal proteins (myosin heavy chain 9, vinculin), chaperones, and calcium binding proteins were identified. Sequence alignment of these 18 S-nitrosocysteine-containing peptides revealed a higher occurrence (50% and 40%) of acidic residues (Asp and Glu, respectively) at positions -3 and -4 relative to the Cys residue. The highest occurrence of basic (Lys, Arg, His) residues was 30% at position 2. There was an occurrence of 10% for acidic residues at position 2 and no occurrence for basic residues at positions 3 and 4. Although primary sequences analyses are useful to determine structural features for specific posttranslational modifications, they do not reveal motifs that result from three dimensional protein structures. *In silico* analyses based on 55 crystalized proteins that have been shown to be nitrosated by ^{*}NO donors were recently performed by Marino *et al.* [61]. They tested first parameters well-known to play a role in post-translational Cys modifications such as pK_a, and the residue exposure. They found that the pK_a values of the Cys residues targeted for S-nitrosation are higher (9.1) than those of redox and non-redox catalytic Cys (5.5). Furthermore, 65% of the Cys-NO sites were exposed. S-nitrosation seems to be favored by hydrophobic regions around the target Cys increasing thiol nucleophilicity [62] and concentrating nitrosating agents like albumin in the plasma [63]. Indeed, Rafikova *et al.* showed that in the presence of a physiological concentration of ^{*}NO, albumin becomes saturated with ^{*}NO and accelerates the formation of low-molecular-weight RSNO, *in vitro* and *in vivo*. This mechanism is due to micellar catalysis of ^{*}NO oxidation in the hydrophobic core of albumin. The hydrophobic region within the modified Cys seems also to be of great importance since Sun *et al.*, showed that the ryanodine receptor was nitrosated on only one Cys (cys3635) out of about 50 [62] and located in a hydrophobic pocket.

RELATIONSHIP BETWEEN THIOREDOXIN AND NITRIC OXIDE DONORS IN CARDIOVASCULAR DISEASES

Cardiovascular diseases like hypertension, atherosclerosis, thrombosis and cerebrovascular diseases are often associated with oxidative stress. Under oxidative stress (for example exposure of cells to sublethal amounts of hydrogen peroxide) [64], Trx-1 is released from cells, and it appears as a good marker of oxidative stress in plasma [65]: 15-30 ng/mL for healthy volunteers, rising to 40-140 ng/mL in patients suffering from diseases related to oxidative stress [66]. Trx-1 has a protective role in different cardiovascular diseases [67]. Indeed, data indicated that serum Trx-1 levels were found to be significantly increased in patients with acute coronary syndromes and dilated cardiomyopathy [68].

As mentioned in the section concerning Trx, this enzyme has been shown to be S-nitrosated at Cys-69 in EC [32]. Interestingly, Tao *et al.* documented that the intraperitoneal administration of S-nitrosated human Trx in a mouse myocardial ischemia model after 30 min of ischemia and 10 min prior to reperfusion, potentiated the cardioprotective effects of human Trx [36]. These results suggested a possible association between Trx-1 secretion and cardiovascular diseases. Literature data on interactions between RSNO and Trx, as

stated in the section concerning Trx, have focused on GSNO, which is the most abundant low molecular weight RSNO inside the cells [27]. In contrast, data on denitrosation of CysNO, S-nitrosohomocysteine (HCysSNO), and SNAP by Trx are rather limited. The V_{max} values for CysSNO, HCysSNO, and SNAP were 1.2, 0.3, and 0.1 nmol of substrate (nmol of Trx)⁻¹ min⁻¹, respectively [23].

Reduced ^{*}NO bioavailability, due to either endothelial dysfunction or eNOS deficiency, seems to be involved into the onset and the development of pathologies cited above [69]. Due to its short half-life (few seconds), ^{*}NO is not administrated *per se* (as a gas) to induce prolonged effect but as ^{*}NO donor such as sodium nitroprusside or organic nitrates. The indications, which are validated worldwide by the Health Regulatory Authorities, concern angina pectoris, chronic cardiac failure and hypertension in emergency cases. Although, a wide panel of beneficial pharmacological properties is attributed to this radical [70] and a reduced level of ^{*}NO has been implicated into the onset and the progression of various diseases, the therapeutical indications of ^{*}NO donors still remain limited. Moreover, RSNO cerebrovascular beneficial effects have already been described. One of the RSNO, i.e. GSNO, was studied in rat under various diseases such as subarachnoid hemorrhage [71], traumatic brain injury [72], and cerebral ischemia [73]. The reported beneficial effects were as follows: increase of cerebral blood flow and improvement of blood perfusion as a consequence of capillaries relaxation; decrease of inflammatory environment; protection *versus* traumatic penumbra in the specific case of the traumatic brain injury; improvement of the overall structure of the tissue with the conservation of the blood brain barrier and the reduction of the post-accident neurological deficit. From a mechanistic point of view, ^{*}NO provided by GSNO was deemed to suppress iNOS induction but also to enhance at the same time eNOS expression, thus explaining the therapeutic effect of the molecule [74].

The therapeutic use of RSNO is relevant based on their unique capacity to deliver ^{*}NO and beneficial effects, particularly in the cerebrovascular system. At this time, only GSNO and NACNO have been involved in several clinical trials (Table 1) demonstrating therapeutic interest. Nevertheless, GSNO is quite unstable in the bloodstream (50 min) [75], one of the reasons being its specific breakdown occurring through catalytic reaction with γ-glutamyl-transpeptidase (GGT) [76]. GGT is a central enzyme involved in the degradation of GSH through its specificity for the γ-Glu amino acid [77]. Furthermore, the structure of RSNO has to be modulated to obtain a greater stabilisation and/or protection *versus* environmental attacks through catalytic action of Trx and PDI, to increase the duration of ^{*}NO delivery in the bloodstream. Thus the potential applications of RSNO require appropriate chemical modification and/or formulation.

RATIONAL DESIGN OF S-NITROSOTHIOLS FOR A SUSTAINED RELEASE

Due to the lability of ^{*}NO and its limited diffusion from 40 to 200 μm [92], this drug cannot be administered *per se* to reach constant systemic effects, thus it has to be carried by a prodrug. Various kinds of carriers have been designed for this purpose; some of them were based on molecular concepts. Indeed, to delay the interaction with environmental factors as well as proteins such as disulfide forming/isomerizing enzymes, the moiety wearing -NO has to confer a high stability to the final molecule. Moreover, macromolecular as well as macroassembly approaches and lastly drug delivery systems were designed in order to increase either the grafting of -NO moieties *per* molecule or the time of drug release (ensuring a good protection of -NO) or both. As it will be later explained, this can guarantee a good protection of the sensitive -NO part. Besides, the biological effect of the drug is also strongly related to the delivered concentration therefore, its sustained release at the targeted site remains a crucial challenge.

Table 1. Main characteristics of clinical trials based on administration of *S*-nitrosoglutathione (GSNO) and *S*-nitroso-*N*-acetylcysteine (NACNO) reported in the literature. In all these studies, a research ethics committee was consulted

Main aim of the study Targeted application	Main conclusion	Number of included patients	References
Reduction of cerebral embolization			
Carotid angioplasty carotid endarterectomy and/or recurrent stroke and/or systemic embolization	Highly significant reductions in the number of Doppler cerebral embolic signals in GSNO groups	42	[78]
	GSNO resulted in a rapid reduction in the frequency of cerebral embolic signals of 95% at 0 to 3 h and 100% at 6 h	16	[79]
		20	[80]
	Significant reduction of cerebral embolic signals in the GSNO group	24	[81]
Inhibition of platelet activation and/or vasodilatation and/or effect on blood flow			
Percutaneous transluminal coronary angioplasty	Significant inhibition of the increase in platelet surface expression of P-selectin and glycoprotein IIb/IIIa without altering blood pressure	13	[82]
Anti-thrombotic effect	GSNO is a relevant drug for anti-thrombotic effect	10	[83]
Coronary artery saphenous vein grafts	GSNO significantly inhibited platelets activation	24	[84]
Severe Preeclampsia	Reduction of maternal mean arterial pressure, platelet activation and uterine artery resistance without further compromising foetal Doppler indices.	10	[85]
Systemic vasodilatation	Intravenous application of GSNO has similar hemodynamic effect as compared to inhaled ¹⁴ NO	10	[86]
Dermal wound dressings and local vasodilation in ischemic tissues	Release of GSNO from films induced local vasodilatation relevant for dermal wound dressings or for promoting local vasodilation in ischemic tissues	7	[87]
Wound healing	Increase of blood flow parallel with ¹⁴ NO delivery after application of GSNO or NACNO hydrogel skin application	4	[88]
Female sexual disorder	Increase of clitoral blood flow	40	[89]
Replenishment of GSNO pool			
Effect on left ventricular function	Positive effects on left ventricular function	12	[90]
Effect on cystic fibrosis	Modest improvement of general oxygenation	20	[91]

Molecular ¹⁴NO Carriers: *S*-nitrosothiols

The ¹⁴NO donors class can be divided into organic nitrates (R-O-NO₂), for instance glyceryl trinitrate (GTN), nitrites (R-O-NO) and *X*-nitroso- derivatives (R-*X*-NO). Moreover, research is cur-

rently focusing on hybrid or dual molecules able to deliver ¹⁴NO associated with various drugs (for example, in the non-steroidal anti-inflammatory drug class, both aspirin modified as an organic nitrate derivative, and diclofenac bearing an *S*-nitroso-moiety were studied; the antihypertensive drug captopril was modified on its

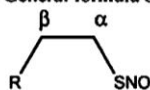
RSH group with the grafting of NO) [93,94]. The strategy developed in these cases will not be detailed here.

Among the *X*-nitroso-compounds, -NO can be linked either to metal ligands (named nitrosyl derivative such as sodium nitroprusside) or to carbon, nitrogen and sulfur atoms. As compared to classical *NO donors, RSNO induce neither oxidative stress nor tolerance phenomenon [95]. Moreover, some RSNO are physiological *i.e.* CysNO, GSNO, and AlbNO and they are considered as an important means of *NO storage in the organism [96]. Due to RSNO unique properties that can be associated to a minimal toxicity, they are nowadays extensively studied. Different clinical trials were also led with GSNO and NACNO as already mentioned (Table 1). Therefore, in this review, only the RSNO family will be described, taking into account the main parameters to stabilise the sulfur-nitrogen bond against environmental attacks (for the other *NO donors, see [97]).

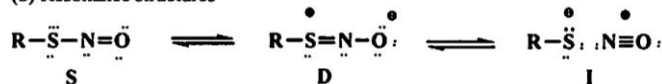
S-nitrosothiols are sensitive to the environment conditions, which may be explained by the weak sulfur-nitrogen bond characterised by a homolytic dissociation energy value of 126 kJ/mol. As a consequence, degradation occurs following a treatment by external factors such as light, temperature and internal factors including cationic ions (Cu^{2+} *via* Cu^+), other sulfur containing compounds such as the gaseous messenger H_2S , ascorbate, and proteins like disulfide forming/isomerizing enzymes. These stress conditions are frequently used to study the stability of newly synthesized products [98]. In order to confer a great stability to the sulfur-nitrogen bond, four main parameters have to be highlighted. Three of them are deeply linked to the three resonance structures described for RSNO (Fig. (4)) [99].

The dominant form S (70 to 80 %) is the usual structure characterised by a single bond between sulfur and nitrogen. In the D form (15 to 25 %) the S-N bond is strengthened since a double bond is

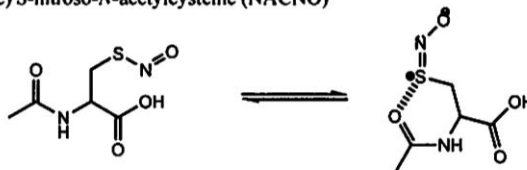
(a) General formula of *S*-nitrosothiol (RSNO)



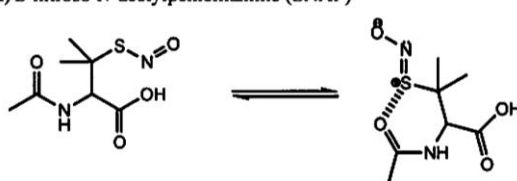
(b) Resonance structures



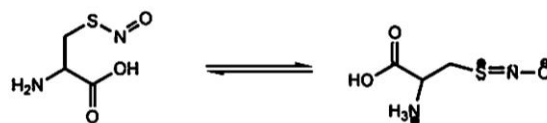
(c) *S*-nitroso-*N*-acetylcysteine (NACNO)



(d) *S*-nitroso-*N*-acetylpenicillamine (SNAP)



(e) *S*-nitrosocysteine (CysNO)



(f) *S*-nitrosogluthathione (GSNO)

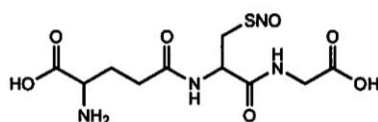


Fig. (4). Structures of low molecular weight *S*-nitrosothiols.

created between these atoms. In contrast, in the ionic form I (5 to 10 %) the S-N bond is weak. As a result, each modification that favors the D structure will increase the stability and decrease the homolytic cleavage probability. This stable form can be generated by (i) the well-controlled synthesis of S-nitrosocompounds, (ii) the chemical substitution of carbon atoms located either at the alpha position of -SNO group or at its beta position. The last parameter that may induce a better stability versus metallic cation attack relies upon the increase of the molecule lipophilicity or its steric hindrance.

A summary of the two physico-chemical as well as vasorelaxant properties of molecules that will be exposed below is proposed in Fig. (5).

Measuring vasorelaxation of isolated and precontracted rat aortic ring [110,111] is now recognized as the most frequently used *ex vivo* model to test efficiency of \bullet NO donors. The high standardisation level of the assay gives rise to less and less variations between research teams, facilitating data comparison. Another *in vitro* cellular model used to evaluate efficiency of \bullet NO donors consists in the measurement of cGMP produced by an enzymatic reaction catalysed by sGC; however, most of cultured cell lines derived from muscular cells express low levels and/or inactive sGC to set the assay in a reliable way [112].

Several chemical procedures have been usually described to synthesize RSNO. In aqueous medium, nitrosation of a thiol is

usually provoked using sodium nitrite in acidic medium, by mixing \bullet NO/O₂ gas or transnitrosation with another RSNO. Nevertheless, in non-stoichiometric conditions between nitrating reagent and thiol, S-nitroderivatives, RSNO₂, can be produced [113,114]. Such products show the same UV-visible spectrum; as a consequence, another methodology have to be operated (e.g. HPLC [113,114]) to facilitate their identification. But this reaction is described not to favor the stability of the product because the D structure formation does not tend to occur. Indeed, Balazy *et al.* estimated the half-life of GSNO₂ (generated with a five-fold excess of nitrating agent) at ca. 30 min in the absence of other destabilising stimuli [113]. The same result was observed in our laboratory not only on the cited product but also after the formation of S-nitro-N-acetylcysteine NACNO₂ (generated by the same excess of nitrite reagent) and was also characterised by a half-life of less than 30 min. Therefore, the operating conditions to obtain RSNO have to be well-defined.

The two carbon atoms located at the alpha and beta positions of the -SNO group (Fig. (4)) are of major importance to design stable RSNO. Firstly, regarding the alpha position, the -SNO stabilisation was reported to be increased with the substitution degree of this carbon atom [14,15]. Nevertheless, the theory was not in accordance with all experimentations, since NACNO (rate of \bullet NO release = 0.025 nmol.mL⁻¹.min⁻¹ in phosphate buffer) was described as more stable than its tertiary substituted derivative, SNAP (0.046 nmol.mL⁻¹.min⁻¹ in phosphate buffer pH 7.4) (Fig. (4) and Fig. (5)) [108]. Recent computational work on these molecules (undergoing

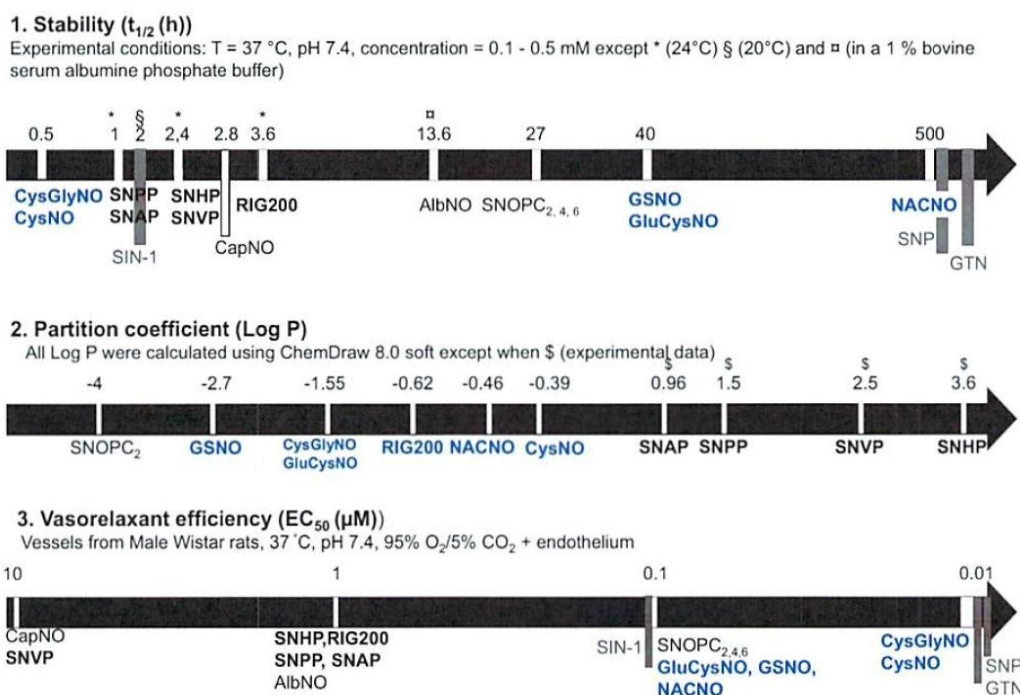


Fig. (5). Classification of main physico-chemical and physiological properties of various \bullet NO donors according to literature data [100-109]: *in vitro* stability, partition coefficient (log P) and vasorelaxant efficiency (corresponding to the half maximal effective concentration EC₅₀ obtained in vasorelaxation measurement of isolated and precontracted rat aortic ring). AlbNO: S-nitrosoalbumin, CapNO: S-nitrosocaptopril, CysNO: S-nitrosocysteine, CysGlyNO: S-nitrosocysteinylglycine, GluCysNO: S-nitroso- γ -glutamylcysteine, GSNO: S-nitrosogluthathione, GTN: Glyceryl trinitrate, NACNO: S-nitroso-N-acetylcysteine, RIG200: N-(S-nitroso-N-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-O-acetyl- β -D-glucopyranose, SIN-1: Linsidomine, SNAP: S-nitroso-N-acetylpenicillamine, SNHP: S-nitroso-N-heptanoyl-D,L-penicillamine, SNOPC₂: S-nitrosophytochelatin-2, SNOPC₄: S-nitrosophytochelatin-4, SNOPC₆: S-nitrosophytochelatin-6, SNP: Sodium nitroprusside, SNPP: S-nitroso-N-propanoyl-D,L-penicillamine, SNVP: S-nitroso-N-valeryl-D,L-penicillamine.

hydrolysis in acidic medium) [114] proved that the sulfur protonation of SNAP is favored by the presence of the two methyl groups and as a consequence, the equilibrium of the resonance is modified towards the I (51 %) and S (49 %) forms which may ease the nucleophilic attack of -NO moiety [115].

Secondly, as far as the beta position is concerned, the role of this carbon atom as a stabiliser was highlighted when comparing *S*-nitrosocysteine (CysNO) and NACNO (half-lives of 0.54 h and > 500 h in phosphate buffer, respectively [107]) (Fig. (4) and Fig. (5)). The substitution by a quaternary ammonium functional group, as seen in CysNO, dramatically decreases the stability of the S-NO bond. On the contrary, the presence of a hydroxyl or an acetamido group such as in the second molecule has the opposite effect. Indeed, in the case of CysNO, the positively charged ammonium group leads to an electrostatic repulsion with the sulfur of the S-NO bond. On the opposite, the acetamido substituent of NACNO has a stabilizing effect by the formation of a 6-membered cycle between the carbonyl function of the acetamido and the sulfur of S-NO bond which lacks electrons (Fig. (4)). As a conclusion, the proportion of the D resonance form is highly increased in the case of NACNO and decreased for CysNO [14]. This also gives an explanation to the high stability of GSNO (Fig. (4) and Fig. (5)) versus its active metabolite *S*-nitrosocysteinylglycine (CysGlyNO) (half-lives of 41 h and 0.5 h in phosphate buffer respectively [107]) after GGT activity or *S*-nitroso- γ -glutamylcysteine (GluCysNO) (half-life of 42 h in phosphate buffer [107]).

Lastly, other chemical modifications of RSNO were directed in order to increase the drug lipophilicity or steric hindrance to improve the drug stability versus metallic ion attack. Two main strategies were undertaken, both being performed on SNAP as the core. On the one hand, a chemical modification was performed with the grafting of a tetra-*O*-acetylglucosamine moiety on the carboxylic function to obtain *N*-(*S*-nitroso-*N*-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl- β -*D*-glucopyranose (RIG200) to modify steric hindrance [104]. On the other hand, a carbon chain was added to the amido group of the molecule: with either a C1 to C4 via a grafted glycine upstream [116] or directly a C3 (*S*-nitroso-*N*-propionylpenicillamine, SNPP), a C5 (*S*-nitroso-*N*-valerylpenicillamine, SNVP) to C7 (*S*-nitroso-*N*-heptanoylpenicillamine, SNHP) [106,117]. The last cited modification led to the establishment of a correlation between measured partition coefficient (log P) and the carbon chain length, from log P = 1.5 for the C3 to 3.5 for C7 versus nearly 1 for SNAP, which is much higher than GSNO (calculated log P = -2.7) or NACNO (calculated log P = -0.46) (Fig. (5)). In each study, the new derivatives were more stable than the mother molecule versus catalytic decomposition by copper ions. Although, the efficient concentration 50 (EC₅₀) was not improved (Fig. (5)), a sustained release of *NO was demonstrated (> 1 h for SNHP compound [106] and > 4 h for the tetra-*O*-acetylglucosamine derivative, RIG200 [104] in presence of vessels). A linear correlation was observed between the lipophilic character of the compound (C3 to C7 RSNO) and the sustained vasorelaxation [106]. As a result, the mechanism of sustained vasodilation was hypothesised to be correlated to retention of the lipophilic RSNO derivatives into the lipid rich domains of the sub-endothelial layers of arteries. Indeed, the effect was obvious into endothelium-denuded vessels. The authors concluded to a selectivity of the compounds towards injured vessels [106].

According to these results, the physico-chemical data of *in vitro* stability and partition coefficient and the vasorelaxant efficiency are difficult to perfectly correlate, and therefore from Fig. (5), no general rule can be formulated. As a consequence, the design of the ideal RSNO appears as a real challenge. Although SNAP and its derivatives showed a delayed degradation through metallic ion attack but a vasorelaxant efficiency lower than GSNO, the sustained release of *NO appears interesting from a therapeutical point of view. Nevertheless, the development of *N*-acetylpenicillamine

derivatives must include some immunological investigations since penicillamine is known to induce autoimmunity [118]. Moreover, the molecules based on a Cys core present a better vasorelaxant effect and for some of them a satisfying stability (GSNO and NACNO) nonetheless, a sustained delivery of *NO has never been described from these derivatives to the best of our knowledge.

Macromolecular Concepts and Macroassemblies for *NO Transport

The design of macromolecular *NO donors is based not only on polypeptides, or proteins, or polymeric structures but also on mixed structures.

Taking the previous chemical data into account and because GluCysNO has a greater stability (half-life of 42 h in phosphate buffer), *S*-nitroso derivatives of GSH were synthesized [107]. In this way, phytochelatins, (γ -glutamylcysteinyl)_n-glycine (n = 2 to 6 for SNOPC_{2, 4, 6}), were nitrosated and characterised [98,109]. The modified-glutathione derivatives showed a *NO release with a half-life of ca. 27 h under *in vitro* conditions. Their efficiency to induce a vasorelaxant effect was in relationship with the number of grafted -NO moiety on the molecule with EC₅₀ of 0.14, 0.08 and 0.06 μ M for SNOPC_{2, 4, 6}, respectively (Fig. (5)). These oligopeptides did not manage to produce a sustained release of the drug and the authors concluded to combine *S*-nitrosophytochelatins to drug delivery systems.

Among *S*-nitrosoproteins, the most studied ones remain AlbNO and its derivatives. As previously mentioned, AlbNO is the main nitrosated protein encountered in the blood [5]. Several procedures are described to obtain the modified protein. Different approaches are usually envisaged: either the simple nitrosation of albumin introducing one -NO moiety on the protein (involving the Cys34) [104,111], or more complex chemical modifications that aim at inserting more -NO groups on albumin via S-NO as well as N-NO bond formation (using an excess of nitrating agent) [119] or with the grafting of chemical groups to anchor more thiols into the protein structure that will then be nitrosated (i.e. Poly-SNO-BSA) [120,121]. Regarding the pharmacokinetic parameters, the half-lives of the nitrosated proteins were improved by the binding of a polyethylene glycol (PEG) group: 147 h for PEG- AlbNO versus 13.6 h for AlbNO in a buffer containing 1 % bovine serum [120] as compared to 5.5 h in human blood under *in vitro* conditions [104]. The chemical modification of albumin with the introduction of more than one -NO group leads to the highest content of -NO (up to 10 mol -NO/mol albumin [120]). Nevertheless, it also deeply modifies the physico-chemistry of albumin [119], its interaction with cells [121] and finally may induce some immunological properties conferred by the nitrosated as well as the denitrosated macromolecule [122].

Dendrimers which are tree-like polymers were used as a strategy to increase the loading of -NO onto a macromolecule. Thus, generation-4 polyamidoamine dendrimers were modified with RSNO, synthesized and characterised [123,124]. Sixty four units of either SNAP or NACNO were grafted onto the dendrimer skeleton. The *in vitro* half-lives in presence of copper (0.2 mM) of the as-synthesized polymers were evaluated at 2.3 min and 106 min for the derivatives of SNAP and NACNO in phosphate buffer, respectively [123]. Using the same concentrations, the dendrimer derivatives of SNAP showed a more pronounced efficiency to inhibit platelet aggregation compared to the unmodified RSNO. As a result, the authors concluded that these dendrimers appeared as a relevant strategy in ischemia-reperfusion injury.

A polypeptide/polymeric mixed macroassembly was also designed in order to control *NO release. Either GSNO or SNOPC was covalently linked to a polymer (poly(vinyl methyl ether-co-maleic anhydride)) and then interpolymer complexes were created with a poly(vinyl pyrrolidone) thanks to hydrogen bonds between both polymers [125]. As concerns GSNO, a 50 % grafting yield was

reached. Moreover, a controlled release of ^{*}NO was effective until day 10 under *in vitro* conditions for both RSNO. The complexes were applied to wound healing with interesting positive results.

Thus, the macromolecular structures cited here improved the loading of –NO and some of them induced a sustained release.

Innovative Drug Delivery Systems Applied to S-nitrosothiols

Various concepts were designed in order to enhance the RSNO stability towards the environment and also to ensure a sustained release of the drug. Studies describing RSNO delivery systems are summarised in (Table 2) and they are based on approaches related to particles, matrix or films or both.

S-nitrosothiol delivery systems manage to protect the molecule and to control the release of ^{*}NO. A complete proof-of-concept with an increase of the vascular flow was provided [133]. ^{*}NO delivery systems (including RSNO delivery systems) have already been extensively reviewed [92,134,135]. Nevertheless, some remarks have to be taken into consideration for further developments:

- The safety and biocompatibility of the formulations have to be fully demonstrated. This includes:
 - the stability of the drug delivery system tested in physiological conditions (temperature, ionic strength, pH) in order not to release some impurities;
 - a total absence of toxicity: since most of the delivery systems are characterised by a release half-life in the range of hours even days to develop a chronic treatment (for example 7 days of release half-life in *in vitro* conditions *i.e.* phosphate buffer [136]);
 - no activation of the immune system with respect of antigen presenting cell phenotype after administration of the formulations, not to induce an inflammatory phenomenon [137].
- Concerning the particle design with a vascular target aim, following points have to be considered:

Table 2. S-nitrosothiol Delivery Systems Formulated as Particles, Matrix or Film, or Both

Drug delivery system	S-nitrosothiol	Physicochemical characterisation	Main conclusions	References
Particle-based systems				
Particles prepared by ionic gelation with alginate/chitosan	GSNO	Diameter > 300 nm	Decrease of GSNO decomposition rate when entrapped into NP especially in the first hours.	[126]
Liposomes	NACNO associated with a photosensitizer	Liposomes (made of dipalmitoyl-phosphatidylcholine) filtered through a 0.45 μm membrane	Photodynamic therapy is possible using such particles.	[127]
Mercaptosilane-based silica	Nitrosation of the mercapto-moieties after NP preparation	Diameters tunable from 240 to 720 nm -SH content from 0.5 to 20 % wt/wt and 55 to 100 % nitrosation efficiency	[*] NO release from the NP exceeded 48 h.	[128]
Particles entrapped into matrices				
S-nitrosothiol-derivatized fumed silica polymer filler particles	CysNO, SNAP, NACNO	Diameters from 7 to 10 nm Total -NO loading from 21 to 138 nmol/mg NP	SNAP derived NP showed the highest –NO loading. Polymeric films containing the NP were prepared and released [*] NO upon photoexcitation	[129]
Liposomes entrapped into a porous silica matrix	SNAP	Liposomes (made of dimyristoyl- <i>sn</i> -glycero-3-phosphocholine, cholesterol and dihexadecyl hydrogen sulphate) diameters of 150 nm Loading equivalent to 35 nM into the final material	Release of [*] NO is controlled by photoexcitation with a rate comparable to a biological active one.	[130]
Matrix and films				
Polyethylene glycol matrix	NACNO	-	Stability enhanced into the PEG matrix. Storage possible for 10 weeks at -20°C	[131]
Polymeric films	GSNO	20.4 wt % into a film made of polyvinyl alcohol and polyvinyl pyrrolidone	Release of [*] NO and GSNO from the films with a half-life of 35.5 min.	[132]
Polymeric films	GSNO	5 to 30 % of GSNO with a loading efficiency of 30 to 70 %	Increase of vaginal blood flow during 210 min with the loaded films compared to 20 min for unformulated GSNO	[133]

- to improve the blood circulation half-life, addition of PEG onto the particle to ensure its stealthiness from the mononuclear phagocytic system may be required [138];
- the size of particle is one major point since particles with a diameter/dimension in the range 100 to 200 nm were shown to be able to accumulate into the inner layers of the arteries [139].

Development of a drug delivery system dedicated to the transport and protection of a ^{*}NO donor is one way to avoid the interaction with degrading factors such as proteins. It was already mentioned that an increase of Trx concentration in the blood can be associated to diseases linked with oxidative stress such as cardiovascular pathologies. In these cases, in which a ^{*}NO donor can be a relevant therapeutic strategy, a particulate system that on the one hand limits Trx ^{*}NO release and on the other hand allows a controlled delivery at the targeted site should be interesting. Moreover, it is noteworthy that drug delivery systems can be internalized by cells. Regarding particles, two major kinds of cells have to be distinguished: antigen presenting cells which are professional phagocytes (since they are the first line of defence and have to screen the entire organism to fight the non-self) and the others for which the phagocytic activity is not the main one. Three main pathways of internalisation are nowadays well described: phagocytosis, endocytosis (including clathrin-mediated endocytosis, caveolae-mediated endocytosis, clathrin and caveolae independent endocytosis and macropinocytosis) [140] and the fusion with cell membrane. The last one is observed in the case of some liposomes or some vesicles [141], and it allows the release of the particle content directly into the cytosol, whereas the other processes may confine the particle inside an organelle (for example endolysosomes). Therefore, using the appropriate drug delivery system, the interaction with proteins can be limited (for example circulating Trx and membrane PDI) to deliver ^{*}NO and/or to nitrosate protein inside the cell. In summary, the assembly of a stable RSNO and a carrier appears the best way to deliver ^{*}NO under a controlled pathway.

GENERAL CONCLUSION

The present review points out the emerging role of RSNO as a new therapeutic class fighting vascular dysfunctions and permitting to overcome drawbacks of actual ^{*}NO donors. This is demonstrated by:

- the actual intense investigation on RSNO interaction with proteins leading to *S*-nitrosation, a translational pathway of main importance in cell signalling leading to physiological action such as vasorelaxation,
- and also by many clinical trials based on administration of GSNO, a physiological RSNO.

As GSNO bioavailability has to be improved, it is necessary to define which parameters should be taken in consideration in order to increase the half-life of new designed RSNO. Structural properties of the molecular skeleton bearing the pharmacophore group SNO as well as the molecule lipophilicity appear interesting to improve their stability and to facilitate their (nano)formulation, respectively, even if no exact relationship between these physico-chemical properties and *in vivo* efficiency has been established at this time. Another real perspective in RSNO design is also to consider their interaction with enzymatically active proteins such as PDI//Trx presently reviewed.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

AlbNO	=	<i>S</i> -nitrosoalbumin
ASK1	=	Apoptosis-regulating kinase-1
CapNO	=	<i>S</i> -nitrosocaptopril
cGMP	=	Cyclic guanosine monophosphate
Cys	=	Cysteine
CysGlyNO	=	<i>S</i> -nitrosocysteinylglycine
CysNO	=	<i>S</i> -nitrosocysteine
EC	=	Endothelial cells
ER	=	Endoplasmic reticulum
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
sGC	=	Soluble guanylate cyclase
GGT	=	γ -glutamyltranspeptidase
GluCysNO	=	<i>S</i> -nitroso- γ -glutamylcysteine
GSH	=	Reduced glutathione
GSNO	=	<i>S</i> -nitrosoglutathione
GTN	=	Glyceryl trinitrate
HcysNO	=	<i>S</i> -nitrosohomocysteine
JNK	=	Jun N-terminal kinase
Lys	=	Lysine
MAPK	=	Mitogen-activated protein kinase
NP	=	Nanoparticles
NACNO	=	<i>S</i> -nitroso- <i>N</i> -acetylcysteine
NADPH	=	Nicotinamide adenine dinucleotide phosphate
NOS	=	Nitric oxide synthases
eNOS	=	Endothelial nitric oxide synthase
iNOS	=	Inducible nitric oxide synthase
nNOS	=	Neuronal nitric oxide synthase
NP	=	Nanoparticles
PDI	=	Protein disulfide isomerase
PEG	=	Polyethyleneglycol
Pro	=	Proline
RIG200	=	<i>N</i> -(<i>S</i> -nitroso- <i>N</i> -acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra- <i>O</i> -acetyl- β - <i>D</i> -glucopyranose
RSH	=	Thiol
RSNO	=	<i>S</i> -nitrosothiol
SIN-1	=	Linsidomine
SNAP	=	<i>S</i> -nitroso- <i>N</i> -acetylpenicillamine
SNHP	=	<i>S</i> -nitroso- <i>N</i> -heptanoyl- <i>D,L</i> -penicillamine
SNOPC ₂	=	<i>S</i> -nitrosophytochelatine-2
SNOPC ₄	=	<i>S</i> -nitrosophytochelatine-4
SNOPC ₆	=	<i>S</i> -nitrosophytochelatine-6
SNP	=	Sodium nitroprusside
SNPP	=	<i>S</i> -nitroso- <i>N</i> -propanoyl- <i>D,L</i> -penicillamine
SNVP	=	<i>S</i> -nitroso- <i>N</i> -valeryl- <i>D,L</i> -penicillamine
TNF α	=	Tumor necrosis factor alpha
Trp	=	Tryptophan

Trx = Thioredoxin
 oTrx = Oxidized thioredoxin
 rTrx = Reduced thioredoxin

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2.3. Conclusion : le choix des *S*-nitrosothiols comme donneurs de NO

Près de 1000 protéines *S*-nitrosables ont été identifiées à l'heure actuelle et ce processus revêt une grande importance physiologique [50]. Il est finement régulé, d'une part car parmi les multiples résidus cystéines disponibles au sein d'une protéine, seulement certains sont modifiés ; et d'autre part car il existe en parallèle des moyens de déplacer ces modifications (transnitrosation) ou de les annuler (dénitrosation).

Les *S*-nitrosothiols de faible masse molaire comme le *S*-nitrosoglutathion sont en équilibre avec les *S*-nitrosothiols protéiques et constituent le réservoir de NO de l'organisme. Grâce à leur existence physiologique, le risque de toxicité après apport exogène de ces composés est faible. De plus, ils ne provoquent pas de phénomène d'échappement thérapeutique contrairement aux dérivés nitrés (Figure 7).

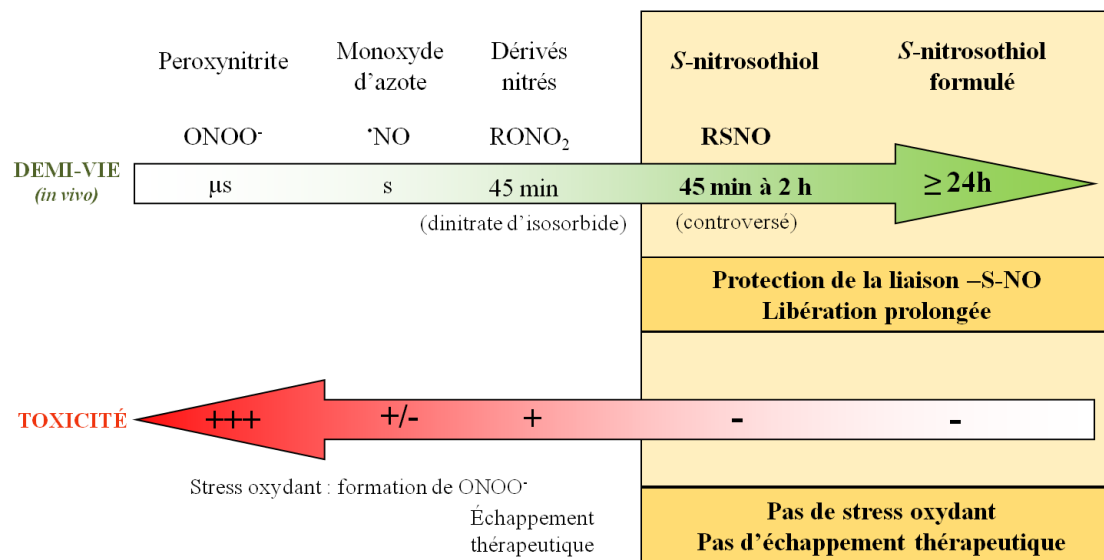


Figure 7 : Rationnel du choix des *S*-nitrosothiols comme donneurs de NO en comparaison aux dérivés nitrés.

Les RSNO représentent donc un choix logique pour le développement de nouveaux donneurs de NO. Il est possible d'obtenir ces composés endogènes par synthèse chimique, mais aussi de modifier leur structure ou de créer de nouveaux composés. Le potentiel thérapeutique est énorme, en particulier dans le domaine cardiovasculaire.

Le RSNO « idéal » devrait posséder les caractéristiques suivantes :

- Disponible en quantité suffisante et peu coûteux et/ou facile à synthétiser et à isoler (facilité de manipulation et de stockage) avec une haute pureté (et un profil d'impuretés bien caractérisé) ;
- Plutôt hydrophobe (augmentation de la pénétration à l'intérieur des cellules et également plus grande facilité de formulation) ;
- Demi-vie suffisante pour l'application thérapeutique visée ;
- Toxicité faible ou nulle ;
- Effet biologique important.

Par conséquent, la conception rationnelle de la structure chimique de ces composés doit intégrer de nombreux objectifs parmi lesquels l'amélioration de la stabilité de la liaison S-N. Un des sous-objectifs du projet NanoSNO devait permettre de synthétiser de nouvelles molécules répondant au cahier des charges établi, en particulier en termes de stabilité et d'hydrophobie. Cependant, seules deux molécules ont pu être isolées et purifiées à ce jour : *S*-nitrosoglutathion et *S*-nitroso-*N*-acétylpénicillamine.

Cette approche est complémentaire de la recherche d'une forme galénique adaptée. Celle-ci doit permettre de protéger les RSNO de l'action des systèmes enzymatiques de transnitrosation/dénitrosation tout en assurant leur libération contrôlée, de façon à mimer la libération physiologique de NO.

3. FORMULATION DES S-NITROSOTHIOLS

3.1. Introduction

Un des objectifs de la formulation galénique repose sur l'optimisation de la disponibilité du principe actif, pour assurer la meilleure efficacité à la dose la plus faible, favoriser l'observance du patient en permettant de diminuer le nombre de prises et améliorer la tolérance.

Dans la section précédente, l'Article 2 a présenté les différentes approches galéniques ayant été testées pour la formulation de RSNO : polymères de RSNO, formulations polymériques dont celles sous forme de matrices. Trois voies d'administration sont principalement concernées : voie inhalée, voie topique et voie parentérale. Dans la majorité des cas, l'application visée est dans le domaine cardiovasculaire : diminution de la pression artérielle, inhibition de l'activation et de l'agrégation des plaquettes, diminution de la prolifération néo-intimale, augmentation du flux sanguin local. La voie parentérale peut alors être privilégiée pour l'administration de formulations à libération plus ou moins prolongée de NO.

En effet, la voie parentérale, bien que parfois mal acceptée par les patients, présente de nombreux avantages d'un point de vue pharmaceutique. D'une part, elle permet un effet rapide du principe actif. D'autre part, l'effet de premier passage hépatique est évité. Par rapport à la voie orale, elle évite la dégradation ou la rétention du principe actif au niveau du tractus digestif. Enfin, des formes permettant une libération prolongée du principe actif peuvent être utilisées. Elles permettent de diminuer le nombre de prises tout en maintenant la concentration plasmatique du médicament dans la zone thérapeutique, sans fluctuations comme observé lors d'administrations répétées de formes à libération immédiate. Ceci est sans doute important dans le cadre de la libération de NO, car il est désormais bien connu que le NO, à faible ou forte concentration (ex : nM vs mM) et en fonction de l'environnement peut exercer des effets différents.

Un des moyens d'obtenir une libération prolongée par voie parentérale est d'incorporer les molécules dans des matrices polymériques, par exemple sous forme d'implants ou de microparticules. En plus d'une libération prolongée, ces systèmes permettent également de protéger le principe actif. Les polymères utilisés peuvent être naturels ou synthétiques. Historiquement, les polymères naturels ont été les premiers développés. Ils sont attractifs car existant de façon endogène dans un organisme vivant, facilement disponibles, assez peu coûteux et pouvant subir des modifications chimiques. Cependant, les polymères synthétiques ont progressivement gagné en popularité grâce à leurs avantages : haute pureté, meilleure reproductibilité d'un lot à l'autre, absence de risque immunogène. Les polyesters aliphatiques synthétiques, en particulier les poly(α -hydroxyacides) comme le poly(lactide) (PLA), le poly(glycolide) (PGA) et leurs copolymères (PLGA), sont largement employés dans des applications de libération contrôlée de substances pharmaceutiquement actives. Ils sont biorésorbables et bioassimilables [70] : les chaînes de polymère sont totalement dégradées par

hydrolyse et les produits de dégradation, l'acide lactique et/ou l'acide glycolique, sont éliminés après métabolisation en eau et dioxyde de carbone *via* le cycle de Krebs (ou directement dans le cas de l'acide glycolique) [71]. Par conséquent, il n'est pas nécessaire de procéder à un retrait chirurgical de ces systèmes, ce qui améliore la compliance des patients. De plus, ces polymères ont montré une excellente biocompatibilité depuis le début de leur usage comme sutures résorbables chez l'Homme dans les années 60 [72]. Cette propriété, en plus des possibilités de modulation de leur temps de dégradation et de leurs propriétés mécaniques, a conduit à leur autorisation réglementaire pour la libération contrôlée de principes actifs (Tableau 1).

Tableau 1 : Polyesters aliphatiques : exemples de formulations parentérales pour la délivrance prolongée de substances pharmaceutiques autorisées sur le marché français. IM : intramusculaire ; SC : sous-cutanée.

Produit	Laboratoire	Forme (voie d'administration)	Principe actif	Indications principales	Fréquence d'injection
Somatuline LP	Ipsen Pharma	Microparticules (IM)	Lanréotide acétate	Acromégalie/ Tumeur carcinoïde	Toutes les 2 semaines
Sandostatine LP	Novartis Pharma		Octréotide acétate	Acromégalie/ Tumeurs endocrines digestives	Tous les mois
Risperdalconsta LP	Janssen Cilag		Risperidone	Schizophrénie	Toutes les 2 semaines
Salvacyl LP	Ipsen Pharma		Triptoréline pamoate	Paraphilie	Tous les 3 mois
Decapeptyl LP		Tous les 1, 3 ou 6 mois			
Enantone LP	Takeda	Microparticules (IM ou SC)	Leuproréline	Cancer de la prostate	Tous les mois
Gonapeptyl LP	Ferring SAS		Triptoréline acétate		
Zoladex	Astra Zeneca		Goséréline acétate		
Bigonist	Sanofi	Implant préformé injectable (SC)	Buséréline acétate		Tous les 1 ou 3 mois
Eligard	Astellas Pharma	Solution injectable (SC) : implant <i>in situ</i>	Leuproréline acétate		Tous les 2 mois
					Tous les 1, 3 ou 6 mois

3.2. Formulations *in situ*

Les formulations de type *in situ* représentent une approche intéressante pour une formulation parentérale à libération prolongée. En effet, elles sont administrées de façon peu invasive (injection d'une formulation liquide), ce qui permet de limiter la douleur : ceci est donc mieux accepté par les patients.

Les formulations *in situ* sont des solutions polymériques contenant le principe actif, qui subissent après injection dans l'organisme des modifications physicochimiques, aboutissant finalement à la formation d'un implant semi-solide ou solide capable de libérer la substance active de façon prolongée. Trois mécanismes de formation sont généralement distingués (Figure 8) :

- ✓ Précipitation *in situ* : le polymère est insoluble dans les conditions physiologiques et sa précipitation se fait par séparation de phase, transition sol-gel à température physiologique ou en réponse à un changement de pH ;
- ✓ Réticulation *in situ* : la réticulation chimique des chaînes de polymère est initiée par un changement de température, de concentration ionique, par photo-irradiation ou par la présence d'enzymes ;
- ✓ Solidification *in situ* : il s'agit de systèmes thermodurcissants qui se solidifient lorsqu'ils sont refroidis à la température corporelle, ou de cristaux liquides lyotropes, qui s'auto-assemblent en solution aqueuse.

Par rapport aux implants et microparticules classiques, les formulations *in situ* permettent un contact optimal avec les tissus environnants puisque le dépôt final s'adapte à l'espace disponible.

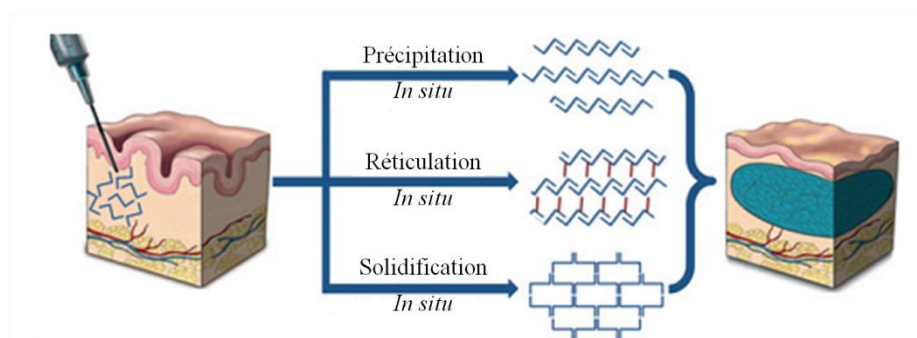


Figure 8 : Représentation schématique du principe de formulations *in situ* et des trois mécanismes permettant la formation d'une matrice polymérique solide ou semi-solide au site d'injection, adaptée d'après [73].

Le système *in situ* « idéal » devrait satisfaire aux conditions suivantes :

- Avoir une faible viscosité (pour faciliter l'injection et diminuer la douleur) ;
- L'ajout du principe actif doit être possible par simple mélange ;
- Les excipients doivent être biocompatibles ;
- Le principe actif doit être stable dans le système ;
- Le système doit présenter une libération du principe actif dans les premiers temps (*burst*) minimale ;
- Le système doit permettre une libération prolongée du principe actif.

Parmi ces systèmes, ce travail s'intéresse à des formulations à base de poly(lactide-*co*-glycolide) (PLGA), générant un dépôt par précipitation *in situ*. D'une part, ce sont les premières formulations *in situ* à avoir été décrites [15,74]. D'autre part, deux ont été mises sur le marché (Atridox et Eligard, uniquement Eligard en France). Enfin, l'équipe d'accueil possède une expertise dans ces formulations innovantes [75].

3.3. Article 3 : « PLGA *in situ* implants formed by phase inversion: critical physicochemical parameters to modulate drug release »

L'Article 3, paru dans *Journal of Controlled Release*, est une revue consacrée exclusivement aux implants *in situ* à base de PLGA, formés par précipitation du polymère lors de l'échange solvant/non-solvant. Les mécanismes de libération de principe actif à partir de matrices polymériques à base de PLGA sont connus comme complexes (Figure 9).

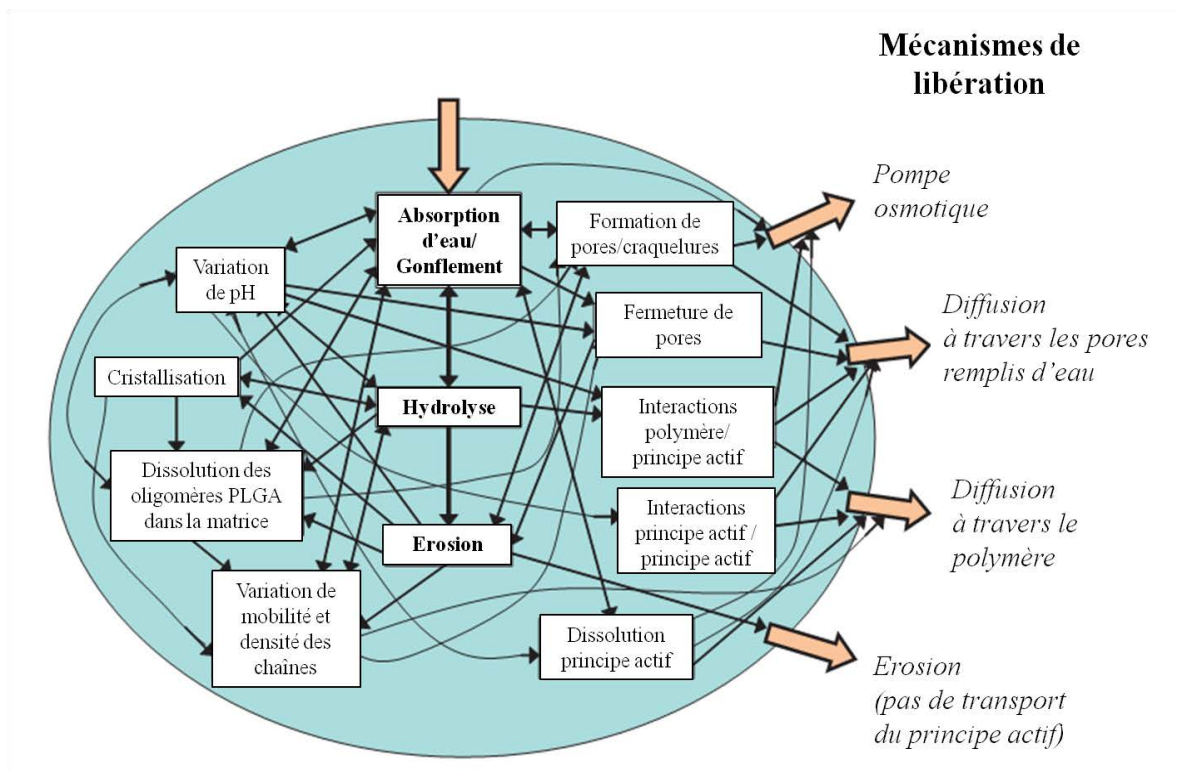


Figure 9 : Représentation schématique de la complexité des procédés physicochimiques ayant lieu dans les matrices PLGA et ayant pour conséquence la libération de principe actif, adaptée d'après [76]. L'influence de chaque procédé physicochimique sur la libération de principe actif et sur les autres procédés est représentée par des flèches, qui dans certains cas pointent dans les deux directions.

L'Article 3 a pour but de faire le point sur les principales étapes de vie des implants *in situ* et leur influence sur la libération de principe actif. Cette base théorique permet ensuite de comprendre en quoi les différents paramètres de la formulation peuvent être modifiés pour moduler la formation et la dégradation de la matrice polymérique ainsi que la libération de principe actif.



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Review

PLGA *in situ* implants formed by phase inversion: Critical physicochemical parameters to modulate drug releaseMarianne Parent^a, Cécile Nouvel^{b,c}, Martin Koerber^d, Anne Sapin^a, Philippe Maincent^{a,*}, Ariane Boudier^a^a Université de Lorraine, CITHÉFOR, EA 3452, Faculté de Pharmacie, BP 80403, F-54000 Nancy Cedex, France^b Université de Lorraine, Laboratoire de Chimie Physique Macromoléculaire (LCPM), FRE 3564, ENSIC, BP 20451, F-54000 Nancy Cedex, France^c Centre National de la Recherche Scientifique (CNRS), Laboratoire de Chimie Physique Macromoléculaire (LCPM), FRE 3564, ENSIC, BP 20451, F-54000 Nancy Cedex, France^d Freie Universität Berlin, College of Pharmacy, Kelchstraße 31, 12169 Berlin, Germany

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ABSTRACT

In situ forming implants (ISI) based on phase separation by solvent exchange represent an attractive alternative to conventional preformed implants and microparticles for parenteral applications. They are indeed easier to manufacture and their administration does not require surgery, therefore improving patient compliance. They consist of polymeric solutions precipitating at the site of injection and thus forming a drug eluting depot. Drug release from ISI is typically divided into three phases: burst during precipitation of the depot, diffusion of drug through the polymeric matrix and finally drug release by system degradation. This review gives a comprehensive overview on (i) the theoretical bases of these three phases, (ii) the parameters influencing them and (iii) the remaining drawbacks which have to be addressed to enlarge their commercial opportunities. Indeed, although some of them are already commercialized, ISI still suffer from limitations: mainly lack of reproducibility in depot shape, burst during solidification and potential toxicity. Nevertheless, depending on the targeted therapeutic application, these shortcomings may be transformed into advantages. As a result, keys are given in order to tailor these formulations in view of the desired application so that ISI could gain further clinical importance in the following years.

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Abbreviations: BA, benzyl alcohol; BB, benzyl benzoate; DMSO, dimethyl sulfoxide; EA, ethyl acetate; ISI, *in situ* forming implants; ISM, *in situ* forming microparticles; GA, glycolide; GRAS, generally recognized as safe; IIG, inactive ingredient; LA, lactide; LD50, lethal dose 50; NMP, *N*-methyl-2-pyrrolidone; PEO, poly(ethylene oxide); PDLA, poly(*D,L*-lactide); PGA, poly(glycolide); PLA, poly(lactide); PLGA, poly(lactide-co-glycolide); PLLA, poly(*L*-lactide); PPO, poly(propylene oxide); PVP, polyvinylpyrrolidone; TA, triacetin; 2P, 2-pyrrolidone.

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1. Introduction

Despite the fact that oral route ought to be considered as highly desirable by the patients it still represents a huge challenge in many applications. Indeed, an increasing number of new active pharmaceutical ingredients belong to the family of peptides or proteins and suffer from low bioavailability after oral administration. Alternative routes of administration (pulmonary, nasal, buccal, transdermal, ocular, and rectal) have also shown drawbacks such as enzymatic degradation or low/variable absorption [1]. As a result, there is a renewed interest in parenteral administration, especially as many improvements have been done in pain reduction. Nevertheless, many drugs are characterized not only by a high activity but also by a short half-life. In this context, sustained release forms are highly desirable to avoid continuous infusions or frequent injections. They display several advantages, such as enhanced patient compliance and the avoidance of peaks and valleys in plasma concentrations, thus allowing a reduction of the total dose and minimizing potential side effects.

The development of new injectable drug delivery systems has received considerable attention over the past few years, and many systems have been developed: e.g. microparticles, nanoparticles, liposomes and micelles [2–4]. Besides, significant advances have been made in the field of implants and microparticles. Implants classically formed by melt extrusion must be implanted into the patients, either surgically or through large diameter needles, and surgically retrieved after use unless they are biodegradable. Microparticles can be injected through smaller needles causing less pain to the patient, but the multi-step production processes are costly, the scale-up more difficult to achieve and the encapsulation efficiencies often low [5]. Therefore new alternatives have been studied to allow simple and painless administration as well as easy manufacturing. One of these alternatives is the development of smart systems, especially *in situ* forming depots, which can be injected into the body in liquid form and then solidify *in vivo* at the place of injection.

In situ forming systems have been commonly classified according to their mechanism of formation as: (i) *in situ* solidifying organogels; (ii) *in situ* cross-linking systems and (iii) *in situ* precipitating systems. Previous papers offered comprehensive reviews of all these *in situ* forming systems [5–8]. By contrast, this review will exclusively focus on the *in situ* forming implants (ISI) based on poly(lactide) (PLA) and poly(lactide-co-glycolide) (PLGA), which precipitate following phase separation triggered by solvent/non-solvent exchange. Among the first been described, these systems have been used for the delivery of various drugs. As they can be easily modulated, cover a wide range of release periods and are based on biodegradable polymers with excellent biocompatibility approved for parenteral administration, they represent a valuable strategy for controlled drug release applications.

The concept of *in situ* forming implants was first introduced by Dunn et al. in the 90's [9,10]. A water-insoluble biodegradable polymer is dissolved in a pharmaceutically acceptable organic solvent, miscible or partially miscible with water. Drug is added to this polymeric solution to form either a solution or a suspension. Following injection into an aqueous medium, a phase separation occurs as the solvent diffuses towards the surrounding aqueous environment while water/body fluids penetrate into the organic phase. This results in polymer precipitation and formation of a depot entrapping the drug at the injection site.

Two products have been commercialized, both using *N*-methyl-2-pyrrolidone (NMP) as solvent and PLA or PLGA as biodegradable biocompatible water-insoluble polymers. On the one hand, Eligard® (Sanofi) contains leuprolide acetate (a luteinizing hormone-releasing hormone agonist) and is injected subcutaneously at 1 to

6-month intervals for the treatment of advanced prostate cancer [11]. On the other hand, Atridox® (Tolmar Inc.) offers a local sustained release of doxycycline over a week after direct injection into the periodontal pocket. It is indicated in the treatment of chronic periodontitis for both human and veterinary purposes [12].

In view of the above, many other PLA- or PLGA-based ISI have been developed for local or systemic delivery of drugs (including peptides, proteins and nucleic acids), covering a wide range of therapeutics, which shows the potential of *in situ* implants in a broad range of indications (Table 1). Furthermore, some tissue engineering applications have been explored [12,13]. Drug release from these systems is generally characterized by an initial burst during the solidification of the matrix, followed by a second period mainly controlled by diffusion processes. Finally, subsequent drug release is driven by the polymeric carrier degradation and erosion [14]. Matrix formation, drug diffusion, matrix degradation and therefore drug release could be modulated varying several parameters. This review contains the theoretical basis of drug release from *in situ* forming PLGA implants, the parameters influencing the drug release characteristics as well as a critical discussion of potential toxicity issues and industrialization challenges. Finally, a decision-scheme is proposed to tailor the formulation depending on the drug and the targeted clinical application.

2. *In situ* implants – fate and drug release kinetics

This chapter reviews the theoretical basis of the solidification process of the *in situ* forming implants, the degradation of the polymeric matrix and the mechanisms of drug release.

2.1. Matrix solidification phase

2.1.1. Phase inversion dynamics

Contact of *in situ* forming PLGA implants into an aqueous medium or body fluids triggers a phase inversion process in the polymer solutions, which finally results in polymer precipitation. Nevertheless, the formation of the final solid/semi-solid depot is not instantaneous but depends on the kinetics of the phase inversion.

The dynamics of non-solvent induced phase inversion have been extensively studied with polymer-membranes designed for purification applications and the underlying thermodynamics and mass-transfer laws as well as corresponding morphologies of the membranes have been elucidated [40]. After immersion of the polymeric solution into an aqueous medium, an exchange between polymer solvent and non-solvent (i.e. water or body fluids) occurs. Induced by this diffusion process the polymer solution turns into a thermodynamically metastable or unstable state. Driven by a decrease of the free energy of the system the homogeneous solution can be separated into two phases of different compositions – a polymer-lean and a polymer-rich phase. Ternary phase diagrams with the composition in solvent, non-solvent and polymer represent a useful tool to follow or predict the phase transitions which occur after injection of the polymeric solution into aqueous environments (Fig. 1A). The phase diagrams visualize compositions where the polymer solution consists of a single homogeneous phase as well as an area representing the liquid–liquid demixing gap delimited by the binodal curve. This demixing gap is again divided into a domain delimited by the spinodal curve representing unstable compositions (Fig. 1A: area II) as well as a domain between the spinodal and the binodal curves representing metastable compositions (Fig. 1A: areas I and III). Demixing of the latter compositions occurs according to a binodal decomposition through the generation and growth of stable nuclei of a polymer-lean phase at higher polymer concentrations

Table 1
Results published on PLA/PLGA-based ISI categorized for indications.

Molecule	Polymer	Solvent	Main outcomes	Ref
<i>Cancer therapy</i>				
Cisplatin	PLGA	NMP	<i>In vivo</i> sustained delivery (rats) for 7 days Increased maximum tolerated dose and tumor suppression effect (mice) vs. free cisplatin	[15]
Fenretinide	PLGA	DMSO	<i>In vivo</i> sustained release (dogs) but lack of effect (inactivation of cisplatin bioactivity by interaction with DMSO)	[16]
	PLGA	NMP	<i>In vitro</i> 1-month sustained release	[17]
<i>Hormonal therapy</i>				
Human growth hormone	PLGA	BB	Sustained serum levels for 28 days (rats)	[18]
Levonorgestrel	PLA	BB/BA	Zero-order drug release <i>in vitro</i> lasting on 90 days	[19]
Testosterone				[20]
Calcitonin				[21]
<i>Immunomodulation</i>				
Betamethasone	PLGA	NMP	<i>In vitro</i> releases from sterilized formulations from 24 to 90 days, depending on the PLGA composition	[22]
Thymosin-1-alpha	PLGA	NMP (\pm TA)	Significant increases of thymic and spleen indexes (immunosuppressive mice)	[23]
Soluble tumor necrosis factor (TNF α) receptor	PLGA	Glycofurol	<i>In vitro</i> : burst (<20%) then continuous release (20 days)	[24]
			<i>In vivo</i> : long-lasting protection against pathological effects of TNF α (mice)	
<i>Anti-infectious therapy</i>				
Ivermectine	PLA	NMP, 2P, TA, BB	<i>In vitro</i> release rates (96 days) with speeds ranked in the order NMP > 2P > TA > BB	[25]
Secnidazole	PLA PLGA	NMP	<i>In vitro</i> release (3 days) with high bursts (>30%, desired) and suitable antimicrobial activity	[26]
doxycycline				
Tinidazole	PLA	NMP	Significant decrease in periodontitis symptoms (dogs, 7-day local delivery)	[27]
HIV-fusion inhibitor	PLGA	DMSO/TA	Drug plasma concentration in the therapeutic range up to 48 h (rats)	[28]
<i>Analgesia/anesthesia</i>				
Aspirin	PLGA	NMP	7-day controlled release <i>in vitro</i> Faster polymer degradation with aspirin	[29]
Ketoprofen	PLGA	NMP	Effective plasma levels maintained about 8 weeks (rats)	[30]
Bupivacaine	PLGA	2P	Reduction of plasmatic concentration (systemic side effects) while local analgesic effect was maintained for 6 h (rats)	[31]
<i>Neurological disorders</i>				
Haloperidol	PLGA	NMP	20–30 days of <i>in vivo</i> release (rats)	[32]
Risperidone	PLGA	BB/BA	Prolonged mean residence time: 32.6 h vs. 5.8 h for risperidone solution (rabbits)	[33]
Risperidone	PLGA	DMSO	3-week sustained release (dogs)	[34]
Paliperidone			Sustained suppressive effect of psychotic behavior during 38 days (mice)	
Naltrexone	PLGA	NMP	30-day sustained release <i>in vitro</i> (burst >40%)	[35]
<i>Metabolic disorders</i>				
Rosiglitazone	PLGA	NMP, TA	Sustained <i>in vitro</i> release up to 8 days with lower burst for TA (<20%) than NMP (20–60%)	[36]
Insulin	PLGA	BB/BA	Better pharmacological response during 15 days after a single injection vs. routine once-a-day administration of insulin (mice)	[37]
<i>Gene delivery</i>				
Model plasmid DNA	PLGA	Glycofurol	2-month <i>in vitro</i> release (burst <20%) with transfection activity maintained <i>In vivo</i> transfection with 10-fold higher protein expression (up to 67 days) vs. plasmid solution (mice)	[38]
Model plasmid DNA-containing PLGA microspheres	PLGA	Glycofurol	<i>In vitro</i> controlled release during 70 days with transfection activity maintained	[39]
<i>Tissular reconstruction</i>				
Bone morphogenetic proteins	PLGA	NMP	Trend without statistical increase in bone formation vs. blank formulations but less inflammatory response (rats)	[13]

NMP: N-methyl-2-pyrrolidone; DMSO: dimethylsulfoxide; BB: benzyl benzoate; BA: benzyl alcohol; TA: triacetin; 2P: 2-pyrrolidone.

(area I) or of a polymer-rich phase at lower polymer concentrations (area III), which affects the morphology of the resulting system significantly (Fig. 1B).

Binodal and spinodal curves meet at only one point, called the “critical composition” (indicated by the cross in Fig. 1A), at which transition from the homogeneous to the unstable domain and hence phase separation occurs spontaneously by spinodal decomposition resulting in a bicontinuous structure of polymer-lean and polymer-rich phase (Fig. 1B, II).

After injection of ISI formulation into aqueous medium, water concentration in the polymeric solution increases until finally the demixing gap is reached. In most cases, the phase inversion process crosses the area I, as polymer concentrations used are commonly above the critical point. Hence, droplets of polymer-lean phase form within the polymer-rich phase during the demixing process. These two liquid phases are in thermodynamic equilibrium and hence evolve in parallel until the

polymer concentration of the polymer-rich phase becomes high enough to solidify the structure (Fig. 1A: solidification region) due to the continuous loss of polymer solvent to the aqueous surrounding.

Key parameters of the phase inversion dynamics of *in situ* forming systems are thus the influx of non-solvent (i.e. water) as well as the outflow of polymer solvent. Several scenarios are possible depending for example on whether the solvent used has high or low miscibility with the non-solvent (Fig. 2).

In case of water-miscible solvents, injection of the polymeric solution into water leads to a fast diffusion of the solvent towards the aqueous medium (e.g. NMP; fast inverting systems). The solution is quenched immediately and the composition path crosses the binodal curve without delay time. This triggers the formation of a solidified polymer layer at the top of the depot (Fig. 2: point D), sublayers still consisting in homogeneous polymer/solvent solution (phase A). Then, the top layer forms a barrier to the entry of water. Following the progressive

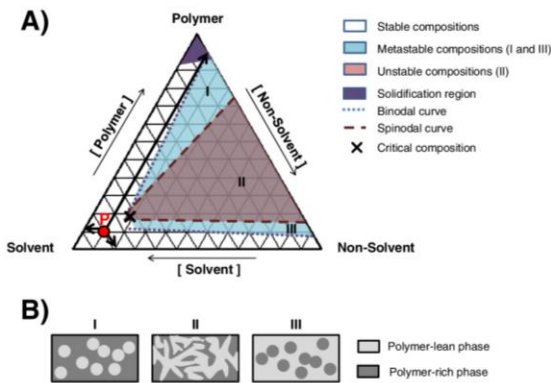


Fig. 1. Model of a ternary phase diagram of a mixture of solvent, non-solvent and polymer (A). As an example, P point corresponds approximately to 8% of polymer, 8% of non-solvent and 84% of solvent. In the areas I and III, demixing occurs through binodal decomposition, with nucleation and growth of a polymer-lean phase (area I) or a polymer-rich phase (area III) (B). Area II, area of absolute instability, direct transition leading to spontaneous formation of bicontinuous structures.

penetration of water, the sublayer composition enters progressively the metastable region leading to phase separation (phases B and C). The core of the nascent pore consists of a water-rich polymer-lean phase (C), while the surrounding is a polymer-rich phase (B). If the solvent has a high affinity for water, demixing requires low quantities of water. The resulting structure is a thin top layer on a pore-rich region. Furthermore, solidification of the pore wall is slow because solvent diffuses rapidly and in large quantities into the nascent pore. As the water penetration front progresses, pores are supplied with both solvent and water. Pore growth is stopped by the solidification of the polymer-rich phase surrounding it (phase D). As penetration of water is fast, the polymer-rich phase part of the pore situated close to the implant surface will solidify while the part far from the surface will stay liquid longer. As a result, typical “finger-like” pore structures (Fig. 3A) are generated [40].

In case of solvents immiscible with water, liquid–liquid demixing is delayed when the formulation is injected into water, as diffusion rates are slower (e.g. TA; slow inverting systems). As a result, the top layer does not solidify so fast and therefore affects in a lower manner solvent and water diffusions from/into the sublayers. Consequently, the final structure is more homogeneous. Solvent exhibiting low affinity for water, nucleation starts later as it requires a higher quantity of penetrated water. Consequently, numerous nuclei are initiated at the same time and because each of them consumes solvent, the growth of each nucleus is limited by its neighbors. Moreover, solidification of the pore wall is fast because a lower proportion of solvent is present in the polymer-lean phase. In this case “sponge-like” implant morphologies (Fig. 3B) are observed [41].

The dynamics of the phase inversion can be tailored by modulating the *in situ* implant composition, i.e. polymer solvent and/or polymer. These changes, however, impact the initial drug release within the first day (burst) directly but also affect the following diffusion- and erosion-controlled release phases, due to effects on the matrix morphology and the degradation of the solidified matrix.

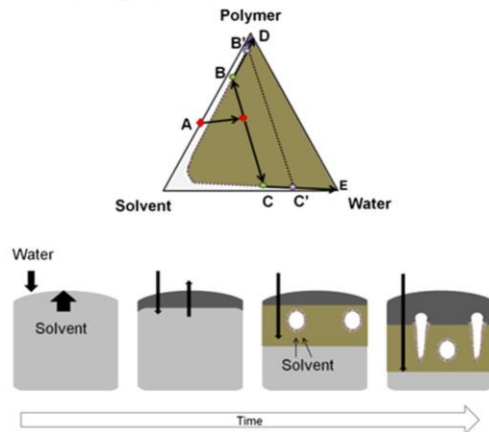
2.1.2. Drug release during matrix solidification

During the first periods after injection of ISI, drug release is essentially occurring by drug transport either through the polymer-rich or through the polymer-lean phase of the forming implants [14,41]. Although, it was recently hypothesized that convection might contribute to the initial drug release of *in situ* forming implant systems [42], a diffusion-

controlled drug and solvent release is generally accepted as the applicable transport mechanisms during the solidification [41].

According to a diffusion-controlled release, the quantity of drug initially released can be attributed to physicochemical constants of the drug such as solubility, diffusivity, partition coefficient, dissociation constant and molecular weight [43,44]. The water-miscibility of the polymer solvent, however, plays a crucial role for drug release, due to its impact on the environment governing drug diffusion.

Fast inverting system



Slow inverting system

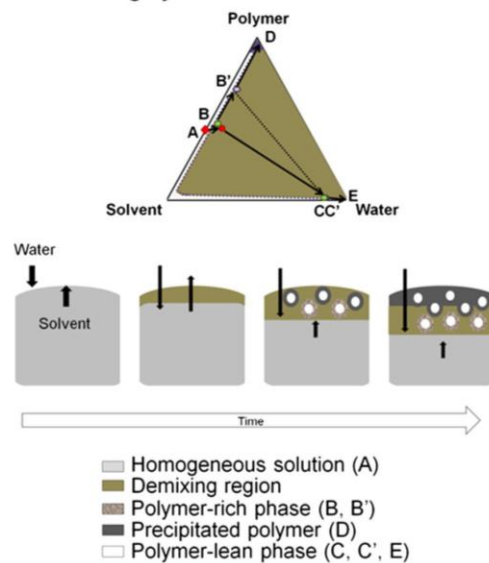


Fig. 2. Typical ternary phase diagrams and matrix structures observed with a fast inverting system (for example when solvent has high water miscibility, e.g. NMP) and a slow inverting system (when solvent is partially water miscible, e.g. TA). Dotted black lines on the phase diagram represent the tie lines between the two compositions in equilibrium. After injection, the single phase mixture A splits into two phases B and C which then evolve through B' and C' and finally to D (in the solidification region) and E. In the first case, relatively large increases in polymer concentration of polymer-rich phase will occur quickly upon the separation, as indicated by the steep slopes of tie lines. In the second case, the tie lines have lower slopes and cross the horizontal axis of the diagram at fixed and low solvent compositions (related to water solubility). Thus, a less concentrated, more fluid polymer-rich phase is generated.

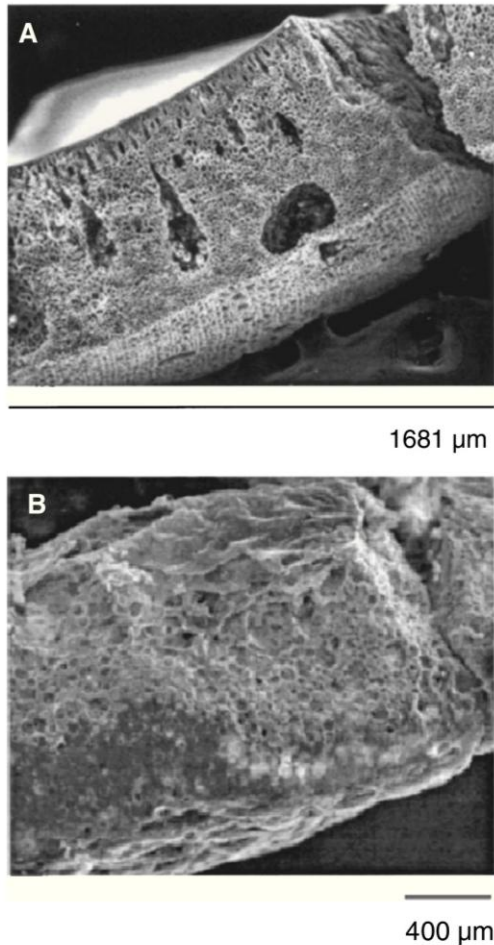


Fig. 3. Examples of “finger-like” structures (A) and “sponge-like” structure (B) in a fast and a slow inverting *in situ* formulation, respectively. Formulation: 40% m/m PLGA (50:50, M_w 10,000) in NMP (A) or TA (B). Reprinted from [41] copyright (1999), with permission from Elsevier.

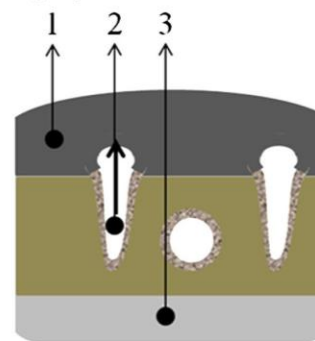
Fast inverting systems based on water-miscible solvents are often characterized by a high drug burst [25]. This is usually attributed to an increased probability for the formation of an interconnected network of polymer-lean phase, representing an implant domain with high drug diffusivity (Fig. 4) [14,41]. Diffusion coefficients are considered to be around 10^{-5} cm²/s in the polymer-lean phase of the nascent implant system (Fig. 2: phase C) and about 10^{-7} – 10^{-8} cm²/s in the corresponding polymer-rich phase (Fig. 2: phase B) as well as in the homogeneous polymer solution (Fig. 2: phase A) [45]. An interconnected network increases the chance for the incorporated drug load to have access to the surface of the solidifying matrix and hence to be rapidly released [46]. Once the porous volume is depleted and the remaining drug is entrapped, drug is released much slower by diffusion through the hardened polymeric matrix (Fig. 4).

As an example, large hydrophilic molecules like peptides and proteins are transported preferentially through the interconnected polymer-lean phase of a fast inverting system and hence released in form of a burst. A retarded diffusion is obtained, however, if these drugs are forced through the polymer-rich phase as reported for slowly inverting *in situ* implant formulation (Fig. 4) [41].

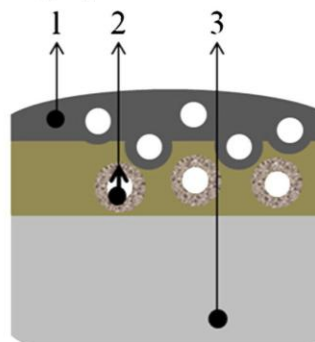
Hence, slow inverting systems are characterized by reduced bursts, because the extent of the formation of a polymer-lean phase is limited by the limited water-miscibility of the systems [14], which causes the depot to stay more or less viscous during a prolonged solidification process and produces a lower porosity in the hardening structure. As a consequence the drug diffusivity is decreased as well as the diffusion path length increased, resulting in a more gradual drug release.

Small or hydrophobic drugs, however, can efficiently diffuse through the polymer-rich and even the hardened polymer phase into the aqueous surrounding [47]. If drugs in close contact to the polymer are ionizable, polymer–drug interactions can modify the diffusion rates further [48].

Fast inverting system



Slow inverting system



Diffusion coefficients (cm²/s)

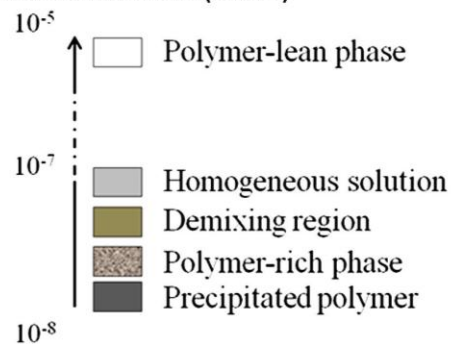


Fig. 4. Release of drugs during implant hardening in relation to their location in fast and slow inverting systems: 1. drug close to the surface = fast release, 2. drug in the polymer-lean phase = fast release, 3. drug in the polymer solution = slow release.

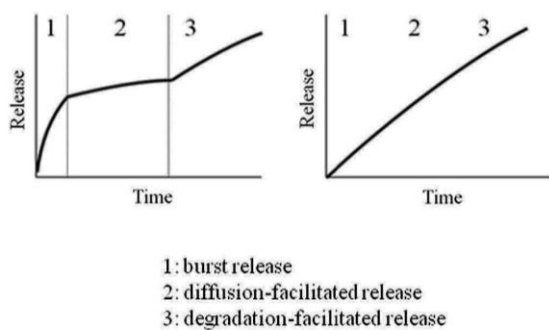


Fig. 5. Typical release profiles of fast (left) and slow (right) inverting systems.

2.2. Drug diffusion phase

Drug diffusion out of the hardened implant needs to proceed according to the same principles as applicable to conventional matrices, like implants and microparticles, consisting of drug and polymer only. According to applicable laws, drug diffusion is a function of the physico-chemical properties of the drug, the polymer as well as the matrix morphology. The main factors affecting the release can thus be derived; being the drug diffusivity within the polymer matrix, the drug solubility, drug loading, but also the matrix dimensions (i.e. surface area) as well as its porosity and the tortuosity of the diffusion pathway [43,49].

In a number of cases the fraction of drug released within this phase is small [17,19,20], which can result in a release plateau after the initial phase and before the erosion-controlled release phase commences (Fig. 5 left) [50].

A careful modulation of the *in situ* implant composition and hence the phase inversion dynamics, however, can lead to continuous diffusion-controlled release (Fig. 5 right) of drugs over time-frames exceeding the hardening phase of the formulations [18,21,23].

2.3. Implant erosion phase

Polyester degradation is the hydrolytic chain scission process cutting polymer chains into oligomers and finally monomers [51]. The degradation of the polyesters poly(lactide) (PLA), poly(glycolide) (PGA) and poly(lactide-co-glycolide) (PLGA) is the prerequisite for the erosion of the polymer, which is the loss of implant mass due to the release of water-soluble PLGA degradation products (critical molecular weight 10^3 g/mol [52]) from the polymer matrix. The monomeric end-

products of the hydrolysis process are lactic and/or glycolic acid, which are eliminated from the body through the Krebs cycle.

Water uptake and hence degradation of the polyesters actually starts immediately after injection of the ISI formulation into aqueous medium (water or body-fluids). However, it is not before water-soluble oligomers are formed that the polymer matrix starts to erode due to the release of the mobilized molecules [53]. Additionally, these products have carboxylic chain ends which are able to autocatalyze the ester bond hydrolysis and hence lead to a faster degradation of the entire polymer matrix.

In case of the PLA, PGA and PLGA water penetration into the polymer is faster than the degradation of polymer bonds (Fig. 6). Hence, the polymer is hydrolyzed over the entire matrix leading to a homogeneous formation of degradation products and finally to bulk erosion [54]. On the contrary, if the chain scission occurs faster than diffusion of water, as for poly(ortho esters) for example [51], the hydrolysis is confined to the matrix surface and the polymer matrix undergoes surface erosion. Although these polymers could be potentially used for biodegradable *in situ* implants, they are not approved for parenteral administrations yet.

The release of the formed oligomers is not necessarily immediate as shown for large polymer matrices [55]. In thick structures lactic acid oligomers can form salts differing from the protonated acids in solubility characteristics [51]. Accumulation of degradation products in the core of such a matrix and the entering of buffer ions from the matrix surface favor a crystallization of insoluble salts in the outer shell of the polymer matrix. Besides the formation of insoluble salts the formation of stereocomplexes between poly(D-lactic acid) and poly(L-lactic acid) oligomers appears to be another reason for the insoluble residual of PL(G)A matrices undergoing “heterogeneous” bulk erosion [51].

Moreover, it was hypothesized that the pH gradient developing between the matrix surface, which is in contact with buffered medium, and an oligomers-enriched inner core results in a slower degradation of the shell vs. the center [56]. This, however, seems to be inconsistent with recent insights into the hydrolytic degradation of PLA oligomers: stability of oligomers is indeed nonlinearly related to the pH with a stability maximum at acidic conditions and not at neutral pH [52]. In such conditions, faster degradation in the center is probably related to the accumulation of -OH and -COOH functions in the core (by random hydrolysis) resulting in increased hydrophilicity [52].

The erosion of the polymer matrix facilitates the release of efficiently entrapped drug molecules, probably situated in the polymer-rich phase during phase inversion [14]. Erosion starts when the degradation is sufficient to result in soluble oligomers forming a porous network, which enables the release of oligomers as well as the entrapped drugs [57].

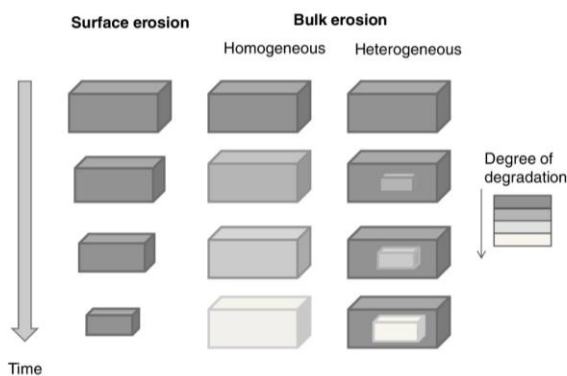


Fig. 6. Different degradation pathways of polymeric matrices.

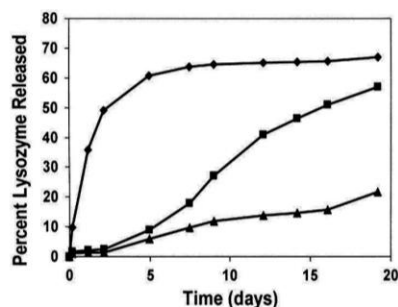


Fig. 7. Typical example of *in vitro* release profiles of a protein illustrating the impact of water-affinity of solvents on drug release, reprinted from [14] Copyright (1999), with permission from Elsevier. (♦) NMP; (■) TA; (▲) ethyl benzoate.

In summary, water uptake is the main trigger of matrix degradation and erosion, resulting in a calculable change of the polymer molecular weight. During degradation, however, physical changes can occur such as softening, pore creation/closure due to the decrease in polymer molecular weight, pH changes due to the formation of acidic degradants and crystallization. All of these events can affect the drug release during this phase making it a complex phenomenon. As for conventional biodegradable matrices, however, modulations are generally feasible by the choice of the polymer grade as well as by addition of additives like pore-formers [58], pH modifiers [59], or plasticizers [57].

It can be concluded, that the drug release from *in situ* forming implants is highly dependent on the matrix structure resulting from the phase inversion process as well as the polymer and drug properties.

3. Key parameters of *in situ* implant composition to obtain a suitable drug release

This part highlights the main aspects currently encountered in the literature. As previously seen, the matrix structure is highly dependent on the solvent used, especially on its affinity for water. Although this has been studied from the very beginning of the ISI concept [9,10], other key parameters such as polymer, drug, additives, or injection site have progressively emerged and will be discussed in this section.

3.1. Solvent

The ideal solvent or solvent blend for *in situ* systems needs to possess suitable properties in terms of water affinity, viscosity, ability to dissolve the polymer and last but not least, safety.

A low water affinity assists controlling the phase inversion/matrix formation and thus the drug burst. Reducing the affinity of solvent for water by replacing part or the totality of the solvent by a water-immiscible one slows the phase inversion rate increasing the chance for a more uniform to zero-order release pattern over an extended time-period [14,41]. This trend has been observed both *in vitro* [19,25,32] and *in vivo* [18,20,23] typically with solvents like benzyl benzoate, ethyl acetate, ethyl benzoate or TA. A good example of the influence of water-immiscible solvents is given in Fig. 7, from the study of Graham et al. [40]. In another *in vitro* study, Ahmed et al. reported haloperidol bursts from a PLGA-based ISI (50:50, M_w 60,000–70,000 g/mol; 20% w/v) of 20% with DMSO, 18% with NMP, 9% with ethyl acetate and only 7% with TA [32]. Durations of release were also affected by the solvents: being 24 and 28 days for DMSO and NMP (water-miscible) vs. a 60 day extended release for ethyl acetate and TA (water-immiscible).

The solvent should further have a viscosity facilitating the easy injection of the formulations (also referred to as “syringeability”). Systems

based on some water-immiscible solvents, for example, could have viscosities making their own injection difficult, which could necessitate implementing a warm-up step before injection, as previously reported [20].

In addition to the viscosity of the solvent itself, syringeability is also facilitated by solvent affinity to the polymer (“good” solvent). This will indeed not only ease the dissolution step but also decrease the viscosity of the overall concentrated polymeric solutions used for *in situ* systems. As a result, in “good” solvents, polymer–solvent interactions predominate over polymer–polymer ones, therefore lowering the viscosity. By contrast, in “poor” solvents of the polymer, polymer–polymer interactions are favored, leading to the formation of aggregates and an increased viscosity [25]. “Good” solvents for the polymer also present the advantage of a reduced injected volume, due to the possibility to achieve higher polymer and drug loadings in the formulations. This is beneficial in two aspects, (i) volume constraints of subcutaneous or intramuscular injections (<1 mL [60]) and (ii) a lower amount of organic solvent administered. Beyond its impact on the injectability of the formulations, viscosity also affects the diffusion of species in the solidifying matrix. In this respect, a high viscosity will slow down the entry of water and the drug diffusion. Therefore it reduces the burst as well as delays polymer degradation.

Thirdly, good compatibility of the solvent with the polymer and the drug is a prerequisite. For instance, Dong et al. reported an influence of the solvent properties on the storage stability of PLGA [61] and Dernell et al. reported an interaction between DMSO and cisplatin, diminishing its biological activity [16].

Finally, biocompatibility or at least low toxicity is required for pharmaceutical acceptance (see also Section 4.1.). Several solvents have already been used for *in situ* depots formulation (Table 2). Of course, none of these gathered all the previously mentioned qualifications. NMP and DMSO seem to be safe due to pharmaceutical precedence in approved parenteral products, but they are freely miscible with water resulting in a rapid solvent and drug burst, as discussed above. One has to consider, that rapid initial release of large amounts of drug and solvent within a short time frame (minutes to hours) is undesirable, since it may result in local tissue irritation or even systemic side-effects not predicted by an estimated average dose, which considers a constant administration over the entire drug delivery period.

On that basis, water-immiscible solvents are attractive but challenge the injectability of the formulation. Hence, the better alternative can be a blend of water-immiscible and water-miscible solvents to obtain acceptable viscosity, suitable phase inversion rate and low burst.

3.2. Polymer

The characteristics of the biodegradable polymer strongly impact the degradation and hence not only the erosion of the matrix but also the phase inversion dynamics.

PLGA is a copolymer of D,L-lactic and glycolic acid obtained by ring-opening copolymerization of D,L-lactide (LA) and glycolide (GA) (Fig. 8) [65]. Polymer grades are available with lactide/glycolide molar ratios 100:0 to 0:100 and molecular weights from below 10,000 up to 200,000 g/mol. As a result, PLGA copolymers provide a wide range of physicochemical and degradation characteristics for controlled drug delivery applications.

The composition of the copolymer is a critical point. LA/GA ratio and their distribution inside the chains (*i.e.* copolymer microstructure) are complex parameters, modulating the hydrophobicity and crystallinity of the system. Indeed, lactide exists in three different forms due to its two asymmetric carbons: *L*-lactide, *D*-lactide or *meso*-lactide (Fig. 8). *L*-lactide and *D,L*-lactide (*i.e.* the racemic mixture of *L*- and *D*-lactide) are the most commonly used forms for drug delivery applications. Poly(*L*-lactide) (PLLA) is a semi-crystalline material while poly(*D,L*-lactide) (PDLA) is amorphous [66]. PGA is also crystalline but

Table 2
Common solvents in ISI formulations and their main characteristics.

Solvents	Water miscibility (mg/mL)	Viscosity (cP) at 20 °C	Classification	LD50 oral rat (mg/kg)
Glycofurofural	miscible in all proportions ^a	8–18 ^a	/	980 ^c
DMSO	miscible ^a	2.19 ^d	ICH class III ^e	14,500 ^c
NMP	1000 ^b	1.89 ^d	ICH class II ^e	3914 ^c
2P	1000 ^b	14.66 ^d	/	328 ^c
TA	64 ^b	19.7 ^d	FDA GRAS ^a	3000 ^c
BA	35 ^c	5.81 ^d	FDA IIG ^a	1 230 ^c
BB	Insoluble ^b	8.67 ^d	FDA IIG ^a	1 680 ^c

NMP: N-methyl-2-pyrrolidone; DMSO: dimethyl sulfoxide; 2P: 2-pyrrolidone; TA: triacetin; BB: benzyl benzoate; BA: benzyl alcohol; GRAS: generally recognized as safe; IIG: inactive ingredients. LD50: lethal dose 50.

^a Data are from [62].

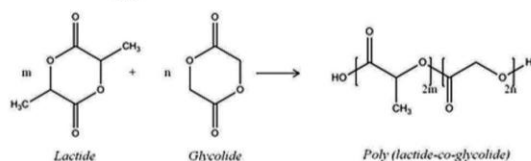
^b Data are from [25].

^c Data are from Material Data Safety Sheet.

^d Data are from [63].

^e Data are from [64].

A) Synthesis of PLGA by ring-opening copolymerization of lactide and glycolide



B) The three isoforms of lactide

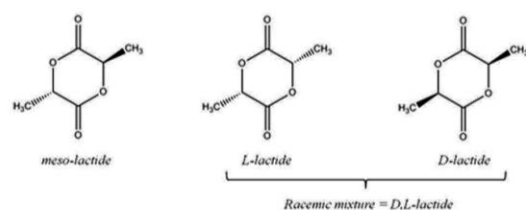


Fig. 8. Principle of PLGA synthesis through ring-opening copolymerization of lactide and glycolide (A) and existence of three isoforms of lactide (B).

PLGA, the copolymer of LA and GA, can be again amorphous depending on the polymer composition and microstructure.

The hydrolysis proceeds faster in the amorphous polymer parts compared to the crystalline ones resulting in a faster onset of polymer erosion and thus erosion-controlled drug release [67]. Degradants of the initially amorphous D,L-PLA, however, can crystallize during the hydrolysis resulting not only in a slower degradation of these domains [68] but also in a compromised biocompatibility of the polymeric device [69]. Crystallization occurs especially if the degree of randomness of the polymer is low, i.e. the segment lengths of monomeric repeat units in the polymer backbone are long. During crystallization, drugs can be excluded from the newly formed domains to the amorphous or already porous region, therefore accelerating their releases [70].

Modulating the polymer hydrophobicity could impact both the burst by influences on the solvent–water exchange rate and the degradation. As GA is slightly more hydrophilic than LA and introduces the more labile GA–LA bonds into the polymer backbone [71], the higher the GA content, the faster is the hydrolysis rate. Due to the crystalline nature of PGA, however, the degradation of PLGA containing 40 to 70% of GA is the fastest. Thus, for drug release applications for up to 2 months, PLGA 50:50 is often used [72].

The molecular weight of the polyester is another factor affecting matrix erosion and also the initial drug release. Several studies showed indeed that smaller bursts occur with low molecular weight PLGA compared with medium or high molecular weight PLGA. Patel et al. [73], for example, reported a burst of 28.2% fluorescein from PLGA implants (50:50, 36.5% m/m in NMP) for low molecular weight PLGA (M_w 16,000 g/mol), vs. 55.1% for high molecular weight PLGA (M_w 60,000 g/mol). Results from Luan et al. [46] followed the same pattern with a burst of leuprolide acetate of 18.8% and 48.1% from implants based on PLGA 50:50 with low or high molecular weight (M_w 7000–17,000 and 24,000–38,000 g/mol; 30% m/m of polymer in NMP), respectively. In both studies a slower diffusion of NMP from the lower molecular weight-PLGA solution and thus a slower phase transition was hypothesized, due the higher hydrophilicity of this polymer. This affinity for water also explains their higher tendency to swell [42,74], probably driven by an osmotic process.

The degradation process is obviously a function of the initial polymer molecular weight, since it is the starting point for the pseudo-first order decrease of the molecular weight over time [75]. Consequently, the higher the molecular weight initially, the longer is the time needed to

produce water-soluble oligomers and hence the induction period until matrix erosion commences [53].

To summarize, low molecular weight-PLGA formulations often exhibit a lower initial release than high molecular weight-PLGA systems, but onset of the erosion-controlled release is accelerated. Intermediate properties can be achieved using a blend of PLGA with various molecular weights [74]. However, instead of a simple average of the release profiles of the pure polymers, the release can be primarily controlled by the polymer with the higher molecular weight during the burst phase, while degradation-facilitated release seemed to be controlled by the lower molecular weight polymer in the first place. Intermediate behavior was maintained until a 10:1 ratio of both polymer types.

The last parameter modulating the hydrophobicity of PLGA is the kind of chemical moieties at the chain ends. PLGA could be provided either with free carboxylic acids at the ends of polymeric backbone chain, or end-capped with alcohols. The end-cappings decrease PLGA hydrolysis rates, because they make the polymer somewhat more hydrophobic [75], leading to a decrease in water uptake and most importantly because capped acids cannot participate to the autocatalysis of the ester bonds. However, reduced burst might also be obtained with uncapped polymers in the case of active drugs having functional groups interacting with polymer acidic ends [46,76]. Covalent modifications of the polymeric backbone itself with a hydrophilic polymers, like poly(ethylene glycol), have been investigated but lack yet of pharmaceutical acceptance [77,78].

The simplest modulation to reduce the burst of *in situ* forming implants is an increase of the polymer concentration, due to the decreased water affinity of the solution, the thicker solidified polymer skin and the generally lower diffusivities in the system, which slows the solvent/water exchange and generates a less porous structure. This approach has been extensively studied [19,23,26,41,79,80]. For example, haloperidol initial release from ISI was decreased, increasing the polymer concentration (20, 30, 40% m/v), whatever solvent was used (NMP, DMSO, TA, ethyl acetate) [32]. Additionally, increasing polymer concentration also extended the duration of release, e.g. 28, 35 and 47 days for ISI–NMP [32]. However, the limitation of this method is the concomitant increase in system viscosity, which could quickly hamper injectability [60].

To conclude, polymer characteristics influence on the one hand the hydrophobicity of the system, which impacts the initial as well as the diffusion-controlled drug release *via* its effect on the phase inversion dynamics, and on the other hand the erosion-controlled release out of the matrix according to the initial molecular weight and the degradation rate.

3.3. Drug

Injectable formulations with high drug loadings have the advantage, that the volume of the formulation can be reduced considering a fixed dose to be administered. This is interesting from an economical point of view and from the patients' point of view since pain exposure times are reduced [60].

The effect of the low loading on release of *in situ* implants was investigated in a number of studies. Wang et al. [81] varied ketoprofen from 4 to 10% m/m (PLGA 70:30, 35% m/m in NMP) without significant effect in drug release *in vitro*. Ravivarapu et al. [82] obtained the same results *in vivo* with leuprolide acetate 3 to 6% m/m (PLGA 75:25, 45% m/m in NMP). Interestingly, Chen et al. [20] observed also the same but at much higher drug loadings of 20–60% (PDLA 5% m/v in BB/BA 85/15 v/v). Although the release was increased in absolute terms (0.33 to 0.85 mg/day), a 3-fold increase of the drug loading led to an about 3-fold increase of the absolute amount of testosterone released within a 3-month period, which means that the fraction of drug released (%) remained the same.

According to the solubility of the drug in the polymer solution and its concentration, the drug can be either dissolved or dispersed in the *in situ*

forming implant. Based on this state, different behaviors have been described. Körber and Bodmeier [83] found a faster release of lysozyme (4% based on polymer) when it was dispersed rather than dissolved in the polymer solution (PLGA 50:50, 40% m/m in DMSO). This was attributed to drug particles sedimentation and heterogeneity of the resulting polymer–solvent–drug mixture. Large drug aggregates were created at the surface of the matrix, and released rapidly. In agreement with this, Brodbeck et al. [18] investigated human growth hormone formulations, which showed a large burst, when the bulky lyophilized powder was suspended in the polymer solution, whereas a low burst was obtained for the material obtained after densification of the lyophilized material, which was attributed to a reduced water uptake of the formulations.

Another point of particular importance is their stability. As previously mentioned and as in any galenic formulation, drug interaction with excipients can also occur in ISI. On one side, a drug can accelerate polymer degradation [13,29,38,61], either because it contains water or possesses H-bond donating functional groups which can interact catalytically with polymer chains and therefore increase the exposition of ester bonds to water. On the other side, polymer and/or solvent can degrade or inactivate a drug [23,29,61]. In this sense, PLGA based-ISI are not suitable for drugs degradable by water or highly sensitive to the acidic environment formed within the degrading polymer matrix. However, stabilization strategies such as the formation of insoluble salts or complexes [18] or addition of basic additives [84] have not yet been fully explored.

3.4. Additives

An alternative method to scale the burst of ISI is the incorporation of additives into the polymer solution. The use of hydrophilic additives is thereby expected to accelerate the liquid–liquid demixing and hence generate higher bursts, as shown for mannitol [85] or polyvinylpyrrolidone (PVP) [41] addition. Interestingly, Graham et al. [41] observed an 8-fold increase in the separation rate with only 3% (m/m) PVP. Accelerating the phase separation permits the faster emergence of zones where species have great diffusion capacity (see Fig. 4). Such additives appear to impact the initial period of release only [85], which could be explained by a lack of changes in terms of water penetration or overall morphology due to the rapid leaching of these additives.

Addition of amphiphilic or hydrophobic additives reduces the burst while also modifying the morphology of the system, with a transition towards a sponge structure. Glycerol monostearate, ethyl heptanoate, stearic acid, ethyl heptanoate, methyl heptanoate and ethyl nonoate, for example, were added to ISI formulations, from 1 to 10% m/m [33,35,86,87]. Whatever the active ingredient, formulations with these additives retained more solvent than control ISI, consisted in less porous and even sponge-like matrices, which allow lower bursts and extended releases. In addition, these effects were proportional to the amount of incorporated additive and to its hydrophobicity. Noteworthy, the highest tested quantities of these additives did not result in increased viscosity as it would be expected for a burst reduction by an increase of the polymer concentration. To illustrate, risperidone burst from ISI studied by Dong et al. [33] (PLGA 70:30, 30% m/v in BB/BA 90:10 v/v) was reduced *in vitro* from 32.2% to 4.7% in formulations containing glycerol monostearate. A higher mean-residence time was also obtained *in vivo* (86.8 h for modified ISI vs. 32.6 h for control ISI vs. 5.8 h for risperidone solution). A very smooth surface was observed for modified ISI, probably because of a specific location of the additive filling the pores on the surface. A similar burst reduction has been reported by DesNoyer and McHugh [58] after addition of Pluronic (triblock copolymer poly(ethylene)oxide/poly(propylene)oxide/poly(ethylene)oxide, PEO-PPO-PEO) in a formulation of PDLA in NMP. Nevertheless in this case, no significant morphological change was observed, yet the protein burst was strongly reduced. This was explained by a preferential segregation of Pluronic to the phase boundary during phase inversion. The

hydrophobic PPO chains are anchored into the polymer matrix, while the hydrophilic PEO ends deploy into the surrounding aqueous environment, both at the implant surface or into the pores. The resulting hydrophilic coating of the surface might improve the biocompatibility of the system and hinder protein adsorption as proposed elsewhere [88]. At the same time, PEO segments can fill the pores thereby creating a diffusion barrier. Optimization of Pluronic concentration or structure (ratio PEO/PPO, PEO chain length) is necessary to displace the balance between increased water absorption (hydrophilicity of the PEO chains) and diffusion barrier. Although authors considered that only this physical interaction is involved, they only studied one model protein (lysozyme) and additional interaction might occur with the drug, impacting its release. Depending on the application aimed, additives may also enhance drug activity, such as in anticancer therapy with some Pluronic potentially exerting chemo-sensitizing action [89].

3.5. External phase to create *in situ* forming microparticles

To address the burst while maintaining a low viscosity of the system, a novel approach has been developed [31,80,90,91]. The drug-containing polymeric solution (inner phase) is thereby emulsified with an oily or aqueous external phase containing a stabilizer. Droplets of the internal phase solidify upon contact with body fluids to form *in situ* forming microparticles (ISM).

One advantage of such a formulation is that it has lower viscosity than ISI formulations, because viscosity is determined by the external phase [63]. Consequently, higher polymer concentrations can be used without rendering injection difficult or painful. Additionally, ISM showed both reduced burst [31,32] and lower myotoxicity [92], as external phase creates a supplementary barrier slowing down solvent/drug leaching and water entry during solidification. Another advantage is the regular shape of formed ISM, which is determined by the size of the previous emulsion droplets and therefore minimizes morphological variations hence providing a more consistent and reproducible drug release than ISI. Nevertheless, as for every emulsified system, despite the use of surfactants, the main drawback could be a lack of emulsion stability. In the case of ISM, as the emulsion is extemporaneously prepared, this does not challenge the injection itself. However the coalescence of emulsion droplets could occur during their solidification [60].

Similarly to ISI, influence of several parameters on ISM systems has been studied [31,32,46,80,90]. In the same manner as ISI, burst decreases depending on the solvent and polymer used, increasing polymer concentration or decreasing drug loading. Additionally, higher viscosity of the external phase, faster emulsification rate or lower internal/external phase ratio can also contribute to burst reduction. In this respect, Li et al. [93] optimized the preparation of vinpocetine-ISM using a spherical symmetric design–response surface methodology considering drug loading, surfactant concentration and internal/external phase ratio. Optimized ISM had a very smooth surface and displayed a very gradual release with low burst *in vitro* (6.64% released the first day).

4. Remaining limitations to be addressed

In situ forming implants are promising galenic tools to administer, protect and release a wide range of compounds in a sustained fashion. They avoid frequent administrations, painful surgical procedures, allow localized or systemic drug delivery and are self-eliminating. And yet only two products have been commercialized until now. This could be potentially explained by the several issues which have to be solved: (i) drug burst release, (ii) safety and tolerability, (iii) reproducibility, and (iv) sterilization and stability. Means of controlling the first point have been extensively developed above; remaining points will be detailed below.

4.1. Safety

Despite the fact that some ISI products have already been commercialized, injection of organic solvent(s) remains a cause for concern. To choose the best solvent is quite difficult, because only little toxicological data concerning their parenteral administration are available (Table 2). However, NMP, DMSO, BA, BB and glycofurol have been used in injectable products for human use [94,95] and 2P and TA in veterinary products [96].

Only few toxicological and histological studies of ISI are reported in the literature, on several animal species and with sometimes contradictory results. For example, Bodmeier et al. used a rat model to evaluate the acute myotoxicity after single intramuscular injection [92,97]. In the first study, they tested *in vitro* NMP, DMSO and 2P as pure solvents: only 2P toxicity was significantly lower than the positive control [92]. Injections of ISI based on these solvents led to toxicities comparable to pure solvents. In the second study, they tested BA, ethyl acetate, propylene carbonate, TA and triethyl citrate and except for ethyl acetate, all solvents caused high muscle toxicity, especially BA [97]. The evaluation of muscle damage during 72 h *in vivo* after intramuscular injection of ISI or ISM based on 2P or ethyl acetate revealed that myotoxicity of ISI was not different from pure solvents, while ISM were found to be much less myotoxic because of the 1 to 10-fold dilution of the solvent with the external phase. Results from a study conducted over a longer time frame on monkeys are not consistent with the foregoing [98]. Authors injected animals both subcutaneously and intramuscularly with ISI formulations based on NMP or DMSO. No safety concern appeared, as animals maintained normal behaviors during the study and histological analysis after 1 month showed tissue reaction similar to those reported for usual preformed biodegradable implants. However, recent concerns about the reproductive toxicity of NMP administered orally and dermally resulted in a tightening of the permitted daily exposure information included in the International Conference on Harmonisation guidelines Q3C (R5) on impurities in 2006.

Finally, in rabbits, ISI based on glycofurol or on a mixture of BB and BA showed no irritation after subcutaneous or intramuscular injections and/or normal inflammatory and foreign body reactions similar to blank and pure drug solutions [33,38,99]. In addition to the modulation of solvent mixture hydrophobicity, the advantage to use BA is its local anesthetic effect, which could avoid or reduce pain during injection, especially for viscous solutions [5].

Overall, ISI formulations appear to be well tolerated. Systems with hydrophobic solvent are thought to be less irritating, as solvent diffuses more slowly into the surrounding tissues. For the same reason, ISM systems are generally regarded as less irritant than ISI, because the external oily phase forms a barrier between the muscle and the internal phase, thus limiting the amount of solvent in immediate contact with the muscle after injection.

To conclude, toxicity evaluations of ISI seem to differ depending on the route of administration, the method employed, the time of evaluation and eventually the animal model. Extensive toxicological studies, with a more harmonized methodology, are really needed to widen the future of ISI. They will represent an important cost, but also a beneficial step for other drug delivery systems, as many of the newly discovered drugs have low water-solubility. New solvents such as low molecular poly(ethylene glycol) (PEG) have been introduced [100–102]. Nevertheless, authors reported that conventional PEG accelerates the degradation of PLGA in solutions [61] by a trans-esterification mechanism [101]. As a result, significant improvement was obtained by alkyl-capping of the polymer ends. Additionally, transient edema was reported with PEG [103].

An alternative approach to enhance the safety of *in situ* systems is to reduce or suppress the need of organic solvents through the development of new polymers. For example, alkyl substituted polylactides were recently synthesized from the monomer 2-hydroxyoctanoic acid [104]. Resulting biodegradable polymers ("hexyl-substituted

polylactides" or "hex-PLA") form viscous solutions, are injectable without or with only small amount of NMP (<5%) and showed good biocompatibility [105].

4.2. Reproducibility

A key feature of drug delivery systems is the reproducibility of the drug release characteristics. This is obviously conditioned by reproducibility in terms of shape, size and structure. Structure of ISI is highly dependent on the components and rate of phase inversion, as discussed above. Concerning shape and size, however, they are influenced by several factors related to the formulation, the administration procedure and the environment [7].

As the solidification of the *in situ* systems takes place at the site of injection, it is easily understandable that it will be particularly sensitive to environmental changes. Accordingly, good correlations between polymer precipitation and drug release have been obtained both *in vitro* and *in vivo* while establishing correlations between *in vitro* and *in vivo* drug releases remains challenging [42,74]. Even between *in vitro* results, comparisons are difficult, as several protocols for drug release assays from ISI are described in the literature. Variations include the composition, volume and pH of the aqueous medium, use of shaking device, dialysis membrane [31] or even home-made structures [106] and agarose phantoms [42].

Only a few studies have been conducted to evaluate how the aqueous environment composition influences the system *in vitro*. Brodbeck et al. found that organic salt or small chain triglyceride in the aqueous bath impact demixing kinetics and lysozyme release (PLGA 50% m/m in NMP, TA or EB) [14]. Formulations based on solvent with high affinity for the non-solvent bath (NMP), however, were less strongly affected. In another study, quenching of a PLGA 50% m/m/DMSO solution with water, a phosphate buffer solution or with horse serum did not result in any change neither in phase inversion dynamic nor in the depot morphology [41]. The use of DMSO, a freely water miscible solvent, in this latter study is a potential explanation for the lack of change.

In addition to potential differences in the external tissue surroundings composition (proteins, salts, acids etc.), one of the main factor of influence *in vivo* is the tissue stiffness. ISI were indeed originally intended to be injected subcutaneously or intramuscularly. Nevertheless, emerging applications are moving towards more unusual sites of injection, such as directly into specific area (the eye [107], the brain [108]), physiological pockets [12,26,27], tumors [109–111] or bone defects [13]. This is achieved due to the capacity of ISI to adjust readily to the surrounding tissue, providing a high level of contact. However, the final shape of the injected implant will affect the diffusion conditions and thus drug release. Patel et al. [110] obtained for instance uniform spherical shape after *in vitro* injection, when flat disc like shape was observed after subcutaneous injection and multi-lobular shape after intratumoral injection. Additionally, they tested three different PLGA in ISI injected subcutaneously; fluorescein burst release was always higher *in vivo* than *in vitro*. They attributed this trend to the limitation of implant swelling and expansion *in vivo*, due to interstitial pressure or compressive forces exerted by the surrounding tissue. Other authors proposed the use of ISM systems to improve reproducibility, as regular shape is ensured by the emulsion droplets solidification [90].

Injection sites might also diverge in terms of tolerance. *In situ* forming formulations are generally described as well tolerated, although a fibrous capsule surrounding the implant has been described [103]. Such a structure would constitute a barrier impeding water/solvent exchanges and drug release. Moreover, entrapment of polymeric oligomers produced during degradation within the capsule might result in a lower pH inside the matrix and thus accelerated matrix degradation. In this respect, non-invasive imagery techniques (electron paramagnetic resonance [112], magnetic resonance imaging [103], and ultrasound imaging [42,74,110]) can be useful for the real-time monitoring of ISI. They provide information about the implant itself (solidification, shape, size,

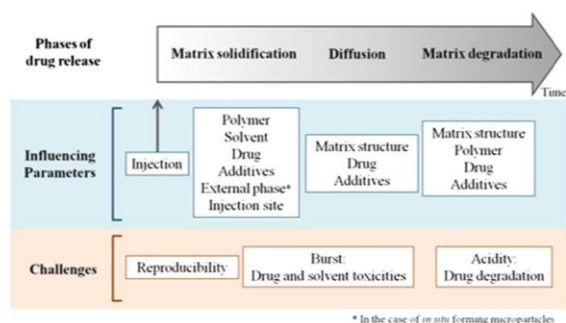


Fig. 9. The three phases of drug release from ISI and critical parameters affecting them.

and disappearance) in addition to the eventual biological response to implantation (edema, inflammation, and encapsulation).

Finally, the conception of injection devices in order to standardize the deepness and the speed of injection might broaden clinical applications of ISI in the future.

4.3. Industrial development

From an industrial point of view, *in situ* forming systems provide several benefits. First, the development of these injectable sustained-release formulations might extend the life cycle of a drug. Second, preparation process is very simple, accomplishes essentially 100% encapsulation efficiency and avoids the use of high temperatures or high shear methods which can be deleterious for fragile compounds. Consequently, a wide range of molecules can be incorporated into ISI, including peptides and nucleic acids.

ISI formulations are intended to be injected into the body and thus have to fulfill parenteral requirements including sterility. Gamma-irradiation is widely accepted for terminal sterilization of biodegradable polymer systems such as ISI since it results only in a slight decrease in molecular weight (e.g. [13,82]). If the drug is not stable into the polymeric solution, it can be provided as a lyophilizate in a separate syringe: sterilization is obtained *via* sterile filtration or aseptic manufacturing. Dong et al. [61] have also proposed to freeze-dry a drug-containing PLGA solution in dioxane or acetic acid. The obtained sponges can be dissolved in the ISI solvent just before injection. However residual dioxane or acetic acid can be a problem for this approach.

Interestingly, delivery time of drugs from ISI can be modulated over a large range. Consequently ISI offer an alternative to oral (24 h), transdermal systems (1–7 days) and conventional implants (months–years).

A key element of the *in situ* systems is the polymer: safe use history, FDA approval and wide range of products available have made PLA and PLGA very popular. Nevertheless, these synthetic polymers are costly (2000–6000 \$/kg) and change in supplier as well as batch-to-batch variations could lead to different properties. For example, copolymer microstructure (i.e. alternation of GA/LA units) is strongly influenced by the polymerization conditions, which determine the importance of the main secondary reactions (redistribution reactions) during the polymerization process [65]. Furthermore, despite PLGA non-toxicity and good *in vivo* degradation properties, polymer–drug interactions or acidic microenvironment created during the degradation may represent additional obstacles. Switch towards other polymers ongoing surface erosion or homogeneous bulk erosion could represent a valid approach.

The use of potentially toxic organic solvents, however, remains a major issue for these formulations. Therefore, toxicological studies and the elucidation of new solvents could help to further develop ISI systems.

Finally, a comprehensive overview of the main points discussed in the different sections of this review is proposed in Fig. 9.

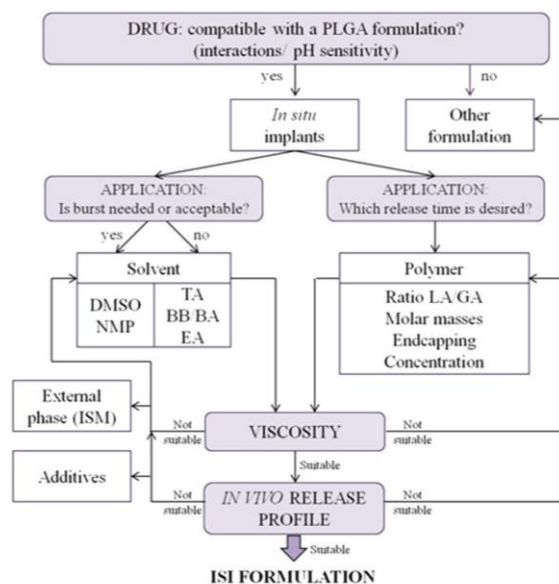


Fig. 10. Schematic representation for a rational design of *in situ* implants. DMSO: dimethylsulfoxide; NMP: *N*-methyl-2-pyrrolidone; TA: triacetin; BB: benzyl benzoate; BA: benzyl alcohol; EA: ethyl acetate; LA: lactide; GA: glycolide; ISM: *in situ* forming microparticles.

5. Conclusion

Polymeric *in situ* implants formed by phase separation are promising minimally invasive parenteral formulations applicable in many therapeutic fields. A loading dose followed by a sustained release at lower concentration may be desirable especially for hormonal castration, anticancer therapy, for antibiotic applications as well as for local anesthesia. Apart from the traditional use as delivery systems for chemical compounds, *in situ* implants also offer potential uses as gene delivery platform or tissue repair scaffold. In the future, one might imagine further applications, for example in vaccination or in allergic desensitization, as already shown with other polymeric forms [113,114]. Finally, composite forms might also be designed, where ISI will allow site-specific delivery of other drug-containing systems. One example is already encountered in the literature: Yehia et al. demonstrated recently the feasibility of ISI-containing lipospheres [115].

This review gives an overview of the actual knowledge about design and performance of *in situ* forming implants and it offers the keys to a rational development of products (Fig. 10) with potential benefits in terms of costs and patient compliance.

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3.4. Conclusion

De nombreuses formulations à base de PLGA précipitant *in situ* ont été développées depuis leur découverte dans les années 1990. Les principes actifs incorporés présentent des propriétés physicochimiques variées (masse molaire, coefficient de partage, constante d'ionisation). Néanmoins, seulement deux formulations ont pour l'instant été mises sur le marché. Ceci s'explique en partie par la nécessité d'optimiser la formulation pour chaque principe actif/application visée, ce qui peut demander un investissement financier et temporel important, étant donné les nombreux paramètres pouvant être modulés.

Cependant, ces formulations pourraient représenter une approche viable pour la délivrance prolongée de RSNO car le procédé d'incorporation du principe actif est simple, doux et se fait de façon extemporanée, ce qui est favorable dans le cadre de molécules sensibles.

4. OBJECTIF DU TRAVAIL EXPERIMENTAL

Le but de ce travail est donc de démontrer la faisabilité de l'utilisation de formulations *in situ* à base de PLGA pour améliorer la biodisponibilité des RSNO.

Un travail de thèse précédemment réalisé au sein de l'équipe (thèse de Javier Camargo soutenue en 2010) avait consisté à optimiser des formes *in situ* de type implants ou microparticules pour la libération prolongée d'ivermectine (un antiparasitaire lipophile pour une application vétérinaire), en faisant varier différents paramètres (type de polymère et sa concentration, type de solvant, ratio phase interne/phase externe pour les microparticules). Ces travaux préliminaires, même s'ils ne portent pas sur un RSNO, ont permis de fixer des paramètres de formulation qui seraient applicables à nos molécules. En effet, une libération prolongée sur 30 jours *in vitro* (7 jours *in vivo*) a été obtenue pour ce principe actif lipophile ($\log P = 3,2$) avec une faible libération initiale (*burst*). Comme l'un des objectifs du *drug design* effectué sur les RSNO était d'augmenter l'hydrophobie des molécules pour atteindre des coefficients de partage de cet ordre de grandeur, il a été décidé de se baser sur les résultats optimisés pour l'ivermectine pour sélectionner les paramètres des formulations testées pour les RSNO. Par conséquent, un seul type de polymère a été retenu pour ce travail (PLGA 50 :50, M_w 7 000 – 17 000 g/mol), à une concentration donnée (20 % m/m dans la solution polymérique), en conservant cependant deux types de formulations (implants ou microparticules) et deux solvants différents (*N*-méthyl-2-pyrrolidone, NMP et triacétine, TA). Deux RSNO ont été incorporés à ces formulations (GSNO et SNAP). En effet, les expériences de *drug design* n'ont pas abouti à d'autres composés, essentiellement à cause de problèmes de pureté et d'isolement des molécules obtenues.

Le travail expérimental doit donc répondre à la question : les formulations *in situ* sont-elles adaptées aux RSNO ? Pour cela, les sous-objectifs soulevés sont les suivants :

- Les formulations *in situ* permettent-elles de libérer de façon prolongée les RSNO *in vitro* ?
- Est-ce que le profil de libération dépend de l'hydrophobie de la molécule ?
- De quelle façon le type de formulation (ISI, ISM) et le type de solvant (NMP, TA) influencent-ils la libération des RSNO ?
- Existe-t-il une différence en termes de formation de la matrice, de structure de celle-ci et de vitesse de dégradation entre l'*in vitro* et l'*in vivo* ?
- Les formulations *in situ* permettent-elles de libérer de façon prolongée les RSNO *in vivo* ?
- L'effet produit est-il local ou systémique ?
- La libération (en termes d'effet ou de concentrations en espèces circulantes) est-elle plus prolongée *in vivo* avec les ISM qu'avec les ISI ?

Pour répondre à ces différentes problématiques, le travail expérimental a consisté dans un premier temps à vérifier la pureté des RSNO isolés obtenus. Dans un deuxième temps, ils ont été incorporés

aux différentes formulations *in situ* et leur libération *in vitro* a été étudiée. Les formulations à base de NMP ont été sélectionnées pour les expériences *in vivo*. Une caractérisation des formulations (morphologie, dégradation du polymère) a été effectuée et a permis de mettre en évidence des différences entre les formulations et entre les environnements *in vitro/in vivo*.

ÉTUDE EXPÉRIMENTALE

1. SYNTHÈSE ET CONTRÔLE QUALITÉ DES S-NITROSOETHIOLS

1.1. Article 4 : « A complete physicochemical identity card of *S*-nitrosglutathione »

Ainsi que précédemment mentionné, la synthèse de *S*-nitrosoethiols comme le GSNO « à façon », c'est-à-dire directement par les utilisateurs, est assez aisée (une seule étape). Ceci entraîne la multiplication des protocoles dans la littérature (ratio thiol/nitrite, temps et température d'incubation, etc.), qui ne comprennent que rarement une étape de purification. De plus, le contrôle qualité de la molécule obtenue, quand il existe, est souvent limité à la mesure de son absorbance à une longueur d'onde variant de 330 à 340 nm selon les auteurs pour GSNO. Cependant, les produits de réaction, les intermédiaires réactionnels ou les produits de dégradation peuvent modifier la pureté, la stabilité et/ou l'activité du RSNO obtenu. De plus, pour envisager une application thérapeutique, il est vital que le composé actif soit parfaitement défini et caractérisé. Aussi, alors que le *drug design* semble constituer l'un des piliers de l'amélioration des RSNO, il est nécessaire de pouvoir évaluer la qualité et la pureté des produits obtenus.

En conséquence, un protocole de synthèse du GSNO avec étapes de précipitation et de purification a été mis en place, ainsi qu'un contrôle qualité de la molécule obtenue, à la fois par mesure de l'absorbance à 334 nm, par la méthode de Saville-Griess et par chromatographie liquide haute performance (Article 4, paru dans *Current Pharmaceutical Analysis*).

A Complete Physicochemical Identity Card of *S*-nitrosoglutathione

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Abstract: *S*-nitrosoglutathione (GSNO) is one of low molecular weight *S*-nitrosothiols occurring in humans. Nowadays, it is widely used as a nitric oxide donor for *in vitro*, *ex vivo* and *in vivo* experiments related to the investigation of its pathophysiological role as well as in clinical trials, aimed at its potential therapeutic use. Despite numerous reports on this physiological molecule, its quality control does not match the criteria required by competent pharmaceutical authorities. Hereby, an extensive physicochemical characterisation of synthesised and purified GSNO is provided for the first time. Indeed, structural identification including spectrometric, thermal and elemental analyses was consistent with the GSNO structure. An ion-pairing reversed phase HPLC system was developed to assess (i) GSNO content with UV detection at 334 nm, and (ii) fingerprint of its impurities coming from synthesis process and/or storage conditions, at 220 nm. The as-synthesised product showed a content of 102.5 %, with respect to a commercially available standard. The identified impurities, *i.e.* chloride, nitrite, nitrate, reduced glutathione and glutathione disulfide, were also quantified basing on pharmaceutical requirements. Main products released during various storage conditions (pH, temperature, dioxygen, ...) were disulfide glutathione and nitrite ion. Recommendations are given for the safe use of GSNO in biological and pharmacological experiments.

Keywords: Content, ICH guidelines, Impurities, Ion-pairing reversed phase HPLC, *S*-nitrosoglutathione synthesis, Stability, structural identification.

1. INTRODUCTION

Nitric oxide (nitrogen monoxide, NO radical, NO[•]) plays an important role in many physiological processes, especially in vascular tone homeostasis. The decrease of its concentration can lead to numerous pathophysiological events; therefore, it is of considerable importance to find and characterise suitable NO[•]-donors that may be used for the treatment of arterial hypertension and cardio-vascular failure.

Nitric oxide exhibits a very short half-life, *i.e.* less than 1 s in blood [1] and must be delivered as a pro-drug such as an organic nitrate (*e.g.* nitroglycerin, isosorbide dinitrate or mononitrate) [2]. These common drugs are known to provide a fast NO[•] release concomitant with the induction of oxidative stress and frequently tolerance. To limit such side effects, research is now focused on innovative NO[•] donors such as *S*-nitrosothiols (RSNO), especially *S*-nitrosoglutathione, GSNO, (*N*-(*N*-*L*- γ -glutamyl-*S*-nitroso-*L*-cysteinyl)-glycine, CAS number: [57564-91-7]) (Fig. 1). This latter is an endogenous NO[•] donor, which is found in human plasma at concentration values in the range of 90 to 270 nM [3]. Due to its safety (since NO[•] release is

accompanied by glutathione production), GSNO appears as a leader molecule both as a tool to investigate biochemical or pharmacological mechanisms of NO[•] action and for therapeutic applications. As a result, GSNO is actually the most studied RSNO, using various *in vitro*, *ex vivo* and *in vivo* models [4-7]. GSNO has also been included in clinical trials (Table 1). In these studies, GSNO was administered *via* parenteral or topical routes. Up to now, only dozens of people were included into each study and a good tolerance of the drug was observed (few side effects on few patients were reported like headache, palpitation and hypotension). All these arguments lead to the conclusion that GSNO may be a drug in the making, or at least a tool to investigate physiological mechanisms.

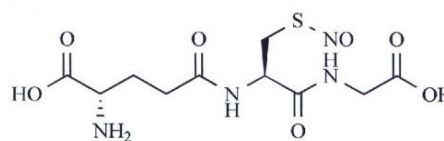


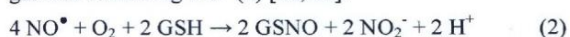
Fig. (1). *S*-nitrosoglutathione structure.

This molecule is commercially available but it can also be easily synthesised according to three main synthetic pathways. The most commonly one uses nitrite ion and reduced glutathione (GSH) in a 1:1 stoichiometric ratio under acidic conditions as follows (1):

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Nonetheless, GSNO can also be produced using either a gas mix containing NO[•] (2) [22, 23]:



or by a transnitrosation reaction with another RSNO (3) [24, 25]:



The yield of the synthesis is commonly provided by a unique physico-chemical test based on the molar absorbance at 334 nm of 922 M⁻¹cm⁻¹, first defined by Hart [26].

S-nitrosoglutathione preparations used in *in vitro/ex vivo* and *in vivo* studies are very frequently not completely characterised and mostly as a non-purified product in home-made solutions without any accurately defined content. Moreover, a discrepancy is noted in the literature concerning the possible pharmacological effect of GSNO impurities, *i.e.* nitrite and nitrate ions [16, 27, 28]. Besides, some clinical studies (Table 1) are now clarifying the role of NO[•] in

parallel with RSNOs in the vascular and cardiac systems. Therefore, as GSNO is on the way to go from bench to bedside, there is a great need to introduce a complete physicochemical control of the drug, based on classical pharmaceutical requirements (International Conference on Harmonisation (ICH) [29, 30] and European Pharmacopoeia 7th ed [31]) to ensure the quality of the administered product.

In this study, an extensive physicochemical characterisation of GSNO is described including structural evaluation, content and both identification and quantification of impurities which may result from synthesis and storage conditions [32]. To the best of our knowledge, it is the first time when numerous experimental data have been gathered, permitting a full characterisation of GSNO for further biological and pharmacological studies, focusing on its content and impurities. Special emphasis is also paid to the impurities fingerprint in order to highlight how important is the purification step. Finally, this work brings some key recommendations to use GSNO as a well-characterised and safe product.

Table 1. Summary of clinical trials described in the literature with administration of GSNO. (In all studies, a research ethics committee was consulted.)

Targeted application	Administered dosage	Number of included Patients	References
Reduction of cerebral embolisation			
Carotid angioplasty, carotid endarterectomy and/or recurrent stroke and/or systemic embolisation	Intravenous infusion at a rate of 2.2 nmol/kg.min and if tolerated, increased to a rate of 4.4 nmol/kg.min after 10 min for 90 min	24	[8]
		28	[9]
		20	[10]
		16	[11]
Inhibition of platelet activation and/or vasodilatation and/or effect on blood flow			
Percutaneous transluminal coronary angioplasty	Intracoronary infusion of 40 nmol/min 10 min before surgery and continued for 10 min after	13	[12]
Anti-thrombotic effect	Intravenous infusion 50 µg/min and increased by 50 µg/min every 5 min to a maximum of 250 µg/min after 20 min. The dosage was maintained for 10 min	10	[13]
Coronary artery saphenous vein grafts	Intravenous infusion of 40 nmol/min	24	[14]
Severe preeclampsia	60 - 90 min intravenous infusion of 50 - 250 µg/min	10	[15]
Systemic vasodilatation	1 - 4 µmol <i>via</i> intravenous route	31	[16]
Dermal wound dressings and local vasodilatation in ischemic tissues	22.5 - 42 µmol loaded into polymer films applied on skin	7	[17]
Wound healing	0.3 mol GSNO/g polymer hydrogel applied on skin	8	[18]
Female sexual disorder	100 µM of a GSNO gel applied on clitoris	40	[19]
Replenishment of GSNO pool			
Effect on left ventricular function	Intravenous bolus of 0.5 µmol	12	[20]
Effect on cystic fibrosis	0.05 ml/kg of a 10 mM GSNO solution aerosolised	20	[21]

2. MATERIALS AND METHODS

2.1. Chemicals and standards

All solvents and chemicals were of high purity (> 99%). Tetrabutylammonium hydrogen sulfate (TBAHS) and GSNO (when used as a standard) were obtained from Sigma-Aldrich (France). Ultrapure deionised water (> 18.2 MΩ cm) was used to prepare all solutions.

2.2. *S*-nitrosoglutathione synthesis

S-nitrosoglutathione was obtained by synthetic pathways either without further purification (i) or as purified product (ii), as follows:

(i) A 10 mM NaNO₂ solution was prepared in deoxygenated water using ultrasound bath. 62 mg of GSH were weighed in a 10.0 mL volumetric flask and dissolved in *ca.* 7 mL of deoxygenated 0.5 M HCl. Then, 1.0 mL of the NaNO₂ solution was added, and the volume was filled up to 10.0 mL with 0.5 M HCl. The resulting mixture was kept in the dark at 4 °C for 1 h. Afterwards, 0.45 mL of a 40 % (w/v) NaOH solution was added and the volume was finally completed to 20.0 mL with phosphate buffer (pH 7.4; 0.5 M). The solution was stored at 4 °C for a maximum period of 7 days.

(ii) 3.1 mmol (960 mg) of GSH were dissolved in 5.0 mL of 0.626 M HCl cooled to 4°C under nitrogen flow. Then 3.1 mmol (215 mg) of NaNO₂ were added and the mixture was stirred at 4 °C for 40 min in the dark. 5 mL of cold acetone were then added to precipitate GSNO. The purification step was performed by filtration of the pink product which was then successively washed with acetone-water (80/20, v/v) (10 mL), acetone (2 × 10 mL), and diethylether (3 × 10 mL). The product was then dried under vacuum (15 mm Hg) for 30 min at room temperature (22 ± 2 °C) and immediately stored at -20 °C under inert argon atmosphere. The nitrosation yield was about 70 %.

2.3. Griess and Saville-Griess methods

Nitrite and GSNO concentrations were determined by using Griess and Saville-Griess methods, respectively [1]. Briefly, samples were diluted with acetoacetic buffer (pH 2.5; 1.75 M) in order to obtain a final volume of 1 mL. Calibration curves were performed in the range 1 - 10 μM, using NaNO₂ and GSNO as standards for Griess and for Saville-Griess assays, respectively. What concerns the Griess assay, 200 μL of sulfanilamide solution (0.6 % (w/v) prepared in 0.4 M HCl) were added to the previously diluted sample. After a 3 min incubation in the dark at room temperature (22 ± 2 °C), 50 μL of *N*-1-(naphthyl)ethylenediamine solution (0.6 % (w/v) prepared in 0.4 M HCl) were added. After a 5 min incubation, absorbance was read at 540 nm. The same operating conditions were used for the Saville-Griess method, but the sulfanilamide solution was added with HgCl₂ (0.2 % w/v).

2.4. Instrumentation

UV-visible spectra were carried out on a double-beam spectrophotometer (UV 1700 model, Shimadzu, France).

Circular dichroism spectrum (solution of GSNO at 10 mM in water) was recorded in a CD6 dichrograph equipped with a CDMax software (Jobin Yvon, Longjumeau, France). Specific optical rotations were determined in a PolAAR 20 polarimeter (Particular Sciences, Ireland). Angles of rotation α of GSH (9.5 mM in water) and GSNO (9.5 mM, in 0.5 M phosphate buffer pH 7.4) were measured at 20 °C with a 1.9009 dm length polarimeter tube. Infrared analysis was realised on a Fourier-Transform InfraRed (FT-IR) spectrophotometer (Vector 22 model, Bruker, Germany) equipped with an ATR system (MIRacle Single Reflection Horizontal ATR, Pike Technologies, France), mass spectrum by using electrospray ionisation (Waters Platform, Canada) and ¹H NMR spectra using D₂O as solvent, on an Avance 300 spectrometer (Bruker, Germany). Multiplicities are reported as follows: s singlet, d doublet, t triplet, and m multiplet. Elemental analysis was performed on a Thermo Finnigan EA 1112 (Thermo Fisher Scientific, USA). Differential scanning calorimetry/thermogravimetry (DSC/TG) analyses coupled with mass spectrometer (MS) were conducted using a STA 409C equipment (NETZSCH-Gerätebau GmbH, Germany). A Karl Fischer coulometer (756 KF coulometer Metrohm, France) was used for titration of water content in purified GSNO (25 mg). A Clark electrode equipped with the Atelier Scientific software (Jeulin, France), was used to evaluate the dioxygen content of solutions. The theoretical dipole moment of GSNO was calculated with the Chemdraw software (CambridgeSoft, Perkin-Elmer, USA) using the MOPAC application.

2.5. HPLC system and operating conditions

The HPLC system consisted of an isocratic pump (L-6000 model, Hitachi-Merck, Germany), an injection valve equipped with a 20-μL loop (7725i model, Rheodyne, IDEX, USA), a column oven (Croco-Cil, Cluzeau, France), a UV detector (LKB Bromma, Sweden), and a data processing software (Azur[®], Datalys, France). The columns (250 × 4 mm I.D) were prepacked with two different stationary phases (5-μm particle size): LiChrospher[®] RP-18e (Merck, Germany) or Nucleosil[®] 100 C18 HD (Macherey Nagel, Germany). The mobile phase consisted of a mixture of methanol and phosphate buffer (10/90, v/v); the phosphate buffer was prepared as follows: 1 mM TBAHS and 0.1 mM EDTA were added to 10 mM KH₂PO₄ and the pH was adjusted to 6.8. Column temperature was 40 °C and flow rate was 0.8 mL/min (resulting back pressure was *ca.* 120 bars). UV detection was operated at either 220 or 334 nm.

GSNO solutions for HPLC analysis were prepared as follows: for the GSNO standard and the as-synthesised product, a 10 mM stock solution was obtained by dissolving the powder in phosphate buffer (pH 7.2; 10 mM KH₂PO₄; 0.1 mM EDTA). For the non-purified GSNO, the solutions obtained after synthesis (*ca.* 10 mM) were considered as stock solutions. Dilutions were performed with mobile phase before injection into the HPLC system. All solutions were protected from light, stored at 4 °C and used within 12 h for the content assay and/or the determination of impurities profile.

2.6. Calculation of GSNO constants

The specific optical rotation, the solubility of GSNO as well as the HPLC parameters were calculated according to the European Pharmacopoeia [31]. The molar ellipticity $[\theta]$ was calculated from delta absorbance (ΔA) given by the software with $[\theta] = 3300 \frac{\Delta A}{lc}$ where c is the concentration of the solution (M) and l is the length the cuvette (cm); $[\theta]$ is given in $^{\circ}\text{cm}^2/\text{dmol}$. The limits of detection (LOD) and quantification (LOQ) were expressed according to the ICH [29] using the slope and intercept of the linear regression of each calibration curve.

3. RESULTS AND DISCUSSION

The full characterisation including identification, determination of the exact content as well as the impurities fingerprint, is always a question that arises when pharmacological or clinical trials using a new product are performed. Therefore, the present study is presenting all these data for GSNO.

3.1. Properties of purified *S*-nitrosoglutathione

Isolated GSNO is a fine pink powder, sparingly soluble in water, practically insoluble in ethanol, acetone, diethylether and methylene chloride. The solubilities of GSNO in tested solvents are in accordance with its calculated dipole moment, *i.e.* 3.9 D.

3.2. Structural identification of purified *S*-nitrosoglutathione

The structural identification of purified GSNO and the characterisation of the sulphur-nitrogen bond were performed using classical physicochemical methods (Table 2 and (Fig. 2)). The molecular structure was fully assessed, first by using mass spectroscopy (MS). The protonated and deprotonated molecular ions of GSNO (calculated $M_r = 336.3$) were observed at either $m/z = 337.0$ or 334.9 , using detection in positive and negative modes, respectively. Signals corresponding to GSNO adducts with H^+ and Na^+ and the pattern of molecule degradation appeared as previously described [33]. Secondly, both elemental analysis and ^1H NMR spectrum were consistent with the expected structure (Table 2 and Fig. 2).

Table 2. Physicochemical properties of GSNO compared to GSH, using spectroscopic, thermal and elementary analyses

Method	GSNO	GSH
UV-visible	Maxima at 334 and 545 nm $\left(\frac{\epsilon_{334}}{\epsilon_{545}} = 61.3 \right)$	No maximum at $\lambda > 220$ nm
Fourier-Transform InfraRed (FT-IR)	Disappearance of the thiol stretching vibration (2525 cm^{-1}); N-O stretching vibration (1520 cm^{-1})	Thiol stretching vibration (2525 cm^{-1})
^1H NMR	4.69 (dd, $^3J = ^3J' = 4.6 \text{ Hz}$, 1H) 4.09 (dd, $^2J = 14.8 \text{ Hz}$, $^3J = 4.6 \text{ Hz}$, 2H) 3.97 (s, 2H) 3.82 (t, $J = 6.3 \text{ Hz}$, 1H) 2.47 (t, $J = 7.4 \text{ Hz}$, 2H) 2.23-2.10 (m, 2H)	4.59 (t, $J = 5.8 \text{ Hz}$, 1H) 4.00 (s, 2H) 3.85 (t, $J = 7.4 \text{ Hz}$, 1H) 3.01-2.90 (m, 2H) 2.58 and 2.57 (td, $^3J = 7.4 \text{ Hz}$, $^4J = 3.0 \text{ Hz}$, 2H) 2.19 (td, $^2J = ^3J = 7.4 \text{ Hz}$, 2H)
Thermal analysis DSC/TG-MS (onset temperature)	Two endothermic events: decomposition at $150.3 \text{ }^{\circ}\text{C}$ with a loss of 8.85 % of initial mass corresponding to a fragment with a $m/z = 30$ melting point at $195.3 \text{ }^{\circ}\text{C}$	One endothermic event: melting point at $194.9 \text{ }^{\circ}\text{C}$
Elemental analysis	Analysis (mean of two independent batches of synthesis) (Theoretical percentages)	Analysis (mean of two independent batches) (Theoretical percentages)
Carbon	34.9 % (35.7 %)	38.9 % (39.1 %)
Hydrogen	4.8 % (4.8 %)	5.6 % (5.6 %)
Nitrogen	15.8 % (16.7 %)	13.6 % (13.7 %)
Sulfur	9.6 % (9.5 %)	10.0 % (10.4 %)
Oxygen	32.4 % (33.3 %)	31.7 % (31.2 %)

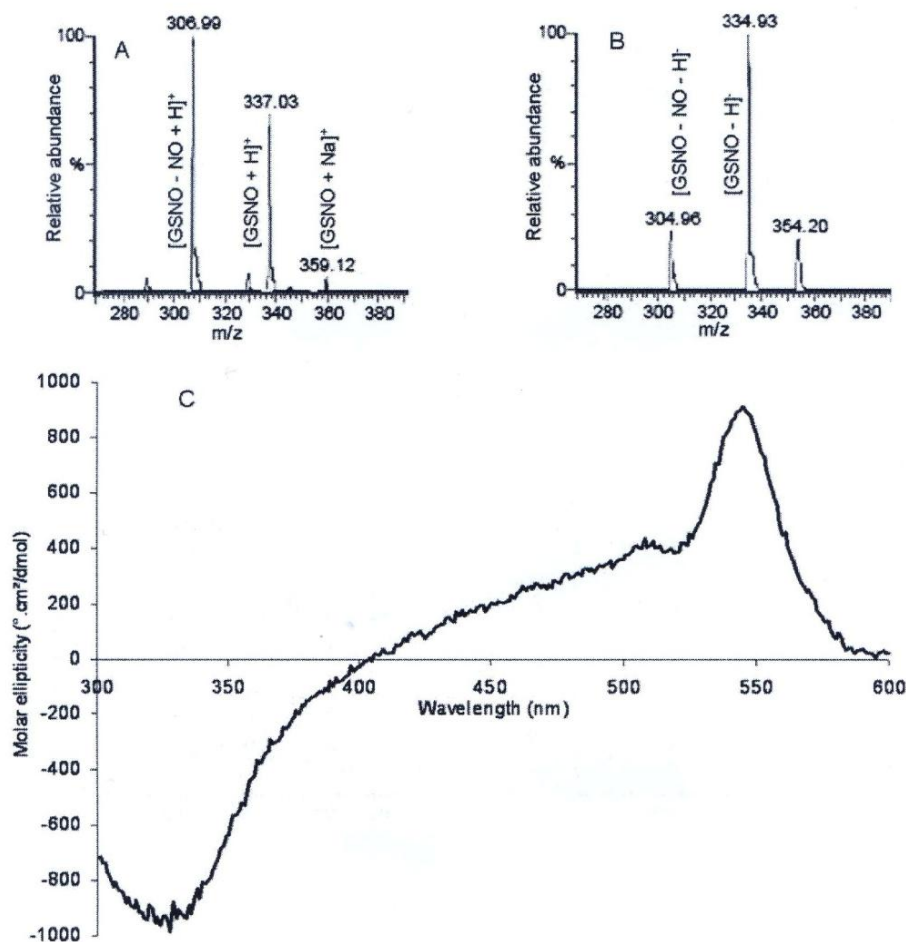


Fig. (2). Mass spectra using (A) positive and (B) negative ionisation modes and (C) circular dichroism spectra of the purified GSNO.

In the ^1H NMR spectrum, the methylene group linked to the thiol function was shifted from 3.01 - 2.90 ppm in GSH to 4.09 ppm after nitrosation. Then, the sulphur-nitrogen bond was considered by UV-visible, circular dichroism and FTIR spectroscopy, as well as thermal analysis. The *S*-nitroso-modification of glutathione was signed by maxima at 334 and 545 nm in the UV-visible spectrum due to $\pi \rightarrow \pi^*$ and $n_{\text{N}} \rightarrow \pi^*$ transitions, respectively [26]. The circular dichroism spectrum of the molecule (Fig. 2) was in accordance with such a modification since two maxima appeared: one negative at 334 nm with a molar ellipticity, $[\theta] = -935 \text{ } ^\circ\cdot\text{cm}^2/\text{dmol}$ and one positive at 545 nm with $[\theta] = 915 \text{ } ^\circ\cdot\text{cm}^2/\text{dmol}$. This latter result was in accordance with the optical rotation evaluated at $63 \text{ } ^\circ\cdot\text{mL}\cdot\text{dm}^{-1}\cdot\text{g}^{-1}$ at 589.3 nm, suggesting again a molecule different than GSH ($15 \text{ } ^\circ\cdot\text{mL}\cdot\text{dm}^{-1}\cdot\text{g}^{-1}$). FT-IR analysis showed on one hand the disappearance of the thiol stretching vibration (at 2525 cm^{-1}) and on the other hand, the appearance of the nitrogen-oxygen vibration at 1520 cm^{-1} . Finally, thermal analyses were performed with DSC and TG coupled with MS. The results

confirmed the grafting of one nitroso group onto GSH, since the first endothermic event at $150.3 \text{ } ^\circ\text{C}$ was parallel with a mass loss of 8.85 %, corresponding to NO molar mass, *i.e.* $m/z = 30$. The melting point of GSH, corresponding to the second endothermic event in GSNO analysis, was recovered at *ca.* $195 \text{ } ^\circ\text{C}$. Altogether, the different analyses were consistent with the structure of GSNO as the main product resulting from synthesis.

3.3. Development of the HPLC method and its suitability test

Liquid chromatographic methods devoted to RSNO have already been reported [3, 4, 6, 34, 35]. Most of them depend on the use of reversed-phase columns eluted with acidic, neutral or slightly alkaline mobile phases and they are mainly aimed at separating various RSNO, especially metabolites of GSNO [6]. An ion-pairing reversed phase HPLC (IP-RP-HPLC) system using a cationic detergent, *i.e.* TBAHS, has already been proposed [35]. In our hands, only this latter system permits to obtain baseline separation of

very polar impurities resulting from GSNO synthesis and degradation, especially inorganic species such as chloride, nitrite and nitrate ions. Indeed, as we were expecting to find these by-products, we made some trials with a commercially available ion chromatographic system but the highly alkaline mobile phase used with the anion column induces GSNO precipitation. Therefore, the method described by Tsikas *et al.* [35] was used with the following modifications: the use of methanol instead of acetonitrile and ten-times lower concentrations of TBAHS and EDTA; a slightly acidic mobile phase (pH value equal to 6.8) was retained in order to preserve integrity of the silica-based stationary phase and the analyte. Although the presently reported HPLC system needs a long equilibration period (about 3 h) to obtain stable and reproducible retention times (corresponding to the maximum

adsorption of TBA on the stationary phase), it seems to be a good compromise as a method to determine both GSNO content and its impurities profile at the same time (using a diode array detector for example). As a matter of fact, it is worthwhile to use a unique separative system from a practical and financial point-of-view to operate both content assay and impurities profile. Two different C18 columns were tested and they gave rise to very similar data concerning the chromatographic behaviour of considered analytes. Detection was operated at 334 nm to evaluate the product content, and at 220 nm to detect impurities. Indeed, the wavelength value of 334 nm corresponds to a specific absorption band exhibited by all RSNO due to the S-N bond (see 3.2). Typical chromatograms are shown in (Fig. 3).

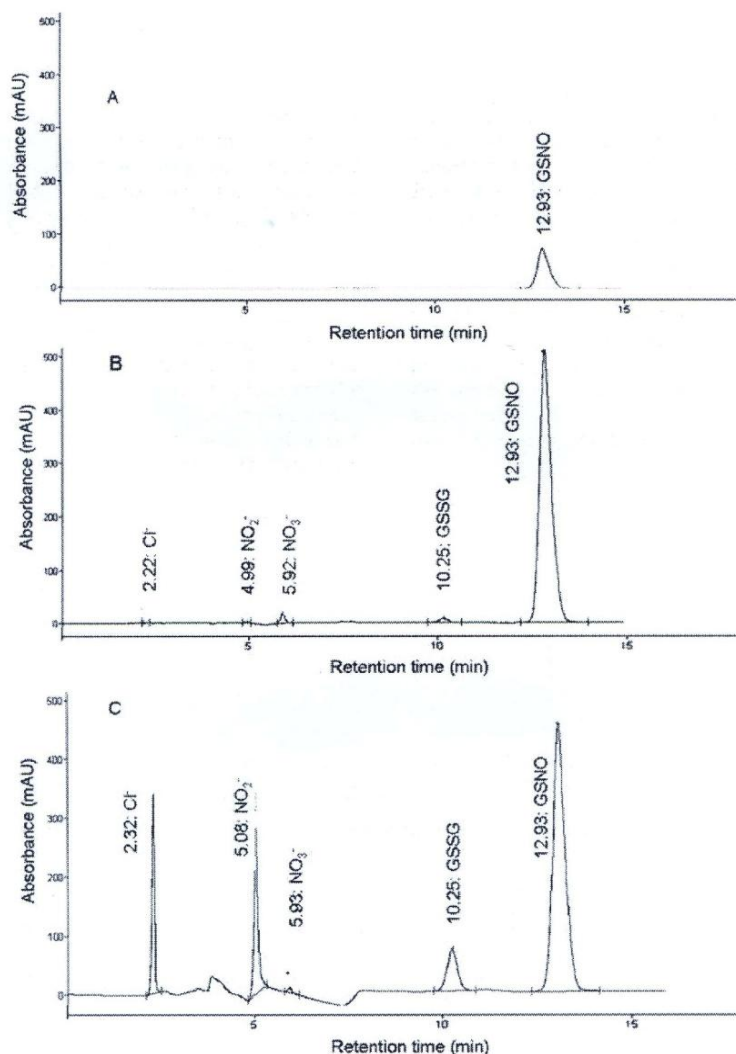


Fig. (3). Typical chromatograms corresponding to either the as-synthesised and purified GSNO with detection wavelength at 334 nm (A), and 220 nm (B), or the extemporaneously obtained product without any purification (C) at 220 nm. Each product was injected at a concentration of 1 mM. Chromatograms were recorded using Nucleosil® 100 C18 HD (similar results were obtained on LiChrospher® RP-18e).

The suitability test was built on three main parameters which appear and play an important role in the method robustness: first, GSNO retention time and column efficiency expressed as number of theoretical plates (N) calculated for GSNO peak, second, resolution factor (R_s) between the impurities characterised by the closest elution times, and third, the relative standard deviation (RSD) of GSNO peak area calculated with triplicate injection. It is well known that use of tensioactive agents as counter-ions in mobile phase leads to a loss of hydrophobic capping on the stationary phase resulting in a decrease of analytes retention. Despite using slightly acidic pH and ten times less counter-ion than Tsikas *et al.* [35], we observed this phenomenon relatively rapidly (over a 150-h period) when using the two different stationary phases, thus it becomes obvious that performances should be daily checked. The suitability test agreement needs: (i) a GSNO retention time higher than 9.5 min (retention factor, k, higher than 2.6) and N higher than 7000, (ii) resolution between peaks corresponding to nitrite and nitrate ions higher than 2.8 (injecting a solution of nitrite and nitrate ions at a concentration of 0.1 mM and operating detection at 220 nm), and (iii) a relative standard deviation (RSD) value for GSNO peak area (injecting GSNO at a concentration of 1 mM and operating detection at 334 nm) lower than 1.2 % (n = 3).

3.4. Content determination

This study proposed to assess GSNO content by using the HPLC method with UV detection at 334 nm. A calibration curve was performed using the standard (water content: 1.4 % (m/m) given by the supplier) and Saville-Griess method as a reference. On the one hand, the linearity of the HPLC method was checked in the range 0.50 - 5.00 mM, with corresponding equation of the regression line: A (peak area of GSNO, 334 nm) = 1 279 000 × [GSNO] (± 9 000) - 2.2 (± 18.7); coefficient of determination, $r^2 = 0.999$ (6 points, n = 5). LOD and LOQ were calculated and the values found were 0.05 and 0.15 mM, respectively. Finally, repeatability, intermediate precision and accuracy of the HPLC method for GSNO measurement were evaluated. The values of RSD were equal to 1.8 % for repeatability (6 determinations at 100 % of the test concentration, *i.e.* 1 mM) and 2.6 % for intermediate precision (determined by 3 injections of the 100 % test solution over 2 days). Accuracy was evaluated at 100.34 ± 1.26 % (5 points, n = 5). On the other hand, the linearity of the colorimetric Saville-Griess method was tested in the range of 1.0 - 10.0 μM, with corresponding equation of the regression line: $A_{540\text{ nm}} = 41400 \times [\text{GSNO}] (\pm 700) - 0.002 (\pm 0.002)$, $r^2 = 0.998$ (6 points, n = 5). The values of LOD and LOQ were 0.2 and 0.5 μM, respectively.

The content of the isolated product evaluated by HPLC was equal to 102.5 % (mean of two synthesis batches of GSNO) as referred to the dry substance (water content: 1.5 %, m/m). Using Saville-Griess procedure, a content of 97.0 % was found. These results were in accordance with the usual requirements of health competent authorities.

3.5. Impurities profile

Using the HPLC method with detection at a low UV wavelength (220 nm), the qualitative and quantitative fingerprint of all GSNO impurities which could result from either synthesis or storage conditions was determined for the first time to the best of our knowledge. Indeed, the question of presence of potential impurities has arisen in the literature in order to prevent their possible influence on the biological or pharmacological results [16, 21, 27, 28, 36]. Based on the International Conference of Harmonisation guidelines for the registration of pharmaceuticals for human use [30] and on the hypothesis of a maximum daily dose of GSNO less than 2 g/day (which agrees with the first clinical studies, Table 1), the impurities were either reported or identified or qualified depending on the percentages found, *i.e.* 0.05, 0.10 and 0.15 %, respectively. Typical chromatograms corresponding to the profile of GSNO impurities are shown in (Fig. 3); the impurities were identified by adding a corresponding standard into a GSNO solution (Table 3). The results assessed the presence of chloride, nitrite, nitrate ions and glutathione disulfide (GSSG). These by-products were expected as they are brought by either the raw material of the synthesis or the possible degradation pattern of GSNO during the process [32, 37]. Finally, the residual level of the organic solvents used to isolate and purify GSNO (acetone and diethyl ether, which are class 3 residual solvents [31, 32]) was checked. In the specific case of diethyl ether, the solvent was mixed and shaken with the mobile phase until saturation before HPLC analysis. Relative retention time *vs.* GSNO was 0.56 for acetone and 1.93 for diethyl ether, and no interference with the other impurities was noted. In the purified batches, neither acetone nor diethyl ether were observed at a level higher than 0.15 %; this value was less than the authorised limit threshold (0.5 % [31]).

Quantification of impurities with abundance higher than 0.15 % was performed, which was the case of nitrite, nitrate ions and GSSG. Chloride was present in large quantities after synthesis but it was removed after isolation and purification of GSNO, leading to very low residual amount, lower than 0.15 %. Therefore, chloride was not quantified. Other impurities, *i.e.* nitrite, nitrate ions and GSSG, were quantified by HPLC using calibration curves and nitrite ion was additionally quantified using the colorimetric Griess method. Results are shown in Table 4. The nitrite ion content measured by Griess and HPLC method was as follows: 0.18 % (m/m) and 0.19 % (m/m), respectively, for the standard, 0.21 % (m/m) and 0.15 % (m/m), respectively, for the as-synthesised and purified GSNO. Besides, amounts of nitrate ion and GSSG were 0.69 % (m/m) and 2.36 % (m/m), respectively, for the standard and 0.18 % (m/m) and 1.83 % (m/m), respectively, for the as-synthesised and purified GSNO. The purification step of GSNO decreased the quantities of impurities found initially in the reaction medium by a factor of *ca.* 4 for nitrite ion and GSSG, and 1.6 for nitrate ion (giving for the unpurified product 0.57 % (m/m), 8.13 % (m/m) and 0.29 % (m/m), respectively). Since nitrite and nitrate ions can induce some vascular effects [16, 27, 28, 36], the importance of the purification step to avoid any pharmacological interference that may be generated by the impurities has to be highlighted.

Table 3. Chromatographic parameters obtained for GSNO at 220 nm. Results (relative retention time (RRT), retention factor (k), resolution between vicinal peaks (R_s) and percentage of area obtained by the sum of all peak areas) are the means of independent analyses of two different batches of GSNO.

	RRT	k	R _s	Percentage of area	
				GSNO standard	Purified GSNO
Chloride	0.18	0	-	0.04	0.02
Nitrite	0.39	0.88	17.09	0.47	0.39
			4.06		
Nitrate	0.46	1.22	6.03	1.44	0.38
			7.59		
GSH	0.53	1.53	-	Not found	Not found
GSSG	0.79	2.82	5.23	0.69	0.56
GSNO	-	3.86	-	96.94	98.51

Table 4. Calibration curves of nitrite and nitrate ions, and glutathione disulfide (GSSG), using HPLC and Griess method (nitrite ion). Data are expressed as mean ± SD of five calibration curves.

Impurity	Range (μM)	Slope	Intercept	r ²	LOD (μM)	LOQ (μM)
Nitrite (Griess)	1 to 10	39 000 ± 1 000	0.003 ± 0.002	0.9990	0.17	0.51
Nitrite (HPLC)	1 to 100	4 600 000 ± 200 000	-0.527 ± 1.277	0.9999	0.92	2.78
Nitrate (HPLC)	1 to 100	4 200 000 ± 350 000	-0.815 ± 3.460	0.9982	2.72	8.24
GSSG (HPLC)	1 to 100	4 300 000 ± 300 000	-0.875 ± 6.300	0.9997	4.84	14.5

3.6. Stability studies: determination of the optimal storage conditions

First, concerning GSNO storage as a solid, it should be stored under inert atmosphere (argon or nitrogen), at -20 °C and in the dark. Under these conditions, GSNO integrity was preserved in our hands over a 6-months period.

Then, it is now well known that a common scheme related to new drug development includes the use of drastic operating conditions (strong acids and alkalis, intense heat and light exposure, ...) to form degradation products of the tested compound. In the case of GSNO, applying such conditions immediately induces full breakdown of this compound as a drug highly sensitive to environment. Indeed, the *in vitro* decomposition of RSNO has already been extensively studied (see for example [38, 39, 40]) and light, temperature, pH, metal ions were pointed out as the main parameters influencing their stability. However these studies were performed over a short term (48 h max., more often a

few hours) period of storage at room temperature. In order to determine the optimal storage conditions of GSNO, some additional investigations were presently made by studying similar parameters under longer storage periods (until 14 days at 4 °C) and considering other parameters, *i.e.* dioxygen tension and metal ions.

The stability of a 10 mM GSNO solution in phosphate buffer (pH 7.4; 0.5 M) in the dark, was monitored as a function of time by varying temperature. At room temperature (25 ± 2 °C), a loss of 43.1 ± 4.8 % (n = 3) was observed at day 7. This result is coherent with previous work [41]. The storage at -80 °C showed no degradation (0.6 ± 1.2 %, n = 3) as compared to -20 °C (45.4 ± 3.2 % loss of GSNO, n = 3) after one week. At 4 °C, the degradation rate of GSNO was very low (less than 4 % loss after 4 days) and reached *ca.* 10 % after 7 days (n = 3). As a result, a temperature of 4 °C was chosen for the storage of GSNO solutions and for further experiments.

Additionally, the effect of pH was evaluated (Fig. 4A): synthesis was performed and 10^{-2} M solution of GSNO was stored at 4 °C either maintaining at pH = 0.3 or after shifting the pH to the physiological value. The results showed that pH effect was not obvious until day 5, but later, the concentration of GSNO in acidic medium dramatically decreased. Therefore, acidic medium which is necessary to the nitrosation process ought to be shifted to pH 7.4 to ensure better storage conditions. Besides, low pH value (lower than 1 unit with respect to physiological one) is not convenient for cell or animal experiments. It has already been stated that there are possible resonance forms between RS-N=O (form S), RS⁻=N-O⁻ (form D) and R-S⁻N=O⁺ (form I). Form D is described as favourable to strengthen the S-N bond and to be more abundant at low pH values [42, 43]. We presently observed that acidic medium did not improve GSNO solution storage over a long period (longer than 5 days). Although most authors consider GSNO to be more stable in acidic conditions, they performed stability studies at temperature equal or higher than 22 ± 2 °C, and often with low purity reagents or with addition of metallic ions. In this way, a previous study of pH effect on *S*-nitrosocysteine stability indicates that it is linked to the presence of metallic ions (traces in low-purity buffer and reagent, or intentionally added to high-purity buffer) [39]. The use of high-purity eliminated the instability of the preparation. Hence, the

influence of 10 μ M copper II added to GSNO solutions (10 mM in phosphate buffer pH = 7.4, 4 °C) was studied (Fig. 4A). In this experimentation, a dramatically high initial rate of degradation was observed during the first day (75 % of the initial concentration remaining), followed by a lower decrease rate. The latter could be attributed to the formation of a complex between Cu²⁺ (or Cu⁺ which is suggested to be the effective agent [44]) and the glutamate residues in the degradation product, GSSG [44]. The addition of the chelating agent EDTA (0.1 mM) inhibited the copper II catalysed degradation (Fig. 4A). In addition, GSNO alone at pH = 7.4 was as stable as the GSNO/EDTA or GSNO/Cu²⁺/EDTA solutions (Fig. 4A), demonstrating that the use of EDTA was not mandatory to obtain satisfying stability of GSNO, probably because of the high purity of our reactants and water. Besides, it is noteworthy that the chelating agents can induce some side effects in cell biology and pharmacological experiments (*e.g.* calcium flux is necessary to maintain the vascular tone in arteries).

At last, the impact of magnetic stirring on the stability of a GSNO solution at pH 7.4 stored at 4 °C was evaluated (Fig. 4B). Vigorous stirring (800 rpm) induced on the one hand oxygenation of the medium (the measured P_{O2} difference between stirred and unstirred solutions was equal to + 37 mm Hg) and on the other hand an increase of the diffusion of species present. With these synergetic effects,

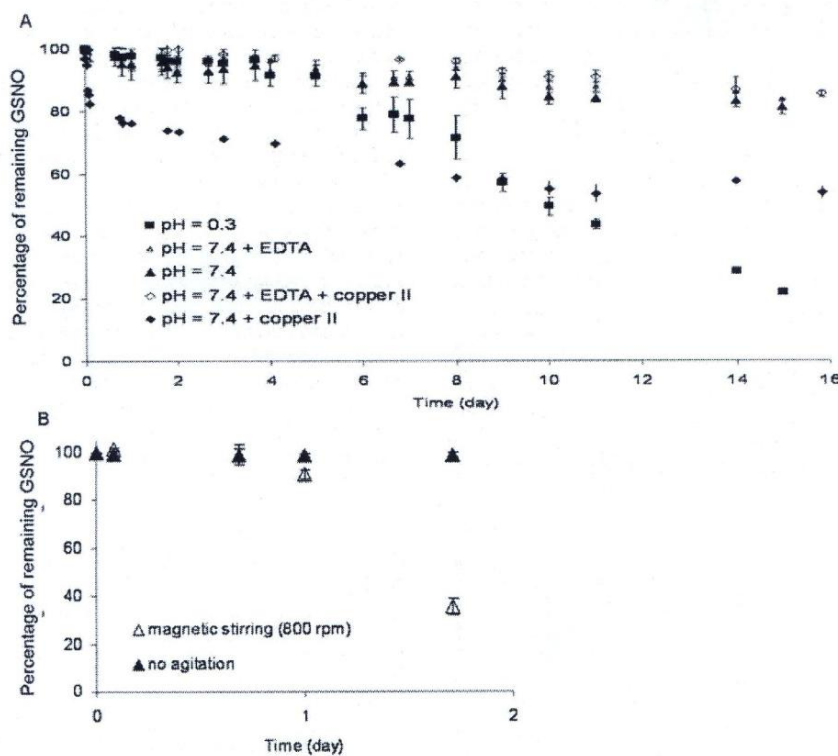


Fig. (4). Stability studies under various conditions: (A) at different pH values (pH = 0.3 corresponds to the reaction medium after synthesis; pH = 7.4 was obtained by neutralising with NaOH and adding phosphate buffer pH 7.4) with or without adding EDTA or copper ions (10 μ M), at 4 °C and (B) at pH 7.4 and at 4 °C with or without stirring. Results are given as a percentage of remaining GSNO (initial concentration of 10 mM) as evaluated by spectrophotometry at 334 nm ($n = 2$).

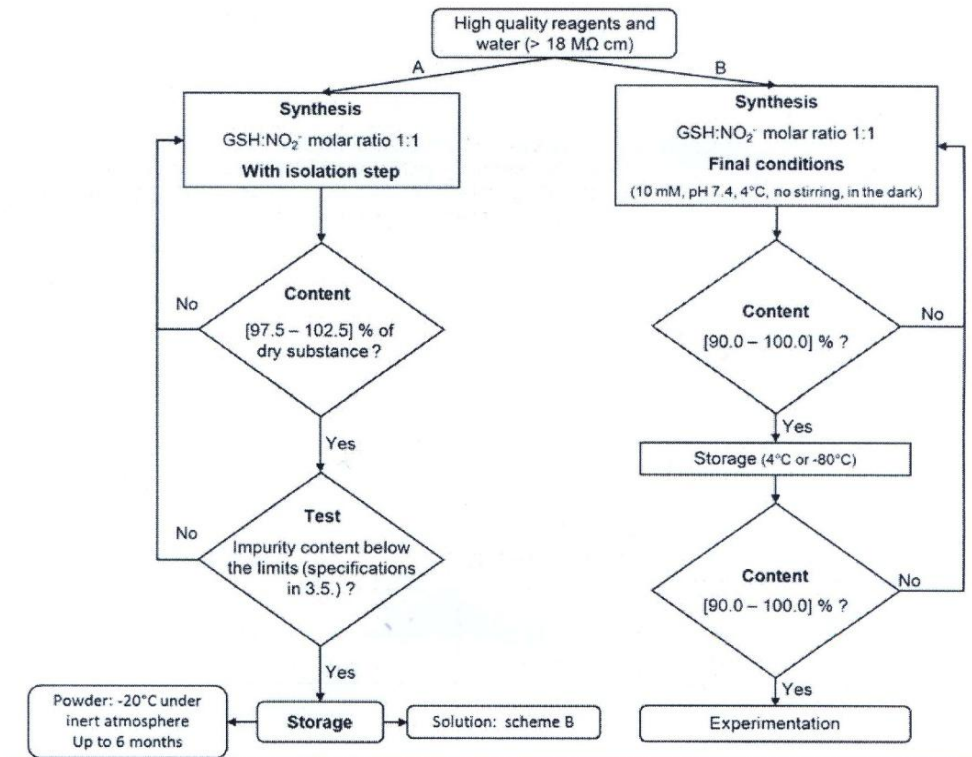


Fig. (5). Scheme summarizing the guidelines for GSNO synthesis with (A) or without the isolation step (B), relevant assays and storage.

GSNO was severely degraded: the phenomenon was observed since the second day of storage. The reaction between RSNO and dioxygen is already known [45]. Moreover, De Oliveira *et al.*, [46] showed that their decomposition, implied by an increase of Brownian motion, is influenced by the initial concentration of RSNO, but this effect was more pronounced for *S*-nitroso-*N*-acetylcysteine and *S*-nitrosocysteine than for GSNO. Therefore, the storage conditions should avoid any handling that can increase the Brownian motion and/or introduce dioxygen into the medium.

After studying the effect of these parameters on GSNO stability, we examined the products of its degradation with the HPLC method. The analysis, performed on a 10-days-old GSNO solution stored at 4 °C in phosphate buffer, pH 7.4, revealed the presence of nitrite as well as GSSG in large quantities (2.9 % (m/m) and 25.2 % (m/m), respectively) and a small amount of nitrate (0.4 % (m/m)). Due to the presently found impurities, *i.e.* GSSG (which is the thermodynamically stable dimer [46]) and nitrite ions, a homolytic decomposition of S-NO bond is strongly hypothesised, followed by a dimerisation of the thiyl radical to form GSSG (4), as follows:



As a conclusion, the GSNO storage conditions are of main importance to avoid the presence of impurities in too high concentrations and to guarantee its unique

pharmacological effects: according to our results, GSNO solutions at pH 7.4 prepared with high purity water and reagents, carefully handled, can be stored in the dark, at 4 °C for 7 days. A summary reporting the critical points of the synthesis with or without the isolation and purification step, assays and storage is proposed in (Fig. 5).

CONCLUSION

S-nitrosoglutathione is extensively studied due to its important role as a physiological NO• donor and a prospective drug. The present work has revisited different synthesis pathways and physico-chemical properties of GSNO in order to define its secured use for further *in vitro/ex vivo* or *in vivo* studies. It is the first time, to the best of our knowledge, that a HPLC method has been developed and validated to evaluate the content of the as-synthesised GSNO as well as to identify and qualify the impurities, based on pharmaceutical requirements. As demonstrated in this work, synthesis using NO₂⁻ and GSH in an 1:1 molar stoichiometric ratio, followed by GSNO isolation and purification, leads to a high product content and a very limited amount of impurities, as required by competent pharmaceutical authorities. The obtained product is also perfectly suited for biological experimentation.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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1.2. Conclusion

Le travail présenté dans l'Article 4 nous a permis de standardiser le procédé de synthèse, isolement et purification permettant d'obtenir un principe actif parfaitement contrôlé.

Malgré le travail engagé dans la tâche 2 du projet NanoSNO se concentrant sur le design et la synthèse de nouveaux RSNO, un seul autre RSNO a pu être synthétisé, isolé et purifié : la *S*-nitroso-*N*-acétylpénicillamine (SNAP). Il n'y a donc eu au final que deux RSNO disponibles pour ce travail. De façon intéressante, ils présentent des caractéristiques assez différentes : d'une part GSNO existe de façon endogène alors que SNAP est purement synthétique, d'autre part la structure de GSNO est basée sur un cœur cystéine contre un cœur pénicillamine pour SNAP. De plus, leurs propriétés physicochimiques diffèrent également : GSNO est plus hydrophile (coefficient de partage calculé, $\text{clogP} = -2,7$ vs $+0,08$ pour SNAP) et vingt fois plus stable *in vitro*, ce qui sous-entend une réactivité différente. Enfin, en conditions *ex vivo*, GSNO est un vasodilatateur plus puissant (*cf.* Article 2). Ces deux composés sont les deux chefs de file (endogène et non endogène) de la famille des RSNO et ont déjà été largement utilisées dans la littérature. Bien qu'elles ne soient pas aussi innovantes ni aussi hydrophobes que le cahier des charges initial le prévoyait, elles ont cependant été formulées dans les implants et microparticules *in situ*.

2. S-NITROSOTHIOLS INCORPORES DANS DES FORMULATIONS *IN SITU*

2.1. Introduction

GSNO et SNAP ont donc été incorporés dans des formulations *in situ*. Le copolymère qui a été retenu pour l'ensemble des études est un PLGA (ratio lactide/glycolide 50 : 50) de faible masse molaire (7 000-17 000 g/mol) aux extrémités de chaîne protégées par des groupements éthers d'alkyle. Dans un premier temps, la preuve de concept d'une libération prolongée *in vitro* des RSNO a été apportée. En parallèle, l'impact du solvant (*N*-méthyl-2-pyrrolidone, NMP ou triacétine, TA) et du type de formulation (implants ou microparticules *in situ*) a été étudié (Article 5). Dans un deuxième temps, la preuve de concept d'un effet *in vivo* prolongé a été apportée, pour SNAP (Article 5) et pour GSNO (résultats complémentaires), en suivant la pression artérielle des animaux après injection grâce à un système de télémétrie (projet « SNOtélém » approuvé par le Comité Régional d'Ethique). Afin d'aider à la compréhension, le paragraphe suivant présente brièvement les déterminants de la pression artérielle et le principe de la télémétrie.

2.2. Pression artérielle et télémétrie

La pression artérielle est nécessaire pour la circulation du sang et donc pour les apports en nutriments et l'évacuation des déchets des différents organes. Il s'agit d'une force exercée sur une surface : elle maintient les parois artérielles distendues, ce qui permet l'écoulement du sang et donc la perfusion des différents organes. La pression artérielle est régulée par différents systèmes, à court, moyen ou long terme [77].

Durant le cycle cardiaque, la pression artérielle oscille entre une valeur maximale, la pression artérielle systolique (P_{sys}) et une valeur minimale, la pression artérielle diastolique (P_{dia}). Le niveau de pression artérielle est généralement décrit grâce à la pression artérielle moyenne (MAP). Néanmoins, celle-ci ne tient pas compte du caractère pulsatile du débit cardiaque. On lui associe donc un autre paramètre, la pression artérielle pulsée (PAP). Elles peuvent être représentées et estimées de la façon suivante (Figure 10) :

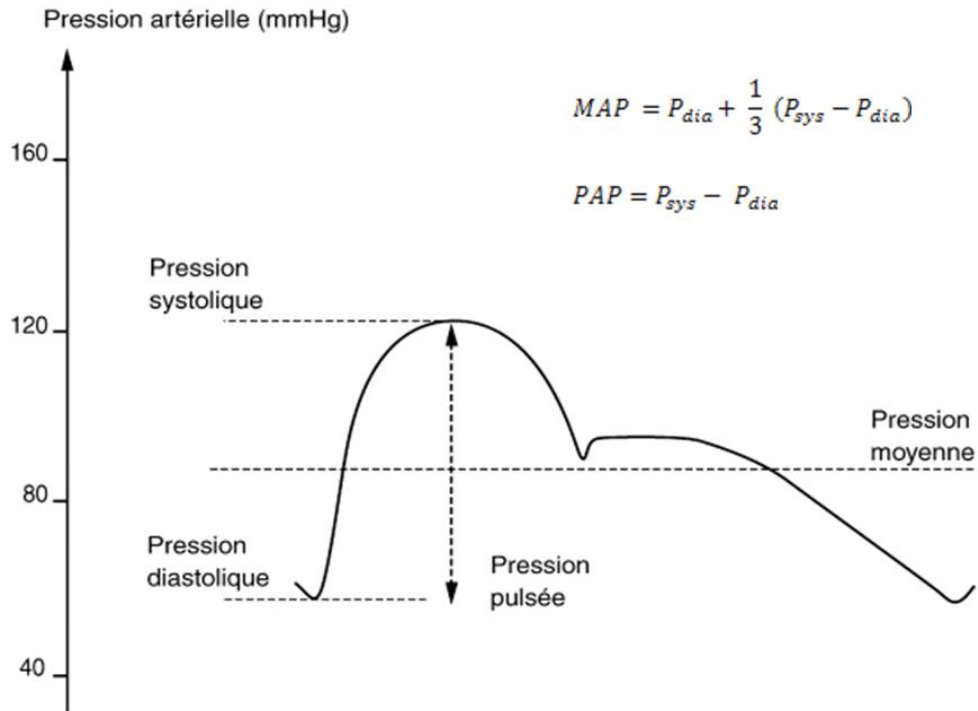


Figure 10 : Représentation schématique de la pression artérielle: composante continue et pulsatile, d'après [77].

Les pressions artérielles moyenne et pulsée dépendent en partie du diamètre des vaisseaux sanguins (Figure 11). Comme NO est un vasodilatateur, les donneurs de NO comme les RSNO exercent également une action vasodilatatrice [78]. Leur libération à partir des formulations *in situ* devrait donc se traduire par une diminution des valeurs de pressions artérielles.

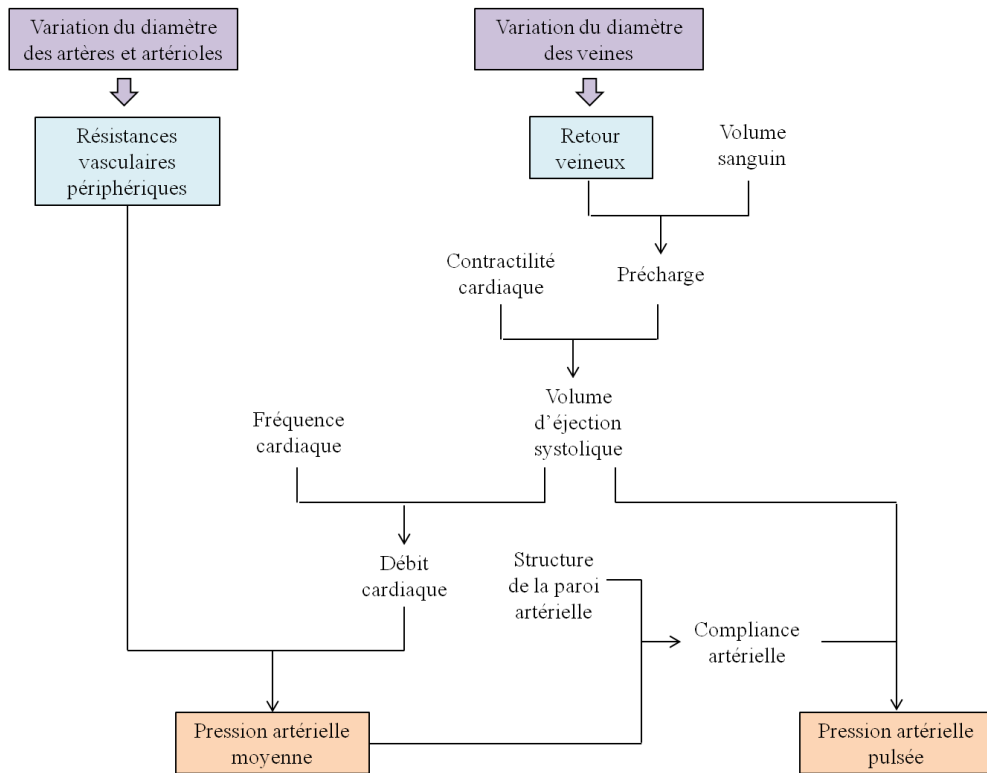


Figure 11 : Déterminants des pressions artérielles moyenne et pulsée. Une vasorelaxation du lit artériel diminuerait la résistance périphérique totale (ce qui se traduirait par une diminution de la MAP) et une vasorelaxation du lit veineux diminuerait le retour veineux et donc le volume d'éjection systolique (impact sur la MAP et sur la PAP).

Il existe différentes méthodes pour mesurer la pression artérielle chez un rongeur (Tableau 2).

Tableau 2 : Avantages et inconvénients des différentes méthodes de mesure de pression artérielle chez le rat.

Méthodologies	Pléthysmographie (manchon caudal)	Cathétérisme artériel classique	Télémétrie
Nécessité d'une intervention chirurgicale	Non	Oui (cathéter artériel, extrémité libre externalisée au niveau de la nuque)	Oui (implantation du capteur de télémétrie)
Récupération après chirurgie	Sans objet (mais temps d'adaptation au dispositif)	≥ 48 h	≥ 2 semaines
Précision de la mesure	+/- (chauffage)	+ (gêne liée au cathéter externalisé)	++
Animal contraint	Oui	Oui : cathéter le reliant aux appareils de mesure	Non (signaux envoyés par ondes radio)
Maintien de l'animal	Autorise des mesures répétées sur le même animal (non-invasif)	Plusieurs jours (risque infectieux/ bouchage cathéter)	Plusieurs mois

Au vu des avantages et inconvénients des différentes méthodes, la télémétrie a été choisie comme méthode pour ces mesures. Elle consiste à implanter au sein de l'animal un capteur (au niveau sous-cutané ou intrapéritonéal) dont l'extrémité est placée à l'endroit adéquat selon le paramètre à mesurer (pour la pression artérielle, le cathéter est inséré dans l'artère fémorale ou dans l'aorte abdominale) (Figure 12). C'est un système sans fil, car les informations recueillies sont transmises par ondes radio à une plaque réceptrice placée sous la cage de l'animal. Ceci permet d'acquérir les données chez un animal éveillé non contraint (ce qui diminue le stress lié à l'expérience) et pendant un temps long (jours, semaines voire mois). Par ailleurs, le risque d'infection est limité de par l'absence de cathéter externalisé. Il est donc possible de conserver l'animal pendant plusieurs mois et de l'inclure dans différentes étapes d'un protocole expérimental, ce qui permet de limiter le nombre d'animaux utilisés. En fonction du capteur utilisé, une pression (ex : pression artérielle, veineuse, intraoculaire, vésicale) ou une activité électrique (électrocardiogramme, électroencéphalogramme, électromyogramme) peuvent être mesurées en plus de la température corporelle et de l'activité de l'animal. Les capteurs utilisés dans cette étude ont enregistré le signal de pression artérielle.

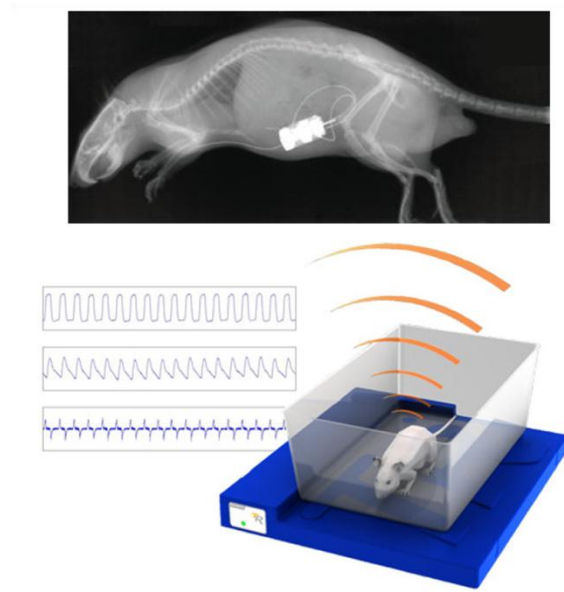


Figure 12 : Principe de la télémétrie : radiographie d'un rat équipé d'un capteur implanté au niveau de la paroi abdominale et schéma illustrant l'enregistrement des paramètres de l'animal libre de ses mouvements. Les images proviennent de fournisseurs de matériel de télémétrie (Data Sciences International, Millar).

2.3. Article 5 : « Are *in situ* formulations the keys for the therapeutic future of *S*-nitrosothiols? »

Cet article accepté dans *European Journal of Pharmaceutics and Biopharmaceutics* comporte dans une première partie les résultats des essais *in vitro* : formation et dégradation de la matrice polymérique, libération des donneurs de NO. Deux solvants différents (NMP et TA) ainsi que deux types de formulations (ISI et ISM) sont étudiés. Dans une deuxième partie, les formulations préparées avec le solvant NMP et chargées en SNAP, qui semblaient être les plus performantes d'après les résultats *in vitro*, ont été testées chez le rat Wistar.



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Research paper

Are *in situ* formulations the keys for the therapeutic future of S-nitrosothiols?

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ABSTRACT

S-nitrosoglutathione (GSNO) and S-nitroso-N-acetylpenicillamine (SNAP) were formulated into *in situ* forming implants (ISI) and microparticles (ISM) using PLGA and either N-methyl-2-pyrrolidone (NMP) or triacetin. Physicochemical characterization was carried out, including the study of matrix structure and degradation. A strong correlation between drug hydrophobicity and the *in vitro* release profiles was observed: whatever the formulation, GSNO and SNAP were completely released after ca. 1 day and 1 week, respectively. Then, selected formulations (i.e., SNAP-loaded NMP formulations) demonstrated the ability to sustain the vasodilation effect of SNAP, as shown by monitoring the arterial pressure (telemetry) of Wistar rats after subcutaneous injection. Both ISI and ISM injections resulted in a 3-fold extended decrease in pulse arterial pressure compared with the unloaded drug, without significant decrease in the mean arterial pressure. Hence, the results emphasize the suitability of these formulations as drug delivery systems for S-nitrosothiols, widening their therapeutic potential.

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1. Introduction

In recent years, S-nitrosothiols (RSNOs) have emerged as one of the most promising families of nitric oxide (NO) donors. Physiological roles of NO include neurological, immune, gastrointestinal, and cardiovascular homeostasis. In this field, NO induces vasodilation and inhibits platelet activation and aggregation, as well as leukocyte adhesion to the endothelium [1–3]. Decreased synthesis and/or bioavailability of NO have been linked to endothelial dysfunction itself linked to cardiovascular diseases [4]. As NO is too short half-lived to be used for systemic therapy, a broad field is opened for NO-donor drugs. Over the past century, organic nitrates have been prescribed for the treatment of angina pectoris. Nevertheless, a therapeutic window has to be respected to avoid tolerance phenomenon and oxidative stress and therefore impairs the enhancement of their therapeutic indications [5].

RSNOs represent relevant candidates in NO-donors development (for reviews on other NO donors, see [6–8]): first, they constitute endogenous storage forms of NO (for instance, S-nitrosoalbumin, and S-nitrosoglutathione, GSNO) [9]. Second,

these physiological compounds can also be synthesized and isolated as pure products, with an adequate pharmaceutical quality control, as previously shown [10]. As a consequence, RSNOs have been already administered in preclinical studies in human volunteers. Local and systemic vasodilations as well as powerful antiplatelet effects have been demonstrated [11,12].

However, the therapeutic use of RSNOs is prevented by the lack of stability of the S–NO bond, as an extensive number of environmental conditions are susceptible to break it: enzymes, reductants, metallic cations and light [13]. The first approach to extend the half-life of the S–NO bond is drug design through appropriate structural changes [14]. Modulations of the molecule will result in changes in the stability, but sometimes also in the potency and/or the hydrophobicity of the molecule. A broad range of molecules can be obtained, as purely synthetic RSNOs (e.g., S-nitroso-N-acetylpenicillamine, SNAP) can also be designed from thiol-containing compounds. The second and complementary approach relies on the use of drug delivery systems as they will protect RSNOs, ensure their controlled release, and eventually enhance the NO payload in the case of macromolecular scaffolds (for reviews, see [15,16]). Among them, conventional polymeric drug delivery systems such as micro- or nano-particles generally require multiple steps and aggressive manufacturing processes (heat, pressure, shear stress), which are not suitable for labile compounds such as RSNOs [17]. As a result, this work is devoted

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to the incorporation of RSNOs, namely GSNO or SNAP, into *in situ* forming implants (ISI) and microparticles (ISM) based on a bioerodible and biocompatible poly(lactide-co-glycolide) polymer (PLGA).

ISI have been introduced as drug delivery systems in the 1990s by Dunn et al. [18]. Drug is extemporaneously added to a solution of PLGA in a pharmaceutically acceptable organic solvent, usually *N*-methyl-2-pyrrolidone (NMP). Subsequent injection of the formulation into an aqueous medium leads to solvent/water exchange and finally to the precipitation of a polymer matrix, entrapping the drug. Sustained release is achieved through drug diffusion and polymer degradation. High initial drug release (i.e., burst) during system solidification can be limited by the use of a less water-soluble solvent, such as triacetin (TA) [19]. However, this approach generally increases the viscosity of the formulation, potentially hindering the injection. To solve this issue, *in situ* microparticles (ISM) have been developed [20]. In this case, ISI formulation is emulsified with an external phase prior to injection, and the solidification of emulsion droplets produces microparticles *in situ*. ISM usually generate lower bursts [21] and the external phase could facilitate injection, as viscosity of the ISM system is determined by viscosity of the external phase [22].

Therefore, the objective of this study was to demonstrate the suitability of *in situ* forming PLGA depots as RSNOs delivery systems. Two solvents were used: NMP, FDA-approved for human parenteral use and TA, FDA-approved for human oral administration and veterinary parenteral uses. *In vitro* studies were carried out with variations in the solvent (NMP or TA), the formulation (ISI or ISM), or the drug (GSNO or SNAP). Resulting modulations in matrix structure, degradation and drug releases have been explored. Moreover, correlations have been established between the hydrophobicity of the NO-donor and the *in vitro* release profile. Finally, SNAP-loaded formulations based on NMP have been subcutaneously injected to Wistar rats, and their impact on arterial pressure monitored with a telemetric device.

2. Materials and methods

2.1. Materials

PLGA 50/50 (Resomer RG 502 S, $M_w = 7000\text{--}17,000$ g/mol, alkyl ether endcapped, inherent viscosity 0.20 dL/g) was obtained from Evonik (Essen, Germany). NMP was purchased from VWR (Fontenay-sous-Bois, France) and TA from Alfa-Aesar (Schiltigheim, France). All other reagents were of analytical grade and were supplied by Sigma-Aldrich (Lyon, France), including GSNO and SNAP when used as standards. Isosorbide dinitrate (ISDN) and isosorbide mononitrate (ISMN) used as model drugs for *in vitro* release assays were kindly supplied by Etypharm (Saint-Cloud, France) mixed with lactose in order to reduce explosive risk (drug/lactose: 25/75 m/m). Isoflurane was purchased from Baxter (Maurepas, France), and all products for surgery and animal welfare were from a veterinary provider (Centravet, Nancy, France). Highly purified water (≥ 18 M Ω .cm) was used for all experiments.

2.2. NO donor syntheses and isolation

GSNO pink powder was synthesized, purified, isolated, and stored as previously described [10]. SNAP green powder was obtained as follows: *N*-acetyl-D-penicillamine (500 mg, 2.61 mmoles) was solubilized in 7 mL HCl 1 M in MeOH. Then, 0.7 mL of concentrated H₂SO₄ and a solution of 450 mg of NaNO₂ in 7 mL of water were added. Reaction was agitated at 25 °C during 35 min under inert atmosphere in the dark. The solid was then filtered, washed with water, dried under vacuum, and stored in the same conditions

than GSNO. Isolated RSNOs had a purity $\geq 99\%$ according to Griess–Saville method in comparison with standards (for detailed protocol, see [10]).

Separation of organic nitrates from lactose was realized as follows: the ISDN/lactose mixture was triturated with ether, then ultrasonicated for 7 min, and filtered. Ether was evaporated under reduced pressure. Enough ethanol was added to dissolve the residue and resulting solution cooled on ice to achieved recrystallization. Finally, the suspension was filtered and crystals were dried in a desiccator. The ISMN/lactose mixture was placed with acetone under magnetic stirring for 5 min and then filtered under vacuum. The filtrate was kept in air oven at 60 °C until evaporation of the solvent and crushed to powder with a glass beater. Purity of obtained solids ($\geq 99\%$) was assessed by RP-HPLC, following the method described below. They were stored at 4 °C, protected from light.

2.3. *In situ* implants (ISI) and microparticles (ISM) preparation

Typical compositions of *in vitro* and *in vivo* formulations are indicated in Table 1. Briefly, polymer solutions were obtained by dissolving PLGA in the solvent (50 °C, 500 rpm, 1 h). A previous size-exclusion chromatography experiment exhibits no degradation of polymer after 5 h in these conditions (protocol: see below; data not shown). Solutions were allowed to cool down to room temperature before use. Drugs were mixed with the polymer solution just before use (300 rpm without heating). Drug loadings for *in vitro* experiments (5% m/m for RSNOs, 20% m/m for organic nitrates) were chosen according to the sensitivity of the method used for quantification.

All formulations were filled into 1 mL syringe (Becton Dickinson, USA) and injected through 21 G needle (Terumo, USA). ISI were obtained by injecting directly the drug/polymer/solvent mixture (ca. 0.3 g of whole formulation) into a physiological aqueous medium (*in vitro* experiments, 0.22- μ m filtered phosphate buffered solution, PBS, 0.148 M pH 7.4) or rats (*in vivo* experiments, subcutaneous injection). For ISM, ca. 0.3 g of the drug-containing polymer solution used for ISI (m_i) was emulsified with ca. 0.3 g of an external phase (m_e) immediately before injection through a polypropylene connector (1.4 mm inner diameter, gift from R. Bodmeier's group, Germany). The external phase for NMP-formulations consisted in a mixture of sesame oil (96% m/m_e), aluminum monostearate (2% m/m_e), and Span 80 (2% m/m_e). For TA-formulations, external phase was constituted of water previously saturated with TA and then added with Pluronic F68 (1% m/m_e). ISM formulations were prepared with a 1:1 (m_i/m_e) phase ratio at a mixing speed of 2 cycles per second; 50 mixing cycles were applied.

Injectability of formulations was evaluated by the facility to inject them through the 21 G needle.

2.4. Characterization of depots structure and degradation

Polymeric structures obtained 7 days after *in vitro* injections were observed with scanning electron microscopy (SEM) (JSM-7600 F, Jeol, Tokyo, Japan), at 1 kV without metallization after lyophilization (24 h, from -56 to $+12$ °C, 4 Pa). Differential scanning calorimetry (DSC) experiments were performed on a DSC Q10 apparatus with a cooling system (TA Instruments, New Castle, USA). Freeze-dried unloaded formulations were analyzed (5–7 mg in aluminum pans) in 2 runs, from -90 to $+100$ °C (heating rate 10 °C/min, cooling rate 20 °C/min). Results from the second run were taken into account. Additionally, characterization of ISM system was made observing the emulsion droplets immediately after emulsification with an optical light microscope equipped with a camera (TI-PS and DS-U2, Nikon, Tokyo, Japan). Then, solidified unloaded ISM

Table 1
Typical compositions of ISI and ISM used *in vitro* and *in vivo*. Drug loadings are indicated in comparison with the total mass of internal phase (m_i).

	Component	<i>In vitro</i>		<i>In vivo</i>
		RSNOs (5% m/m_i)	Organic nitrates (20% m/m_i)	SNAP (2.5% m/m_i) ^a
ISI	$m_i = 300$ mg	PLGA	57 mg	48 mg
		NMP or TA	228 mg	192 mg
		Drugs	15 mg	60 mg
ISM	m_i emulsified with 300 mg of external phase (m_e)			

^a SNAP loadings for *in vivo* experiments were decreased because control injection of unloaded drug beyond this dose could have led to unethical hypotension (see Section 3).

were removed from the *in vitro* medium 7 days after injection and resuspended in 2 mL of an aqueous solution of Tween 80 (1% m/v) under magnetic stirring (300 rpm for 48 h). Mean diameters were calculated from the optical microscopy pictures with the Saisam 5.4.7 software (Microvision Instruments, Evry, France).

Polymer degradation was evaluated through size-exclusion chromatography (SEC) after retrieval of formulations from *in vitro* release medium after 14, 21, or 28 days. Samples were dissolved in tetrahydrofuran (~ 10 g/L), and 100 μ L of solution was filtered and injected in SEC system. SEC analyses were performed in tetrahydrofuran eluent at room temperature using a Merck HPLC pump (L-6200 A, Germany) equipped with a DG-1310 degazer, two PL-gel 5 μ m columns (100 Å, 300 \times 7.5 mm; 1000 Å, 300 \times 7.5 mm and guard columns 50 \times 7.5 mm – all columns from Polymer Laboratories, Amherst, USA). Elution (0.7 mL/min) was monitored by a differential refractometer (Merck RI-71). Number average molecular weight (M_n) and polydispersity index (PDI) were calculated by reference to a calibration curve obtained with polystyrene standards (linear regression of $\log(M_n)$ in function of elution volume, M_n of standards in the range 580–28,770 g/mol). Results are expressed in polystyrene equivalents.

Release of L-lactic acid from the formulations within time was also investigated as another way to evaluate polymer degradation. Briefly, *in situ* formulations were formed *in vitro* as described previously and the medium harvested at days 14, 21, 28, 35, 42, 50, 59, and 70. A kit assay for the determination of L-lactic acid (R-Biopharm, Darmstadt, Germany) was used according to the manufacturer instructions, and suitable calculations were made to obtain the cumulated percentage of total lactic acid (L-lactic acid + D-lactic acid) release.

2.5. *In vitro* release assays

Approximately 0.3 g of ISI and 0.6 g of ISM formulations were injected into glass hemolysis tubes (VSM, France) filled with 5 mL of preheated PBS. The tubes were incubated in a horizontal shaker at 75 rpm and 37 °C (22L WNB and SV 14/22, Memmert, Germany). The release medium was carefully removed and replaced with 5 mL freshly prepared and prewarmed buffer at each sampling point (1 h, 4 h, 7 h, and then day 1, 4, 7, 10, 14, 21, 28, 35 and 37).

In vitro drug releases were assessed as follows. For GSNO and SNAP, concentrations were determined with the colorimetric Griess–Saville and Griess methods according to previously reported protocols [10]. As regards ISDN and ISMN, a RP-HPLC method was used. The HPLC apparatus (Shimadzu, Japan) was equipped with a C18 reversed phase column (LiChrospher 100 RP-18, 250-4, 5 μ m endcapped, Merck, Germany) kept at 40 °C. The mobile phase was a mixture of acetonitrile and ultrapure water (40:60 v/v) at a flow rate of 0.8 mL/min. 50 μ L of each diluted sample was injected, and the detection wavelength was set at 210 nm. The retention times were ca. 11.8 min for ISDN and 3.4 min for ISMN. HPLC method was validated for ISDN and ISMN according to ICH Q2 (R1). Specificity was assessed injecting standards from

European Pharmacopeia, matrices, lactose, nitrite, and nitrate ions. Linearity was assessed in the range 10^{-3} – 5×10^{-6} M (6 points, $n = 3$). For both compounds, detection and quantification limits (based on standard deviation and slope) were 2 and 5×10^{-6} M, respectively. Finally, repeatability, intermediate precision, and accuracy of the HPLC method were evaluated. Values of mean relative standard deviations were $\leq 1\%$ for repeatability (6 determinations at 100% of the test concentration, i.e., 4×10^{-4} M) and $\leq 1.5\%$ for intermediate precision (determined by 3 injections of the 100% test solution over 2 days). Accuracy was evaluated at $100.28 \pm 0.87\%$ for ISDN and at $98.95 \pm 1.05\%$ for ISMN (6 points, $n = 3$). This method was used for the determination of percentage of drug released in the medium at each time point, after assessing in twelve samples the full correlation of the results with the determination of remaining drug inside the depots (dissolution of the formulations in pure acetonitrile before dilution in mobile phase and HPLC determination) (not shown).

A correlation has been established between the hydrophobicity (partition coefficient P , calculated with the Chemdraw 8.0 software, CambridgeSoft, Waltham, USA) and the release profiles of the NO donors.

2.6. *In vivo* experiments

All experiments were performed in accordance with the European Community guidelines (2010/63/EU) for the use of experimental animals and for the respect of the 3 Rs' requirements for Animal Welfare (I. Lartaud permit n° 54-5, F. Dupuis n° 54-105, French Ministry of Agriculture, Paris, France). The protocols and procedures were approved by an advisory regional ethical committee on animal experiments (CELMEA-2012-0006, project "SN0té-lém"). Animals had mild restriction to standard rat chow (A04, Safe, France, ca. 20 g per day) in order to maintain their body weight at around 500 g, and drinking water (Aqua-clear®226, Culligan, USA) *ad libitum*. During the experiments, neither change in the weight nor in the behavior of the animals was observed.

Two groups of male normotensive outbred Wistar rats (Rj/Han:Wi; Janvier, France, 4 month old) were used for the experiments. Rats of the first group ($n = 8$) received injections of non-loaded formulations to study the *in vivo* degradation of matrices. After sacrifice, depots were surgically removed and placed 1 h in a trypsin-EDTA solution at 37 °C. After 2 days of drying at 22 ± 2 °C, samples were analyzed by DSC or SEC with the same protocols than for *in vitro* experiments. Rats of the second group ($n = 7$) were equipped with telemetry devices (PA-C40, Data Sciences International, New Brighton, USA) under isoflurane anesthesia and aseptic surgery. They were then allowed 1 month for recovery with careful post-surgery watching, before starting arterial pressure measurements. The latter were conducted during the next 5 months. PBS was used as control in the experiments to avoid the washout period needed for polymeric matrix degradation. Indeed, preliminary studies indicated that injection of unloaded formulations did not impact significantly arterial pressures vs PBS (not shown). Each rat was used as its own control and thus received PBS and several

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doses of unformulated SNAP (random order, separated by a 2-day washout period), then the 2 formulations (random order) at distinct injection sites separated by a 1-month washout period. Drugs and formulations were injected subcutaneously in the dorsal region under transient isoflurane anesthesia (3 min for induction +5 min for shaving and injection) in order to standardize the protocol and to reduce stress due to injections. All the injections were performed between 11 and 12 a.m.

Signals of arterial pressure were recorded using Ponemah 5.1 software (Data Sciences International, New Brighton, USA). Mean arterial pressure (MAP) was calculated as the area under the pressure wave for each cardiac cycle and pulse arterial pressure (PAP) as the difference between P_{sys} and P_{dia} , (P_{sys} and P_{dia} being the systolic and diastolic arterial pressure values, respectively, determined as the maximum and minimum pressure values of each pressure wave).

Before each injection, MAP and PAP were recorded for each rat during 30 min and averaged on the last 5 min to obtain baseline MAP and PAP.

For unformulated SNAP, MAP and PAP were recorded continuously for 4 h in animals which were awake. Variations of MAP (Δ MAP) and PAP (Δ PAP) vs baseline values were averaged on 1 min every 5 min and then expressed as means \pm s.e.m. vs time.

For SNAP-loaded formulations, MAP and PAP were recorded continuously for 3 days and Δ MAP and Δ PAP were averaged on 5 min every 15 min then expressed vs time (means \pm s.e.m.). Areas under the curve (AUC) were calculated for each 5 min section and then averaged for each day (D) or night (N) period.

Formulations were prepared as for *in vitro* experiments, using sterile bottles. Exact received doses (16 ± 1 mg/kg, $n = 14$ injections) were determined by weighing the syringe/needle system before and after injection.

At the end of the experiments, animals were euthanized by pentobarbitone overdose (120 mg/kg).

2.7. Statistical analyses

For *in vitro* experiments, results are given as mean \pm s.d. of 3 experiments. Correlation coefficients have been compared to a Fisher table (2 degrees of freedom, $p < 0.05$ considered as significant).

For *in vivo* experiments, results are given as mean \pm s.e.m. Significant differences between PBS and the doses of unformulated

SNAP were determined by a two-way ANOVA (variables: “time” and “dose”) followed by a post hoc Bonferroni test. Significant differences between PBS and SNAP-loaded ISI or ISM data were determined by one-way ANOVA performed on AUC, followed by a post hoc Bonferroni test.

The duration of effect was evaluated as the last time point which still showed significant difference from the corresponding PBS effect. The null hypothesis was rejected at $p < 0.05$. Statistical analyses were performed using the GraphPad Prism software (GraphPad Software, San Diego, USA).

3. Results and discussion

3.1. Influence of solvent and formulation type on the formation and the degradation of the systems

In situ implants and microparticles have been used as drug delivery systems in a wide range of applications [23,24]. Moreover, some *in situ* formulations have already fulfilled all the preclinical and clinical regulatory requirements and are already marketed for human use (e.g., Eligard®). Nevertheless, burst (i.e., rapid and massive drug release) remains the main limitation to the development of further commercialized products. Classical approaches to reduce the burst are the use of either hydrophobic solvents (e.g., TA [19]), or ISM [20]. Burst reduction observed in these cases is generally attributed to a delayed phase inversion. This induces morphological changes in the polymeric matrix and therefore might also impact its degradation. Indeed, water which enters into the matrix (during polymer precipitation and after) is the trigger of the polymer degradation. As a result, the influence of solvent (NMP or TA) and formulation type (ISI or ISM) on the injectability, the formation, and the degradation of the systems were studied in the first part of this work.

First, *in vitro* injections of formulations through a 21 G needle were easily obtained, except for TA-based ISI. This was probably in relation to the high viscosity of the solvent. By contrast, TA-based ISM, with an aqueous external phase, was much easier to inject, as expected [22]. For NMP-based formulations, passage through the syringe/needle was more difficult for ISM than for ISI, because in this case the oily external phase of ISM was more viscous than the solvent [22]. Finally, formulations were ranked according to their facility of injection as follows: TA-based ISM > NMP-based ISI > NMP-based ISM > TA-based ISI.

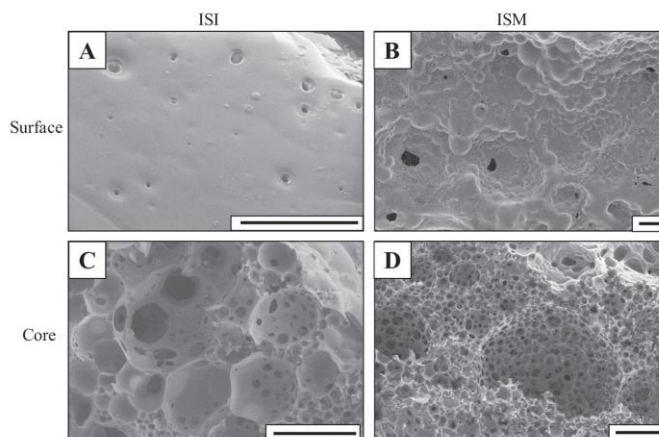


Fig. 1. Scanning electron microscopy pictures of unloaded ISI and ISM obtained *in vitro* with NMP as solvent, 7 days after injection. Scale bars correspond to 100 μ m.

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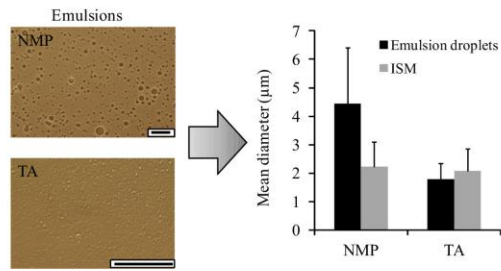


Fig. 2. Pictures of unloaded ISM emulsions before injection and mean diameter of the emulsions droplets and of the obtained *in vitro* ISM ($n=200$). Scale bars correspond to 50 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

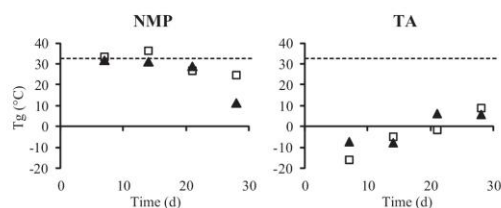


Fig. 3. Differential scanning calorimetry studies: evolution of the glass transition temperature (T_g) of the polymer in unloaded ISI (□) and ISM (▲). Dotted line indicates T_g value of the native polymer powder.

Then, morphological evaluations were conducted on the depots retrieved from the aqueous medium 7 days after injection. This time was selected to ensure complete depot formation before analysis. SEM pictures of NMP-based ISI showed a smooth surface with few pores (Fig. 1A) and a porous core with large honey-comb structures (Fig. 1C). NMP-based ISM did not appear as completely isolated microparticles, but rather as an agglomerate of microparticles, as indicated in Fig. 1B. A more delicate and more porous honey-comb structure compared with ISI was observed inside the ISM depot (Fig. 1D).

Nevertheless, the existence of microparticles during the ISM injection was proved by optical microscopy (Fig. 2). Mean diameter of the mother emulsion droplets was 4.4 ± 2.0 µm for NMP-based ISM and 1.8 ± 0.6 µm for TA-based ISM. In both cases, regular spherical microparticles with a narrow distribution of diameters centered on 2 µm were observed.

Then, glass transition temperature (T_g) of the native polymer powder was determined at 35 °C (Fig. 3). Unlike the “native” material, samples have been freeze-dried before DSC measurements. This process was able to remove almost all the water from formulations. The results thus mainly revealed the plasticizing effect of the remaining solvents. For NMP-based formulations, T_g values at days 7 and 14 were similar to value of native polymer: this can be explained by a fast and complete leaching of NMP from the formulations. Decrease in T_g after day 14 corresponds to the polymer degradation. For TA-based formulations, T_g values at day 7 were much lower than for native polymer (-16 °C for ISI and -7 °C for ISM): this indicates the persistence of the solvent inside the formulations. T_g values increased slowly after day 7 but remained low compared to initial values: this probably results from both progressive TA leaching and polymer degradation. Slow solvent release might explain why TA-based depots became viscous solutions after freeze-drying and were therefore not suitable for SEM observation. This problem has already been mentioned by other authors [19,25] and attributed to a high amount of residual solvent, which is coherent with the DSC results. After freeze-drying (and thus water removal), the residual solvent allows the redissolution of polymer, probably facilitated in this study by the low polymer concentration.

Finally, degradation of the formulations was investigated. As similar trends were observed for both solvents, only results of NMP formulations are presented (Fig. 4). Number average molecular mass (M_n) of the native polymer was evaluated at 5400 g/mol with a polydispersity index (PDI) of 1.2. Degradation of both types of *in situ* forming depots followed the same pattern: following indicated values correspond to ISI. During the first 14 days after injection, a very slight decrease in M_n was observed (5300 g/mol) as PDI increased (1.6), which could indicate a slow onset of matrix degradation. Then, M_n started to decrease whereas PDI continued to further increase (at day 21 M_n at 3900 g/mol and PDI at 1.8). Finally, degradation was accelerated after 21 days, with values at day 28 being 1400 g/mol for M_n and 3.6 for PDI.

In addition to these results, release of lactic acid was detected between the 14th and the 21st days for both ISI and ISM (Fig. 4).

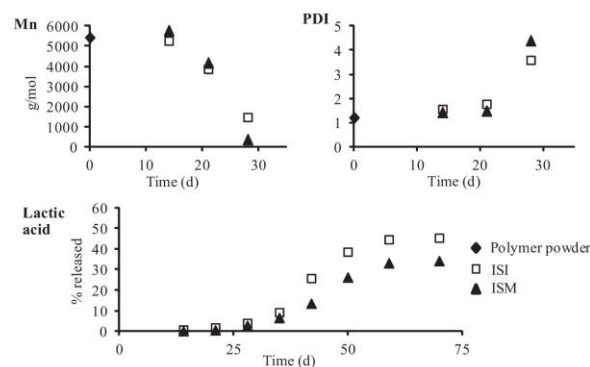


Fig. 4. Degradation of unloaded NMP-based formulations monitored by the number average molecular mass (M_n) and the polydispersity index (PDI) of the polymer, as well as by the cumulative lactic acid release.

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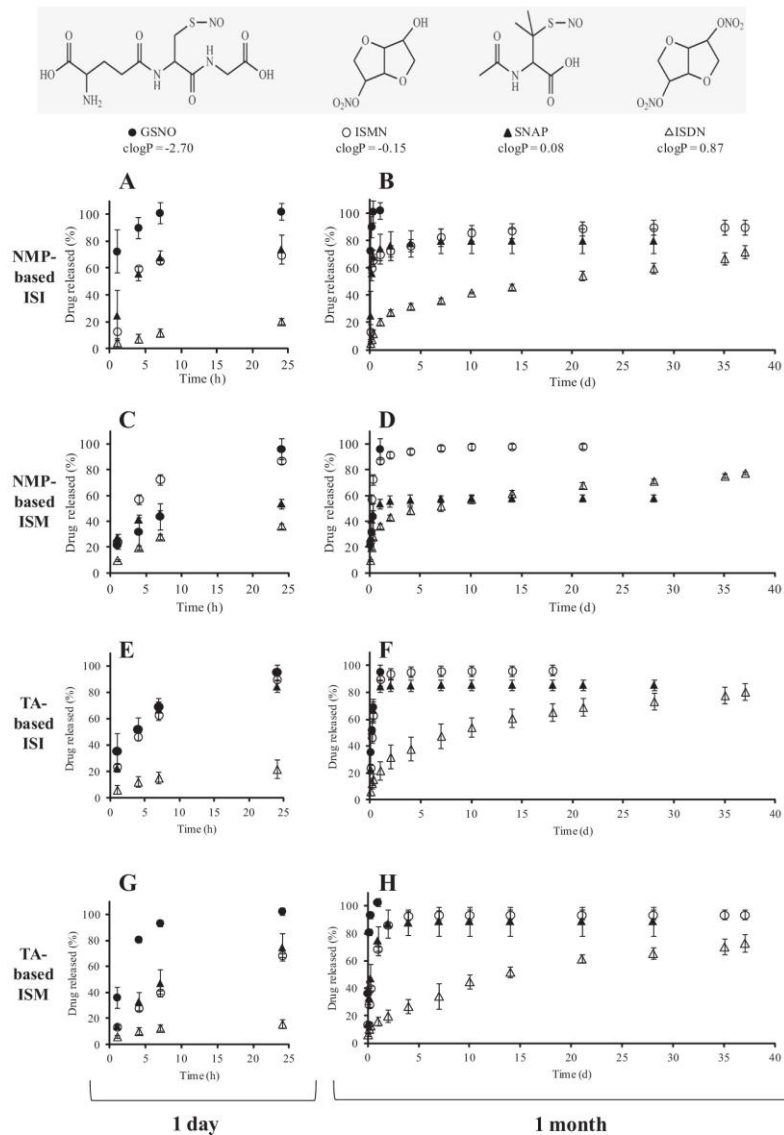


Fig. 5. *In vitro* releases of NO donors from *in situ* formulations ($m \pm sd$, $n = 3$).

Then, lactic acid concentration increased, reaching a plateau (native PLGA: 53%, ISI: 45%, ISM: 34%) between day 60 and day 70. The totality of the lactic acid was not recovered probably because of the experimental procedure: indeed, all the media were changed at each sampling; thus, PLGA oligomers may have been retrieved before they finally degraded into lactic acid. Nevertheless, these results confirmed the onset of the *in vitro* degradation between day 14 and day 21. Additionally, a complete disintegration of the system was noticed at day 70 *in vitro*.

Introduction of an external phase (ISM formation) is also a common approach to slow down the phase inversion [21]. Comparing ISI and ISM based on the same solvent, no significant difference was observed neither in the T_g evolution nor in the degradation profile. This might rely on the potential agglomeration of the ISM during solidification, as suggested by SEM pictures of NMP-based formulations (Fig. 1B).

With the polymer, the solvent represent one of the main parameter influencing the ISI behavior. Especially, solvents with

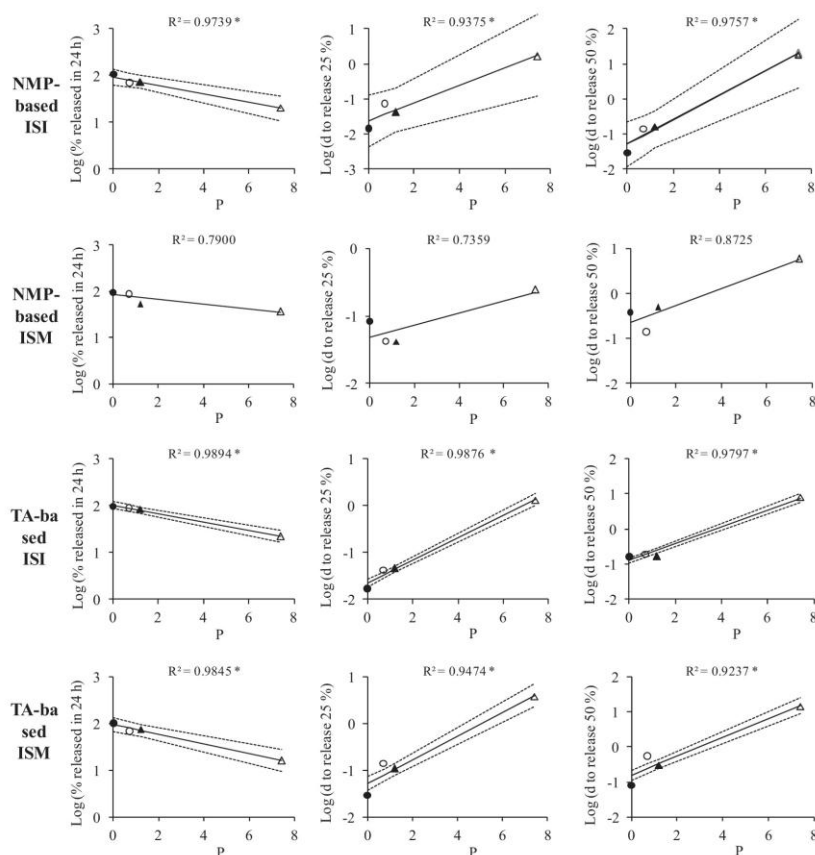


Fig. 6. Correlations between hydrophobicity of NO donors (partition coefficient P) and their *in vitro* release profiles from *in situ* formulations ($*p < 0.05$; dotted lines delimit the confidence interval (95%). No statistical significance was obtained for NMP-based ISM.

Table 2

In vivo degradation of unloaded NMP-based formulations monitored by the number average molecular mass (M_n), the polydispersity index (PDI), and the glass transition (T_g) of the polymer.

	Day 7	Day 14	Day 21	Day 28
ISI				
M_n	/	5300	4200	4000
PDI	/	1.5	1.8	1.9
T_g (°C)	34	34	33	30
ISM				
M_n	/	5500	5300	3800
PDI	/	1.3	1.4	2.0
T_g (°C)	34	34	33	32

low miscibility with water, such as TA, are known to slow the phase inversion and usually give rise to more homogeneous and more hydrophobic matrices [19], which could lead to a slower entry of water. As water is the trigger of polymer degradation, a slower degradation rate was also expected with these solvents. Nevertheless, in contrast to this hypothesis, results showed that for such formulations, solvent type indeed affected injectability

of formulation but not the degradation rate of polymeric matrices. Even if SEM photographs of TA-based formulations were not obtained, one can assume according to the literature [26] and DSC results that phase inversion process also is affected by solvent type. Differences in drug release at least during initial phase of release were therefore expected.

3.2. *In vitro* drug release

To evaluate the influence of drug hydrophobicity on the release profile, four drugs were tested. Besides GSNO and SNAP, two commercial NO-donors belonging to the organic nitrate family, i.e., isosorbide mononitrate (ISMN) and isosorbide dinitrate (ISDN), were also incorporated into NMP- or TA-based ISI and ISM as model drugs and to enlarge the log P scale toward more hydrophobic compounds (Fig. 5). GSNO was fully released from all formulations during the first 24-h period. However, NMP-based ISI and TA-based ISI offered a more gradual profile, with only $22 \pm 4\%$ and $36 \pm 14\%$ released after the first hour, and less than 50% and 70% released after 7 h, respectively. In comparison, NMP-based ISI and TA-based ISM released $72 \pm 16\%$ and $36 \pm 8\%$ of GSNO after the first hour and more than 90% of the drug during the first 7 h. Except for

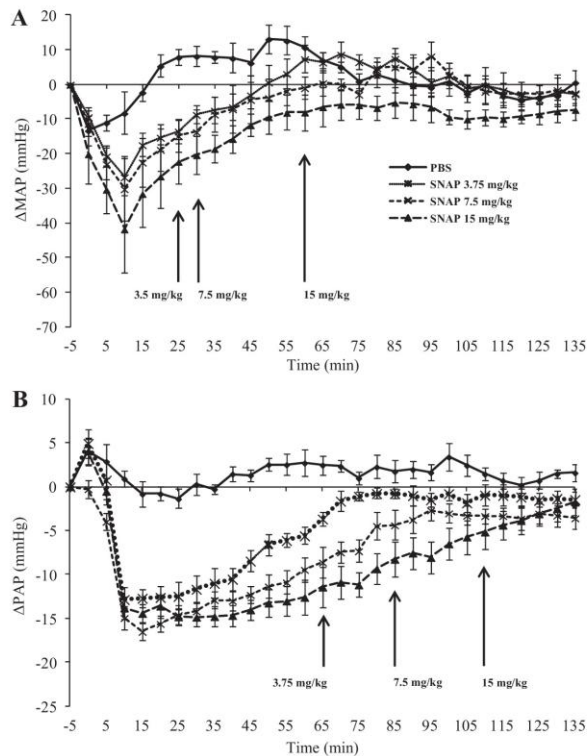


Fig. 7. Impact of unformulated SNAP on MAP (A) ($p_{\text{interaction}} = 0.018$, p_{dose} and $p_{\text{time}} < 10^{-4}$) and PAP (B) ($p_{\text{interaction-pdose}}$ and $p_{\text{time}} < 10^{-4}$) ($n = 6$). Arrows indicate for each dose the last time point showing significant difference vs PBS.

NMP-based ISM, ISMN and SNAP had close release profiles, with burst at 24 h higher than 60% and a complete release in 4–10 days. For SNAP, the most gradual releases are observed with ISM formulations, with $27 \pm 3\%$ and $14 \pm 1\%$ released after 1 h and $46 \pm 3\%$ and $47 \pm 10\%$ after 7 h for NMP- and TA-based ISM, respectively. For all formulations, burst of ISDN was low (at 24 h: $21 \pm 3\%$ released from NMP-based ISI, $37 \pm 2\%$ from NMP-based ISM, $22 \pm 7\%$ from TA-based ISI and $16 \pm 3\%$ from TA-based ISM) and followed by a sustained release over a month.

According to these results, switch to a less water miscible solvent and/or add an external phase to obtain ISM instead of ISI generally decrease the burst. However, the best formulation choice also relies on the characteristics of the incorporated drug.

Three endpoints of the release profiles were chosen to establish the potential links between drug hydrophobicity (P) and the release patterns: percentage of drug released during the burst (i.e., the first 24 h) and times to release 25% and 50% of the drug, expressed as their logarithms. Significant correlations were obtained for these parameters for all formulations except NMP-based ISM (Fig. 6). As a result of this, for each formulation, the higher the hydrophobicity of the NO-donor, the lower was the burst and the more sustained was the release.

Both ISMN and ISDN were used as model drugs in this study. Although ISDN gave the best release profiles, with low bursts and gradual release over a month, organic nitrates cannot be used in therapeutic for sustained release of NO. Indeed, tolerance (i.e., a loss of efficiency) appears under continuous administration. This

phenomenon has been well described since the beginning of their use in the treatment of angina pectoris [5]. Furthermore, in this study, preliminary *in vivo* assays showed that ISDN did not induce any pressure effect when injected subcutaneously, while inducing hypotension when injected intravenously (not shown). In this context, previous studies established the advantage of RSNOs, which do not induce tolerance when continuously used [27,28]. One possible explanation for this difference between both families of NO donors might rely on the mechanisms of NO release. Organic nitrates are indeed deeply dependent on enzymatic metabolism, with few enzymes involved, while NO release from RSNOs can proceed by either enzymatic or non-enzymatic reactions [29]. *In vitro* experiments indicated that the hydrophobicity of the NO donor directly impact its release from *in situ* formulations, even if no statistical significance was reached for NMP-based ISM. Hence, drug design theoretically represents a useful tool to increase both the stability and the hydrophobicity of RSNOs, and enhance their release profile from *in situ* formulations.

3.3. *In vivo* degradation of matrices

DSC and SEC experiments were also performed on samples subcutaneously injected to rats. Results (Table 2) indicated that solvent was quickly removed *in vivo* from both formulations (T_g value close to the native material) and that degradation was slower *in vivo* than *in vitro* (slower decrease in M_n /increase in PDI). Injection sites did not present any visible sign of inflammation.

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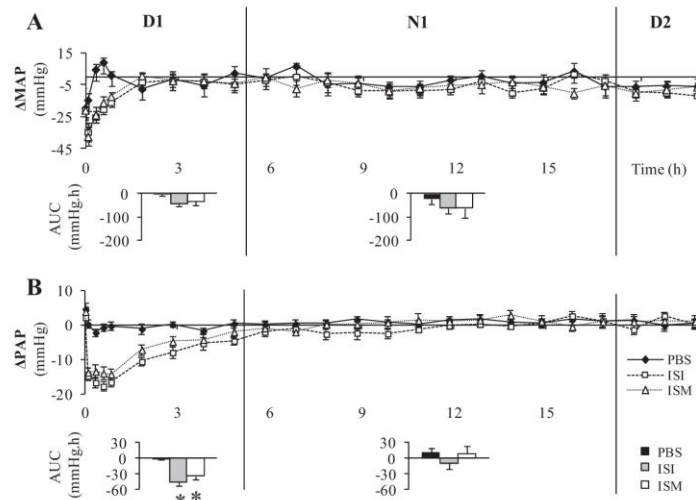


Fig. 8. Impact of SNAP-loaded NMP-based formulations (15 mg/kg) on MAP (A) and PAP (B) ($n=7$). Nycthemera are indicated in days (D1, D2) and nights (N1) periods. * $p < 0.05$ vs PBS. For the sake of clarity, after the first hour, only one point for each hour was represented.

3.4. In vivo effect of SNAP-loaded ISI and ISM on arterial pressure

To obtain the proof of concept that these *in situ* formulations were able to sustain the *in vivo* effect of RSNO, formulations based on NMP and containing SNAP have been selected on the basis of the *in vitro* experiments. First, SNAP release from *in situ* formulations was more extended than GSNO release. Second, NMP is the solvent used in already marketed *in situ* formulations and thus has fulfilled all the requirements for parenteral injection. Finally, viscosity of the ISI TA solution raised potential concern on *in vivo* injection. SNAP-loaded NMP-based formulations were therefore subcutaneously injected in rats and arterial pressure was followed in order to evaluate their vasorelaxant effect.

Injection of unformulated SNAP induced a dose-dependent fall in mean arterial pressure (MAP) vs baseline (from -27 ± 5 mmHg for 3.75 mg/kg to -42 ± 12 mmHg at 15 mg/kg) with a dose-dependent length of effect ranging from 25 to 60 min (Fig. 7A). No dose higher than 15 mg of SNAP/kg was administered, because of the high variability of the arterial pressure value observed at the hypotensive peak, which could have led to unethical hypotension in some of the animals. The fall in pulse arterial pressure (PAP) (ca. -15 mmHg) reached a plateau from the first dose of 3.75 mg/kg, but this fall lasted longer than that of MAP, with a dose-dependent manner from 65 to 110 min (Fig. 7B).

Injections of SNAP-formulations induced a transient (ca. 1.5 h) MAP hypotensive peak (-35 ± 6 and -37 ± 6 mmHg for ISI and ISM, respectively), but the AUC did not significantly differ from the control (Fig. 8A). SNAP-formulations led to a significant fall in PAP (peak at -18 ± 1 and -14 ± 1 mmHg for ISI and ISM respectively) during 5 h for ISI and ISM (Fig. 8B). As a result, both formulations extended SNAP effect on PAP 3-fold compared to unformulated drug, without significantly affecting MAP.

In the literature, studies of RSNOs formulations are generally limited to *in vitro* experiments [14] and only few of them are interested in the prolongation of RSNOs *in vivo* effect [30,31]. In this work, a sustained *in vivo* vasorelaxant effect of SNAP incorporated into NMP-based ISI and ISM was demonstrated

through a significant reduction of PAP during 5 h (see below for pharmacological interest). These formulations, based on a bioerodible FDA-approved polymer with a long history of safe use, are therefore promising for a sustained NO release therapy. Safety concerns raised by the use of an organic solvent, NMP, are lowered by its approval for parenteral use in the already marketed ISI. Despite this, the duration of vasorelaxant effect is low compared to the *in vitro* release period and especially compared to the life span of the polymeric matrix (>1 month). The telemetry system represents a unique tool to follow arterial pressure non-invasively and continuously during prolonged periods. As a result, arterial pressure was the parameter chosen to assess the systemic effect of SNAP. Nevertheless, vasorelaxant effect requires high doses of NO compared to other physiological effect such as inhibition of platelets activation and aggregation [32]. This latter will be investigated in the future, as it could be modified for a longer time than arterial pressure by the sustained release of NO from RSNOs-loaded ISI or ISM. Moreover, a local effect might also be targeted with *in situ* formulations, for example to enhance blood flow in an ischemic region. In this case, a lower drug payload should be used to avoid any systemic action. Finally, discrepancy between *in vitro* and *in vivo* results with *in situ* formulations has been recently highlighted and attributed in particular to differences in the compressive forces of the surrounding environment [33]. In this study, *in vitro-in vivo* differences in terms of polymeric matrices degradation have been demonstrated (see Figs. 3 and 4, Table 2). Nevertheless, the duration of effect of loaded formulations on arterial pressure (5 h) is in the same range as the time of *in vitro* release before reaching the plateau. As this time increases with drug hydrophobicity, the use of RSNO with higher clogP (from drug design) could improve *in vivo* results. This, as well as the fact that penicillamine (the chemical core of SNAP) is known to induce autoimmunity [34], suggests that other RSNO or NO donor will be a better choice for a therapeutic application. For example, use of NONOates, whose hydrophobicity is easily tunable, might represent an alternative approach [35].

Finally, the reduction of PAP without change in MAP suggests a higher impact of SNAP on venous than arterial bed. Physiologically, a decrease in PAP is attributable to a fall in stroke volume and/or an increase in elastic properties of the aortic wall. This latter can be excluded, as NO donors do not directly change aortic wall elastic properties in rats [36]. Therefore, the direct effect on venous bed more probably explains the long lasted decrease in PAP, certainly via a decrease in venous return and then stroke volume. As a result, therapeutic impact of these RSNO-formulations will be investigated in the future in an animal model of cardiac infarction.

4. Conclusion

This study reports for the first time the integration of RSNOs into ISI and ISM. Formation and degradation of the matrices were only slightly affected by the solvent or the type of formulation used. Interestingly, *in vitro* releases of NO donors were well correlated with the hydrophobicity of the compounds. *In vivo* results showed a 3-fold significant extension of SNAP effect on PAP when formulated in ISI or ISM vs unformulated drug. In conclusion, ISI and ISM were shown to be suitable for NO-sustained delivery, although some optimizations are still needed, for example improvements of RSNOs chemical structure through drug design, or the use of other NO donors. Further studies will evaluate whether these RSNOs formulations might be useful in cardiac or ischemic diseases.

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3. RESULTATS COMPLEMENTAIRES

Dans cette section sont présentés des résultats complémentaires à ceux de l'Article 5.

En premier lieu, en ce qui concerne les résultats *in vitro*, la comparaison des résultats de libération obtenus par différentes méthodes de dosage est effectuée. En outre, plus de détails sont apportés sur la libération des donneurs de NO sous leur forme native ou leurs produits de dégradation.

En second lieu, des résultats supplémentaires sont présentés en ce qui concerne les différences de morphologie des matrices polymériques obtenues *in vitro* et *in vivo*.

En dernier lieu, des résultats complémentaires *in vivo* sont apportés, avec d'une part les expériences menées pour suivre la pharmacocinétique des RSNO, et d'autre part les résultats de télémétrie avec les formulations chargées en GSNO. Finalement, les expériences préliminaires concernant l'inhibition de l'activation et l'agrégation plaquettaire par les RSNO chez l'homme sont décrites, ainsi que le protocole retenu pour la future étude chez le rat après administration des formulations chargées en RSNO.

3.1. Cinétiques de libération *in vitro*

3.1.1. Prélèvements

Comme indiqué dans l'Article 3, différentes méthodes peuvent être utilisées pour réaliser les essais de libération *in vitro*. Afin de diminuer le nombre d'implants à réaliser, une méthode « indirecte » de dosage (quantification dans les milieux de libération) a été retenue. Une étude a cependant été menée pour vérifier l'adéquation entre les résultats fournis cette méthode et ceux donnés par une méthode « directe » de dosage (quantification dans les formulations), pour des formulations contenant du dinitrate d'isosorbide.

La préparation des formulations et la méthode « indirecte » de dosage étaient similaires à celles décrites dans l'Article 5. L'essai de libération était conduit pendant 10 jours, sans changement de milieu, puis une analyse « indirecte » (milieu de libération) et une analyse « directe » des formulations étaient réalisées en parallèle sur le même échantillon. La méthode « directe » de dosage consistait à dissoudre les formulations lyophilisées dans 25,0 mL d'acétonitrile. Après 5 minutes au bain à ultrasons et 1 h 30 sous agitation magnétique (500 rpm), une dilution dans la phase mobile et une centrifugation de 20 min à 42 000 g étaient effectuées avant injection de 50 μ L de la solution dans le système CLHP (identique à celui décrit dans l'Article 5). Les résultats n'ont pas mis en évidence de différence significative entre les deux méthodes (test des rangs signés de Wilcoxon ; $\alpha = 0,05$; $n = 3$) (Figure 13).

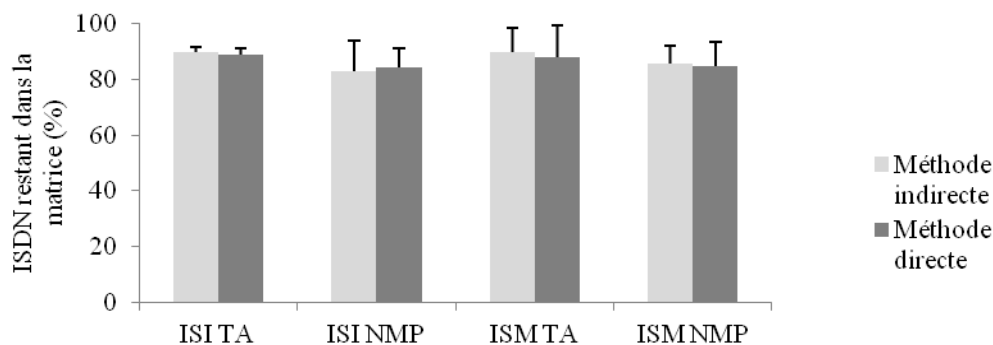


Figure 13 : Comparaison entre les méthodes de dosage du dinitrate d'isosorbide. La méthode « indirecte » détermine la concentration de l'espèce dans le milieu de libération, la méthode « directe », celle dans la formulation (après extraction). ISI : implants *in situ* ; ISM : microparticules *in situ* ; NMP : *N*-méthyl-2-pyrrolidone ; TA : triacétine.

3.1.2. Libération *in vitro* de *S*-nitrosothiols : sont-ils dégradés ?

Dans l'Article 5, une libération *in vitro* prolongée de 4 donneurs de NO (GSNO, SNAP, ISDN et ISMN) a été montrée. Bien que cela n'ait pas été développé dans l'article, ce sont principalement les RSNO ou les nitrates organiques qui sont retrouvés dans le milieu de dégradation et non leurs produits de dégradation. En ce qui concerne l'ISDN et l'ISMN, aucun produit de dégradation (ions nitrate) n'a été mis en évidence au cours de l'étude (résultats non montrés). Pour les RSNO, GSNO est majoritairement retrouvé, bien que des ions nitrite et nitrate ainsi que du glutathion oxydé aient également été mis en évidence (*cf.* équation 4 de l'Article 4) (Figure 14).

En ce qui concerne SNAP, la molécule parente était également majoritaire, même si les produits de dégradation (ions nitrite et nitrate) étaient présents en plus forte proportion (Figure 15), ce qui est cohérent avec une stabilité *in vitro* plus faible du SNAP (voir l'étude de stabilité Figure 16). Cependant, il est possible que la dégradation des RSNO n'ait pas eu lieu à l'intérieur des matrices polymériques mais dans le milieu de libération.

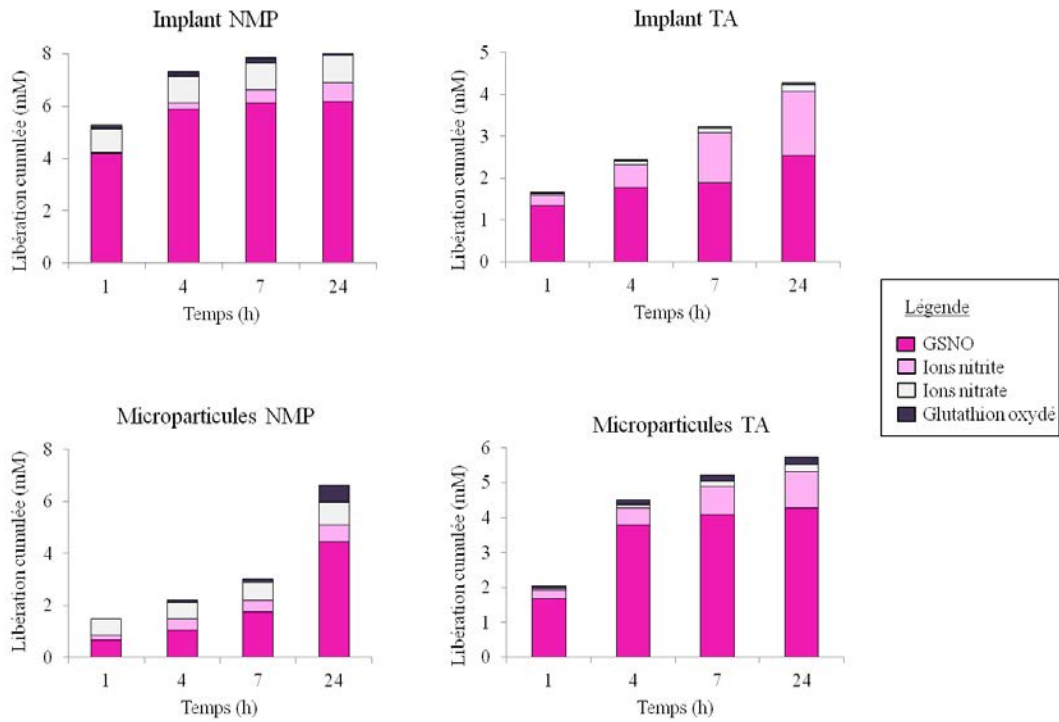


Figure 14 : Libération *in vitro* du GSNO et de ses produits de dégradation à partir des formulations *in situ*. Le dosage des 4 espèces a été réalisé en simultanément par la méthode de chromatographie liquide développée dans l'Article 4.

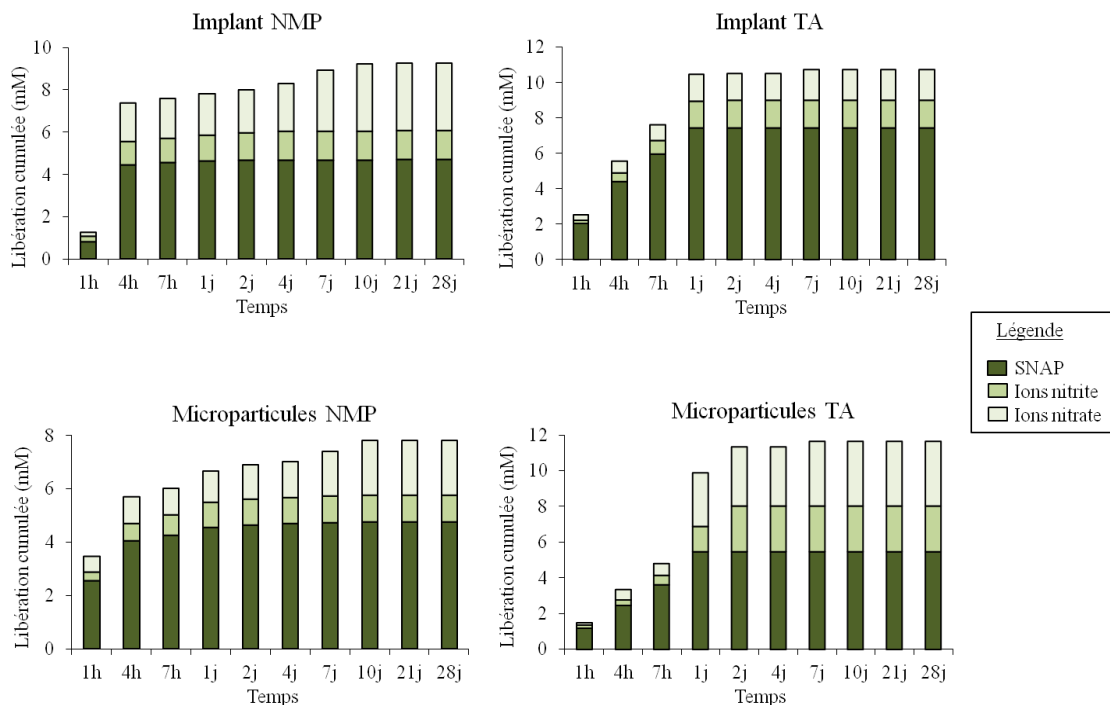


Figure 15 : Libération *in vitro* du SNAP et de ses produits de dégradation à partir des formulations *in situ*. Le dosage de SNAP et des ions nitrite a été réalisé par colorimétrie (Saville-Griess et Griess) et celui des ions nitrate grâce au kit « Total NO/Nitrite/Nitrate Immunoassay » (R&D Systems : réduction des nitrates en nitrites par une nitrate réductase, puis colorimétrie).

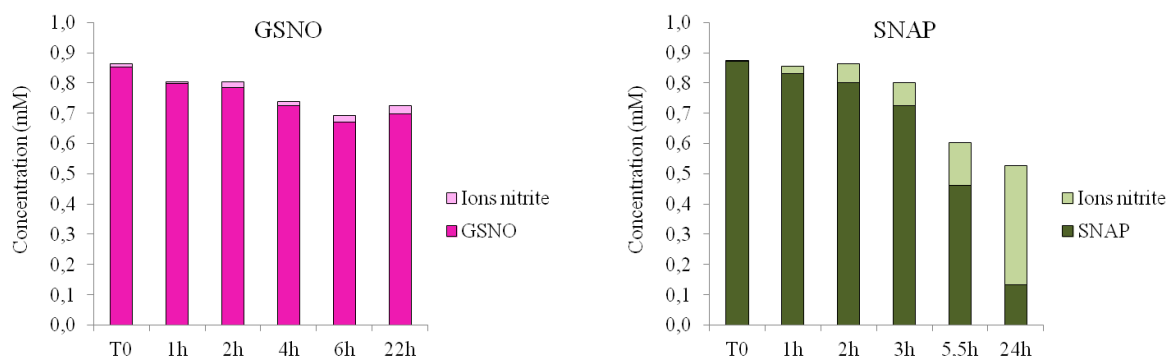


Figure 16 : Stabilité des RSNO dans les conditions des essais de libération *in vitro*. Des solutions de RSNO dans le milieu de libération ont été préparées (à partir des RSNO isolés) et incubées dans les conditions des essais de libération. Les concentrations en RSNO et ions nitrite ont été mesurées par colorimétrie (Saville-Griess ou Griess).

3.2. Morphologie des matrices polymériques : différences *in vitro* et *in vivo*

La microscopie a montré des surfaces plus rugueuses et plus hétérogènes *in vivo*, ainsi que des structures internes plus denses et moins poreuses, pour les ISI (Figure 17) comme pour les ISM (même tendance). Par ailleurs, les formulations *in vitro* précipitaient au fond des tubes et se présentaient au 7^{ème} jour comme des petits cylindres avec un volume calculé de 0,3 cm³ pour les ISI et 0,8 cm³ pour les ISM. *A contrario*, les formulations *in vivo* étaient plus plates, en ellipse, avec au 7^{ème} jour un volume de 0,1 cm³ pour les deux formulations. Ces observations mettent en évidence l'impact de l'environnement sur la solidification de la matrice polymérique. Ils sont cohérents avec ceux de Patel *et coll.* [79], qui ont montré que les formulations à base de PLGA de faible masse molaire (18 000 g/mol) ont tendance à gonfler *in vitro*, mais qu'*in vivo* leur gonflement est limité par la barrière physique constituée par les tissus environnants.

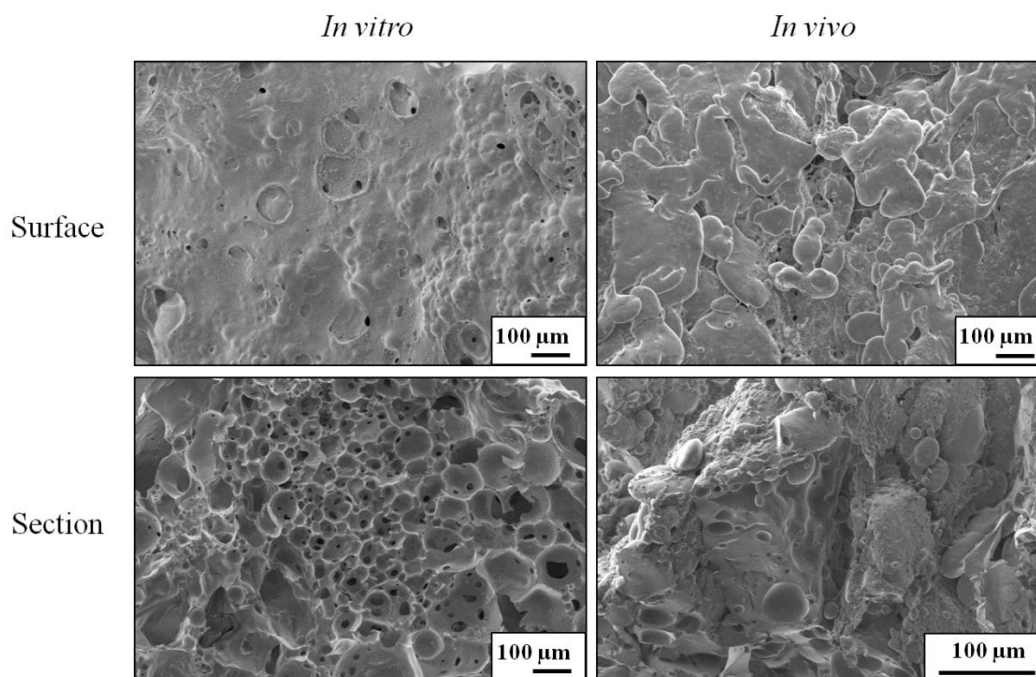


Figure 17 : Microscopie électronique à balayage de la surface et de la section d'implants *in situ* chargés en GSNO 7 jours après formation *in vitro* ou *in vivo*.

3.3. Libération et effet prolongés *in vivo*

3.3.1. Pharmacocinétique

Classiquement, les études *in vivo* s'intéressent à la fois à l'effet (pharmacodynamie) et à la libération (pharmacocinétique) du principe actif. Néanmoins, dans le cas de l'administration de RSNO, il est très délicat de réaliser des études pharmacocinétiques. En effet, l'existence de RSNO endogènes et les phénomènes de transnitrosation ne permettent pas de suivre le devenir exact de la molécule injectée (sauf radiomarquage éventuel). D'une part, les concentrations plasmatiques des dérivés du NO (RSNO, NO_2^- , NO_3^-) sont faibles et controversées (du pico- au micro-molaire selon les études, les méthodes et les conditions de prélèvements car certaines peuvent induire une inflammation et donc l'activité des iNOS) [69,80–82]. D'autre part, elles sont sujettes à des variations inter- et intra-individuelles. Enfin, la libération progressive des RSNO à partir des formes *in situ* pourrait générer des variations de concentrations plasmatiques trop faibles pour être détectables avec les méthodes à disposition du laboratoire.

Un essai préliminaire (approuvé par le comité d'éthique régional, projet « SNOtélém ») a cependant été mené sur des rats pour évaluer la faisabilité d'une étude pharmacocinétique, en mesurant les concentrations plasmatiques en RSNO, ions nitrite et nitrate ainsi qu'en méthémoglobine

(molécule induite par la réaction d'un donneur de NO sur l'hémoglobine). Les animaux utilisés pour cette étude étaient des rats Wistar mâles non consanguins (540 ± 40 g, Rj/HAN : Wi ; Janvier, Le Genest-St-Ile, France) hébergés en conditions standards avec nourriture et boisson *ad libitum* (AO4 ; Villemoisson-Epinay-Sur-Orge, France et système d'osmose inverse, Culligan, Bruxelles, Belgique). 24 à 48 h avant les expériences, un cathéter en polyéthylène était posé au niveau de l'artère fémorale gauche et externalisé entre les omoplates (sous anesthésie à l'isoflurane : induction à 4 % v/v dans O₂ à 2 L/min ; maintien à 2 % v/v dans O₂ à 1 L/min). Le cathéter était rempli d'une solution héparinée de glucose 30 % m/v et les animaux étaient placés en cages individuelles pour la récupération (24 h minimum, paracétamol 60 mg/kg/24 h dans l'eau de boisson). Le sang total était recueilli dans des tubes contenant l'EDTA comme anticoagulant (en raison d'une interférence de l'héparine avec les méthodes analytiques) et les analyses effectuées dans l'heure suivante [83,84]. 25 μ L du sang total étaient immédiatement dilués dans 4 mL d'eau ultrapure afin de calculer la méthémoglobinémie (MetHb) après analyse en spectrophotométrie selon la formule suivante :

$$MetHb\% = \frac{A_{630} - A_{650}}{A_{576} - A_{650}} * 100$$

Où A_{630} correspond au maximum d'absorbance de la méthémoglobine (630 nm), A_{576} à celui de l'oxyhémoglobine (576 nm) et A_{650} au bruit de fond (650 nm).

Le reste du sang était rapidement centrifugé (2 600 tours/min, 10 min, 4 °C) pour récupérer le plasma. Les RSNO et les ions nitrite étaient dosés par une technique fluorimétrique. Brièvement pour le dosage des ions nitrite, 60 μ L d'une solution de 2,3-diaminonaphtalène (105 μ M dans HCl 0,6 M) étaient ajoutés aux 600 μ L d'échantillon dilué. Après 10 min d'incubation à 37 °C et à l'abri de la lumière, 40 μ L de NaOH 1,2 M étaient ajoutés avant de mesurer l'intensité de fluorescence (Fluorimètre Hitachi F-2000, $\lambda_{exc} = 375$ nm et $\lambda_{ém} = 415$ nm). Selon le même protocole, l'utilisation d'une solution de 2,3-diaminonaphtalène supplémentée avec du chlorure de mercure (1,05 μ M) permettait de doser les ions nitrite et les RSNO.

Le kit « Total NO/Nitrite/Nitrate Immunoassay » (R&D Systems) était utilisé pour le dosage simultané des ions nitrite et nitrate (NOx), selon les instructions du fabricant.

A la fin des expériences, les animaux étaient sacrifiés par overdose de pentobarbital (250 mg/kg injectés en intrapéritonéal).

Dans un premier temps, la mesure des paramètres physiologiques chez des rats non traités a donné les résultats présentés dans le Tableau 3. Les prélèvements étant fréquemment hémolysés, l'utilisation du kit pour le dosage des NOx n'a pas été possible dans la majorité des cas (mesure à 570 nm, proche de l'absorbance maximum de l'oxyhémoglobine à 576 nm, donc forte interférence).

Tableau 3 : Détermination des valeurs physiologiques des espèces dérivées du NO chez le rat Wistar mâle : méthémoglobinémie (MetHb), concentrations plasmatiques en S-nitrosothiols (RSNO), en ions nitrite (NO_2^-) ainsi qu'en ions nitrite et nitrate (NOx).

	Résultats expérimentaux			Littérature		
	n	Moyenne	Ecart-type	Moyenne	Ecart-type	Référence
MetHb (%)	13	0,6	0,1	1,5	0,2	[85]
RSNO (μM)	7	0,6	0,5	0,12	0,05	[80]
NO_2^- (μM)	7	2,0	0,3	1,95	1,96	[86]
NOx (μM)	2	53,2	/	34	2	[87]

Bien que montrant une variabilité importante, les résultats obtenus étaient cohérents avec ceux de la littérature. L'étape suivante a donc consisté à injecter aux animaux un bolus sous-cutané de 30 mg de GSNO/kg. Des prélèvements de sang étaient effectués 5 min avant l'injection puis 5, 15, 30, 60 et 120 min après l'injection.

L'injection d'un bolus sous-cutané de GSNO à 30 mg/kg a provoqué une légère augmentation des concentrations plasmatiques de RSNO par rapport aux valeurs physiologiques. Cette augmentation était transitoire, maximale à 30 min (Figure 18). La concentration plasmatique des ions nitrites était augmentée selon le même profil, sans que la méthémoglobinémie ne soit influencée. Sur les prélèvements ne présentant pas d'hémolyse (seulement 2 rats), la concentration des NOx semble augmenter progressivement après l'injection de GSNO. Pour l'ensemble des paramètres mesurés, une grande variabilité des résultats est à nouveau retrouvée.

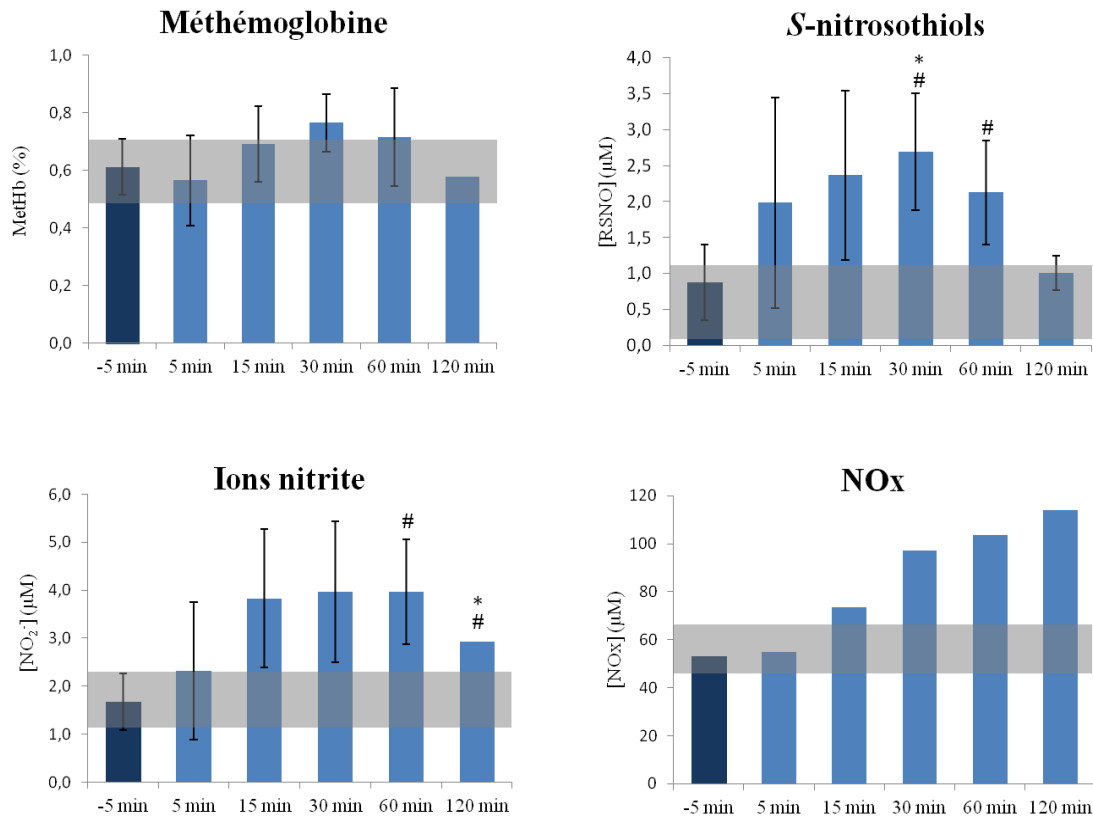


Figure 18 : Evolution des différents paramètres pharmacocinétiques après injection sous-cutanée d'un bolus de 30 mg GSNO/kg (n = 5 sauf pour les NOx où n = 2) ; * p < 0,05 vs t_{5 min} avant injection ; # p < 0,05 vs valeurs physiologiques précédemment déterminées (et représentées par les bandeaux grisés).

Etant donné la faible amplitude dans les variations de ces paramètres et la largeur des écart-types observés, il était probable que cette méthodologie ne permettrait pas de mettre en évidence une libération progressive de la même dose de GSNO à partir des implants *in situ*.

Par conséquent, il n'a pas été possible de prouver directement l'obtention d'une libération prolongée des RSNO à partir des formulations *in situ*, grâce à une étude pharmacocinétique. Cependant une preuve indirecte a été apportée, *via* la prolongation des durées de baisse de la MAP et de la PAP (étude pharmacodynamique) avec des formulations chargées en SNAP (Article 5) ou en GSNO (ci-après).

3.3.2. Résultats obtenus par télémétrie : formulations à base de NMP chargées en GSNO

L'article précédent a montré que l'administration de formulations *in situ* à base de NMP chargées en SNAP (15 mg/kg) permettait d'augmenter de façon importante la durée de son effet sur la pression artérielle pulsée, et ce sans modifier significativement la pression artérielle moyenne (ni la fréquence cardiaque).

Le choix de tester les formulations SNAP s'est fait par rapport aux résultats *in vitro* car ce RSNO plus hydrophobe était libéré de façon plus progressive et prolongée à partir des formulations *in situ*. Néanmoins, comme indiqué dans la revue 3, les profils de libération *in vitro* et *in vivo* sont rarement corrélés car le devenir de ces formulations est très dépendant de l'environnement (*cf.* résultats complémentaires, différences *in vitro* et *in vivo* dans le devenir des matrices).

De plus, bien que Islam *et coll.* [88] aient également obtenu avec des implants *in situ* un *burst in vitro* plus faible avec un principe actif hydrophobe qu'avec un principe actif hydrophile, Deadman *et coll.* [89] ont montré qu'*in vivo*, l'hydrophobie de la molécule n'était pas forcément le principal facteur modulant sa libération à partir de telles formulations. Enfin, le GSNO est le RSNO qui est le plus largement étudié dans la littérature, représentant en quelque sorte le RSNO « modèle », existant par ailleurs de façon endogène, contrairement au SNAP. Par conséquent, les formulations (ISI et ISM) à base de NMP et chargées en GSNO (30 mg/kg) ont également été testées *in vivo* grâce au système de télémétrie, selon les mêmes protocoles que ceux utilisés dans l'étude avec SNAP.

Dans un premier temps, l'injection sous-cutanée chez le rat Wistar de solutions de GSNO a montré une diminution dose-dépendante de la pression artérielle moyenne (MAP) par rapport aux valeurs initiales (de -24 ± 3 mm Hg pour la dose de 3,75 mg/kg jusque -56 ± 6 mm Hg pour la dose de 30 mg/kg), avec un effet durant jusque 40 min pour la dose la plus forte (Figure 19A). Cette hypotension était associée avec une tachycardie de même durée (Figure 19C). Ceci était attendu car l'augmentation de la fréquence cardiaque est l'un des mécanismes de régulation permettant de revenir à une pression artérielle normale (baroréflexe). En parallèle, la pression artérielle pulsée (PAP) a chuté d'environ -17 mm Hg (soit -50 % par rapport à la valeur de base), quelle que soit la dose de GSNO administrée (Figure 19B). Cet effet a duré plus longtemps que ceux de la MAP ou de la fréquence cardiaque (HR), pendant 60 min pour la dose de 3,75 mg/kg jusque 125 min pour la dose de 30 mg/kg.

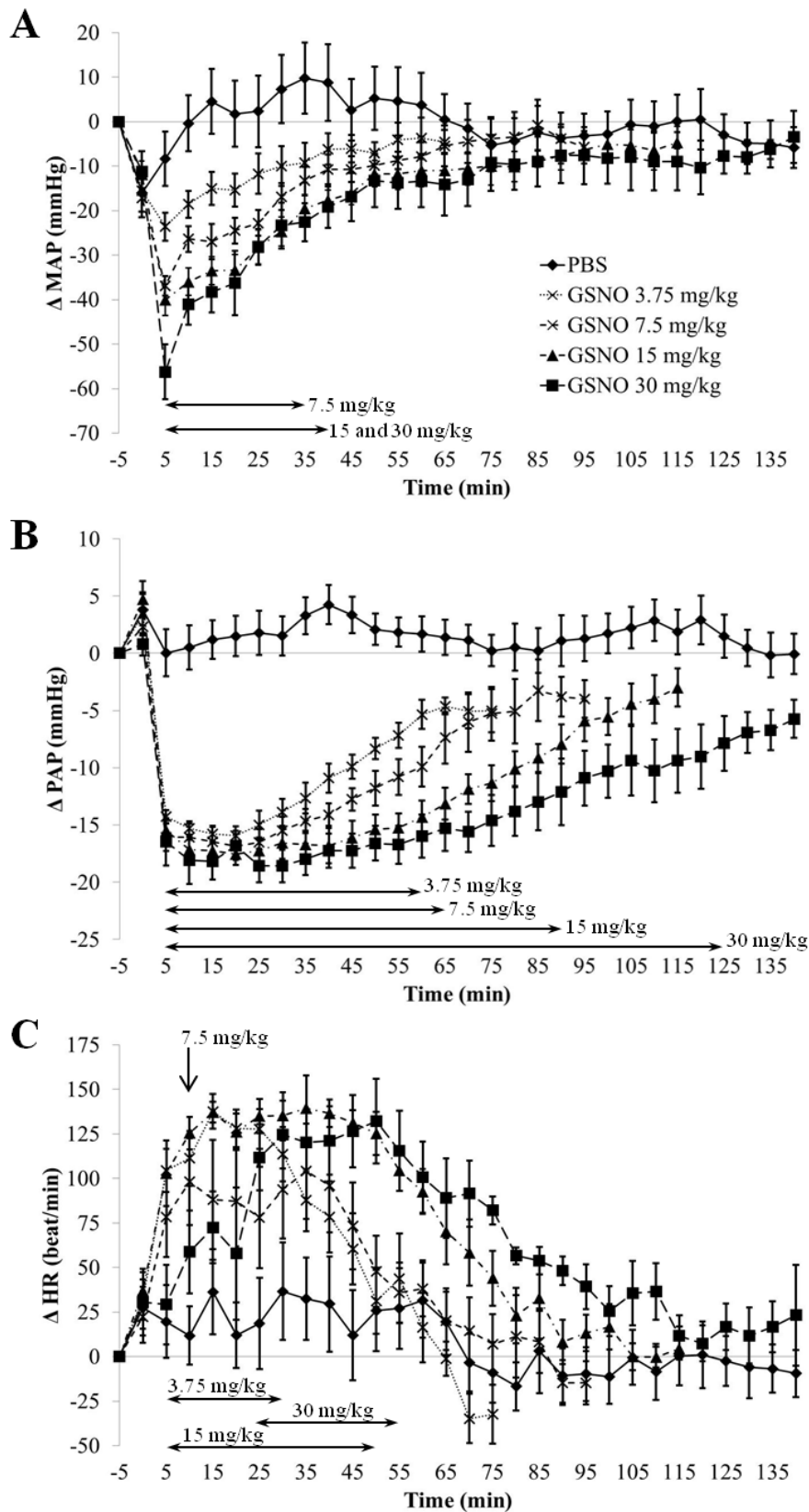


Figure 19 : Effet du GSNO (non formulé) injecté en sous-cutanée chez le rat sur A) la pression artérielle moyenne, B) la pression artérielle pulsée et C) la fréquence cardiaque. $p_{interaction}$, p_{dose} et $p_{temps} < 10^{-4}$ pour les 3 paramètres ($n = 7$). Les flèches indiquent, pour chaque dose, le temps durant lequel un effet significativement différent du PBS est observé.

Les deux types de formulations chargées en GSNO (30 mg GSNO/kg) ont généré une hypotension modérée (environ – 10 mm Hg soit – 15 % par rapport à la valeur de base) mais prolongée (Figure 20A). Ceci a été confirmé par des aires sous la courbe plus faibles que celles du contrôle, à la fois le jour et la nuit. L'analyse statistique indique que cette diminution de MAP est significative pendant 18 h, soit 18 fois plus longtemps que celle obtenue avec la même dose de GSNO injectée sous forme de solution. Par ailleurs, les deux formulations réduisent la PAP avec la même intensité que le GSNO en solution (environ – 15 mm Hg) (Figure 20B). Cependant, leurs effets sont plus longs : 30 h pour les ISI et 42 h pour les ISM, soit une prolongation de 15 fois (ISI) et 21 fois (ISM) par rapport à la solution de GSNO. Enfin, aucun des deux types de formulations ne modifie significativement la fréquence cardiaque, malgré une augmentation transitoire dans les premiers temps après l'injection (Figure 20C).

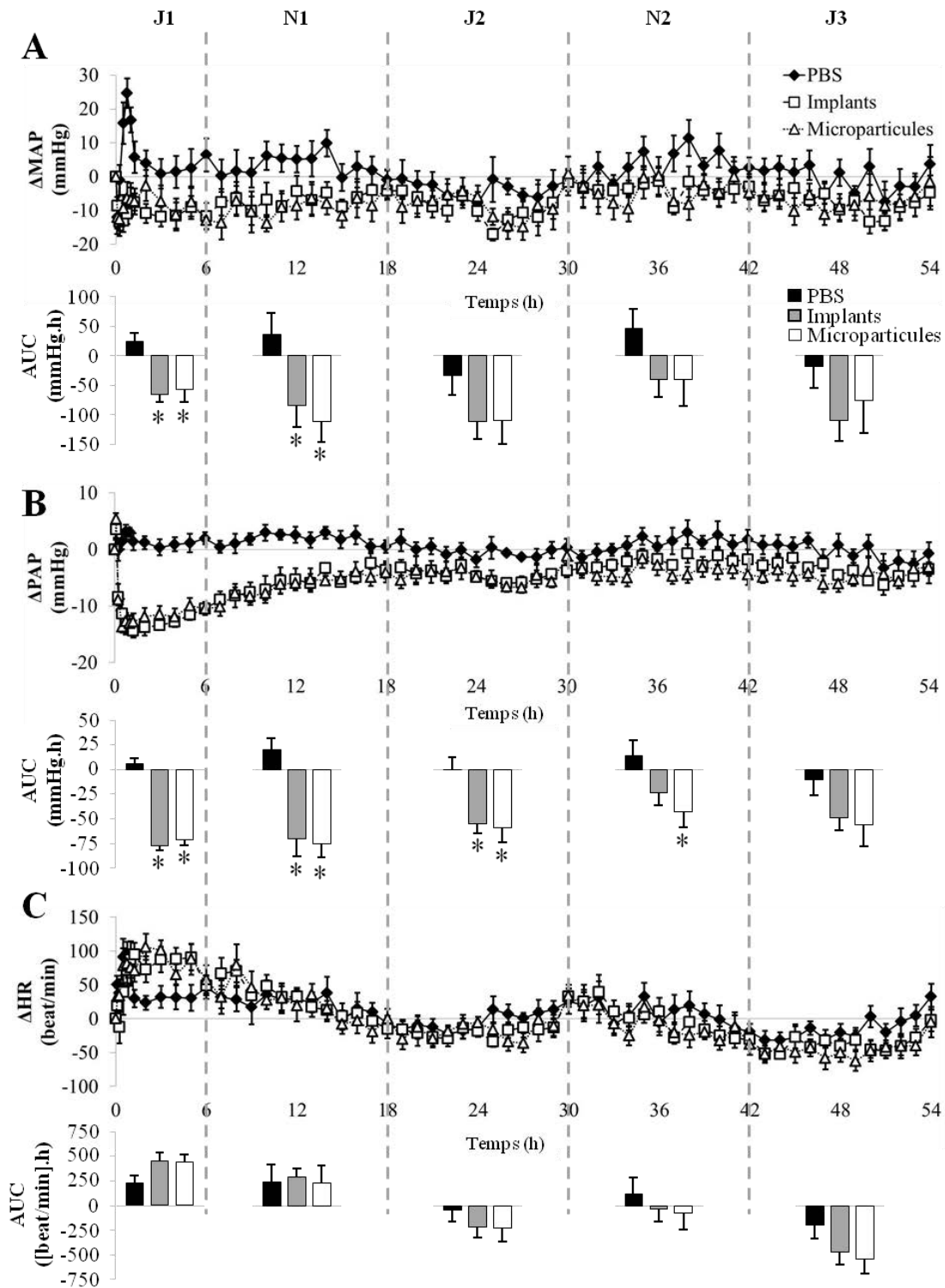


Figure 20: Effet des formulations à base de NMP chargées en GSNO (30 mg/kg) injectées en sous-cutanée chez le rat sur A) la pression artérielle moyenne, B) la pression artérielle pulsée et C) la fréquence cardiaque (n = 7). Les périodes de jour (J1, J2, J3) et de nuit (N1, N2) sont indiquées. * p < 0,05 vs PBS. Pour des raisons de clarté, après la 1^{ère} heure, seulement un point toutes les heures est représenté sur chacun des graphiques.

Les doses de RSNO dans les formulations testées peuvent être considérées comme similaires. En effet, 30 mg GSNO/kg (MM GSNO = 336 g/mol) équivalent à 9×10^{-5} moles de GSNO/kg et 15 mg SNAP/kg (MM SNAP = 220 g/mol) équivalent à 7×10^{-5} moles de SNAP/kg et les deux molécules ne peuvent libérer qu'un équivalent NO. Par conséquent, contrairement à ce qui a été proposé à la suite de l'étude *in vitro*, les formulations chargées en GSNO semblent plus intéressantes que celles chargées en SNAP *in vivo*. En effet, elles sont plus efficaces pour obtenir une réduction prolongée de la PAP (30 h et 42 h pour les ISI et ISM GSNO, contre 5 h avec les formulations contenant SNAP). Cependant, cette diminution de PAP s'accompagne dans le cas des formulations GSNO d'une hypotension significative. Dans le futur, il est prévu de tester des formulations chargées avec 7,5 mg GSNO/kg pour essayer de moduler l'effet sur la pression artérielle, en conservant la diminution de PAP sans altération de la MAP. En effet, l'effet plus prolongé des RSNO sur la PAP que sur la MAP est probablement liée à une dilatation préférentiellement veineuse, qui se traduit par une diminution de la précharge et donc du volume d'éjection systolique (diminution du travail cardiaque) (*cf.* Figure 11). Cette propriété est intéressante sur un plan thérapeutique, par contre une hypotension trop importante pourrait être délétère (malaise, diminution de perfusion des organes). Des expérimentations complémentaires sont nécessaires afin de confirmer l'effet sur le travail cardiaque. Ceci est envisagé rapidement par des mesures du volume d'éjection systolique par échocardiographie. Ces résultats seront valorisés par la suite.

3.3.3. Agrégation plaquettaire

D'un point de vue pharmacodynamique, l'effet des RSNO sur la pression artérielle a été choisi en tant qu'indicateur d'une libération prolongée car il s'agissait d'un paramètre facile à suivre grâce au système de télémétrie. Néanmoins l'effet sur la pression artérielle nécessite des doses assez fortes de RSNO (ex : 300 nmol/kg en bolus intraveineux [90]). Comme le NO et les RSNO sont également connus pour inhiber l'activation et l'agrégation des plaquettes, y compris à concentration plus faible que celle induisant une vasodilatation [91], il serait intéressant de vérifier dans quelle mesure ces propriétés sont conservées/modifiées avec les formulations *in situ*. Ceci permettrait d'une part de confirmer les observations faites sur la pression artérielle et d'autre part, l'effet sur les plaquettes pourrait durer plus longtemps que l'effet sur la pression artérielle, en réponse à une libération de RSNO plus faible mais encore présente après les premiers jours.

Des essais préliminaires d'agrégométrie ont donc été effectués *in vitro* afin de vérifier le potentiel de GSNO et SNAP sur les fonctions plaquettaires humaines. Cette méthode turbidimétrique est basée sur la mesure de la variation d'absorbance (à une longueur d'onde de 950 nm) d'un plasma riche en plaquettes (PRP) (37 °C, sous agitation magnétique) auquel on ajoute un agoniste d'activation

plaquettaire (Figures 21 et 22). Le PRP est opalescent au départ puis se clarifie au fur et à mesure de la formation d'agrégats plaquettaires.

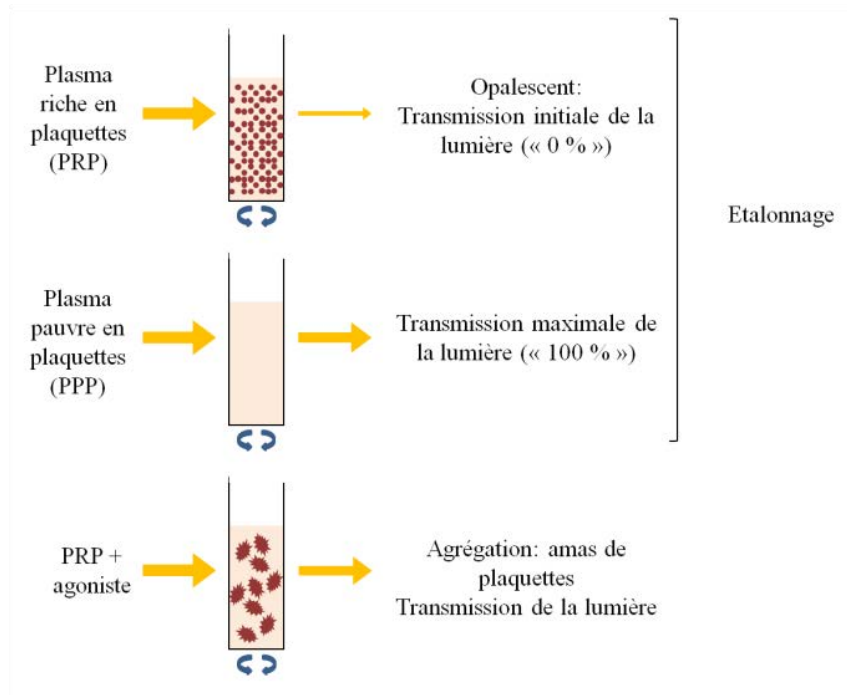


Figure 21 : Principe de l'évaluation de l'activation et agrégation des plaquettes par agrégométrie.

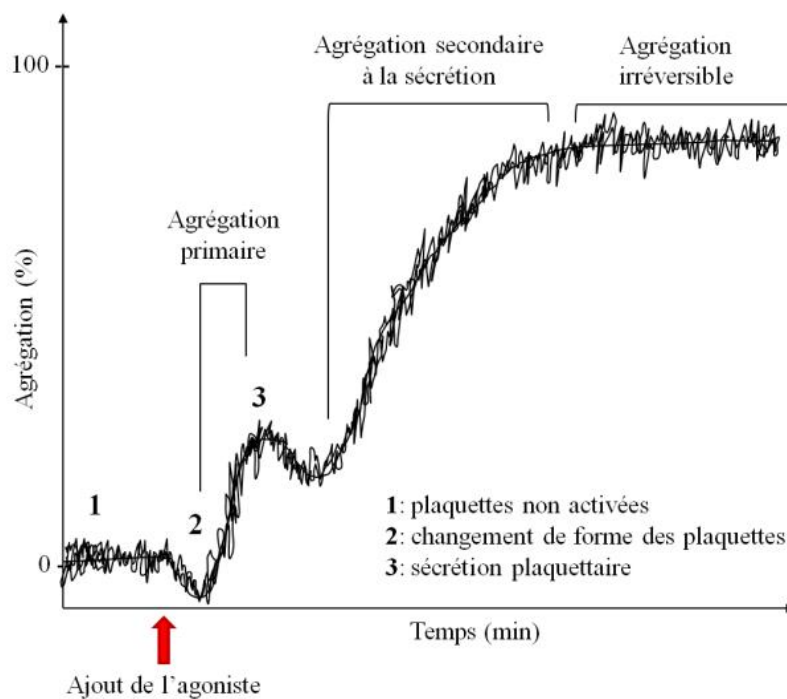


Figure 22 : Représentation schématique d'un profil d'activation/ agrégation plaquettaire obtenu par agrégométrie.

Brièvement, le protocole était le suivant : environ 20 mL de sang veineux étaient prélevés chez un donneur volontaire, sur un tube contenant du citrate de sodium comme anticoagulant. Une première centrifugation ($172 \times g$, 10 min, température ambiante) permettait de récupérer le PRP (phase supérieure). Celui-ci était analysé (automate Advia 2120 Siemens, 300 μ L) pour obtenir la numération plaquettaire. Une deuxième centrifugation des tubes ($2\,500 \times g$, 10 min, température ambiante) permettait de récupérer le plasma pauvre en plaquettes (PPP) (phase supérieure). Le PPP était utilisé pour étalonner l'agrégomètre (Soderel Medical, 8 voies, logiciel Thrombo 1.1.) et pour diluer le PRP afin d'ajuster la numération plaquettaire à 300 G/L. Une solution mère à 10^{-2} M de RSNO était obtenue par remise en solution extemporanée des RSNO isolés dans du NaCl 0,9 %. Des solutions filles aux concentrations désirées étaient obtenues par dilutions successives.

Ensuite, 270 μ L de PRP étaient thermostatés dans l'agrégomètre (à 37 °C sous agitation magnétique) puis 30 μ L de NaCl 0,9 % ou de solution de RSNO étaient ajoutés. Après la durée d'incubation souhaitée, l'activation et l'agrégation plaquettaires étaient déclenchées par l'ajout de différents agonistes : adénosine diphosphate (ADP), collagène, *Thrombin-Receptor Agonist Peptide-6* (TRAP). Pour chaque agoniste, deux concentrations finales ont été testées, suivant les protocoles habituels du laboratoire d'hémostase : ADP 5 ou 10 μ M, collagène 2 ou 10 μ g/mL, TRAP 12,5 ou 25 μ M.

La Figure 23 montre des exemples de résultats obtenus en incubant le PRP humain avec des solutions de GSNO à différentes concentrations pendant 5 minutes avant l'ajout des agonistes.

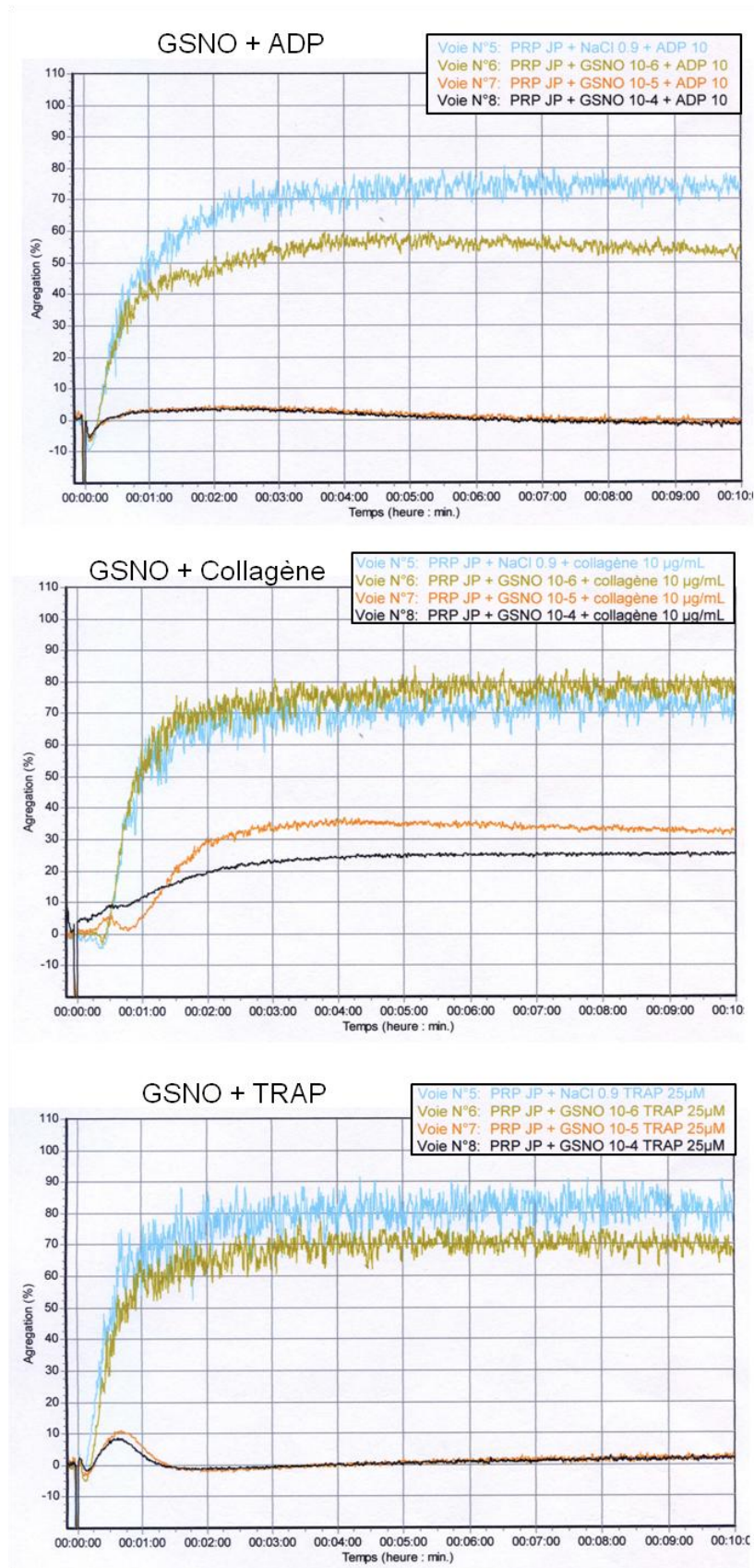


Figure 23 : Profils d'agrégation plaquettaire obtenus lors de l'incubation du PRP avec du GSNO pendant 5 minutes avant l'ajout de l'agoniste: ADP 10 µM, collagène 10 µg/mL ou TRAP 25 µM.

La diminution de l'activation et de l'agrégation plaquettaire par GSNO est dose-dépendante. Elle implique plusieurs voies de signalisation des plaquettes [92] car elle est retrouvée quel que soit l'agoniste utilisé. De plus, l'effet de GSNO est assez puissant car maintenu même avec l'utilisation de fortes concentrations d'agonistes. Des résultats similaires ont été obtenus avec SNAP. Par ailleurs, une expérience complémentaire a montré que l'effet de GSNO (10^{-4} M) est maintenu lorsque la durée d'incubation du PRP avec le *S*-nitrosothiol est augmentée de 5 jusque 60 min avant l'ajout de l'agoniste (ADP 10 μ M).

Ces résultats préliminaires confirment donc que les RSNO testés ont un effet *in vitro* sur la fonctionnalité des plaquettes humaines et les données obtenues sont cohérentes avec celles décrites dans la littérature [93–96].

Par conséquent, d'autres expériences seront conduites prochainement chez l'animal afin d'évaluer l'impact des formulations *in situ* de GSNO sur la fonction plaquettaire. Pour des raisons éthiques, le volume et le rythme des prélèvements de sang nécessitent de travailler en sang total, et non en plasma riche en plaquettes. Le protocole expérimental proposé est le suivant : 5 groupes de 5 rats Wistar mâles (\approx 500 g) recevront par voie sous-cutanée des formulations sans principe actif, des ISI chargés en GSNO (30 mg GSNO/kg et 7,5 mg GSNO/kg), des ISM chargées en GSNO (30 mg GSNO/kg et 7,5 mg GSNO/kg). Des prélèvements de sang total (environ 1 mL recueilli sur tube contenant l'hirudine comme anticoagulant) seront effectués par incision à la queue 24 h avant l'injection, puis à 1, 24, 48 et 72 h après l'injection. Le test d'agrégation plaquettaire sera réalisé immédiatement par turbidimétrie sur sang total (Multiplate, Roche, France) avec 2 agonistes différents (ADP et collagène).

3.4. Conclusion

Les travaux précédemment décrits ont montré que les ISI et ISM sont des formes galéniques permettant la libération *in vitro* prolongée de RSNO. *In vivo*, sur des modèles physiologiques, un effet prolongé sur la pression artérielle a été prouvé, traduisant une libération progressive des RSNO. Ces formulations semblent donc relativement bien adaptées à ces principes actifs, même si une optimisation sera nécessaire pour faire coïncider de manière plus parfaite les temps de libération des RSNO et celui de dégradation des matrices polymériques. La suite logique du travail est la transition vers un modèle pathologique. Par conséquent, une des perspectives de ce travail consiste en l'étude de l'effet bénéfique de ces formulations de RSNO dans un modèle d'infarctus du myocarde.

4. APPLICATION DANS L'INFARCTUS DU MYOCARDE

4.1. Introduction

4.1.1. Physiopathologie de l'infarctus du myocarde

L'infarctus du myocarde (IDM) est une nécrose ischémique du muscle cardiaque qui survient à la suite de l'occlusion brutale d'une artère coronaire. Cette occlusion est le plus souvent causée par une thrombose sur une plaque d'athérome rompue, érodée ou fissurée (IDM de type I, 85 % des cas) (Figure 24). Son incidence est élevée avec 120 000 cas par an en France. Grâce aux progrès réalisés dans la prise en charge de l'IDM durant les dernières décennies, la morbi-mortalité a été réduite, cependant le pronostic reste grave, avec 10 % de décès lors de la crise et 15 % dans l'année suivante [97]. A l'échelle mondiale, les cardiopathies ischémiques représentent la première cause de mortalité, avec 7,2 millions de décès d'origine coronaire sur 50 millions de décès annuels mondiaux [98].

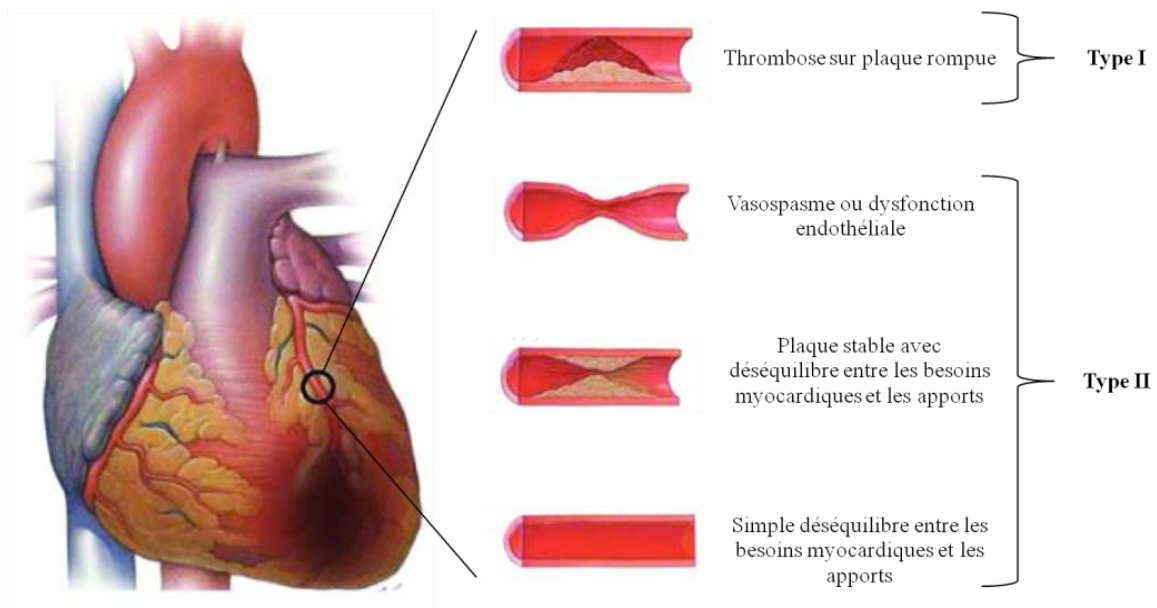


Figure 24 : Schéma d'un infarctus du myocarde, avec les différents états possibles des artères coronaires déterminant la classification de l'infarctus, adapté d'après Thygesen *et coll.* [99].

L'occlusion brutale d'une artère coronaire entraîne un déséquilibre entre les besoins métaboliques du tissu cardiaque et l'apport en nutriment et en oxygène par le sang artériel. Le résultat est une ischémie myocardique, réversible, se traduisant classiquement par une douleur intense. Après 20 min à 4 h d'ischémie [100], la nécrose myocardique (irréversible) débute (le plus souvent la localisation est le ventricule gauche), entraînant la mort cellulaire de la plupart des cardiomyocytes de la zone infarctée.

Quelques myocytes viables persistent cependant, de façon éparsée dans la zone nécrosée. La nécrose se développe progressivement et atteint son maximum 3 à 6 h après le début de l'ischémie. Un processus inflammatoire se met en place dès les premiers jours, puis en quelques semaines un processus cicatriciel avec fibrose et atrophie de la paroi cardiaque concernée.

Les conséquences sur la fonction cardiaque sont à la fois d'ordre mécanique et électrique. La zone infarctée ne se contracte plus (akinésie) et les parois non touchées doivent compenser son manque d'activité par un surcroît de travail. Ceci déclenche un remodelage ventriculaire adaptatif dès les premiers temps de l'ischémie : le ventricule gauche se dilate afin de maintenir le volume d'éjection systolique par l'augmentation des contraintes pariétales. Cette dilatation étire les cardiomyocytes, ce qui modifie leur expression génique et entraîne progressivement leur hypertrophie et ainsi l'augmentation de la masse du myocarde non nécrosé. En parallèle, la zone infarctée en cours de cicatrisation s'amincit par détersion [101]. A terme, l'augmentation des contraintes mécaniques peut provoquer une insuffisance cardiaque, la constitution d'un anévrisme au niveau de la zone nécrosée voire la rupture du ventricule. Les complications comprennent également des troubles du rythme : arythmies, extrasystoles, tachycardie et fibrillation ventriculaires.

4.1.2. De nouvelles pistes de traitement

Brièvement, les thérapies actuelles comprennent le traitement d'urgence de l'IDM avec revascularisation par thrombolyse et/ou angioplastie ainsi que la prévention de la récurrence et la réduction de la morbidité par le contrôle des facteurs de risque de l'athérome (diabète, hypercholestérolémie, hypertension...) *via* les mesures diététiques et le traitement pharmacologique (antiplaquettaires, dérivés nitrés, bêtabloquants, inhibiteurs de l'enzyme de conversion, statines...) [99]. Néanmoins, il n'existe pour l'instant aucun moyen de rendre la zone infarctée à nouveau fonctionnelle. Cette constatation a mené, d'une part, au développement de la médecine régénérative pour la réparation cardiaque, avec plusieurs axes de recherche à l'étude : soit la stimulation de la régénération cardiaque à partir de cellules souches endogènes ou des quelques cardiomyocytes viables restants dans la zone infarctée, soit l'apport « exogène » de cellules souches ou progénitrices. D'autre part, un autre axe concerne le développement de nouveaux principes actifs dont les donneurs de NO et en particulier les RSNO.

4.1.2.1. Médecine régénérative pour la réparation cardiaque

Premièrement, il existe un consensus dans la littérature pour dire que de nouvelles cellules cardiaques peuvent être générées *in situ* par la division de cardiomyocytes ou à partir de cellules progénitrices, mais que ce processus est extrêmement lent [102]. Il serait vraisemblablement possible de le stimuler, en utilisant des facteurs de croissance [103] ou par thérapie génique [104]. De même,

les fibroblastes présents au niveau de la cicatrice de l'infarctus pourraient potentiellement être reprogrammés en cardiomyocytes par des stimuli adaptés [105].

Deuxièmement, plusieurs essais cliniques ont démontré la faisabilité de la thérapie cellulaire chez l'Homme, avec l'injection de cellules soit directement dans la zone infarctée, soit en bordure de celle-ci, à distance de la phase aiguë de l'infarctus. Différents types de cellules ont été utilisés, avec des résultats variables en terme d'efficacité, mais aussi d'immunogénicité [106]. Le challenge est de maintenir, au niveau de la zone endommagée, des cellules viables et convenablement différenciées. Une stratégie alternative consiste alors à associer ces cellules avec des biomatériaux, qui leur fournissent un support en trois dimensions, améliorant ainsi leur survie et éventuellement guidant leur différenciation. Un biomatériau est couramment défini comme un matériau non vivant, utilisé dans un dispositif médical à des fins thérapeutiques ou non, et destiné à interagir avec des systèmes biologiques [107]. Idéalement, le biomatériau doit être biocompatible, c'est-à-dire exercer une fonction spécifique sans entraîner de réponse inappropriée de la part de l'organisme hôte. Au-delà d'un simple support, il peut également délivrer des principes actifs et ainsi générer un microenvironnement plus favorable pour l'installation des cellules dans la zone nécrosée. Différents types de biomatériaux, naturels ou synthétiques, ont déjà été proposés dans la reconstruction myocardique et parmi eux certains sont à base de PL(G)A. En effet ces polymères biocompatibles, bioérodibles et bioassimilables bénéficient d'un recul important, puisqu'ils ont été utilisés dès les années 60 comme sutures résorbables puis en chirurgie orthopédique comme alternative aux fixations métalliques, leur caractère dégradable évitant un retrait invasif et permettant la reconstruction osseuse [108]. Leur utilisation comme support pour la reconstruction cartilagineuse a également été étudiée chez l'animal [108]. En parallèle, ces polymères ont été agréés par les différentes autorités de santé pour la délivrance de médicaments après administration parentérale [76]. Pour finir, le Tableau 4 présente une vision générale des études utilisant du PLA ou du PLGA dans des applications cardiovasculaires (à l'exclusion des formulations classiques injectables par voie parentérale).

Tableau 4 : Utilisation de supports à base de PL(G)A pour des applications cardiovasculaires

Libération de principe actif et/ou support	Polymère et forme	Application visée	Cellules cultivées	Modèles animal d'implantation	Référence
Support de thérapie cellulaire	Films de PLGA découpés au laser	Greffé vasculaire (microvaisseaux)	Cellules endothéliales bovines	/	[109]
	Implant poreux PLGA	Patch cardiaque*	« Cardiomyocytes » dérivés de cellules souches osseuses	Rats (poche péritonéale)	[110]
	Fibres PLGA obtenues par electrospinning		Cardiomyocytes	/	[111]
	Mèches tubulaires de PGA/PCL/PLA	Greffé vasculaire	Cellules musculaires lisses et endothéliales	Agneaux (veine cave inférieure)	[112]
Délivrance de principe actif	Microparticules de PLGA contenant du facteur de croissance de l'endothélium vasculaire (VEGF)	Angiogenèse au niveau de l'infarctus		Rats (injection intramyocardique en bordure de l'infarctus)	[113]
	Microsphères de PLGA contenant un NONOate, greffées sur un stent	Inhibition de la resténose sur stent	/	Lapins avec régime hypercholestérolémiant (pose du stent dans l'aorte abdominale)	[114]
	Stent recouvert de PLGA et de tyrophostine	Inhibition de la prolifération néointimale après pose de stent		Mini-pores (pose du stent dans l'artère coronaire gauche descendante)	[115]
Support mécanique et délivrance de principe actif	Tube de PLGA et de facteur de croissance fibroblastique	Régénération cardiaque	/	Mini-pores (paroi du ventricule infarci)	[116]
	Microsphères de PLGA libérant de l'oxygène, dans un hydrogel		Test <i>in vitro</i> : survie de cellules souches en conditions mimant l'infarctus		[117]
	Matrices composites PLA + nanoparticules de PLGA contenant un NONOate	Valves cardiaques	Test <i>in vitro</i> : prévention de la calcification de cellules interstitielles de valve aortique de porc		[118]

* : « patch » formé de cellules cultivées sur un biomatériau, destiné à être implanté à la surface de la zone infarctée. PCL : poly(ϵ -caprolactone) ; PGA : poly(glycolide) ; PLA : poly(lactide) PLGA : poly(lactide-*co*-glycolide).

De façon intéressante, le critère principal permettant d'améliorer la réponse de l'organisme envers un implant ne serait pas le type de matériau utilisé ou ses propriétés physicochimiques (hydrophobie, charge de surface, ...) mais plutôt la topographie de sa surface [119]. En effet, plus le système est lisse, plus il déclenche une réaction inflammatoire importante, avec constitution d'une capsule fibreuse autour de l'implant. Au contraire, une surface présentant des pores ou des stries provoquerait moins d'inflammation et améliorerait la colonisation cellulaire et la vascularisation.

4.1.2.2. Place des *S*-nitrosothiols dans la thérapie de l'infarctus du myocarde

NO, apporté à de faibles concentrations, peut représenter une cible thérapeutique particulièrement importante dans le domaine cardiovasculaire et en particulier dans l'infarctus du myocarde. Des études ont par exemple montré que :

- La déficience en eNOS entraîne l'apparition précoce d'infarctus du myocarde [120] ; à l'inverse, une surexpression d'eNOS par les cardiomyocytes limite la dysfonction et le remodelage du ventricule gauche après infarctus [121] ;
- Les patients avec un infarctus du myocarde aigu présentent une dysfonction endothéliale vasculaire après la reperfusion thérapeutique (faible activité eNOS, diminution de NO et augmentation End-1) [122] ;
- La diminution de la biodisponibilité de NO contribue aux dommages subis lors de l'ischémie-reperfusion cardiaque ; ceci a été mis en évidence par l'effet cardioprotecteur d'inhibiteurs de l'enzyme arginase (compétition avec la synthèse de NO car utilise le même substrat que les NOS, la *L*-arginine) [123–125].

Les effets cardioprotecteurs de NO sont assurés par :

- Un effet vasodilatateur, y compris au sein d'un myocarde ischémié, permettant d'améliorer la perfusion tissulaire [126] ;
- Un effet anti-inflammatoire, en particulier avec l'inhibition du recrutement leucocytaire, diminuant ainsi les lésions au niveau de l'endothélium et du myocarde [127,128] ;
- Un effet anti-activation et anti-agrégation plaquettaire ; en parallèle de l'effet sur les leucocytes, il permet d'atténuer les phénomènes d'obstruction de capillaires (diminution des lésions « à distance » de la zone ischémiée) [129] ;
- La participation au recrutement de cellules progénitrices au niveau des tissus ischémiques [130].

Etant donné l'effet cardioprotecteur de NO, il est logique que des stratégies d'apport exogène aient déjà été testées : NO inhalé [131], différents donneurs de NO en bolus, perfusion ou administrations répétées [132–136]. Toutes ont montré une réduction de la taille de l'infarctus et une amélioration des paramètres fonctionnels. Une étude clinique (NITRITE-AMI) vient d'ailleurs d'être mise en place (Avril 2013) pour évaluer si l'injection d'ions nitrite en intracoronaire réduit la taille de l'infarctus chez les patients ayant un infarctus et subissant une angioplastie primaire [137].

Concernant les RSNO, ces molécules ont montré des effets bénéfiques dans les pathologies ischémiques au niveau cardiaque et également au niveau cérébro-vasculaire. Ainsi, la *S*-nitroso-2-mercaptopropionylglycine a montré une cardioprotection dans un modèle d'ischémie-reperfusion de la

même importance qu'un effet de préconditionnement (*cf.* Article 1) [135]. Dans cette étude, la *S*-nitrosation du complexe 1 de la mitochondrie a montré un rôle clé. Par ailleurs, GSNO et SNAP ont particulièrement été étudiés dans des modèles d'ischémie-reperfusion cérébro-vasculaire et de traumatismes crâniens (pathologie dans laquelle 90 % des patients souffrent également d'ischémie cérébro-vasculaire [138]). Suite à un traitement avec ces molécules, les travaux ont mis en évidence [139–143] :

- Une préservation de l'intégrité de la barrière hémato-encéphalique ;
- Une amélioration histologique (diminution de la taille de la lésion, moins de perte de myéline et de cellules neuronales et une promotion de la réparation neuronale) ;
- Un effet anti-oxydant et anti-inflammatoire (diminution de l'expression de iNOS ; moins d'infiltration macrophagique ; diminution du taux de nitrotyrosine, d'ions peroxy-nitrite et de peroxydation lipidique et augmentation du taux de glutathion réduit) ;
- Une augmentation de la concentration en NO dans la zone ischémisée ;
- Une amélioration du score neurologique.

Finalement, les auteurs ont conclu à une neuroprotection des RSNO dans les pathologies d'ischémie-reperfusion et de traumatisme. L'efficacité des RSNO a également été montrée comme supérieure aux autres donneurs de NO testés : le nitroprussiate de sodium, deux composés de la famille des NONOate et la 3-morpholino-sydnonimine [140].

Pour conclure, NO et les donneurs de NO représentent une approche prometteuse dans les pathologies ischémiques au niveau cérébral ou cardiaque.

4.1.3. Objectif de l'étude

Les chapitres précédents ont montré la capacité des systèmes *in situ* à protéger et délivrer de façon progressive des principes actifs, y compris fragiles comme les RSNO. Aucun problème de toxicité n'a été mis en évidence après administration par voie sous-cutanée chez le rat.

L'objectif des travaux présentés dans ce chapitre concerne le développement d'un implant injectable, administré directement dans le muscle myocardique, comblant *in situ* le déficit au niveau de la zone nécrosée et servant ainsi de support pour le recrutement et l'hébergement de cellules (soit endogènes, soit injectées en parallèle) tout en libérant des molécules cardioprotectrices, les RSNO (Figure 25). L'intérêt des formes *in situ* chargées en RSNO repose non seulement sur leur capacité à libérer ces molécules de manière prolongée mais encore sur le fait qu'elles présentent une porosité de surface (*cf.* Figure 17, *in vivo*). Or, ainsi qu'il a été décrit, cette porosité de surface doit permettre une colonisation cellulaire plus facile. Cette porosité augmentera progressivement vers l'intérieur de la structure pendant la dégradation de la structure (Article 3).

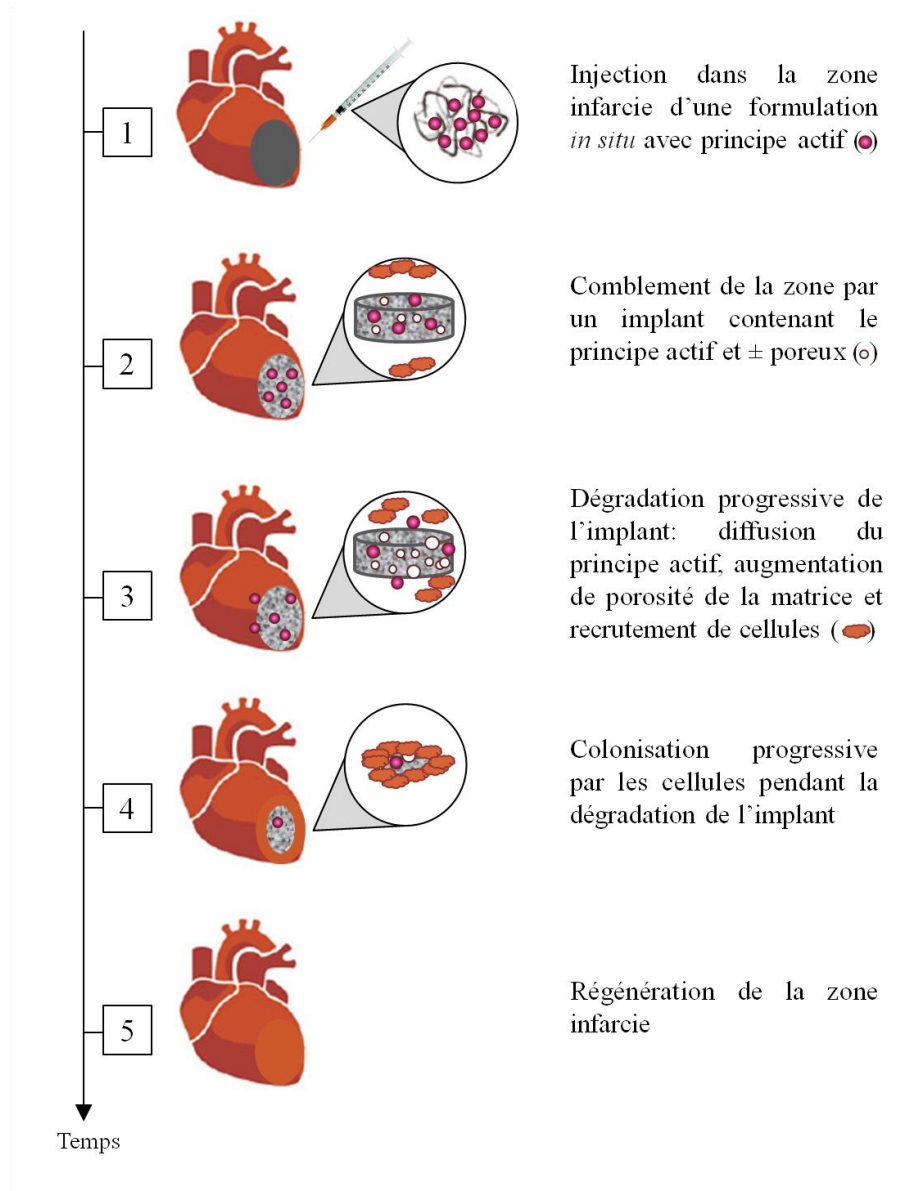


Figure 25 : Rationnel de l'étude présentée dans ce chapitre.

Cette étude a été séparée en deux parties. Dans un premier temps, la colonisation par des cellules d'implants *in situ* libérant des donneurs de NO a été étudiée *in vitro*. Dans un deuxième temps, des essais préliminaires ont montré la faisabilité de l'injection de formulations *in situ* directement dans le muscle cardiaque chez des rats.

4.2. Etude *in vitro* de la colonisation d'implants *in situ* par des cellules

4.2.1. Introduction

L'objectif de ce travail est de vérifier *in vitro* si des implants *in situ* peuvent être colonisés par des cellules. Les observations en microscopie électronique à balayage précédemment réalisées ont montré une disparité morphologique entre la surface des implants *in situ* formés *in vitro* et *in vivo*, avec une surface beaucoup moins lisse et beaucoup plus poreuse après injection *in vivo* (diamètre des pores de 50 à 100 μm , cf. Figure 17). Dans le but de se rapprocher de la surface offerte par des implants *in situ* injectés *in vivo*, leur porosité de surface a été augmentée grâce à l'utilisation d'un agent porogène (dans le cas présent du chlorure de sodium). Ceci permet également de modéliser l'augmentation de la porosité (en surface et interne) qui se produit lors de la dégradation des implants *in situ* (Article 3).

Des implants poreux à base de PL(G)A ont déjà été étudiés dans la littérature comme support d'ingénierie tissulaire délivrant des principes actifs [144,145] : ils sont formés par évaporation du solvant organique (type dichlorométhane) d'un mélange polymère/solvant/agent porogène puis rincés longuement pour éliminer le porogène. Dans notre étude, durant les 30 h de formation des implants *in situ* poreux (ISPI), le milieu aqueux a été fréquemment changé afin d'éliminer la majeure partie du chlorure de sodium et du solvant. Pour ce dernier, le solvant NMP a été retenu car il permet la solidification quasi instantanée du système et devrait être rapidement extrait de l'implant grâce à sa grande miscibilité à l'eau [146]. En ce qui concerne le polymère, le même PLGA que précédemment a été employé, seul ou en mélange avec de la poly-(ϵ -caprolactone) (PCL), afin d'augmenter la durée de vie et la résistance de la matrice polymérique. Les implants poreux obtenus ont ensuite été mis en contact avec des cellules de la lignée A-10 (cellules musculaires lisses de rat).

Les résultats obtenus jusqu'à présent ont montré que le chlorure de sodium était extrait de façon satisfaisante durant la période de lavage et que les structures ainsi obtenues pouvaient donc être mises au contact de cellules sans générer d'hypertonie du milieu. Les quantités de principes actifs libérées à la fin du processus étaient susceptibles d'avoir un impact sur les cellules. Les premiers résultats ont montré la présence de cellules viables 24 h et 48 h après un ensemencement direct sur les structures polymériques. Il reste à démontrer que les étapes de lavage permettent d'extraire totalement le solvant NMP des structures, car ce solvant organique pourrait être toxique pour les cellules. Il faut de plus prouver que les cellules arrivent à pénétrer à l'intérieur des matrices (analyse histologique en cours) et enfin que leur phénotype et leur statut redox ne sont pas altérés.

Bien que ces travaux ne soient pas complètement finalisés, ils sont présentés en anglais, afin d'en faciliter la publication ultérieure.

4.2.2. Abstract

Biodegradable polymeric implants already possess various applications as drug delivery systems. Emerging field of applications concerns regenerative medicine and especially heart tissue engineering. This study evaluated PLGA- and PLGA/PCL-based *in situ* porous implants (ISPI) loaded with nitric oxide donors (isosorbide mononitrate, ISMN and *S*-nitrosoglutathione, GSNO) as scaffolds for tridimensional cell seeding and proliferation. Surface and porosity of prepared ISPI studied by scanning electron microscopy showed great properties for cell seeding, especially for PLGA/PCL-based scaffolds. As expected, PLGA/PCL blend improves ISPI durability and resistance to cell culture conditions as showed with low mass loss and volume decrease during incubation in cell culture medium. In presence of cells, formulations were still able to release NO donors. Cytocompatibility was assessed with smooth muscle cells from rat aorta (A-10 cell line): all ISPI formulations allowed cell survival and growth. Nevertheless, it was demonstrated that GSNO-loaded PLGA/PCL-based formulations were more favorable for cell growth than the others. To conclude, ISPI releasing NO donors may represent good candidates as drug delivery scaffolds for tissue engineering. Still, further experiments have to be performed to evaluate the potential oxidative and nitrosative stress caused by the NO donors and their impact on physiology of other cell lines.

4.2.3. Introduction

Myocardial infarction and heart failure are leading causes of death and disability. Current experimental strategies to prevent or reverse tissue damages and negative left ventricular remodeling process following myocardial infarction, rely on cell transplantation and/or tissue engineering [147]. This field is indeed a growing multidisciplinary research area that aims to develop biological substitutes in order to restore, maintain or improve tissue function [148]. A classical approach consists in using porous scaffolds to control the three-dimensional tissue neo-formation. In addition to three-dimensional template, they must also provide a favorable environment for cell attachment, proliferation, differentiation and neo-tissue growth. Desired features of scaffolds are high porosity with interconnected pores to facilitate cell seeding, adhesion and penetration. Furthermore, appropriate mechanical properties and degradation rate are also critical [149]. For cardiac engineering, two strategies have been explored, either “cardiac patches” (*in vitro* engineered cell scaffolds prior implantation into the body) or injectable scaffolds [147]. These latter (with or without cells) are directly injected inside the heart wall to the site of damage. In these applications, surface topography of scaffolds seems to be the most important parameter as compared to other physicochemical properties. Thus, surface porosity represents the key point in order to improve cell attachment and vascularization without inflammation on the contrary of a smooth surface [119].

In situ implants are well known as drug delivery systems for drug sustained release after intramuscular or subcutaneous injections (*cf.* Article 3). They are liquid outside the body and form a solid depot at the site of administration. Indeed, the water-insoluble polymer (generally a

bioresorbable and biocompatible poly(lactide-*co*-glycolide) copolymer (PLGA) precipitates upon contact with body fluids. The drug is entrapped in the resulting polymeric matrix and then progressively released during its degradation. A wide range of active pharmaceutical ingredients have already been incorporated into *in situ* implants, including growth factors for tissue engineering applications [150] or nitric oxide donors (*cf.* Article 5).

Nitric oxide (NO) is a gaseous radical involved in many physiological processes, especially for its vasodilation, anti-inflammation as well as anti-platelet properties [151]. Furthermore, NO is a key signaling molecule for myocardial homeostasis [152–154] and its endogenous production is often lowered in pathological conditions [155]. As a result, administration of exogenous NO might be beneficial in a context of myocardial infarction and heart failure, through inhibition of inflammation, promotion of angiogenesis and cardiomyocyte growth [156]. As NO is too short lived [8], prodrugs are necessary. Organic nitrates (*e.g.*: isosorbide mononitrate, ISMN) are NO donors used for a century in angina pectoris treatment, but they are known to induce oxidative stress and tolerance phenomenon (Article 1). Emerging compounds that include *S*-nitrosothiols (among them *S*-nitrosoglutathione, GSNO) have already been tested in humans in several clinical trials (*cf.* Article 2) and are devoided of tolerance [157]. Moreover, these molecules have shown beneficial properties not only on cerebral ischemia pathologies in terms of anti-oxidative and anti-inflammatory effects and equally decrease of lesion size [139], but also on myocardial infarction with a cardioprotection [135]. Interestingly, *S*-nitrosothiols induced a better improvement on ischemia compared to classical NO donors (sodium nitroprusside or organic nitrate) [140].

Therefore, the aim of this study was to investigate the potential of *in situ* implants loaded with nitric oxide donors (ISMN or GSNO) as scaffolds for tissue engineering for a future application in myocardial infarction. Formulations were prepared with PLGA or a PLGA/poly(ϵ -caprolactone) (PCL) blend in *N*-methyl-2-pyrrolidone (NMP). PLGA is indeed the polymer of choice for *in situ* implants and has been approved by authorities for sutures and parenteral drug delivery thanks to its biocompatible and bioresorbable properties [158]. PCL was added to PLGA to increase implant durability and stress crack resistance [159]. Despite its potential toxicity on cells, NMP was used as solvent because its high water miscibility allows a fast solvent/water exchange and therefore a fast solidification of polymeric matrix [160]. Scaffolds were obtained *in vitro* after a phase inversion process and NaCl which is commonly used as porogen due to its low toxicity and high water solubility [161,162] was added to improve surface porosity. Indeed, a previous work showed that *in situ* implant did not present the same topography *in vitro* and *in vivo*. *In vivo* a more important porosity was observed than *in vitro* (*cf.* Figure 17); this trend will also occur in the core of the scaffold during its degradation (Article 3). As a result, in order to mimic the *in vivo* conditions, NaCl was added to the formulation to improve surface porosity, in order to work on *in situ* porous implants (ISPI) characterized by a porous network connected to the surface, suitable for cell colonization. Surface

morphology after preparation was examined by scanning electron microscopy. NO donors releases were monitored and cell proliferation was evaluated, using vascular smooth muscle cells (A-10).

4.2.4. Material and methods

4.2.4.1. Material

NO donors, PLGA and NMP were obtained as in Article 5. All other reagents including PCL (10 000 g/mol) were purchased from Sigma-Aldrich, except NaCl (Prolabo) and phosphate buffer solution (PBS, PAA).

4.2.4.2. Methods

***In situ* porous implants preparation**

Two different formulations were prepared, either containing only PLGA or a blend of PLGA and PCL (75:25 m/m). Polymers were dissolved (20 % m/m) in NMP (500 rpm, 50 °C, 15 min). After cooling and immediately before injection into the aqueous medium, NO donors were added in the polymeric solution, 20 % m/m for ISMN and 5 % m/m for GSNO. Then, for all experiments, NaCl crystals (size $\leq 400 \mu\text{m}$) were dispersed (2:1 m/m) into the polymeric solution. After homogenization, about 1.2 g of the resulting mixture was put into a 2 mL syringe without tip and injected into 5 mL of water in a crystallizer ($\varnothing 5\text{cm}$). Upon contact with water, a phase inversion occurred, leading to the formation of a solid porous scaffold as the organic solvent (NMP) and the porogen (NaCl) diffused into the water. A washing period was instituted, in which aqueous medium was changed 1, 2, 3, 4, 5, 6, 7, 24, 25, 26, 27, 28, 29 and 30 h after injection, to allow both porogen and solvent leachings. After preparation, some scaffolds were freeze-dried and pictures were taken with a scanning electron microscope (HITACHI S-4800, Japan) under 1 kV without metallization.

NaCl and NO donors leachings during ISPI preparation

Porogen leaching was determined in each aqueous medium using flame photometry (range 1.7 to 20 mg/L) (PFP 7 JENWAY, England). ISMN concentration in medium was assessed using the HPLC system previously described (see Article 5). GSNO release was determined immediately after sampling by colorimetry (Saville-Griess assay) or fluorimetry (using 2,3-diaminonaphthalene probe) (see Article 4 and Complementary Results-Pharmacokinetics, respectively).

A-10 cell culture conditions and seeding on scaffolds

Vascular smooth muscle cells derived from embryonic rat aorta (A-10 cells, ATCC) were cultured according to the manufacturer instructions (37 °C, 10 % CO₂) on 75 cm² culture flasks in Dulbecco's Modified Eagle Medium (DMEM) with phenol red supplemented with 4.5 g/L of glucose, 10 % (v/v) of fetal bovine serum, 2 % (v/v) of glutamine (200 mM), 100 U/mL of penicillin, 100 µg/mL of streptomycin and 1 mM of sodium pyruvate. Medium was changed every 3 days and cells were

subcultured every week. For this study, cells were used between passages 27 and 31. After preparation, ISPI (unloaded or loaded with NO donors) were washed twice with 1.5 mL of PBS and then surgical glue (Vetbond Tissue Adhesive, 3M) was used to fix them in a 12-wells microplate. In parallel, cells were washed two times with 10 mL of PBS before trypsination (2 mL of 0.1 % (v/v) trypsin-EDTA solution without phenol red, 5-min incubation at 37 °C) and counted (trypan blue 1:9 v/v). Finally, 100 µL of cell suspension (2×10^6 cells/mL) were seeded directly onto each ISPI (2×10^5 cells/scaffold). After a 3 h-incubation (37 °C, 10 % CO₂), 2 mL of complete medium without phenol red was carefully added into each well to avoid cell detachment.

Degradation tests

Two parameters were monitored to follow scaffold evolution: their mass and their volume.

After preparation (including washing steps), ISPI were incubated into 5 mL of PBS or 5 mL of cell culture medium without cells, at $37 \pm 1^\circ\text{C}$ and under an atmosphere of 10 % CO₂. After 24, 48 or 72 h, samples were freeze-dried and the mass of each sample was determined by gravimetry. Relative mass loss was calculated for each sample according to the theoretical mass injected during ISPI preparation. Results were compared with a control sample, which was freeze-dried immediately after preparation.

An estimation of volumes occupied by ISPI was realized after 24 and 48 h of incubation in culture medium after cell seeding (see above). The two diameters (a and b) and the height (h) of the elliptic shapes were measured with a caliper and the volume (V) was calculated as follows: $V = \pi \times a \times b \times h$. Results were expressed as percentage compared to the volume of medium in the wells (surface area of the well multiplied by the height of medium).

NO donors release in cell experiments

GSNO and nitrite ions concentrations in the cell culture medium were determined by fluorimetry as described previously. However, sample preparation ISMN release determination was slightly modified. Proteins of medium were first precipitated with acetonitrile (1:1 v/v) and samples were then centrifuged 10 min at $8\,000 \times g$. Supernatant was centrifuged again ($42\,000 \times g$, 20 min) and diluted before injection (50 µL) in the HPLC system (Article 5). Nitrate ions concentration was determined using a Total NO_x kit (see Complementary Results-Pharmacokinetics). According to manufacturer instructions; proteins were in this case previously removed by mixing the medium with zinc sulfate (10 % m/v in NaOH 0.5 M) 1:1 v/v, and centrifugation ($8\,000 \times g$, 10 min) after a 20 min-incubation.

Cell viability: modified MTT assay

Metabolic activity of cells was assessed with a MTT assay. 24 or 48 h after cell seeding onto scaffolds, medium of each well was replaced by 1 mL of fresh medium without phenol red containing 250 µL of MTT (2.5 mg/mL). After 3 h of incubation, scaffolds and formazan crystals were dissolved with 500 µL of acidified isopropanol (36 µL HCL 1M/ 1 mL isopropanol) under shaking during 1 h.

Absorbance was measured at $\lambda = 570$ nm (EL-800, Bio-TEK instruments, USA) and results were expressed as percentage compared to PLGA-based ISPI after 24 h of incubation. All data are expressed as mean \pm standard deviation.

4.2.5. Results and discussion

In this study, two kinds of formulations (containing 20 % (m/m) PLGA or 15 % PLGA + 5 % PCL (m/m)) were characterized.

4.2.5.1. Scaffold characterization

NaCl and NO donors releases in aqueous medium during ISPI preparation

First, porogen release from formulations was monitored as a function of time (Figure 26). For both formulations, a plateau was reached after 7 h, corresponding to 76.7 ± 7.8 % of NaCl released for PLGA and 57.0 ± 0.3 % for PLGA/PCL-based ISPI. NaCl concentrations in the last media collected for analysis were $2.8 \pm 1.3 \times 10^{-5}$ M and $8.1 \pm 3.7 \times 10^{-5}$ M for PLGA- and PLGA/PCL-based ISPI, respectively. As a result, cells will not be affected by NaCl released from the scaffolds after preparation and washing steps, because these concentrations are very low comparing to physiological Na^+ concentration in extracellular fluid (1.5×10^{-1} M).

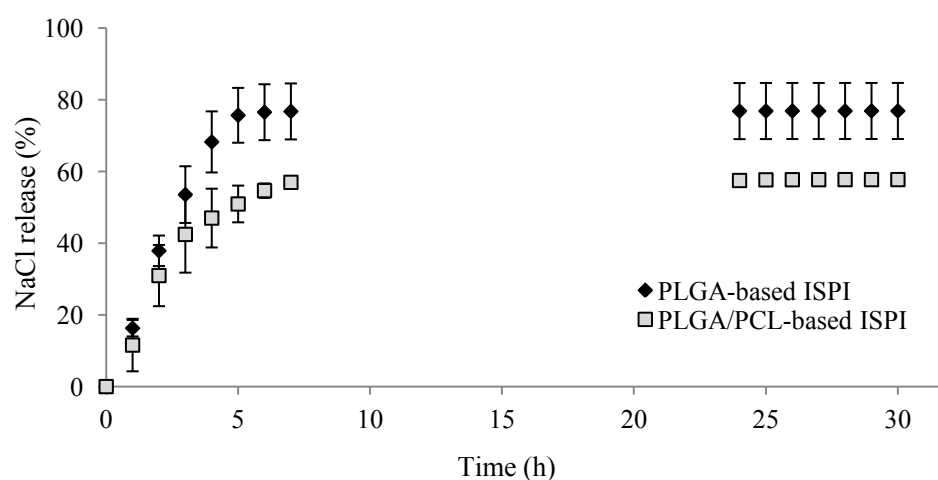


Figure 26 : NaCl leaching during ISPI preparation (n = 3).

Second, concentrations of NO donors released were evaluated at the end of ISPI preparation. As far as ISMN is concerned, the concentrations in the last washing media were $1.7 \pm 1.0 \times 10^{-4}$ M and $0.8 \pm 0.4 \times 10^{-4}$ M for PLGA- and PLGA/PCL-based ISPI, respectively, without any degradation product. *S*-nitrosoglutathione concentrations in the last media were evaluated at $5.9 \pm 0.1 \times 10^{-7}$ M and $3.6 \pm 0.1 \times 10^{-7}$ M for PLGA- and PLGA/PCL-based ISPI respectively, according to Saville-Griess reaction, and only *ca.* 5 % of GSNO incorporated into ISPI was recovered as nitrite ions. Such NO donors concentrations have already been described as sufficient to induce cell proliferation [163]. To

complete this study, a quantification of NMP release should be further performed since the solvent may induce some side effects.

Scaffolds morphology

SEM pictures showed structures with pores at the surface for both non-loaded and loaded formulations (Figure 27A). Surface pore diameters were in the range of 100 – 400 μm and thus should allow cell attachment [164]. Polymeric matrices also offered interconnected pore networks (Figure 27B) which may-permit cell migration through the scaffold. However, surface morphology of implants seemed to be modified by the incorporation of NO donors (Figure 27A). This may be explained by the fact that the drug could have changed the solvent/water exchange rate during scaffolds solidification. Finally, loaded and non-loaded PLGA-based ISPI showed smoother surfaces than corresponding PLGA/PCL-based ISPI. A rougher surface has already been described as more favorable since it was shown to improve cell colonization [119]. As a consequence, PLGA/PCL-based ISPI may be more appropriate in the targeted application than PLGA ones.

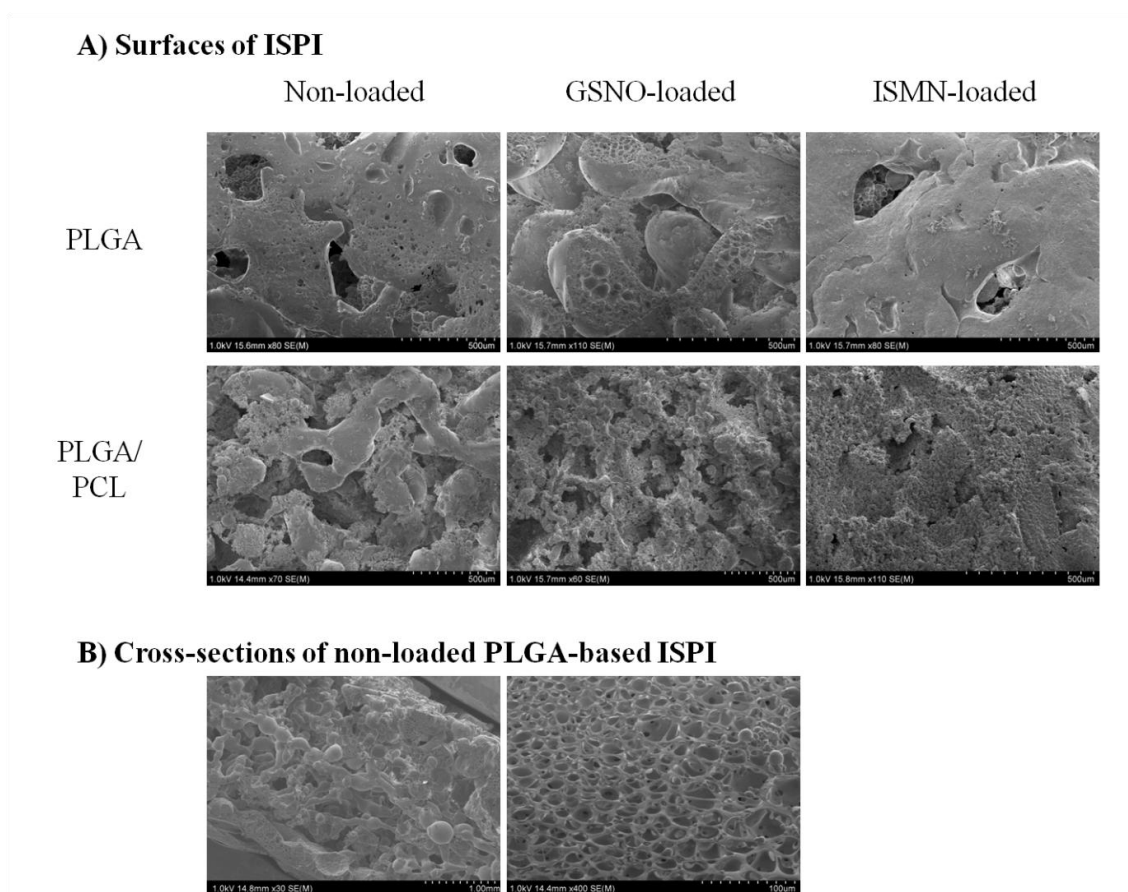


Figure 27 : SEM pictures of ISPI morphology after preparation.

4.2.5.2. Cellular assays

Degradation tests

Such ISPI are designed to be completely replaced by neo-formed tissue during their degradation. However, PLGA 50:50 degradation is known to be quite fast in a cell culture medium [165]. Thus PCL was added to ISPI formulation in order to increase the degradation time. The degradation process was followed monitoring both mass loss and modification of volume.

Firstly, after preparation, ISPI were incubated 24, 48 or 72 h either into PBS or complete cell culture medium (without cells) and the evolution of their masses was evaluated. Results presented in Figure 28A indicated only minor mass losses (< 15 %) in all conditions. As expected, mass losses were more important for PLGA-based ISPI than for PLGA/PCL-based ISPI. It was also the case for each formulation in cell culture medium than in PBS.

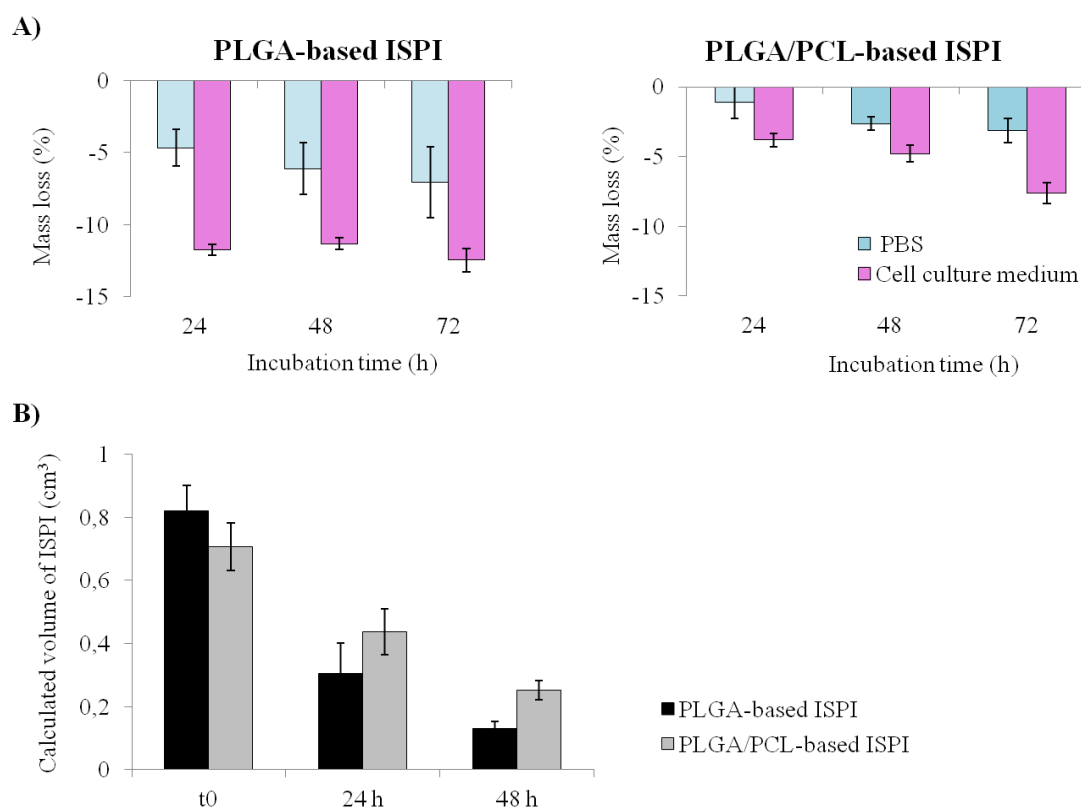


Figure 28 : Evolution of mass and volume of non-loaded ISPI: A) mass loss during incubation into PBS or cell culture medium (n = 3); B) volume evolution in cell experiment conditions (data were analyzed with a two-way Anova followed by a post-hoc Bonferoni test: $p_{\text{interaction}}$, $p_{\text{formulation}}$ and $p_{\text{time}} < 0.05$ (n = 6)).

Secondly, a study was designed to evaluate the impact of cell culture environment on ISPI volume. At the beginning of the experiment, PLGA/PCL-based formulations had slightly lower volumes than PLGA ones ($0.71 \pm 0.08 \text{ cm}^3$ vs $0.82 \pm 0.08 \text{ cm}^3$). Then, volumes of both formulations decreased while

the incubation time increased (Figure 28B). This trend was more marked for PLGA-based ISPI (volume $0.13 \pm 0.02 \text{ cm}^3$ after 48 h) than for PLGA/PCL-based formulations ($0.25 \pm 0.03 \text{ cm}^3$ after 48 h). As PLGA/PCL blend is less hydrophilic than PLGA alone, its swelling during the preparation step was probably lower, thus explaining the lower volume at the beginning of the experiment and the lower volume reduction during incubation in cell culture medium.

NO donor release assay

After implant preparation and seeding of cells, NO donor releases in cell culture media were measured in order to confirm that ISPI were still able to deliver the drugs. In cell culture medium, only nitrite ions could be detected, at a very low concentration ($1.0 \pm 0.0 \times 10^{-7} \text{ M}$) and neither the presence of cells nor the unloaded formulations modified this result. Then, in the presence of GSNO-loaded ISPI, an increase in both nitrite ions and RSNO concentrations in the medium was noticed (Figure 29): as a result, scaffolds were still releasing GSNO. Interestingly, GSNO and nitrite ions concentrations increased with incubation time and RSNO concentration was even more important in the presence of cells. As far as ISMN is concerned, the product was mainly released without an important degradation. Again, the drug was released in higher concentrations in presence of cells than without them; this may be explained by an enhanced degradation of the polymeric matrix and/or cell metabolism. Even if found concentrations of NO donors (range: 10^{-6} M for GSNO and 10^{-4} M for ISMN) are sufficient to have an impact on cell proliferation [163], their impact have to be further investigated in term of protein *S*-nitrosation.

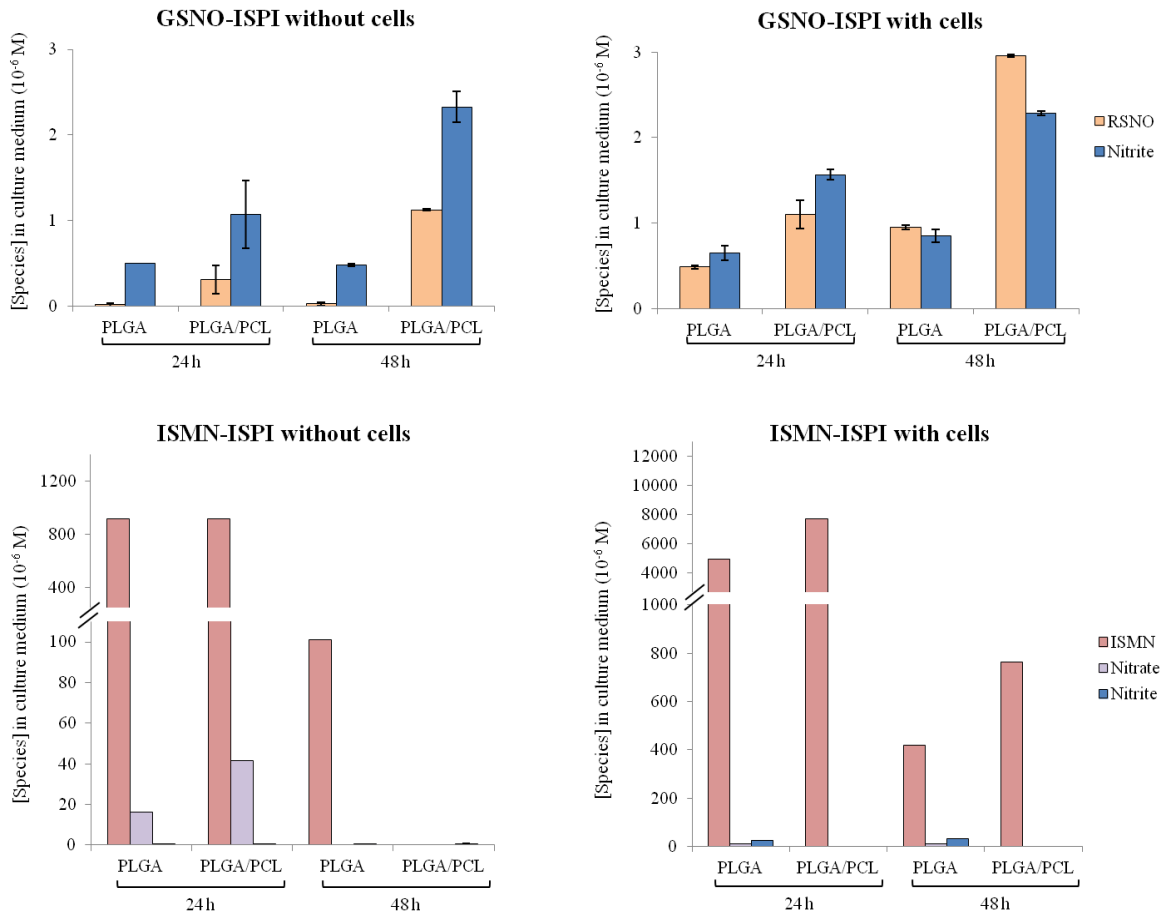


Figure 29 : NO donors and degradation products released in the cell culture medium from GSNO (n = 3) or ISMN-loaded ISPI (n = 2), 24 or 48 h after cell seeding.

Cell proliferation onto scaffolds

Finally, a viability test was performed in order to determine the possibility of cell attachment and growth on ISPI (Figure 30).

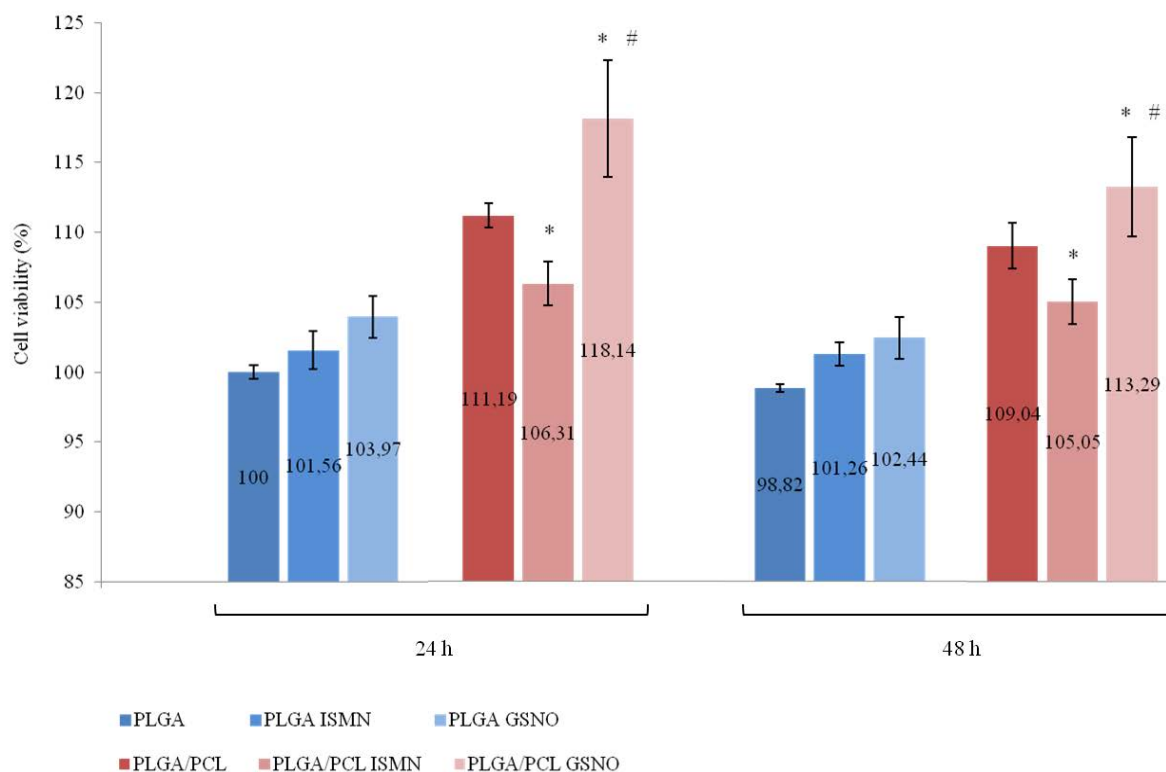


Figure 30 : Cell viability 24 h and 48 h after cell seeding on ISPI. . At each incubation time, data were analyzed with a two-way Anova followed by a post-hoc Bonferoni test: $p_{\text{interaction}}$, p_{polymer} and $p_{\text{drug}} < 0.05$ ($n = 3$). * $p < 0.05$ vs non-loaded PLGA/PCL-based formulation at the same time; # $p < 0.05$ vs ISMN-loaded PLGA/PCL-based formulation at the same time.

First of all, whatever the condition, no dramatic decrease of cell viability was noticed: as a consequence, the formulations did not induce any toxicity. Moreover, in all conditions, PLGA/PCL-based ISPI allowed greater cell growth (10 to 15 %) than PLGA ones. This may be explained by the rougher surfaces of PLGA/PCL-based ISPI as previously observed by SEM, probably more favorable to cell attachment. For PLGA-based ISPI, loading with NO donors (ISMN or GSNO) did not significantly increase cell viability compared to non-loaded PLGA-based ISPI, at 24 or 48 h of incubation. On the contrary, for PLGA/PCL-based ISPI, ISMN loading decreased cell growth while GSNO loading increased it. This may be explained by the fact that ISMN may have induced an oxidative and/or nitrosative stress (Article 1). This will be further investigated by the intracellular measurement of both antioxidative defenses (reduced glutathione) and oxidatives species (reactive oxygen species and rate of protein nitration). Moreover, a formulation containing ISMN in the same amount than GSNO should also be tested.

Finally, to complete this study, some histological analyses are currently under development in order to check whether cells are able to colonize the core of the implant.

4.2.6. Conclusion

In this study, vascular smooth muscle cells were used as models to evaluate cytocompatibility of ISPI. Preliminary results obtained here showed that GSNO loaded PLGA/PCL-based ISPI seemed to be the formulation for which a rough surface was observed and allowing cell growth. Nevertheless, in view of the objective of cardiac regeneration, it would be desirable to test these systems with cardiomyocytes and mechanical constraints similar to those of the myocardium. Indeed, these scaffolds will constitute a mechanical support to the damaged area and should therefore exhibit suitable mechanical properties. Scaffold appropriate stiffness is still a matter of debate, but it is generally recognized that it should be around tens of kPa to match myocardial one (20 kPa to 500 kPa for human myocardium, 0.1 to 140 kPa for rat myocardium [147]). Mechanical properties of the presented scaffolds have therefore to be further studied.

4.3. Injection intramyocardique d'implants *in situ*

Les résultats de l'étude *in vitro* avec les implants *in situ* poreux montrent qu'il est possible pour des cellules musculaires lisses de coloniser leur surface. En parallèle de ces expériences, la faisabilité de l'injection de formulations *in situ* dans le muscle cardiaque de rats Wistar a été testée. Pour cette étude préliminaire, les formulations de type implant (simplicité de préparation et d'administration) à base de NMP (solidification rapide) ont été retenues.

Afin de réaliser l'injection directement dans le muscle cardiaque, les animaux ont été anesthésiés sous isoflurane, puis une mini-thoracotomie a été réalisée par un chirurgien (Dr N. Tran, Ecole de Chirurgie de Nancy) afin d'exposer le myocarde de la façon la moins invasive possible (incision cutanée de 2 cm environ, dilacération des muscles pour accéder à la cavité thoracique). L'injection a été effectuée avec une aiguille de 23 G dans le myocarde à l'apex du ventricule gauche, sur cœur battant.

Les premiers essais effectués chez des rats sains ont permis de déterminer qu'environ 50 μ L (\approx 50 mg) de formulation (sans principe actif) pouvaient être injectés dans le myocarde sans causer le décès de l'animal. Néanmoins, l'injection (Figure 31A) entraînait l'apparition d'un fin réseau blanc (voir flèches blanches Figure 31B) autour du point d'injection, probablement causé par la diffusion de la solution polymérique dans les capillaires environnants avant solidification. Ceci s'est quelquefois traduit par le noircissement de la zone concernée (Figure 31C).

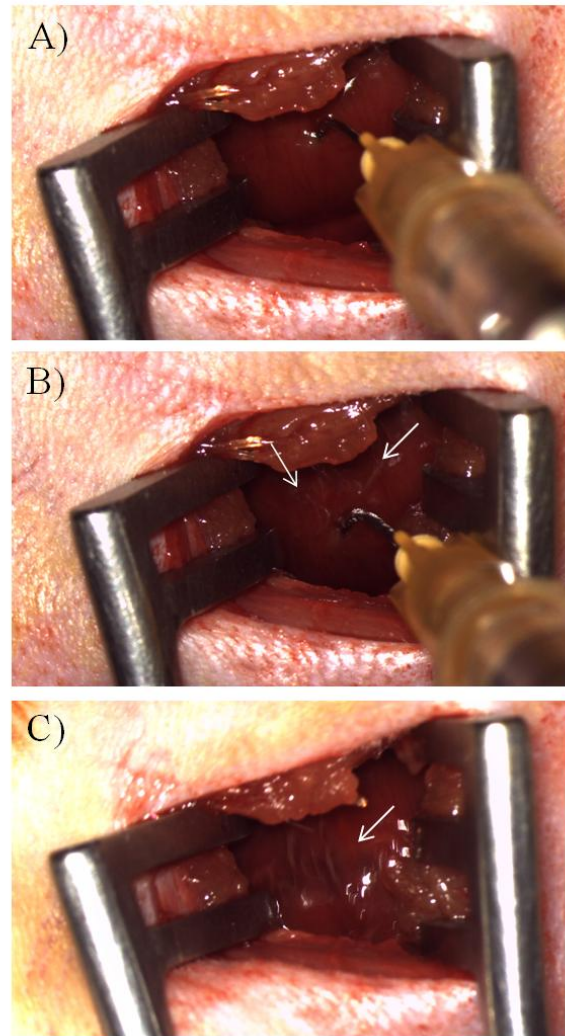


Figure 31 : Injection d'implants *in situ* (sans principe actif) à base de NMP dans le myocarde de rats sains. Photographies prises A) avant injection, B) juste après l'injection ; les flèches blanches indiquent le réseau blanc qui apparaît suite à l'injection, et C) exemple de noircissement de la zone d'injection observé dans certaines expériences. L'incision cutanée mesure environ 2 cm de longueur.

Par conséquent, chez les rats sains, l'injection intramyocardique semble produire un mini-infarctus. Néanmoins, étant donné que la paroi myocardique est modifiée après un infarctus (zone plus fine, plus creuse et akinétique), l'étude a été poursuivie chez des rats ayant un infarctus suite à une ligature coronaire définitive selon un protocole classiquement utilisé à l'Ecole de Chirurgie [166].

A l'heure actuelle, sept rats (Wistar mâles, 500-600 g) ont été opérés et traités avec injection au niveau de la zone infarctée. Deux rats ont reçu une solution de GSNO dans du NaCl 0,9 % (2,5 mg dans 100 μ L soit 5 mg/kg), deux rats ont reçu 50 μ L de formulation sans principe actif et trois rats ont reçu 50 μ L de formulation contenant 5 % m/m de GSNO (5 mg/kg). La ligature coronaire était effectuée 1 mois avant l'injection et une imagerie par Tomographie par Emission de Positrons associée à l'imagerie (TEP-SCAN, plateforme Nancyclotep) était réalisée 2 semaines après la ligature et 2 semaines après l'injection pour voir l'évolution des lésions. Les résultats sont à interpréter avec

une grande prudence étant donné le faible nombre d'animaux, mais semblent néanmoins encourageants : comme attendu, l'injection de la solution de GSNO ou d'un implant sans principe actif ne semble pas améliorer la perfusion cardiaque par contre, celle d'un implant avec GSNO serait bénéfique (Figure 32).

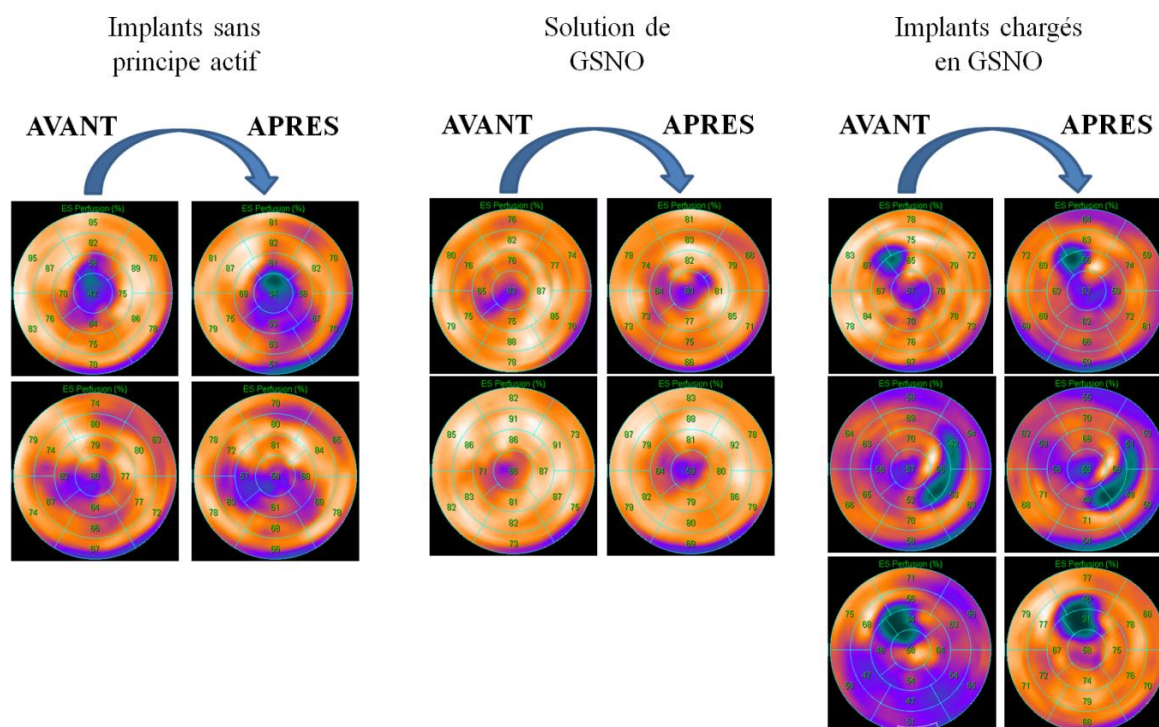


Figure 32 : Imagerie TEP-SCAN de cœurs de rats ayant un infarctus du myocarde suite à une ligature coronaire définitive : à gauche (« avant »), deux semaines après la ligature et à droite (« après »), deux semaines après l'injection dans le myocarde infarcté de: d'implants *in situ* sans principe actif, d'une solution de GSNO (5 mg/kg), ou d'implant *in situ* contenant du GSNO (5 mg/kg). Cette technique d'imagerie permet de mettre en évidence les anomalies de perfusion au niveau cardiaque (les résultats présentés sont obtenus durant la systole cardiaque) : moins le territoire est perfusé, moins la sonde (^{18}F -déoxyglucose) est présente donc plus la zone apparaît bleue.

La médecine régénérative est une discipline en plein essor, en particulier pour la réparation cardiaque. L'approche primitivement développée consistant à apporter de nouvelles cellules capables de se différencier en cardiomyocytes au niveau du territoire infarcté a montré des limitations [102,147,149] : difficulté pour identifier la source optimale de cellules, nécessité d'apporter un grand nombre de cellules, qui sont expulsées du lieu d'injection par les battements cardiaques ou qui meurent avant d'avoir pu proliférer (la zone infarctée étant pauvre en oxygène et en nutriments). L'utilisation de supports en trois dimensions, d'origine naturelle ou synthétique, permet de remplacer temporairement (le temps de la dégradation du support/formation du nouveau tissu) la zone défaillante, et de favoriser la colonisation par les cellules. Enfin, ils peuvent assurer une délivrance

progressive de facteurs de croissance, cytokines ou molécules d'adhésion, afin d'améliorer le recrutement, l'adhésion et la prolifération de cellules. Dans ce cadre, les implants *in situ* chargés en GSNO pourraient représenter une nouvelle plateforme d'ingénierie tissulaire pour la reconstruction myocardique après infarctus. Les résultats obtenus *in vitro* montrent qu'ils peuvent être colonisés par des cellules musculaires (de type A-10) et les résultats préliminaires obtenus *in vivo* indiquent que l'injection intramyocardique est possible, et pourrait entraîner une amélioration de la perfusion cardiaque. Dans le futur, cette thérapeutique pourrait être associée à l'administration conjointe de cellules souches. Néanmoins, une exploration complète de la fonction cardiaque devra être réalisée pour démontrer ou non un effet positif de cette stratégie expérimentale.

DISCUSSION

Avant les années 1980, NO était essentiellement considéré comme un polluant, présent dans la fumée de cigarette et les échappements automobiles. Cette vision a changé dans les années 80 lors de la découverte de la libération par l'endothélium d'un facteur labile, l'*endothelial-derived relaxing factor* (EDRF), causant la relaxation de vaisseaux quand les cellules musculaires lisses étaient stimulées par des vasodilatateurs, puis l'assimilation de l'EDRF au monoxyde d'azote. Ces découvertes ont été récompensées par le prix Nobel de Physiologie/Médecine en 1998 (Ignarro, Furchgott, Murad) [151].

Par la suite, il est apparu peu à peu que NO était un médiateur impliqué dans tous les principaux systèmes de l'organisme, conservé dans le règne animal comme dans le règne végétal [167,168]. Pendant des années, plusieurs molécules ont indifféremment été utilisées dans les études comme « donneurs » de NO avant que ne soient établies la complexité de sa chimie ainsi que la présence de plusieurs formes (NO , NO^+ , NO^-) et de nombreuses espèces dérivées (NO_2^- , NO_3^- , RSNO de faible ou forte masse molaire, ONOO^- ...) pouvant conduire à différentes réactivités.

Dans la littérature, NO est souvent décrit comme un Janus, une molécule à double face, à la fois bénéfique et délétère [47,169–173]. Ce n'est probablement pas seulement sa concentration qui fait pencher la balance d'un côté ou de l'autre, mais un ensemble de paramètres, comme par exemple de façon non exhaustive : sa concentration [163], les espèce(s) dérivée(s) produite(s), le microenvironnement tissulaire (pH, pO_2 , statut redox) [174,175], la localisation intracellulaire et la durée de sa production, les mécanismes de sa destruction, les liens avec les autres médiateurs gazeux (O_2 , CO , H_2S) [176,177]. Tout ceci participe vraisemblablement à activer ou inhiber des voies de signalisation différentes, de manière finement régulée, ce qui explique à la fois les effets pléiotropiques du NO et la difficulté de cibler cette molécule pour une action thérapeutique. En particulier, les phénomènes de S-nitrosation (c'est-à-dire la fixation d'un résidu NO sur le groupement thiol d'une molécule), transnitrosation et dénitrosation jouent un rôle critique de régulation, y compris au niveau cardiovasculaire [157,178,179].

Les effets thérapeutiques d'un apport de NO contrôlé seraient innombrables et toucheraient toutes les disciplines médicales (Tableau 5). Dans ce cadre, l'utilisation de précurseurs de NO est rendue nécessaire par sa très faible demi-vie. Bien que les dérivés nitrés utilisés aient été utilisés depuis près d'un siècle dans le traitement de l'angine de poitrine, leurs inconvénients (tolérance, stress oxydant) empêchent l'élargissement de leurs indications (Article 1).

Tableau 5 : Potentielles applications thérapeutiques d'une formulation délivrant un donneur de NO de façon prolongée.

Applications thérapeutiques	Références
Système cardiovasculaire	
• Modulation de l'hémostase	[91,180]
• Resténose sur stents	[181]
• Dysfonction endothéliale/athérosclérose	[182]
Troubles métaboliques	
• Ostéoporose	[183]
• Insuline : apport exogène, sensibilité	[184,185]
• Diabète	[186]
Système gastro-intestinal	
• Ulcères	[187]
• Entérite	[188]
Système respiratoire	
• Mucoviscidose	[189]
• Asthme	[190]
Système immunitaire	
• Infections bactériennes	[191,192]
• Infections virales	[193,194]
• Infections parasitaires	[195,196]
Thérapie anticancéreuse	
• Tumeurs multi-résistantes	[197]
• Thérapie adjuvante	[198]
Divers	
• Transplantation : protection des organes	[199,200]
• Transfusion sanguine	[201,202]
• Cicatrisation	[203,204]
• Promoteurs d'absorption orale	[205,206]

De nouvelles familles de composés donneurs de NO sont donc à l'étude. Dans l'idéal, en plus d'une absence de toxicité, ces composés devraient avoir deux caractéristiques majeures :

- Une demi-vie suffisante ;
- Une libération contrôlée (de NO à partir du précurseur).

Ces objectifs peuvent être atteints notamment en modulant la structure chimique du précurseur. La protection des composés par une formulation galénique adaptée est également à envisager.

Comme précédemment mentionné, il existe différentes familles de donneurs de NO (nitrates organiques, NONOates, composés hybrides divers, complexes nitrosyl, etc...). Récemment, plusieurs études ont montré que ces différents donneurs de NO n'étaient pas égaux en termes de *S*-nitrosation [207–209]. Au regard de ces données, l'utilisation de donneurs de NO naturellement présents dans l'organisme semble être la meilleure alternative pour le développement de composés améliorant la biodisponibilité de NO. Ils sont en effet susceptibles d'avoir une action proche de la physiologie et une toxicité faible voire nulle. Bien que certains auteurs proposent d'utiliser les anions inorganiques nitrite ou nitrate (car apportés naturellement dans l'alimentation et l'eau de boisson), ces composés ne

peuvent être transformés en NO que dans certaines conditions et doivent être apportés en grandes quantités pour une action thérapeutique, ce qui peut induire un risque carcinogène [210–212]. La seule autre famille comprenant des composés existant de façon endogène est celle des *S*-nitrosothiols, qui sont la forme majoritaire de stockage et transport de NO [13]. Ils ne sont pas toxiques, n'induisent pas de phénomène de tolérance ou de stress oxydant et sont relativement faciles à synthétiser. Malgré leurs avantages indéniables, les RSNO souffrent cependant d'une demi-vie et d'une stabilité plutôt faibles [157]. Des modifications de leur structure ont déjà été effectuées et ont permis de moduler leur hydrophobie, leur stabilité ou leur activité [95,157,213–217], ou d'obtenir un ciblage de la mitochondrie [134,218]. En ce qui concerne leur formulation, de multiples possibilités ont été décrites dans la littérature présentant des résultats intéressants *in vitro* (Article 2), mais très peu d'entre elles ont également été testées *in vivo*.

Le but de ce travail était d'évaluer l'intérêt de formulations *in situ* pour la libération prolongée de *S*-nitrosothiols.

La fabrication de ces formulations fait appel à un procédé simple, peu agressif, particulièrement compatible avec les RSNO. De plus, elles sont basées sur un copolymère d'acide lactique et glycolique bioassimilable et biocompatible, dont la large utilisation dans des produits parentéraux à libération prolongée de principes actifs garantit la sécurité d'emploi. Enfin, comme présenté dans l'Article 3, de nombreux paramètres sont modifiables afin d'ajuster le profil de libération. Pour cette étude, il a été fait le choix de ne travailler qu'avec un seul copolymère (PLGA 50 :50 de faible masse molaire et dont les extrémités terminales étaient protégées) à une concentration donnée (20 % m/m). En revanche, deux types de formulation *in situ*, implants et microparticules, ont été testés, en faisant varier le type de solvant (NMP et TA). L'utilisation d'un solvant organique demeure l'inconvénient de ces formulations, bien que des formulations à base de NMP aient déjà été admises pour l'administration parentérale.

Ces formulations forment une matrice polymérique solide par la précipitation du polymère lors de la mise en contact avec un milieu aqueux. Elles nécessitent donc de disposer de principes actifs isolés, exempts de toute trace d'eau. Dans le cadre du projet « NanoSNO », plusieurs molécules ont pu être synthétisées : GSNO, SNAP, *S-S'*-dinitrosobucillamine, esters nitrosés de la *N*-acétyl-cystéine (plus hydrophobes). Seules deux d'entre elles ont pu être précipitées et purifiées pour obtenir des produits de qualité pharmaceutique (*cf.* Article 4) : l'une naturelle, GSNO et l'autre synthétique, SNAP. Au-delà de leur structure (cœur cystéine ou pénicillamine), ces molécules diffèrent fortement en termes de stabilité, d'hydrophobie (*cf.* Article 5) et légèrement en ce qui concerne leur efficacité (*cf.* Article 2).

Dans un premier temps, la libération *in vitro* de ces deux RSNO à partir des différentes formulations a été étudiée (*cf.* Article 5). Quelle que soit la formulation, GSNO était libéré en moins de 24 h, alors que SNAP était libéré plus progressivement (3 jours) avec cependant une libération massive (*burst*) pendant les 24 premières heures. Le *burst* est un des inconvénients classiquement

décrits pour ces systèmes *in situ* : utiliser un solvant plus lipophile (ex : TA) ou une phase externe (ISM) sont des procédés classiques pour lutter contre ce phénomène. En effet, globalement, l'utilisation de TA a ralenti la libération des RSNO (sauf pour les ISM à base de TA chargées en GSNO). De plus, la libération de GSNO et de SNAP pendant le premier jour à partir de formulations à base de NMP a été plus progressive pour les ISM que pour les ISI. Pour les formulations à base de TA, la libération de GSNO est accélérée par l'utilisation de la forme ISM alors que celle de SNAP est ralentie. Les comportements attendus sont donc retrouvés. En effet, la libération rapide de GSNO à partir des ISM à base de TA peut s'expliquer par la constitution de la phase externe (eau saturée en triacétine), qui présente une faible viscosité (donc un effet barrière peu important) et pour lequel GSNO a vraisemblablement une forte affinité étant donné son caractère hydrophile.

Le type de formulation et le solvant utilisés ont donc montré une influence sur la libération initiale *in vitro* des RSNO. L'observation de la morphologie des dépôts obtenus n'a pu être effectuée que pour les formulations NMP, et a montré une différence entre les ISI et les ISM. En revanche, l'étude de la dégradation des systèmes a pu être effectuée pour toutes les formulations : quels que soient le solvant et la formulation, les mêmes profils ont été observés. Par conséquent, le type de formulation (et aussi le solvant utilisé d'après la littérature [160]) conduit bien à des structures différentes. Par contre, contrairement à l'hypothèse de départ, avec le polymère utilisé à la concentration utilisée, il n'y a pas de différence notable en termes de profil de dégradation entre les différentes formulations.

Cette étude *in vitro* a également permis de déterminer l'importance de l'hydrophobie du principe actif dans sa libération à partir des formes *in situ*. En utilisant deux autres donneurs de NO isolés de la famille des nitrates organiques, des corrélations significatives ont pu être établies entre le profil de libération *in vitro* de ces molécules et leur hydrophobie.

Etant donné les résultats précédents et dans le but de restreindre le nombre de formulations à tester *in vivo*, il a été choisi de conserver les formulations à base de NMP. En effet, bien que l'utilisation de TA diminue le *burst*, elle augmente également la viscosité du système, ce qui peut poser problème lors de l'injection à des animaux.

Premièrement, les résultats ont montré que l'environnement (*in vitro* vs *in vivo*) jouait un rôle prépondérant dans la morphologie et la dégradation des matrices polymériques obtenues avec les formulations à base de NMP. Ces données venaient conforter une étude précédemment réalisée [79] et impliquaient que les résultats de libération de donneurs de NO obtenus *in vitro* ne seraient pas forcément transposables *in vivo* (car la libération du principe actif est dépendante des phases de formation/dégradation de la matrice, *cf.* Article 3).

Deuxièmement, les formulations à base de NMP, chargées en GSNO ou en SNAP, ont été injectées par voie sous-cutanée à des rats Wistar. Il n'a cependant pas été possible d'effectuer une étude pharmacocinétique complète. En effet, les résultats préliminaires ont montré une grande variabilité des concentrations plasmatiques des espèces dérivées du NO, qui sont de plus faiblement modifiées suite à l'injection d'un bolus sous-cutané de GSNO. Le NO apporté par les RSNO administrés peut être

transféré à différents composés ou transformé en d'autres espèces (Article 2), ce qui rend multiple les espèces à suivre et diminue l'amplitude de variation de leurs concentrations sanguines et plasmatiques. Seules des méthodologies de radiomarquage de l'azote permettraient de suivre le NO apporté. Par ailleurs, il faut noter qu'il n'existe pas encore de consensus réel dans la littérature au sujet des concentrations physiologiques de ces différentes espèces, bien que la tendance actuelle consiste à les reconnaître très faibles (nM- μ M) [80], ce qui nécessite de disposer de méthodes extrêmement sensibles.

Troisièmement, l'incorporation des RSNO dans les formulations *in situ* a permis d'augmenter la durée de leur effet sur la pression artérielle des animaux par rapport aux solutions contrôles. *In vivo*, l'hydrophobie du principe actif ne semble pas être un facteur déterminant de la libération, car les résultats étaient meilleurs avec GSNO (jusque 42 h de diminution de la pression artérielle pulsée) qu'avec SNAP (5 h), alors que GSNO était pourtant libéré très rapidement *in vitro*. Comme les doses injectées sont équivalentes en termes de nombre de moles de NO, cela signifie que des phénomènes sont différents au niveau de la libération des composés ou de leur action. Le premier point aurait pu être résolu si l'étude pharmacocinétique avait pu être menée. Pour le deuxième point, quelques éléments de discussion peuvent être apportés en rappelant que GSNO contrairement à SNAP est un composé endogène, et qu'il présente une plus grande demi-vie (40 h vs 2 h, *cf.* Article 2), lui permettant peut-être une circulation plus longue une fois libéré des formulations. Enfin, les deux molécules ne sont pas égales en termes de dégradation, qu'elle soit chimique ou enzymatique (Article 2). D'une part, la structure du composé influe sur la stabilité de la liaison S-N et peut aussi limiter la dégradation par chélation des espèces. D'autre part, plusieurs systèmes enzymatiques peuvent réaliser des réactions de transnitrosation et de dénitrrosation, mais il existe un système spécifique du GSNO, la gamma-glutamyl-transférase [78]. Cette enzyme est présente à la fois dans la circulation et en surface des membranes cellulaires. Pour finir, bien que les résultats en conditions *ex vivo* montrent une différence faible de leur efficacité vasorelaxante (*cf.* Article 2), il est également possible que dans un animal entier, GSNO soit un vasorelaxant encore plus efficace que SNAP. L'étude de l'impact de ces formulations chargées en RSNO sur l'agrégation plaquettaire pourrait également révéler des différences entre les actions des deux composés. Une meilleure connaissance du devenir *in vivo* de ces RSNO est donc nécessaire.

En résumé, les formulations *in situ* permettent de remplir deux des trois conditions du cahier des charges défini au début de cette discussion : demi-vie augmentée et libération contrôlée. Le *drug design*, en réalisant la synthèse de RSNO à partir d'un choix rationnel de leur structure chimique, pourrait permettre d'améliorer encore ces résultats et potentiellement aussi d'atteindre le troisième objectif, le ciblage.

Les formulations *in situ* sont donc des plateformes potentielles pour la délivrance de donneurs de NO avec de nombreuses applications thérapeutiques, même si leur utilisation semble limitée par les

phénomènes de *burst* et l'effet qui reste de courte durée par rapport à la vitesse de dégradation de la matrice polymérique. Une optimisation du système (ex : concentration plus importante de polymère, polymère de plus haute masse molaire, mélange de solvants, *cf.* Article 3) serait à envisager pour leur utilisation en traitement de pathologies chroniques.

Néanmoins, ces formulations pourraient représenter un outil efficace dans le cadre d'une administration ponctuelle par exemple lors d'un événement aigu (ex : infarctus du myocarde [219,220]), lors d'opérations chirurgicales à risque [91,221,222] ou en thérapeutique adjuvante (ex : traitements anticancéreux [223–225]).

Dans cette perspective, des expériences préliminaires ont été réalisées, pour l'injection à distance de l'infarctus, d'implants à base de NMP chargés en GSNO, directement dans le myocarde, au niveau de la zone infarctée. Dans l'idéal, les dépôts obtenus devraient d'une part améliorer la résistance mécanique de la paroi cardiaque en comblant la zone amincie par la nécrose et d'autre part améliorer la recolonisation par des cellules et donc la régénération tissulaire par la libération progressive de NO. Les formulations *in situ* sont majoritairement injectées par voie sous-cutanée. Bien que des injections aient été effectuées en intramusculaire [16,226,227], cela n'a jamais été réalisé à notre connaissance dans le muscle cardiaque. Une seule étude fait part d'une injection d'implants *in situ* dans un tissu « sensible » : Nasongkla *et coll.* ont montré la faisabilité de l'injection d'implants *in situ* à base de glycofurol dans le cerveau de rats, pour la délivrance intracérébrale de principes actifs [228].

Un système pour la régénération cardiaque doit être non toxique, biocompatible, si possible fournir un environnement favorable aux cellules, mais aussi satisfaire aux contraintes mécaniques du cœur. L'avantage des implants *in situ* est de remplir la zone infarctée en s'adaptant à l'espace disponible, de façon la moins invasive possible et avec une taille permettant l'oxygénation et la nutrition des cellules potentiellement recrutées. Ces implants en cours de dégradation dans le tissu seraient susceptibles de favoriser la croissance cellulaire sous réserve d'une absence de toxicité ainsi que du maintien d'un statut inflammatoire et redox approprié. Les premiers essais *in vivo* ont montré la faisabilité de l'injection intramyocardique des formes *in situ*. Les implants non chargés semblent présenter une certaine toxicité, cependant ils pourraient améliorer la perfusion du cœur infarcté lorsqu'ils sont chargés en GSNO chez des rats souffrant d'ischémie myocardique. Le statut redox et le phénotype des cellules isolées (au niveau des expériences *in vitro*) ou au sein d'un tissu (après injection intramyocardique) seront à explorer afin d'assurer une libération de NO dans des conditions favorables.

Toujours dans le cadre de l'infarctus du myocarde, une autre étude est envisagée : l'injection par voie sous-cutanée d'implants à base de NMP, chargés en GSNO, dans les premiers temps après l'infarctus. Ici, le but ne serait plus de régénérer le tissu à distance de l'évènement, mais plutôt de moduler les réactions inflammatoires, limiter les lésions et le remodelage post-infarctus, et donc l'insuffisance cardiaque à long terme. Par ailleurs, la co-encapsulation de principes actifs pourrait être envisagée.

Pour conclure, il a été montré que les formes *in situ* permettent une libération prolongée *in vitro* et un effet prolongé *in vivo* des RSNO chez des animaux sains. La suite de ce travail s'oriente donc logiquement vers l'utilisation des formes *in situ* contenant des RSNO dans un modèle pathologique.

CONCLUSION

Le monoxyde d'azote est un messager physiologique incontournable, tant dans le règne végétal que dans le règne animal. L'administration de NO ou de précurseurs constitue une stratégie thérapeutique prometteuse dans les pathologies cardiovasculaires, notamment ischémiques.

Dans ce travail, des formulations *in situ* pour la libération prolongée de *S*-nitrosothiols (*S*-nitrosoglutathion et *S*-nitroso-*N*-acétylpénicillamine), des précurseurs de monoxyde d'azote de faible demi-vie, ont été préparés et testés *in vitro* et *in vivo*. Ces formulations ont déjà prouvé leur capacité à libérer de manière progressive et prolongée différents principes actifs. Deux d'entre elles ont d'ailleurs été autorisées en thérapeutique humaine. Dans cette étude, un seul polymère (poly(lactide-*co*-glycolide) 50 :50 avec extrémités protégées), mais deux solvants (*N*-méthyl-2-pyrrolidone et triacétine) et deux types de formulations (implants *in situ* et microparticules *in situ*) ont été utilisés.

Dans un premier temps, les RSNO étudiés ont été synthétisés, isolés et purifiés selon des critères pharmaceutiques. Dans un deuxième temps, une caractérisation physicochimique des formulations obtenues *in vitro* ou *in vivo* a été effectuée. Il a été montré que la morphologie et la dégradation des matrices polymériques obtenues dépendaient fortement de l'environnement d'injection (*in vitro* ou *in vivo*). Enfin, une libération *in vitro* prolongée des RSNO a été obtenue grâce aux formulations, avec une corrélation entre le profil de libération de la molécule et son hydrophobie. Par ailleurs, un effet *in vivo* prolongé (jusque 42 h pour GSNO 30 mg/kg vs 5 h pour SNAP 15 mg/kg) sur la pression artérielle a également été démontré avec les formulations à base de NMP. Des expériences complémentaires sont programmées, d'une part pour étudier l'effet de formulations plus faiblement chargées sur la pression artérielle, d'autre part pour évaluer l'impact des formulations chargées (doses fortes ou doses faibles) sur l'activation et l'agrégation plaquettaire.

Les systèmes *in situ* étudiés représentent donc une approche adaptée à la libération prolongée de RSNO. Cependant, la présence d'un solvant organique, la libération initiale massive (burst) de principe actif ainsi que la durée de vie de la matrice polymérique constituent des limites à leur utilisation en vue d'un traitement chronique. Des optimisations sont à envisager en adaptant le cahier des charges à l'application visée.

Les résultats montrant une libération sur quelques jours des RSNO permettent d'ouvrir le champ vers des stratégies thérapeutiques « ponctuelles », par exemple dans des pathologies ischémiques aiguës, type infarctus du myocarde. Ainsi, ces formulations ont commencé à être évaluées dans un modèle animal d'infarctus du myocarde. En effet, ces systèmes pourraient exercer une action multiple : combler le déficit de matière au niveau de la zone infarctée, moduler le microenvironnement par la libération progressive d'un ou plusieurs principe(s) actif(s) et recruter des cellules afin de

reconstituer le tissu lésé. Les premiers résultats *in vitro* (culture cellulaire) et *in vivo* (modèle animal) semblent encourageants mais doivent être confortés.

La preuve de concept de libération prolongée des RSNO par les formulations *in situ* a été apportée dans cette étude. Ceci permet d'ouvrir des perspectives vers de nouvelles stratégies thérapeutiques, en particulier dans les pathologies cardiovasculaires et ischémiques.

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Formulations *in situ* de donneurs de monoxyde d'azote

Les systèmes injectables précipitant *in situ* sont des formulations de préparation facile permettant la libération prolongée de principes actifs. Ils se présentent sous la forme de liquides (solutions ou émulsions) à base de polymère et de solvant organique pharmaceutiquement acceptable, auxquels est ajouté le principe actif. Après injection sous-cutanée ou intramusculaire, leur entrée en contact avec les fluides corporels aqueux entraîne la précipitation du polymère, insoluble dans l'eau, et la formation de dépôt sous forme d'implant (ISI) ou de microparticules (ISM) qui se dégradent progressivement en libérant le principe actif. Dans ce travail, des ISI et des ISM réalisés à partir d'un copolymère d'acide lactique et glycolique (PLGA, biocompatible et biorésorbable) ont été développés pour la libération prolongée de *S*-nitrosothiols, des donneurs de monoxyde d'azote de faible demi-vie. Deux solvants (*N*-méthyl-2-pyrrolidone et triacétine) ainsi que deux *S*-nitrosothiols (*S*-nitrosoglutathion et *S*-nitroso-*N*-acétylpénicillamine) ont été utilisés.

Comme attendu, le type de formulation et de solvant permettait de moduler la libération des principes actifs *in vitro*, mais les expériences ont montré que le profil de libération était également fortement influencé par l'hydrophobie de la molécule. La structure et la dégradation des matrices polymériques étaient plus affectées par l'environnement (*in vitro* ou *in vivo*) que par le type de solvant utilisé. Les expériences *in vivo* ont prouvé un effet prolongé des *S*-nitrosothiols sur la pression artérielle chez le rat par leur incorporation dans les formes *in situ*. Néanmoins, la différence importante entre la durée de l'effet observé (maximum 42 h) et le temps de dégradation des formulations (> 1 mois), nécessite une optimisation de celles-ci pour une application de longue durée.

Cependant, comme une libération progressive pendant plusieurs heures de donneurs de monoxyde d'azote est bénéfique dans des situations ischémiques, le potentiel de ces formulations comme thérapeutique ponctuelle dans un modèle d'infarctus du myocarde a commencé à être évalué. D'une part, la faisabilité de leur injection directement dans le myocarde au niveau de la zone infarctée a été démontrée. D'autre part, les tout premiers résultats semblent indiquer que ces implants chargés en principes actifs permettraient d'améliorer la perfusion du myocarde. Enfin, la porosité de ces systèmes augmente durant leur dégradation, ce qui rend la matrice susceptible de recruter et d'héberger des cellules. Une étude menée *in vitro* avec des implants dont la porosité augmentée a été simulée a montré qu'ils permettaient l'adhésion et la prolifération de cellules musculaires.

Les formulations *in situ* représentent donc des formulations galéniques adaptées aux *S*-nitrosothiols.

Mots-clés : Monoxyde d'azote, *S*-nitrosothiols, libération prolongée, implants *in situ*, microparticules *in situ*

In situ formulations of nitric oxide donors

In situ forming injectable systems are easy to prepare formulations for sustained drug release. They are liquids (solutions or suspensions) based of a polymer and a pharmaceutically acceptable organic solvent, to which drug is added. After subcutaneous or intramuscular injection, contact with aqueous body fluids triggers polymer precipitation (as the polymer is water-insoluble) and the formation of a depot as implant (ISI) or microparticles (ISM). This matrix degrades then slowly while releasing the drug. In this work, ISI and ISM made of a copolymer of lactic and glycolic acid (PLGA, biocompatible and bioerodible) were developed for sustained release of *S*-nitrosothiols, prodrugs of nitric oxide with low half-lives. Two solvents (*N*-methyl-2-pyrrolidone and triacetin) as well as two *S*-nitrosothiols (*S*-nitrosoglutathione and *S*-nitroso-*N*-acetylpenicillamine) were used.

As expected, formulation type and solvent modulated *in vitro* drug releases, but results also showed a strong influence of drug hydrophobicity on their release profiles. Structure and degradation of polymeric matrices were more affected by the *in vitro* or *in vivo* environment than by the solvent used. *In vivo* experiments proved a sustained effect of *S*-nitrosothiols on arterial pressure of rats when incorporated into *in situ* systems. However, considering the discrepancy between the duration of effect (max 42 h) and matrix life-span (> 1 month), there is a need for formulations optimization in view of an application requiring a long lasting release.

Nevertheless, evaluation of therapeutic potential of a single injection of these formulations has begun in a model of cardiac infarction, as a several hours sustained release of nitric oxide donors could be beneficial in such ischemic situation. First, the feasibility of a direct injection into the infarct area has been established. Second, first results seem to indicate that these implants loaded with active pharmaceutical ingredients could enhance heart perfusion. Finally, porosity of these systems increases during their degradation, allowing cell recruitment and colonization of resulting matrix. An *in vitro* study conducted on implants with porosity artificially increased showed adhesion and proliferation of muscular cells seeded onto the systems.

As a result, *in situ* formulations represent suitable drug delivery systems for *S*-nitrosothiols.

Key-words: Nitric oxide, *S*-nitrosothiols, sustained release, *in situ* implants, *in situ* microparticles