



**Polymeric formulations for innovative drug delivery  
systems of nitric oxide in the treatment of inflammatory  
bowel diseases: formulation and bioavailability  
assessment**

Shefaat Ullah Shah

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Par **Shefaat Ullah SHAH**

*Formulations polymériques pour l'administration par voie orale de vecteurs originaux d'oxyde  
nitrique dans le traitement des maladies inflammatoires de l'intestin : mise au point et  
évaluation de la biodisponibilité.*

Date : **3 Novembre 2015**

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## List of abbreviations

CD: Crohn's disease

cGMP: cyclic guanosine 3'5'-monophosphate

CV: cardiovascular

DAN: 2, 3-diami-nonaphthalene

DTNB: 5, 5'-dithiobis (2-nitro benzoic acid

EDAC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride

EDRF: endothelial derived relaxing factor

FTIR: Fourier Transform Infrared

GALT: gut-associated-lymphoid-tissue

GC: guanylate cyclase

GFR: glomerular filtration rate

GIT: gastro intestinal tract

GSH: glutathione

GSNO: S-nitrosoglutathione

GTN: glyceryl trinitrate or nitroglycerine

GTP: guanosine 5'-triphosphate

IFN $\gamma$ : interferon- $\gamma$

IgG1: Immunoglobulin G1

ILF: isolated lymphoid follicles

ISMN: isosorbide mononitrate

LTP: long term potentiation

LTD: long term depression

MWCO: molecular weight cut off



NADPH: nicotinamide adenine dinucleotide phosphate

NANC: non-adrenergic non-cholinergic

NED: sulfa-nilamide, N-(1-naphthyl) ethylenediamine

NHS: N-hydroxysuccinimide

NOS: Nitric oxide synthase

PXRD: Power X-ray diffraction patterns

RSNO's: S-nitrosothiols

SNA: S-nitrosoglutathione-alginate

SEM: scanning electron microscope

SNOC: S-nitrosoglutathione-oligosaccharide-chitosan

TNF $\alpha$ : tumor necrosis factor- $\alpha$

VSMC's: vascular smooth muscle cells

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# **Présentation synthétique de la thèse (français)**

Ce projet de thèse a été réalisé au sein du laboratoire « EA 3452 / CITHEFOR » de l'Université de Lorraine (France). Le laboratoire CITHEFOR met notamment au point des formulations permettant d'administrer des *S*-nitrosothiols (implants, microparticules, nanoparticules).

La plupart des essais *in vivo* ont eu jusqu'à présent pour objectif d'évaluer les effets cardiovasculaires des *S*-nitrosothiols. Toutefois, on sait maintenant que le *S*-nitrosoglutathion, exerce un effet protecteur sur la barrière intestinale qui pourrait être très bénéfique dans le traitement de la maladie de Crohn (Savidge et al., 2007).

L'objectif de cette thèse était de mettre au point des formulations qui puissent être administrées par voie orale et libérer le monoxyde d'azote (NO) au niveau intestinal. Nous recherchons tout particulièrement à protéger les *S*-nitrosothiols de la dégradation chimique et à libérer progressivement le monoxyde d'azote au contact de la lumière intestinale.

Trois types de formulations ont été mises au point :

- Des polymères constitués de chitosane sur lesquels nous avons fixé du *S*-nitrosoglutathion
- Des polymères constitués d'alginate sur lesquels nous avons fixé du *S*-nitrosoglutathion
- Des microparticules de copolymère d'acrylate et d'amonio-metacrylate (Eudragit® RS30D) préparées par Spray Drying.

## **Bibliographie**

La première partie est une introduction générale sur le NO et sur son rôle au sein de l'organisme.

Nous avons également présenté les différentes molécules « donneuses de NO » actuellement commercialisées ou au stade de l'essai clinique en détaillant notamment leur indication potentielle. Nous avons ensuite étudié les différents types de formulations et de thiomères qui pourraient être utilisés dans notre travail.

Enfin, dans un dernier paragraphe, nous expliquons pourquoi les S-nitrosothiols pourraient être intéressants dans le traitement de la maladie de Crohn et quelles formulations pourraient être proposées.

*Ce chapitre contient aussi une brève introduction en français.*

## **Résultats et Discussion**

Cette partie est constituée de trois publications centrées sur des systèmes à libération prolongée de NO. Un de ces articles, intitulé « Synthesis and characterization of S-nitrosoglutathione-oligosaccharide-chitosan as a nitric oxide donor », est déjà publié dans « Expert Opinion on Drug Delivery » (Shah et al., 2015a). Les deux autres articles [1) Synthesis of S-nitrosoglutathione alginate for prolonged delivery of nitric oxide in intestines, 2) Spray dried microparticles of glutathione and S-nitrosoglutathione based on Eudragit® FS 30D polymer] ont été soumis.

*Un résumé en français est présenté avant chaque publication.*

## **Discussion générale (anglais), conclusions et perspectives (français)**

En conclusion, nous avons réussi à synthétiser deux polymères couplés au glutathion réduit (oligosaccharide chitosane – GSH et alginate-GSH) qui ont été nitrosés avec du nitrite de sodium ( $\text{NaNO}_2$ ) pour donner naissance à 2 polymères que nous avons appelé SNOC (S-nitrosoglutathione-oligosaccharide chitosane) et SNA (S-nitrosoglutathione-alginate). Ces 2 nouveaux S-nitrosothiols ont été caractérisés, ce qui a permis de préciser notamment la stabilité du groupement S-NO et la libération *ex vivo* (c-à-d dans des chambres de Ussing) du NO au contact de l'intestin. Le SNOC et le SNA ont été comparés à la molécule de référence : le S-nitrosoglutathion (GSNO).

Les 2 polymères se sont montrés stables plus longtemps que le GSNO.

Les études de libération *in vitro* ont démontré que ce sont de bons candidats pour le développement de nouvelles formulations polymériques (ex : microparticules).



# **Introduction générale (français)**

Le monoxyde d'azote (NO) est un radical libre endogène qui joue un rôle important dans la bio-régulation de voies de signalisation intra- et extracellulaire parmi lesquelles on trouve le contrôle du tonus vasculaire et l'activation plaquettaire (Al-Sa'doni and Ferro, 2000; Friedman et al., 2008). Dans les dernières décennies, les nitrites organiques (de structure R-O-NO<sub>2</sub> appelés aussi dérivés nitrés) ont été utilisés comme donneurs de NO dans le traitement de l'angine de poitrine (Riseman et al., 1958). Plus récemment, le gaz NO (INOmax®) a été proposé pour la dilatation de l'arbre pulmonaire. Il peut ainsi être utilisé pour le traitement des nouveau-nés souffrant d'insuffisance respiratoire associée à de l'hypertension pulmonaire. Enfin, certaines industries pharmaceutiques ont développé des donneurs de NO pour des applications spécifiques telles que le glaucome (Cavet, Vittitow *et al.* 2014) ou la dystrophie musculaire (Malik, Rodino-Klapac *et al.* 2012).

Actuellement, des molécules endogènes appelées S-nitrosothiols (RSNO, par exemple le S-nitrosoglutathion et la S-nitrosoalbumine) font l'objet de recherches intensives. Ces composés ont la propriété spécifique de stocker le NO par couplage sur une fonction thiol (Al-Sa'doni et Ferro 2000, Carini, Aldini et al. 2004) et offriraient de nombreux avantages par rapport aux donneurs de NO existants sur le marché. On sait notamment qu'ils n'entraînent pas de phénomène de tolérance. Un certain nombre d'études cliniques suggèrent que cela pourrait être bénéfique dans les pathologies cardiovasculaires (Everett, Wilkinson et al. 2014). Ces RSNO pourraient être utilisés dans le traitement de l'ischémie cérébrale (Khan, Im et al. 2009), le cancer (Furuhashi, Sugita et al. 2011), la mucoviscidose (Servetnyk, Jiang et al. 2011), ou la cicatrisation des plaies (Georgii, Amadeu et al. 2011).

Le GSNO a été proposé comme un traitement potentiel pour prévenir la défaillance de la barrière intestinale muqueuse dans la maladie de Crohn (Savidge, Newman et al. 2007). Il a été observé que le GSNO augmente de manière significative la fonction de protection de la barrière intestinale muqueuse chez les patients atteints de la maladie de Crohn. A l'inverse, les tissus intestinaux provenant des patients non atteints ne sont pas sensibles au GSNO.

A la suite de cette observation, les auteurs ont émis l'hypothèse que cette spécificité tissulaire peut se rapporter à la perturbation du réseau des cellules gliales entériques chez les patients

atteints de maladie de Crohn. Ceci conduit à des concentrations faibles en NO (Savidge, Newman et al. 2007). Pour contrer ceci, on peut facilement imaginer que de hautes doses de S-nitrosothiols pourraient être utilisées (Katsumi, Nishikawa et al. 2009).

Quelques études suggèrent que les vecteurs polymériques (microparticules, nanoparticules) dans lesquels ont été incorporés des donneurs de NO ( $\text{trans-[RuCl(15)ane(NO)]}^{2+}$ ) bénéficient d'une meilleure stabilité et peuvent libérer progressivement la substance active (Saraiva et al., 2011).

Les S-nitrosothiols sont relativement difficiles à incorporer dans des particules polymériques car ce sont des substances très hydrophiles et peu stables.

Deux stratégies ont été étudiées dans ce travail dans le but d'améliorer la quantité de NO fixé et d'obtenir une libération adaptée *in situ*.

- La fixation directe de GSNO sur des polymères polyosidiques : le chitosane et l'alginate. Ce travail a été réalisé d'abord en suivant les techniques publiées de fixation du GSH. Une nitrosation du groupement thiol a été faite ensuite pour obtenir un polymère de type chitosane – GSNO ou alginate – GSNO.

- La deuxième stratégie a conduit à utiliser le spray drying (séchage par atomisation) qui présente un intérêt pour les principes actifs hydrophiles.

# **Présentation synthétique des méthodes utilisées**

## **1. Synthèse de donneurs de NO**

### **1.1. Synthèse de SNOC (*S*-nitrosoglutathione-oligosaccharides de chitosan)**

La synthèse du SNOC a été réalisée en deux étapes : 1) liaison du chitosan avec le glutathion (méthode des carbodiimides), 2) nitrosation du groupement thiol situé sur le GSH conjugué par le nitrite de sodium.

L'analyse structurale des conjugués synthétisés a été réalisée par RMN et FTIR.

La quantité de thiol fixée a été mesurée par la méthode de Ellman, tandis que la quantité de NO / NO<sub>2</sub> a été mesurée à l'aide de la méthode de Griess et Saville.

Nous avons ensuite pu déterminer les meilleures conditions opératoires afin d'obtenir le « SNOC ». Celui-ci a subi ensuite des tests de stabilité et nous avons finalement évalué ses caractéristiques de transporteur de NO *ex vivo* dans des Chambres de Ussing (un morceau d'intestin de rat était monté entre 2 chambres contenant une solution tampon).

### **1.2. Synthèse de la SNA (*S*-nitrosoglutathione-alginate)**

La synthèse de SNA était globalement basée sur la même méthodologie mais avec de l'alginate. L'objectif de cette deuxième série de synthèses était d'obtenir un polymère moins toxique et plus stable que le SNOC. L'alginate a été fixé au GSH par la méthode de carbodiimides et une nitrosation des thiols a permis de former le SNA. Tout comme dans le cas du SNOC, la quantité de thiols disponibles a été mesurée par la méthode d'Ellman et la méthode de Griess et Saville a été utilisée pour mesurer la quantité de NO / NO<sub>2</sub>.

Nous avons ensuite pu déterminer les meilleures conditions opératoires pour préparer les lots qui ont été testés.

## **2. Microparticules préparées par spray drying : GSH /Eudragit® FS30D et GSNO/Eudragit® FS30D**

Le spray drying est une technique de transformation d'une forme liquide (suspension, émulsion ou dispersion) en une forme solide : le liquide est pulvérisé en fines gouttelettes, dans une enceinte cylindrique verticale au contact d'un courant d'air chaud afin d'évaporer l'eau.

La poudre obtenue est entraînée par le flux de chaleur jusqu'à un cyclone ou un filtre à manche qui vont séparer l'air de la poudre.

C'est un procédé qui peut être utilisé pour la microencapsulation de substances actives. Cette technique est utilisée couramment par les industries cosmétiques et alimentaires et aussi utilisée par l'industrie pharmaceutique pour différents produits médicamenteux sous forme sèche (Patel, Shah et al.). Cette technique représente une méthode prometteuse pour contrôler la libération de principes actifs hydrophiles tels que la citrulline (Bahri, Zerrouk et al. 2014) et pour améliorer leur stabilité physicochimique (Guterres, Berck et al. 2009). La technique de « Spray Drying » est décrite dans de nombreuses publications (Kristmundsdóttir, Gudmundsson et al. 1996, Pignatello, Vandelli et al. 1997, Esposito, Roncarati et al. 2000, Durrigl, Kwokal et al. 2011) pour faire des microparticules de polymères acryliques insolubles dans l'eau et à faible perméabilité parmi lesquels on trouve les copolymères de méthacrylate et d'aminométhacrylate (Eudragit®). Il a été rapporté (Esposito, Roncarati et al. 2000, Rassu, Gavini et al. 2008) que l'augmentation de la température était indirectement proportionnelle à la taille des particules mais aucune corrélation n'a été retrouvée avec le taux d'encapsulation du principe actif. D'autres paramètres, comme le débit d'air et du spray sont également très importants pour la formulation finale (Mishra et Mishra 2011). La température d'entrée, la température de sortie, la pression de l'air et le débit du solvant sont fréquemment étudiés.

Dans cette étude, nous avons préparé des microparticules de glutathion (GSH) et de S-nitrosoglutathion (GSNO) par spray drying. Le polymère utilisé était un poly(acrylate de méthyle -co- méthyle méthacrylate-co-acide méthacrylique), commercialisé sous le nom d'Eudragit® RS 30 D et qui a la particularité d'être gastrorésistant et soluble au-delà de pH 7,0.

Différents lots ont été préparés en faisant varier les paramètres du spray dryer (température d'entrée 80-150 °C, débit du solvant 5-10 ml / min).

La taille des particules et leur forme ont été étudiées par microscopie électronique à balayage, (SEM). La quantité de principe actif a également été étudiée (méthodes d'Ellman et de Griess-Saville).

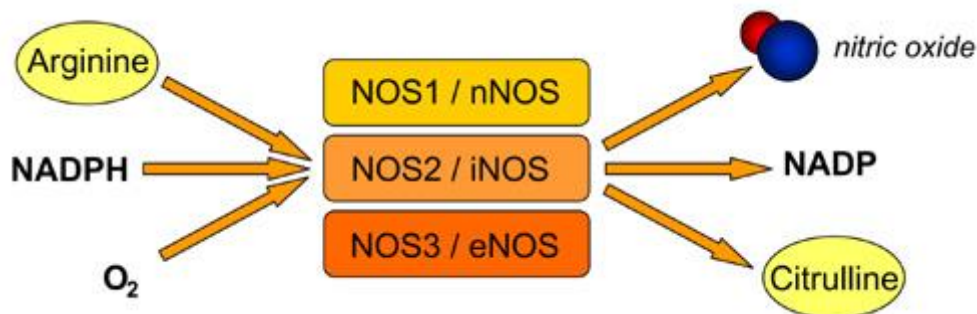
Ensuite, la dispersion des principes actifs (GSH et GSNO) dans le polymère a été mesurée par diffraction X sur poudre (PXRD). La stabilité des médicaments dans des microparticules a été évaluée par spectroscopie infrarouge à transformée de Fourier (FTIR). Enfin, les études de libération du médicament ont été effectuées dans un tampon phosphate (6 ; 6,8 et 7,4), et condition acide de pH 1.2 et 3.

# Bibliography



## 1. Nitric oxide

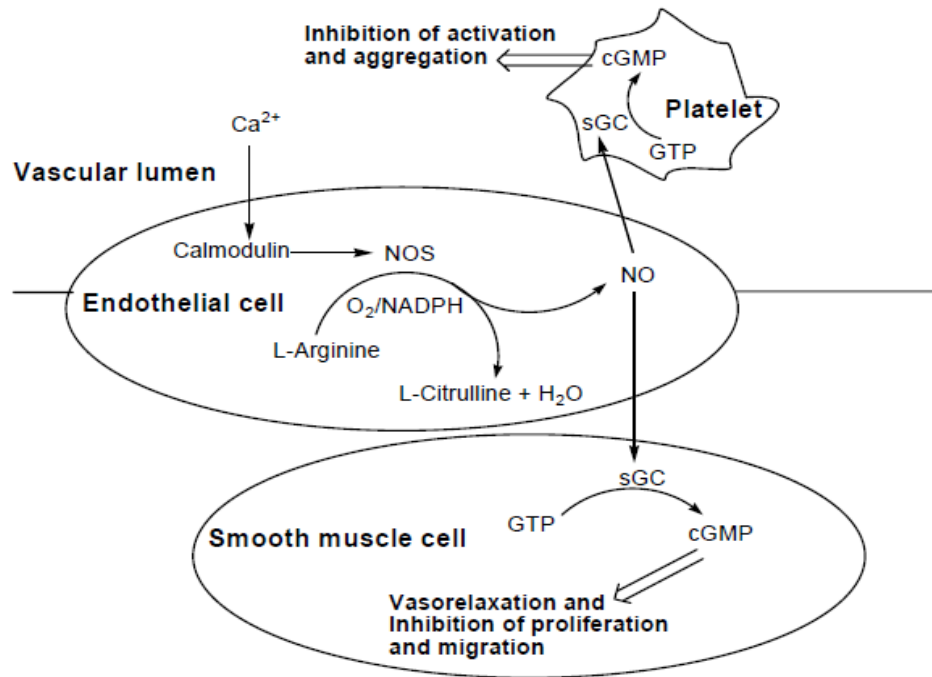
Nitric oxide (NO) is a small endogenous compound (Saraiva et al., 2011) composed of one atom of nitrogen and one atom of oxygen ( $\text{NO}^\bullet$ ); it is also a free radical with an unpaired electron (Lowenstein et al., 1994). These properties make it an ideal messenger which can diffuse freely across membranes. It was first introduced in 1987 (Ignarro et al., 1981). It is highly reactive, it has a very short half-life of 2 to 20 seconds and converts to its degradation product nitrite after transmitting signals (Lowenstein et al., 1994). Endogenous  $\text{NO}^\bullet$  is generated from arginine by a family of three distinct calmodulin dependent enzymes called nitric oxide synthase (NOS), which has three isoforms [NOS from endothelial cells (eNOS), from neurons (nNOS) and calcium independent inducible NOS (iNOS)] (Bredt, 1999). All these three NOS use NADPH as an electron donor and engage five enzyme cofactors to catalyze a five electron oxidation of arginine to  $\text{NO}^\bullet$  along with the formation of citrulline (Bredt, 1999) (Figure 1). It is released either as  $\text{NO}^\bullet$  or is bound to  $-\text{SH}$  groups containing carrier molecules which stabilizes its release (Moncada et al., 1991).



**Figure 1.** *In vivo* formation of NO (University of reading)

NOS are being considered as primary source of NO in biological systems. In endothelial cells, NOS (eNOS) catalyzes the conversion of L-arginine and oxygen to L-citrulline and  $\text{NO}^\bullet$  with the help of activated calmodulin and NADPH. The generated  $\text{NO}^\bullet$  from eNOS diffuses into the surrounding blood and smooth muscle cells and binds to a soluble hemoprotein guanylate cyclase (sGC) [(Denninger and Marletta, 1999; Nakane, 2003)]. Because of this binding event, this enzyme is activated, which catalyzes the conversion of guanosine 5'-triphosphate (GTP) to cyclic guanosine 3'5'-monophosphate (cGMP) (Figure 2). The production of this  $\text{NO}^\bullet$ /cGMP

pathway regulates platelet adhesion and aggregation as well as smooth muscle cells proliferation and migration (Grant and El-Fakahany, 2004; Michiels, 2003).



**Figure 2.** Scheme of the NO production by NOS in endothelial cells (eNOS): In the vascular lumen, NO circulates and inhibits platelet activation and aggregation. In the smooth muscle cells, NO diffuses and cause vasorelaxation and inhibition of proliferation (Hwang, 2007)

Nitric oxide (NO) is considered to be the most important physiological regulators and is involved in many biological roles like vasoprotection (Hwang and Meyerhoff, 2008). NO has many biological roles, which reflects its unique highly reactive characteristics (Schäffer et al., 1996). Some important biological functions (Figure 2) include inhibition of platelet aggregation, inhibition of smooth muscle cell proliferation (Hwang and Meyerhoff, 2008), prevention of microbial growth (Mancinelli and McKay, 1983) and is also involved in wound healing (Schäffer et al., 1996). In inflammatory and mesenchymal cells, the production of large amounts of NO has been shown in response to lipopolysaccharides and cytokines (Lorsbach et al., 1993; Schäffer et al., 1996). This production was the result of the effect of iNOS on arginine. It also plays an important role as a mediator of non-specific immune reaction, vasodilation and neurotransmission (Lowenstein et al., 1994).

Some detailed activities of nitric oxide are given below.

### **1.1. Nitric oxide in central nervous system and its action as a neurotransmitter:**

NO, because of its radical nature can readily diffuse and it was suggested that NO might mediate neuronal plasticity, which can affect the development and information storage in brain. NO involvement in synaptic plasticity has been thoroughly investigated. It is suggested that at the cellular level, NO• signaling is necessary for two types of neuronal plasticity: long term potentiation (LTP) in the hippocampus and long-term depression (LTD) in the cerebellum. Repeated neuronal stimulation in cellular models produces long lasting changes in synaptic strength, while NOS inhibitors prevent these changes (Bredt, 1999; Schuman and Madison, 1994; Shibuki and Okada, 1991). NO involvement in various neurological disorders is well established. Recently, studies are being done to explore the presumed link between NO and Alzheimer's disease (AD). Although existing evidences of a linkage between NO and AD are not abundant, but emerging data shows AD related changes in the NOS system and it appears that NO might be related to many of the AD pathological mechanisms. It should be also mentioned that the relation between NO and AD may not be related to NO-mediated neurotoxicity but the potential neuroprotective properties of NO should also be considered (Law et al., 2001).

Several studies show that NO is involved in neurotransmission in the central and peripheral nervous systems. Neurons present in the gastrointestinal tract (GIT) release NO and cause gastric relaxation in response to food bolus and relaxation of ileocolonic junction and anal sphincter (Lowenstein et al., 1994). Some further studies show that NO is a physiologic mediator of erectile function where small doses of NOS inhibitors eradicated electrophysiologically induced penile erections (Burnett et al., 1992). NOS containing neurons are also found in the cerebellum, which might be involved in balance and coordination.

### **1.2. Nitric oxide in hypertension**

Hypertension is an increasing global health problem. Its awareness and control is varying considerably. Data from a large number of studies show that diastolic and systolic hypertension

is associated with increased risk for cardiovascular (CV) diseases and stroke. The pulse pressure is a difference between systolic and diastolic blood pressures; it is also a predictor of CV problems but this is controversial or doubtful. However, it may help in identifying risk factors associated with systolic hypertension. Hypertension therapy reduces the risk factors of CV and stroke by up to 40%.

Endothelial dysfunction is the first step in the development of atherosclerotic disease and is present in the early stages of all types of CV risk factors. It is characterized by impaired bioavailability of NO. NO is predominantly synthesized in the vascular endothelium and is generated from L-arginine by endothelial NO synthase (eNOS), which metabolites L-arginine to NO. NO stimulates guanylyl cyclase to form 3',5' -cyclic guanosine monophosphate which as a result dilates vascular smooth muscles, prevention of platelet adhesion and aggregation and exertion of anti-inflammatory and antimigratory effects on endothelial cells, leukocytes and vascular smooth muscle cells, consequently provides protection from atherosclerosis.

In this context, impaired NO bioactivity plays a key role in endothelial dysfunction and thus the regulation of NO could be used in treating hypertension and can reduce risk factors of CV diseases (Hermann et al., 2006). An improvement of the endothelial function could be obtained by the administration of S-nitrosothiols and an increased bioavailability of NO may be of particular interest.

A study (Klahr, 2001) shows that pharmacologically induced elevations in blood pressure results in the release of increased amounts of NO in the circulation, whereas a fall in systemic pressure causes a decreased production of NO. NO production and nitric oxide synthase (NOS) activity have been shown greater compared to normotensive controls in a genetic model. It was suggested in these studies that high blood pressure up-regulated NO production, however the mechanisms involved are not clear. It was also suggested that blood flow effects, shear stress and other related mechanical stimuli increases the production of NO and the expression of endothelial NOS (eNOS). In some patients with essential hypertension the production of NO was reduced as compared to normotensive individuals. Similarly, the acute administration of L-arginine to normal animals caused a rapid elevation of blood pressure and a decrease in glomerular filtration rate (GFR) and renal plasma flow. One other study (Hermann et al., 2006)

also suggests the importance of NO in hypertension. It was suggested that NO plays an important role in regulating blood pressure and that impaired NO bioactivity is an important component of hypertension. Clinical studies showed that hypertensive patients had blunted arterial vasodilatory response to infusion of endothelium dependant vasodilators such as acetylcholine and was also stated that inhibition of NO raises blood pressure (Hermann et al., 2006).

### **1.3. Nitric oxide in immune system**

NO has been recognized as one of the most important contributor to the immune system. It is actively involved in the pathogenesis and control of infectious diseases, tumors, autoimmune processes and chronic degenerative diseases. NO has a variety of reaction partners including DNA, proteins, low molecular weight thiols, prosthetic groups, reactive oxygen intermediates (ROI) ; its activity is influenced by their concentration. The exact function of NO in immune system is not known, however similar protective and toxic effects are seen frequently (Bogdan, 2001). According to studies NO can directly or indirectly modulate the immune response through diverse mechanisms like “mediating microbicidal effects” of NO derived free radicals mostly within macrophages and other phagocytes (Pavanelli et al., 2010). NO entered immunology between 1985 and 1990 and was simply defined as a product of macrophages activated by cytokines, microbial compounds or both; it is derived from the amino acid (L-arginine) degraded by the enzymatic activity of iNOS and it functions as a tumoricidal and antimicrobial molecule (Nathan, 1992). This basic effect is still accepted, but it has been recognized in the past decade that NO contributes to many more roles in the immune system (Bogdan, 2001).

### **1.4. NO and regulation of intestinal function**

The role of NO in regulations of intestinal functions has also been investigated recently. Neuron derived NO was first demonstrated in the GIT tract (Bredt, 1999). In 1960's after the

development of adrenergic blocking agents, it was suggested that several actions of the autonomic nervous system are mediated by non-adrenergic, non-cholinergic (NANC) nerves. This NANC nerves plays an important role in smooth muscle relaxation, in the cerebral circulation, gastrointestinal, urogenital and respiratory tracts (Bredt, 1999; Burnstock, 1981). Several other studies showed that NANC transmitter, in many of these pathways, is identical to the endothelial derived relaxing factor (EDRF). Actually, axon varicosities of myenteric neurons are selectively concentrated in nNOS and the adjacent intestinal smooth muscle cells contain an NO receptor, the soluble guanylyl cyclase. It was suggested that during intestinal peristalsis, myenteric neurons fire action potentials and the resulting calcium influx activates calmodulin, which in result stimulates nNOS. The resulting NO diffuses into the adjacent smooth muscle cells and increase accumulation of cGMP, which initiates intestinal relaxation. (It is available only in text form).

Some clinical studies have also suggested that NO can be proposed as a potential treatment to prevent mucosal barrier failure in Crohn's disease. It was observed that NO-donors significantly promote human intestinal mucosal barrier functions in Crohn's disease patients but not in intestinal tissues without inflammatory bowel disease (Savidge et al., 2007)

### **1.5. Nitric oxide and progression of renal disease**

The use of NO in cardiovascular diseases and renal physiology has gained a tremendous scientific interest in the recent years. A variety of actions are known to be mediated by NO in the kidney including transport of water and electrolytes, maintenance of acid-base balance, modulation of glomerular and interstitial functions, renin-angiotensin activation and the regulation of kidney's immune system. NO might also have a role in several disease conditions of the kidney such as acute renal failure, inflammatory nephritides, diabetic nephropathy and transplant rejection (Sharma, 2004). A study conducted by Garvin and Hong suggested that NO inhibited respiration in renal tubules and isolated renal mitochondria, which shows that NO modulates mitochondrial respiration (Garvin and Hong, 1999). In another study, it was suggested that NO inhibits solutes and water reabsorption and plays a key role in acid-base balance. A split-drop micropuncture was used to determine the effect of NO-donor (sodium nitroprusside, SNP) in the rat proximal tubule. The left kidney was prepared for micropuncture

and after 1-2h equilibration, shrinking split-drop microperfusion was performed in midproximal convoluted tubule segments visible on the kidney surface. It was found that NO reduce the proximal tubular fluid absorption. NO also inhibited angiotensin II-stimulated fluid absorption, which is a mediator of the  $\text{Na}^+/\text{H}^+$  exchange in the nephron (Eitle et al., 1998). In a study, a complete restoration of blood flow and GFR was observed in ureteral obstruction rats when L-arginine was administered before the ligation of the ureter (Klahr, 2001; Reyes et al., 1992). Similarly, a marked decrease in macrophage infiltration in obstructed kidney was observed when L-arginine was administered to rats with unilateral ureteral obstruction. In similar studies, the administration of L-arginine in the drinking water blunted a significant increase in the intestinal volume, monocyte infiltration, intestinal collagen IV deposition and  $\alpha$ -smooth muscle actin expression in the kidney with ureteral obstruction (Klahr, 2001). In the same study (Klahr, 2001), a rat model with subtotal nephrectomy was examined. All of the rats were having a high blood pressure, greater proteinuria and lower plasma albumin levels. L-arginine (1% in drinking water) was given and it was observed that the GFR and renal plasma flow was increased. The administration of L-arginine had also a decreasing effect on proteinuria and glomerular hypertension in rats with subtotal nephrectomy. In summary, all the NO functions in kidney are (Sharma, 2004):

1. Renal macrovascular and microvascular dilation
2. Regulation of mitochondrial respiration
3. Modulation of renal medullary blood flow
4. Stimulation of fluid,  $\text{Na}^+$  and  $\text{HCO}_3^-$  reabsorption in the proximal tubule
5. Inhibition of  $\text{Na}^+$  conductance in the cortical collecting duct (CCD)

#### **1.6. Nitric oxide in cancer**

In recent years, NO has gained an interest in carcinogenesis and tumor growth progression. It has tumoricidal as well as tumor promoting effects depending upon the timing, location and concentration. It has been suggested to be involved in modulating different cancer related events including angiogenesis, apoptosis, cell cycle, invasion and metastasis (Choudhari et al., 2013). Diverse actions of NO in cancer have been demonstrated (Choudhari et al., 2013) :

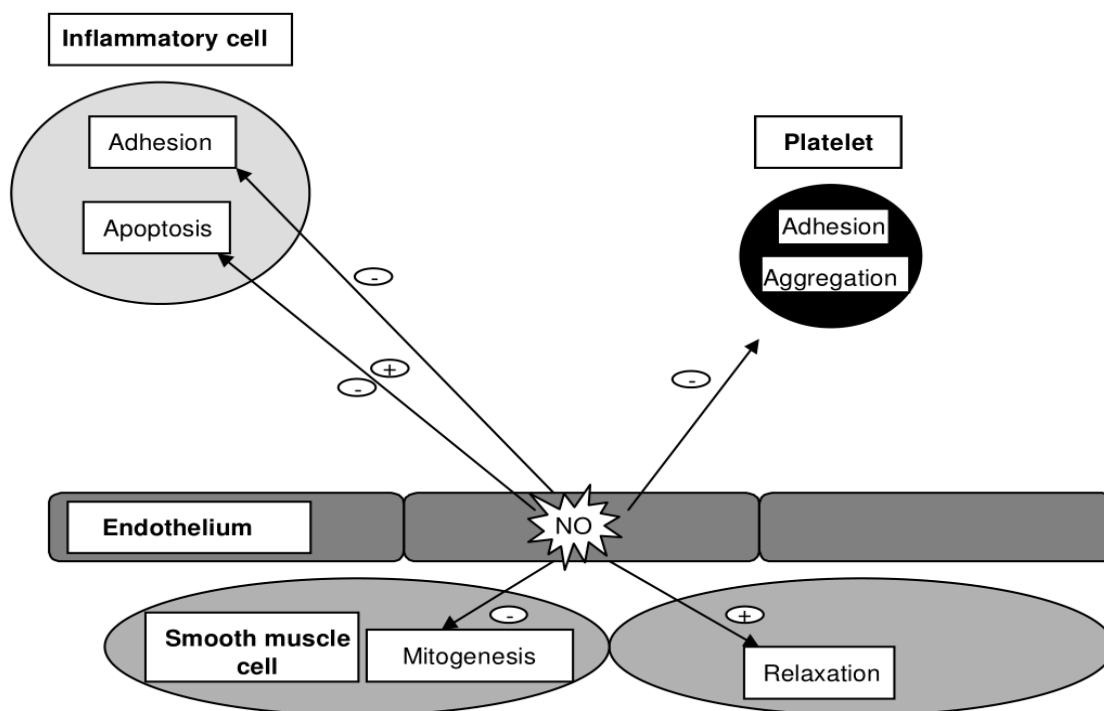
1. NO suppresses cellular respiration and also shifts iron metabolism
2. Suppression of DNA Synthesis
3. Proapoptotic modulator by activating caspase family proteases, up-regulation of p53, alteration in expression of apoptosis-associated proteins

The most common cancer among women is breast cancer. Although a number of reports showed tumor promoting effects of NO, however, some reports suggests that NO derived from macrophages, Küpffer cells, natural killer cells and endothelial cells has a tumoricidal effect against many types of tumors (Li et al., 1991; Shang and Li, 2005). It was suggested that NO plays an important role in tumor cells lysis mediated by cytokine activated endothelial cells (Li et al., 1991). It is suggested that NO may exert a biphasic response. When NO levels are higher than a critical concentration, it would be suitable for tumor growth; hence, the growth arrest or apoptotic pathways are initiated. These effects are studied therapeutically in clinical models of tumors to enhance the efficacy of chemotherapy and radiotherapy and also to slower the tumor growth (Singh and Gupta, 2011).

### **1.7. Anti-inflammatory effects of NO**

NO plays an important role in mediating inflammatory responses. It is an effector molecule of cellular injury, which can also act as antioxidants. It can modulate the release of several inflammatory mediators from a number of cells participating in inflammatory responses such as leukocytes, macrophages, mast cells, endothelial cells and platelets. It is suggested that it can modulate blood flow, took part in the adhesion of leukocytes to the vascular endothelium, which can have an impact on inflammatory responses. In the current years, a number of NO-donors have been developed which exhibits powerful anti-inflammatory effects (Wallace, 2005). In the recent years, the release of NO in inflammatory conditions has gained more attention with the development of NO releasing anti-inflammatory drugs which are considered to be capable of enhancing the anti-inflammatory benefits of conventional anti-inflammatory drugs (Hyun et al., 2004).





**Figure 3.** The major vascular functions of endothelium derived NO (Megson and Webb, 2002)

Chronic lung infection with *pseudomonas aeruginosa* makes more complications in long-term lung diseases including bronchiectasis, cystic fibrosis, chronic obstructive lung disease and mechanical ventilation. It was demonstrated that in chronic airway infections, the inhibition of NO production worsened lung damage whereas augmenting endogenous NO production acted as anti-inflammatory behavior (Hopkins et al., 2006). NO releasing derivatives of cyclooxygenase inhibitors exhibit enhanced anti-inflammatory activities and improved efficacy in a rat model with colitis. This activity was attributed to the enhanced ability to suppress leukocyte infiltration and maybe to scavenge peroxynitrite ( $\text{ONOO}^-$ ) (Wallace et al., 1999).

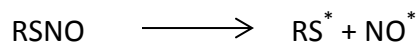
## 2. NO donors

Some compounds are able to carry NO and are called NO donors. In recent years, the first objective was to get compounds able to release NO and that could be used *in vitro* or *in vivo* to evaluate different biological and physiological functions of NO. In this context, exogenous sources of NO donors are required but NO donors can also be considered as potential new

medicines for the treatment of diseases that we have described in previous paragraphs (Saraiva et al., 2011).

NO donors have gained a potential interest recently due to their ability of to release NO at right place and time. A number of NO carriers or donors have been developed; however most of them have problems of toxicity, compatibility and/or stability. They have few additional problems like poor NO storage and unstable release. New NO carriers able to overcome these problems are required.

In recent years, *S*-nitrosothiols were intensively studied: they were pharmacologically active and had the ability to release NO (Saraiva et al., 2011). Some *S*-nitrosothiols (ex: *S*-nitrosoglutathione, *S*-nitrosoalbumine) are endogenous compounds and are involved in a variety of reactions of bioregulatory system based on NO transfer (Singh et al., 1996b). *S*-nitrosothiols are also considered to be the most stable form of NO and some *S*-nitrosothiols are considered to be the major endogenous source of NO in mammals (Josephy et al., 1984; Seabra et al., 2005). In such type of *S*-nitrosothiols the NO is covalently attached to a sulfur atom in C-S-NO molecule, which release NO by S-N bond cleavage and transfer NO to specific receptors in body for different *in vivo* biological effects (Seabra et al., 2005).



Chemical synthesis of *S*-nitrosothiols involve reaction of NO with compounds having –SH groups such as glutathione and albumin. After synthesis these compounds are transported to the site of reaction where they release NO (Singh et al., 1996a). *S*-nitrosothiols can also be linked to biopolymers by a chemical reaction which than make site specific NO delivery (Saraiva et al., 2011).

NO exhibits both therapeutic and protective effects in different disease conditions, so the direct delivery of NO is predictable to be effective in hypertension, stroke, coronary artery diseases (CAD), vascular complications of diabetes and other disorders involving the vascular system. It is known that NO has both beneficial and harmful effects (e.g. it reacts with superoxide anion that is produced by activated macrophages and other cells to form ONOO<sup>-</sup>). That's why, the tissue levels of NO must be well controlled to obtain its therapeutic effects (Herman and Moncada, 2005). The different pathways, which lead to the formation of NO are significantly

different among individual NO-donor classes. Some of these compounds require enzymatic catalysis while some requires non-enzymatic production of NO. Similarly, some NO-donors require interaction with thiols (-SH) to release NO while some undergoes reduction/oxidation. They can also lead to the formation of different end products during decomposition and metabolism (Herman and Moncada, 2005). Moreover, NO reacts with oxygen in a third order kinetic process; therefore, the oxidation of NO to nitrite and nitrates is exquisitely sensitive to the local oxygen tension and cause side reactions such as nitration and/or nitrosation of biomolecules (Ignarro et al., 2002).

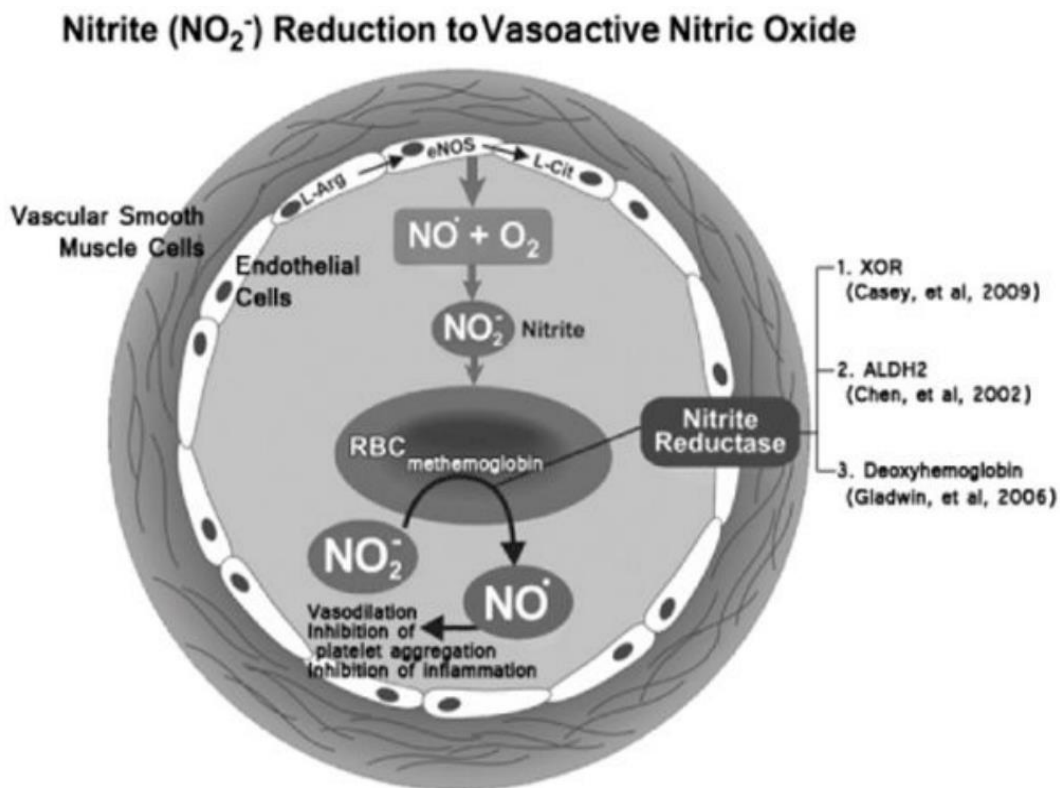
### **2.1. NO donor drugs**

The pharmacological agents possessing a nitroso (R-NO) or nitrosyl functional group are called “direct NO-donors”. S-nitrosothiols are the most common members of this class (Ignarro et al., 2002). These agents spontaneously release NO. On the contrary organic nitrate (R-NO<sub>2</sub>, e.g. nitrite esters: R-O-NO<sub>2</sub>) require metabolic bio-transformation for the release of NO and are called “indirect NO-donors”. This group includes nitroglycerine, amyl nitrite, isosorbide dinitrite, isosorbide 5-mononitrate and nicorandil (Ignarro et al., 2002; Martelli et al., 2006).

### **2.2. Organic nitrates and nitrites**

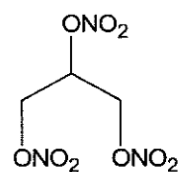
In clinical practice, organic nitrates (R-O-NO<sub>2</sub>) are the nitric acid esters of mono and polyhydric alcohols. Most of them are sparingly soluble in water. The stable stock solutions of these esters can be prepared in ethanol or dimethyl sulfoxide (DMSO) and can be stored in sealed brown glass containers for months and years. They are susceptible to alkaline hydrolysis but are stable at neutral or weakly acidic aqueous mediums (Feelisch, 1998). Organic nitrates and nitrites are the classic vasodilators and include nitroglycerine, amyl nitrite, isosorbide dinitrite, isosorbide 5-mononitrate and nicorandil. These are used since many years for the treatment of cardiovascular diseases (Gruetter et al., 1979; Ignarro et al., 2002). The clinical use of organic nitrates and nitrites was studied by Brunton in 1867 (Lauder Brunton, 1867) where the benefits of nitroglycerine in angina pectoris were revealed. Their main action is vasorelaxation mediated by guanylyl cyclase activation and direct inhibition of non-specific cation channels in vascular smooth muscle cells (VSMC's). This class is limited by potentially adverse hemodynamic effects,

drug tolerance and lack of selectivity and limited bioavailability. Nevertheless, they are considered as main therapeutic agents for patients with ischemic heart diseases. Despite of its wide therapeutic use since centuries, the exact mechanism of action is not known (Feelisch, 1998). But, the majority of their biological effects are attributed to the formation for NO and thiols play a major role in their bio-activation. These agents require enzymatic or non-enzymatic bio-activation for the release of NO. It is suggested that multiple intracellular and extracellular pathways contribute to the formation of NO but the individual metabolic system is poorly understood (Feelisch, 1998). It is also suggested that the therapeutic effect of these drugs involves the release of NO from nitrite (Figure 5), activation of guanylyl cyclase and relaxation of blood vessels. The bio-activation pathways of both nitrites and nitrates are different. In the case of organic nitrates (e.g. nitroglycerine), the formation of NO occurs if thiols are present as a cofactor. In case of nitrites (e.g. amyl nitrite and sodium nitrite), the activation of NO occurs by the reduction of nitrite anion to vasoactive NO (Figure 4).

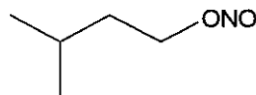


**Figure 4.** In vivo transformation of nitrites to vasoactive NO (Nossaman et al., 2010)

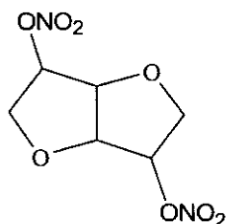
Nitrates are still widely used for the treatment of ischemic heart diseases but recent studies suggests that nitrites have a beneficial effect in protecting heart and other organs from cardiac conditions like ischemia (Nossaman et al., 2010).



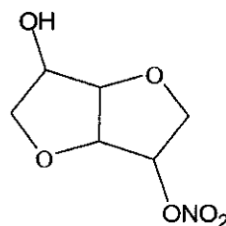
Nitroglycerin



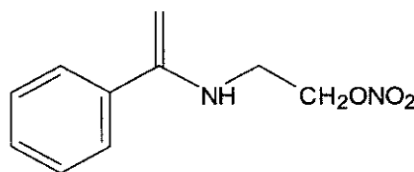
Amyl nitrite



Isosorbide dinitrate



Isosorbide mononitrate



Nicorandil

**Figure 5.** Structure of conventional organic nitrate and nitrite esters (Ignarro et al., 2002)

### Pharmacokinetics

The pharmacokinetic profiles of organic and inorganic nitrates are significantly different (Table 3). This difference was mentioned by Bodo in 1928; he noted that although both classes of

compounds have the same vasodilation effect but the magnitude and duration of effects were different (Bodo, 1928). Several organic nitrates/nitrites such as GTN and amyl nitrite have poor oral bioavailability as they undergo first pass metabolism (e.g. in GTN, 80-90% of drug is metabolized by liver). However, the high lipophilicity of organic nitrates provides them other routes of administration like sublingual and transdermal routes. Other organic nitrates such as ISMN, ISDN, and nicorandil have better bioavailability with less than 25% metabolism by the liver. They have rapid onset of action within 1-3 min after administration into the blood but are also rapidly cleared from plasma (15-30min), which gives them a rapid offset. Inorganic nitrates and nitrites are hydrophilic salts in nature and do not undergo first pass hepatic metabolism, therefore they can be readily administered orally (Omar et al., 2012).

Table 1. A comparison of pharmacokinetic properties of organic and inorganic nitrites/nitrates (Omar et al., 2012)

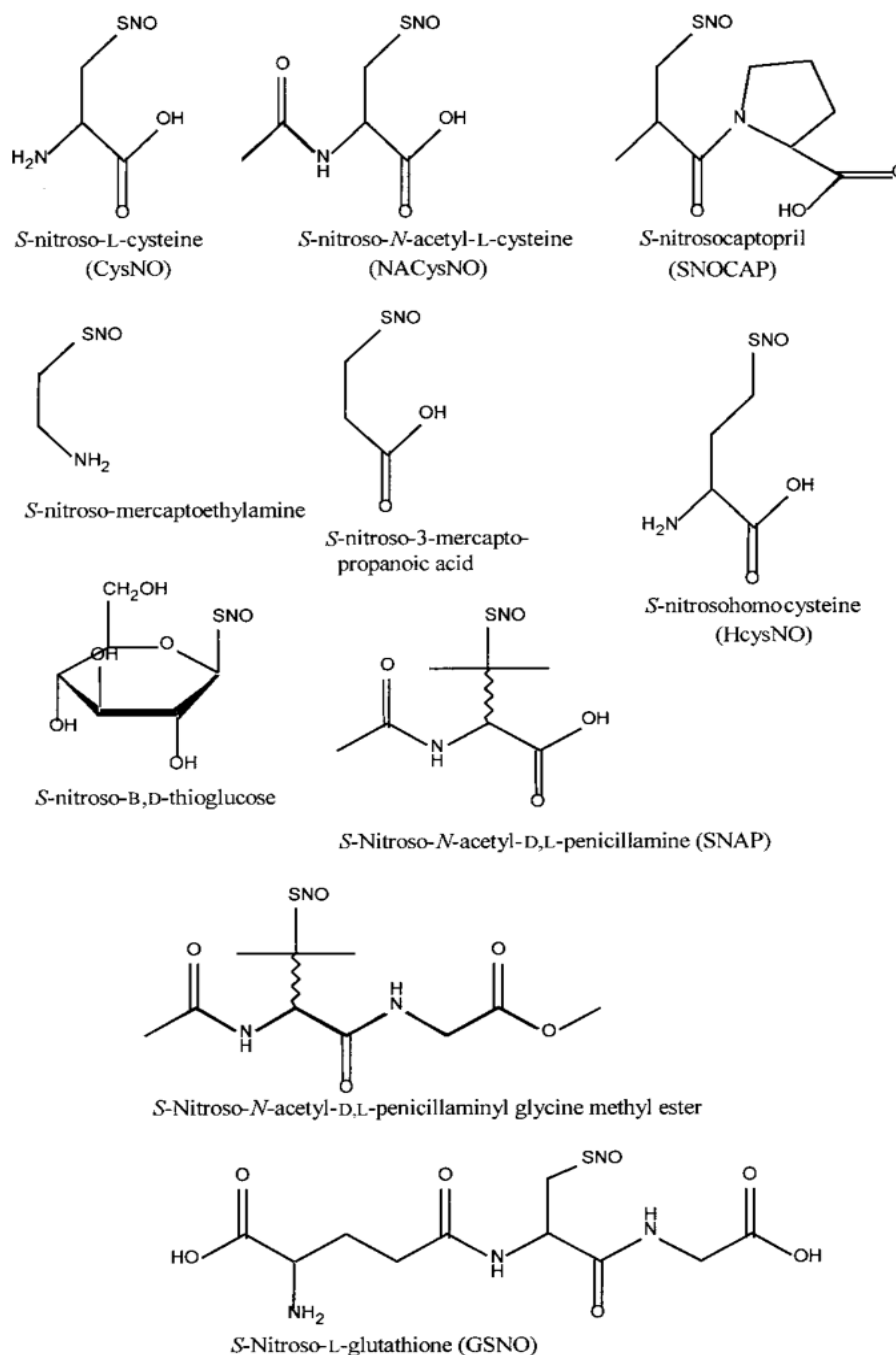
	Organic nitrates/nitrites	Inorganic nitrate/nitrite
Absorption	<ul style="list-style-type: none"> <li>• Readily absorbed via buccal, sublingual, trans-dermal, rectal, routes ; also via inhalation</li> <li>• Variable oral bioavailability due to variable rate of hepatic first pass metabolism</li> <li>• Organic nitrates with poor bioavailability tend to be more potent</li> </ul>	<ul style="list-style-type: none"> <li>• Readily absorbed across the upper GI tract ; also via inhalation</li> <li>• Do not undergo first pass metabolism</li> </ul>
Distribution	<ul style="list-style-type: none"> <li>• Widely distributed in the body via the circulation</li> <li>• Blood levels rise rapidly with onset of action within 1-3 min</li> </ul>	<ul style="list-style-type: none"> <li>• Widely distributed in the body via the circulation</li> <li>• 25% of absorbed nitrate is concentrated in the salivary glands</li> <li>• Following ingestion there is a sharp rise in salivary, plasma and urinary levels of nitrate and subsequently nitrite</li> <li>• Serum nitrite levels start to rise at about 1 h and peak at about 3 h</li> </ul>
Metabolism	<ul style="list-style-type: none"> <li>• Rapidly metabolised by various enzymes leading to rapid off-set within 15-30 min</li> <li>• Cytochrome P450 system: low potency nitrate and high doses of high potency nitrates</li> <li>• ALDH-2: low doses of high potency nitrates</li> <li>• Also: haemoglobin, xanthine oxidase, glutathione-S-transferase, and glutathione dependant reductase</li> </ul>	<ul style="list-style-type: none"> <li>• Via the nitrate-nitrite-NO pathway:</li> <li>• Enterosalivary circulation: nitrate reductase activity of bacteria on tongue generates nitrite:</li> <li>• Nitrite is metabolised to NO in the stomach and circulation</li> <li>• Circulation: haemoglobin, myoglobin, xanthine oxidase, eNOS, Aldehyde oxidase, ALDH2, carbonic anhydrase, ascorbate, polyphenols</li> </ul>
Excretion	<ul style="list-style-type: none"> <li>• Renal failure does not markedly influence kinetics of ISDN</li> </ul>	<ul style="list-style-type: none"> <li>• 65% of absorbed nitrate is excreted by the kidney</li> </ul>

### 2.3. S-nitrosothiols

S-nitrosothiols (Figure 6), is a class of NO-donors represented by general formula (RSNO), they are synthesized by the nitrosation of reduced thiols [(sulfhydryl's) (Megson and Webb, 2002)]. The cysteine residue of several proteins can also be nitrosated (Feelisch, 1998). They contain a

single chemical bond between the thiol group (R-SH) and the NO moiety (Miller and Megson, 2007). These are green or red solids with characteristic absorption peaks at 340 and 520 nm. Several endogenous S-nitrosothiols have been identified including S-nitrosoglutathione (GSNO), albumin and haemoglobin. It is generally believed that high molecular S-nitrosothiols (e.g. S-nitrosoalbumin and S-nitrosohaemoglobin) are present in higher concentrations in blood (i.e. 50 – 200 nM) while low molecular weight S-nitrosothiols (e.g. GSNO) are present in low concentrations in blood (>25 nM). Protein nitrosothiols are usually more stable as compared to S-nitroso- derivatives of low molecular weight thiols and small peptides. Both have been detected in biological fluids as a result of endogenous NO metabolism and exogenous administration of NO. S-nitrosothiols groups in proteins are considered to serve the metabolism of NO and cellular function regulation [(e.g. transportation and targeting NO groups to specific thiol-regulatory effector sites including enzymes and signaling proteins) (Megson and Webb, 2002)]. These are considered to have powerful antiplatelet (Salas et al., 1998) and vasodilator properties (de Belder et al., 1994). NO-donors like GSNO are able to achieve selective antiplatelet and potentially antithrombotic effects (de Belder et al., 1994). S-nitrosothiols have several potential advantages over the other NO-donors like tissue selectivity (e.g. GSNO is selective for arteries over veins, giving them a different haemodynamic profile of action than those of organic nitrates). Similarly, S-nitrosothiols are potent antiplatelet agents, and they inhibit aggregation at doses that do not influence vascular tone (de Belder et al., 1994; Miller and Megson, 2007). Stability of S-nitrosothiols varies in solutions as a function of temperature, pH, oxygen pressure, nucleophiles, redox-active species and trace metal content of the incubation medium [e.g. the half-life of Cys-NO can vary from seconds to hours at mild basic conditions pH 7.4 and depending upon the nature of buffer (Feelisch, 1998)]. It has been suggested recently that the hemolytic decomposition of S-nitrosothiols, which leads thiyl radicals (RS<sup>\*</sup>) and NO, is catalyzed by small amounts of ferrous (Fe<sup>2+</sup>) and cuprous (Cu<sup>2+</sup>) ions (McAninly et al., 1993). In a similar study it was suggested that the effect of thiols and other reductants on RSNO's can be explained by reduction of Cu<sup>2+</sup> (present in the water used to prepare the buffer solution) to Cu<sup>+</sup> (Gorren et al., 1996). Therefore, in order to achieve reproducible results with S-nitrosothiols, careful control of metal content of buffers is required. S-nitrosothiols are light sensitive but their stock solutions can be kept on ice for several hours in the dark. Metal chelating agents like EDTA (Ethylenediamine Tetra Acetic acid) and DTPA

(Diethylene triamine pentaacetic acid) can be added to increase the stability, if they were compatible with the assay (Feelisch, 1998).



**Figure 6.** Some synthetic and naturally occurring *S*-nitrosothiols (Al-Sa'doni and Ferro, 2000)



## Pharmacokinetics and stability

A very few studies are available on the pharmacokinetics and the stability of S-nitrosothiols. In a study presented by Katsumi et al 2004 (Katsumi et al., 2004); the pharmacokinetic parameters of a newly synthesized S-nitrosothiol (NO-BSA, NO-bovine serum albumin) were evaluated after an intravenous injection in mice at a dose of 1 mg/kg body weight. It was observed that the NO-BSA slowly disappeared from plasma but showed greater accumulation in liver and kidney during the first hour after injection.

In a similar study by Katsumi et al 2005 (Katsumi et al., 2005); pharmacokinetic parameters of PEG-poly-SATA-BSA [a newly synthesized S-nitrosothiol by conjugating SATA (*N*-Succinimidyl-S-acetylthioacetate) with polyethylene glycol and Bovine serum albumin] including AUC, total body clearance and volume of distribution were calculated after an intravenous injection in mice at a dose of 1 mg/kg body weight (Table 4).

Table 2. Pharmacokinetic parameters of BSA and PEG-poly-SATA-BSA after intravenous injection in mice at a dose of 1 mg/kg (Katsumi et al., 2005)

	AUC	CL <sub>total</sub>	V <sub>d</sub>
	% of dose h / ml	μl / h	ml
BSA	473	211	1.5
PEG-poly SATA-BSA	824	121	1.2

A variety of protein nitrosothiols and S-nitroso- derivatives of low molecular weight thiols on small peptides have been synthesized but protein nitrosothiols are usually more stable than S-nitroso- derivatives. Only a small number of RSNO's have been isolated in solid form including S-nitroso-*N*-acetyl-DL-penicillamine (SNAP), S-nitrosoglutathione (GSNO) and S-nitrosoalbumin.. As a solid form, SNAP can be stored for years at room temperature but provided it is kept dry and protected from light. GSNO can be stored in dark at -20 °C for at least 6 months while being desiccated while the more unstable nitrosothiols must be prepared shortly before use. Its stability in solution also depends on temperature, pH, oxygen pressure, nucleophiles, redox active species and trace metal content in the incubation medium (Feelisch, 1998). Freez-drying

can also be used to obtain S-nitrosothiols in dry form but no specific literature was found on freeze-dried S-nitrosothiols.

### **3. Polymeric carriers for NO**

A polymeric carrier is interesting for the delivery of NO to its specific site of action. Because of the short half-life of NO and the side effects of several NO-donors, there is a need to develop polymeric delivery systems which could deliver NO to the site of action for a long period of time and avoiding harsh GIT conditions by inclusion in polymers.

In this thesis, we used thiolated polymers also known as thiomers because they have been investigated for therapeutic purposes. Since, thiolated polymers have a free thiol group, NO could be linked and can be delivered for its specific site of action. Thiomers are meant to be used for mucoadhesion purposes because the free thiol group of thiomers can link with the glycoprotein of the mucous in GIT. This occurs via disulfide bonds but we can also utilize this free thiol group to link NO and can be the final compound to be used as a NO carrier. We also used non-biodegradable polymers (Eudragit® FS 30D) for direct encapsulation of NO-donor (GSNO) for specific intestinal delivery.

#### **3.1. Micro/nanoparticles**

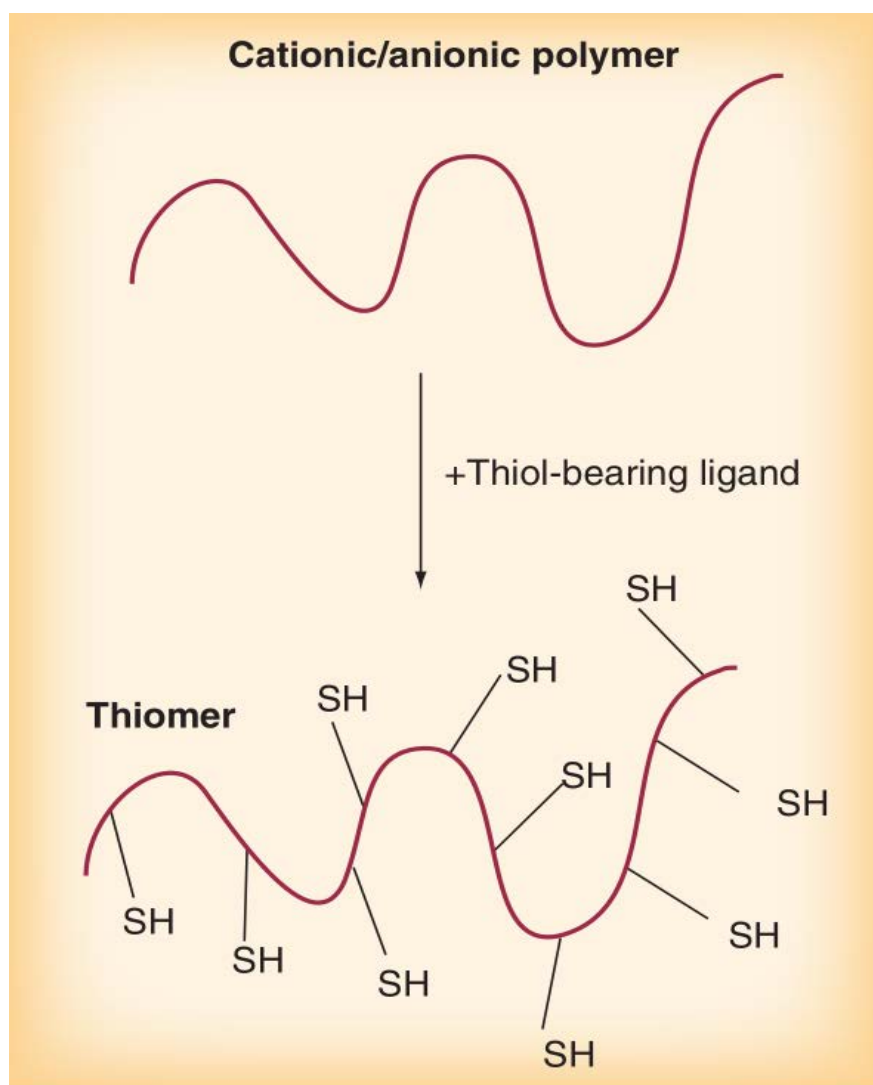
Pharmaceutical companies widely used drug delivery technologies to expand market for already established products. It has been realized in the past two decades that nanotechnology is a fundamental part of drug development, which results in designing a wide range of drug delivery systems. It also progressively increase the number of commercially available nanotechnology based drugs which can reduce side effects, facilitate drug administration, improve patient compliance, decrease drug toxicity, enhance bioavailability of drugs and can be used as site specific therapeutic targeted delivery systems (Saraiva et al., 2011). Nanoparticle drug delivery systems has become a widely studies strategy for increasing solubility and tissue specificity (Polizzi et al., 2007). Furthermore, incorporation of drug in nanoparticulate system can reduce renal and hepatic clearance thus optimizing the drug's pharmacokinetics properties and biodistribution (Orive et al., 2005). In biomedical applications, the encapsulation of drug in

nanoparticles is a significant subject because it can increase the drug stability and solubility. Furthermore, it prolongs the drug release rates and decrease toxicity. A variety of nanostructures are used for these purposes including polymeric nanoparticles, solid-lipid nanoparticles and liposomes etc.; that's why, polymeric nanoparticles appears as an interesting carrier for NO-donors (D Marcato et al., 2013b). Hence, nano-carriers may facilitate the development of multifunctional systems for targeted drug delivery of therapeutic outcome (Bae et al., 2005).

Recent studies showed extensive investigations on the use of polymeric nanoparticles. It is suggested that polymeric particulate systems can modulate drug activity by passive or active targeting to different tissues. Biodegradable polymers can degrade into non-toxic monomers inside the body, which are usually highly stable in biological fluids as well as during preparation and storage (Kanayama et al., 2010; Saraiva et al., 2011). Some reports have been published on the polymeric delivery of NO (Marquele-Oliveira et al., 2010). The therapeutic effects of NO prodrugs can be enhanced by incorporation into nanoparticles composed of polystyrene-b-PEG (Jain et al., 1998). In another study, NO was efficiently delivered to the vaginal mucosa by using PLGA, which results in improved blood perfusion and having an implication on female sexual dysfunction (Yoo et al., 2010).

### **3.2. Thiomers**

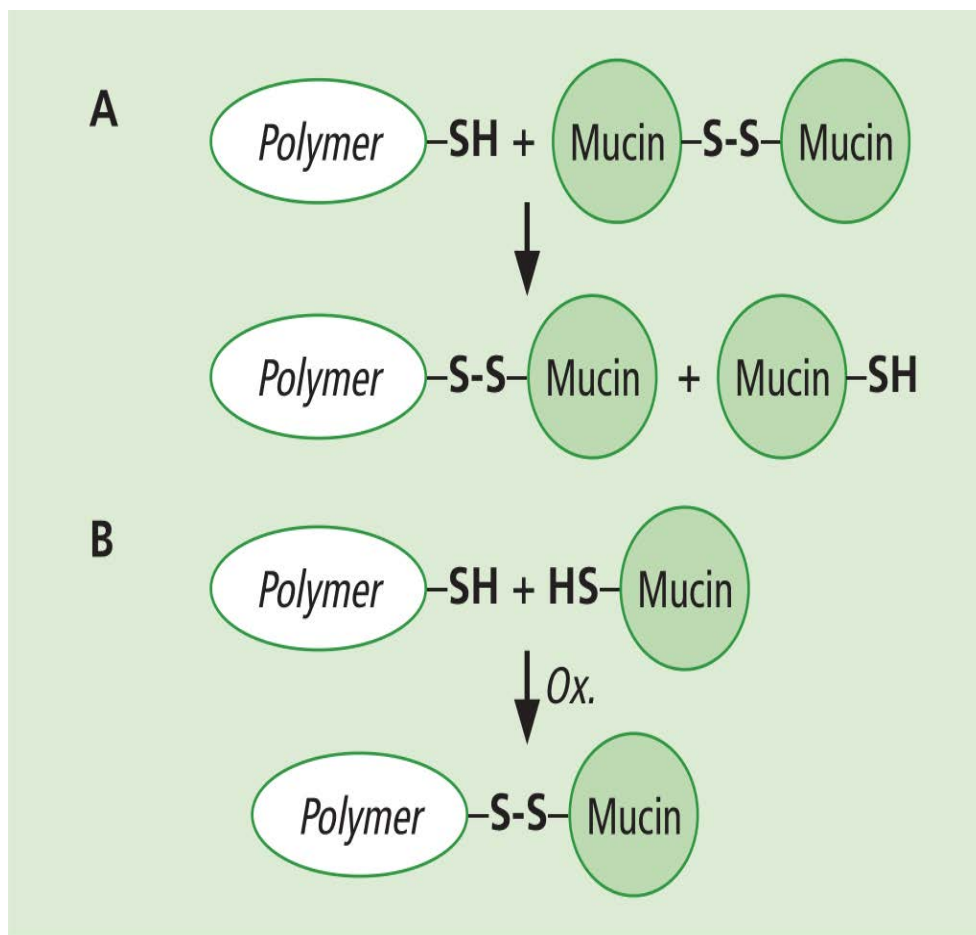
Thiomers (thiolated polymers with an –SH group) are the polymers modified for mucoadhesive or additive properties by incorporating thiol groups (-SH) in the backbone. The reaction (carbodiimide) can be done by substitution or simple oxidation reactions (Figure 7). Thiomers were mainly proposed for poorly soluble drugs with decreased permeability. These drugs could be complexed with thiomers for increased absorption through the mucosal membrane because of their increased contact time and prolonged stay in body due to mucoadhesion [e.g. peptide delivery by coupling cysteine with alginate ((Bernkop-Schnurch et al., 2001))] (Hanif et al., 2015). Thiol groups increase the mucoadhesion properties of the modified polymer (Hanif et al., 2015). Thiomers are believed to interact with cysteine-rich subdomain of mucous glycoproteins leading to the formation of disulfide bonds between the mucous layer and the mucoadhesive polymer (Bernkop-Schnurch et al., 2001).



**Figure 7.** Thiolated polymer or thiomers (Laffleur and Bernkop-Schnürch, 2012)

The mucous layer covering the gastro intestinal epithelia consists of mucous glycoproteins. These glycoproteins consist of a central region rich in O-linked oligosaccharides chains and two flanking cysteine rich subdomains on both sides. The cysteine rich subdomains consisted of 10% cysteine in their primary structure, which are involved in linking mucin monomers into oligomers *via* disulfide bonds and hence building a three dimensional network of mucous gel layer. The mucolytic activity of thiols (e.g. *N*-acetyl-cysteine) is based on disulfide exchange reactions between the mucolytic agent and mucin glycoproteins in mucous. Due to these exchange reactions, intermolecular as well as intramolecular disulfide bridges within the glycoprotein structure is cleaved, which leads to the breakdown of mucous. It was observed

that the mucolytic agent was covalently bound to mucin glycoproteins in the mucous, so other thiol carrying groups or thiomers (Figure 8) should also be covalently linked to the mucous (Bernkop-Schnürch et al., 1999).



**Figure 8.** Formation of covalent bonds between thiomers and mucin glycoproteins of GIT, A) *via* thiol/disulfide exchange reaction, B) *via* an oxidation process (Kafedjiiski and Franzens, 2004)

Thiomers controlled the delivery of active pharmaceutical ingredients in the body. A number of polymers are modified into thiomers by thiolation including chitosan, sodium alginate, polyacrylic acid, sodium carboxymethyl cellulose and guar gum etc. Thiomers can be prepared by using different analytical methods like covalent crosslinking, *in situ* gelation, radical emulsion polymerization and emulsification (Hanif et al., 2015).

In a study reported by Bernkop Schnurch et al (Bernkop-Schnurch and Thaler, 2000), polycarbophil was covalently attached to cysteine moieties. The reaction was mediated by a

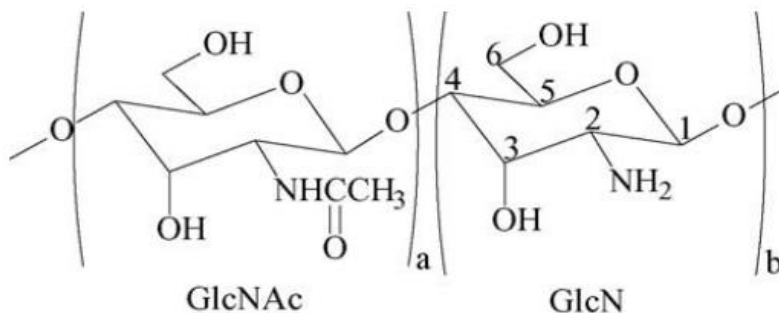
carbodiimide and results in the formation of amide bonds between the primary amino group of the amino acid and the carboxylic acid moieties of the polymer. The conjugate displayed 35 to 50  $\mu\text{M}$  sulfhydryl groups per gram of polymer. This new polymer with thiol groups represents a new generation of thiomers. In a similar study, polycarbophil was attached to cysteine by the formation of amide bonds between the primary amino group of the amino acid and carboxylic acid group of the polymer and the amount of covalently attached cysteine (and the formation of disulfide bonds within the modified polymer) was determined by quantifying the share thiol groups on the polymer conjugates. Polymer and cysteine in a ratio of 1:4 displayed  $142.2 \pm 38.0$   $\mu\text{mol/g}$  of thiol groups immobilized on polymer back bone (Bernkop-Schnürch et al., 1999).

We used two polymers in this thesis (i.e. chitosan and sodium alginate) to be modified into thiomers by linking with GSH. The detailed properties of chitosan and sodium alginate are given below.

### **3.2.1. Chitosan**

Naturally occurring polymers especially polysaccharides like chitosan and alginates have been widely studied as therapeutic carriers for protein molecules etc. Chitosan and its derivatives have permeation enhancing effects, enzyme inhibitory effects and mucoadhesive effects which makes it a good candidate for oral peptide delivery systems (Elgindy et al., 2011). Chitosan is a cationic polymer and has gained a particular interest recently. Naturally, chitosan is a biopolymer and is commercially manufactured on large scale by alkaline N-deacetylation of chitin, an abundant biopolymer isolated from the exoskeleton of crustaceans such as crabs and shrimps (Rampino et al., 2013). Chitin and chitosan are closely related since both are linear polysaccharides containing 2-acetoamido-2-deoxy-glucopyranose (GlcNAc) and 2-amino-2deoxy-D-glucopyranose (GlcN) joined by  $\beta$  (1, 4) glycosidic bonds but can be distinguished by the content of the mentioned units as given in (Figure 9). The GlcNAc proportion in relation to the GlcN units is defined as the degree of n-acetylation (DA) of the biopolymer and is an important parameter which not only provides its physical and chemical properties but also its biological, biomedical and food applications. All of these applications are strongly dependant on physical and chemical properties of chitin/chitosan such as solubility, which strongly depends

on the DA. Therefore, accurate determination of the DA is very important in order to define the application of such type of materials (Guinesi and Cavalheiro, 2006).



**Figure 9.** Chemical structure of (a) 2-acetoamido-2-deoxy-glucopyranose (GlcNAc) and (b) 2-amino-2deoxy-D-glucopyranose (GlcN) joined by  $\beta(1,4)$  glycosidic bonds (Guinesi and Cavalheiro, 2006)

Due to the amino groups present in the chemical structure of chitosan, it can be dissolved in diluted aqueous-acid solutions (El-Hefian et al., 2010). It has a unique polymeric cationic character as well as gel and film forming properties and is an excellent biodegradable and biocompatible polymer. It has been extensively examined in pharmaceutical industries for the development of drug delivery systems. It has been studied widely for its applications in wound healing, drug delivery systems, coatings, tissue engineering and its applications in food, cosmetics and agricultural industries. It has gained increased importance in pharmaceutical field because of its good biocompatibility, low toxicity and biodegradability. Its degradation products are non-toxic, non-immunogenic and non-carcinogenic (Ibezim et al., 2011). It can enhance the absorption of hydrophilic molecules by stimulating structural reorganization of the tight junction-associated proteins. Chitosan derived thiolated chitosan were shown to further improve the mucoadhesive properties due to the formation of disulfide bonds with cysteine-rich domains of mucous glycoproteins (Atyabi et al., 2008).

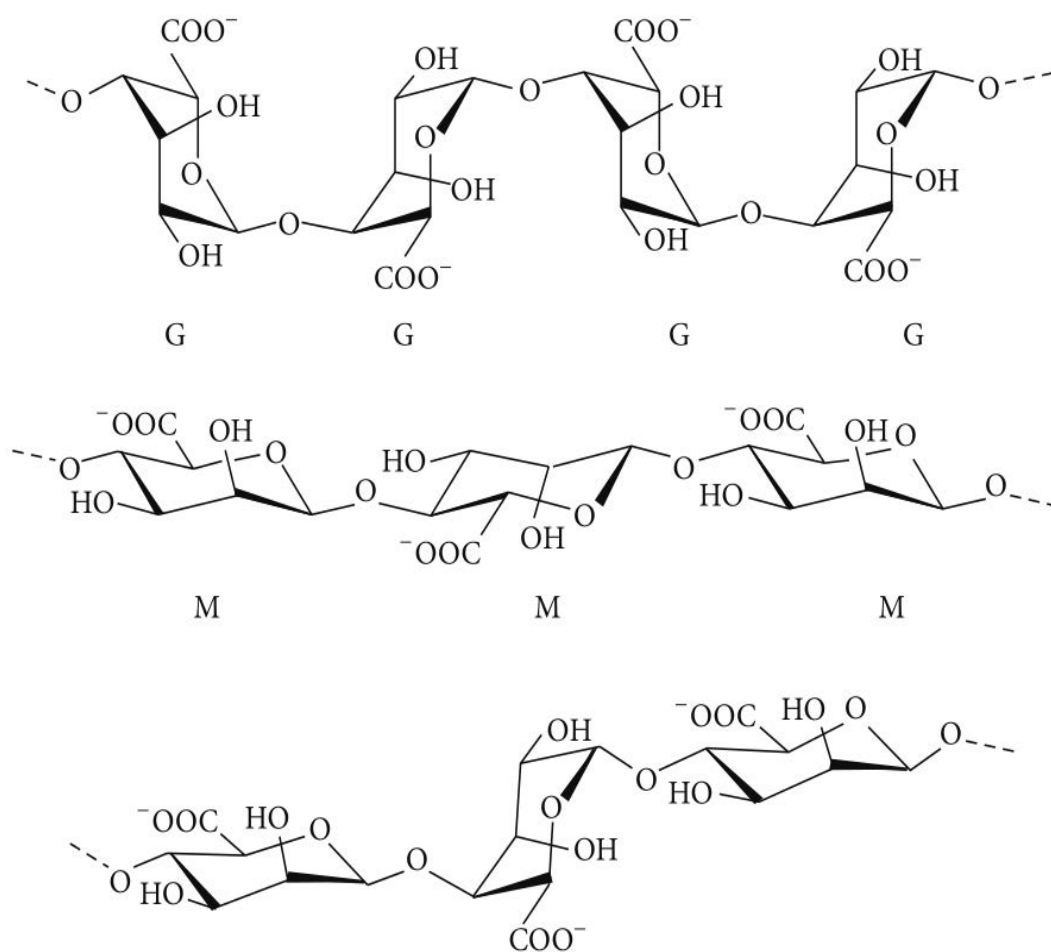
In a study performed by Kafedjiiski et al, (Kafedjiiski et al., 2005), a new generation of thiomers was synthesized by directly immobilization of GSH on chitosan with improve permeation enhancing properties. While focusing on the structure of GSH, the presence of free thiol group in the tripeptide structure and high negative redox potential, the new chitosan-GSH conjugate might exhibit higher permeation enhancing properties among the rest of thiomers. The novel conjugate was toxicologically harmless and because of higher molecular weight it





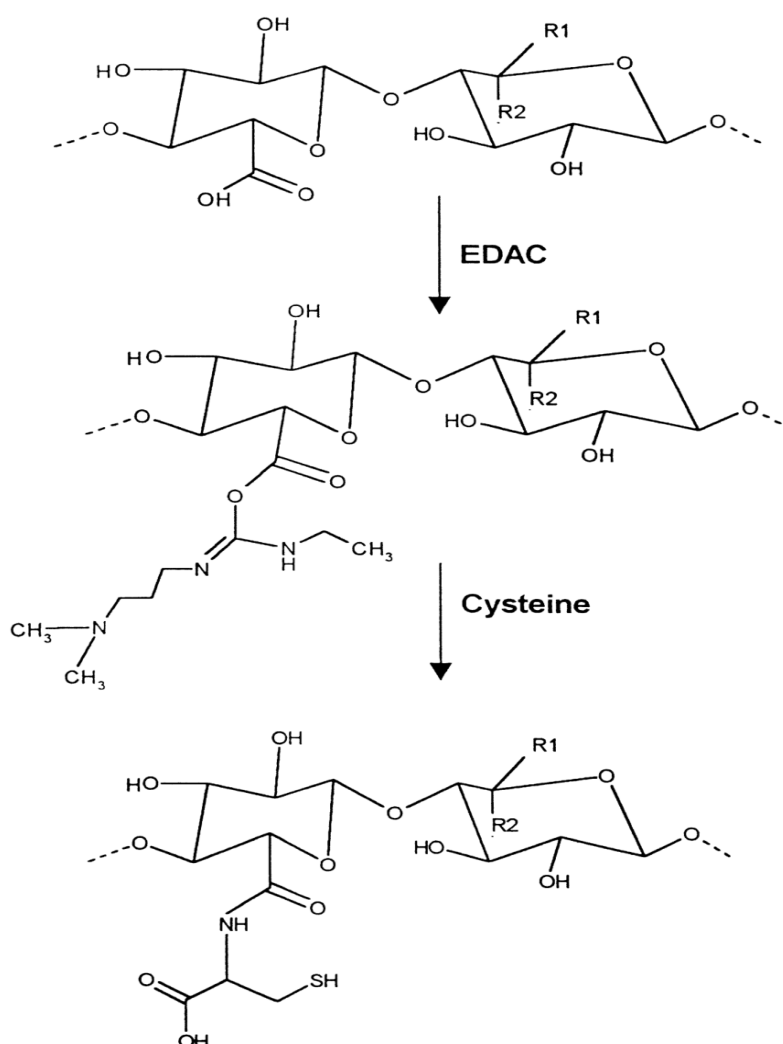
blocks made of random or alternating units of mannuronic and guluronic acids (Tonnesen and Karlsen, 2002).

Alginic acid, sodium and potassium alginates (ALG) are among the most extensively explored mucoadhesive biomaterials having good cytocompatibility, biocompatibility, biodegradation and chemical versatility, which make them suitable for further modifications to its properties (Sosnik, 2014). The molecular weights are between 32 and 400 kg/mol and it can have different chain arrangements compositions based on the source of extraction and the age of the algae. This results in the commercialization of over 200 alginate types. The rheological and drug delivery performance of alginates are also related to the molecular weight, concentration and pH of the medium. Alginates are widely used in pharmaceuticals, cosmetic and food industries. Since alginates are extracted from natural sources, a variety of different impurities may be present including heavy metals, proteins and endotoxins. Ultrapure grades have a controllable level of pyrogenicity (Sosnik, 2014). Although its biocompatibility has been evaluated extensively both *in vivo* as well as *in vitro*, there is still a debate on the impact of alginate composition and this confusion mainly relates to the varying levels of purity in the alginate studies by different scholars (Lee and Mooney, 2012). It was reported that alginates with high M content were immunogenic and approximately 10 times more potent in inducing cytokine production compared to G alginates (Otterlei et al., 1991). The immunogenic response at the site of injection or implantation are attributed to the impurities remaining in the alginate (Lee and Mooney, 2012), however, the alginates purified by a multi-step extraction procedure to a very high purity did not induce any significant foreign body reaction when they were implanted into animals (Orive et al., 2002).



**Figure 11.** Alginate block types: G = guluronic acid, M = mannuronic acid (Tonnesen and Karlsen, 2002)

In a study reported by Bernkop Schnurch et al, a mucoadhesive thiolated polymer was synthesized and characterized by linking alginate to cysteine (Figure 12). Alginate was chosen because of its good mucoadhesive properties and its wide use as excipient in drug delivery systems. Since, alginate displays carboxylic acid groups; the sulfhydryl compound cysteine was easily covalently attached. The resulting conjugate was having improved mucoadhesive properties. It was observed to have high binding capacity for mucin glycoproteins by the formation of covalent bonds. The resultant polymer-cysteine conjugate displayed  $340.4 \pm 74.9$   $\mu\text{mol}$  of thiol groups per gram of polymer. This study showed that the thiolated alginate is a useful excipient for various drug delivery systems providing an improved stability and prolonged residence time (Bernkop-Schnurch et al., 2001).



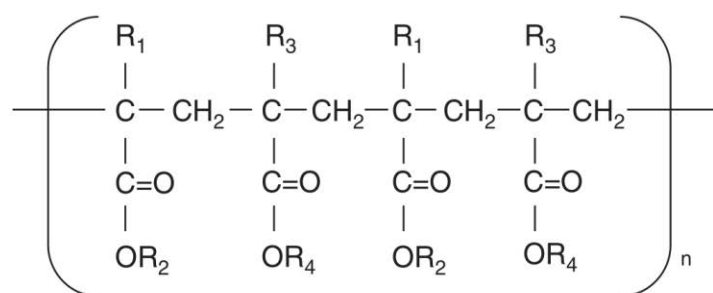
**Figure 12.** Synthetic scheme for the generation of alginate-cysteine conjugate (Bernkop-Schnurch et al., 2001)

We thought that the free thiol groups in alginate-cysteine conjugate could be nitrosated to synthesize of S-nitroso-cysteine-alginate, which could be used as a carrier for NO for local as well as systemic delivery.

### 3.3. Non-biodegradable polymers : Eudragit®

The polymers that are stable in biological system are known as non-biodegradable polymers. These are used as drug carriers and include polymethylmethacrylate, polysilicones and ethylene-vinyl acetate copolymers (Galaev and Mattiasson, 2007).

Eudragit® is the brand name for a variety of copolymers based on polymethacrylates marketed by Evonik Industries, Germany. It was first introduced by Rohm & Hass GmbH, Darmstadt in 1953 as an alkaline soluble material for drug coating purposes resistant to strong stomach acids. Later on, this brand diversified gradually to anionic, cationic as well as neutral copolymers based on methacrylic acid and methacrylic or acrylic esters or their derivatives in varying ratios. These polymers exhibited varying degree of pH dependant and/or independent solubility's. Eudragit® polymers are extensively used for pharmaceutical coatings, matrix formers in common granulation techniques and in direct compression (Thakral et al., 2013). Different types of Eudragit® are commercially available and could be obtained as dry powder, an aqueous dispersion or as an organic solution (Kola and Kumar). These copolymers acts as polyelectrolytes and are suitable for soluble gastro-intestinal drugs delivery as well insoluble but swellable delivery systems (Animesh et al., 2012).

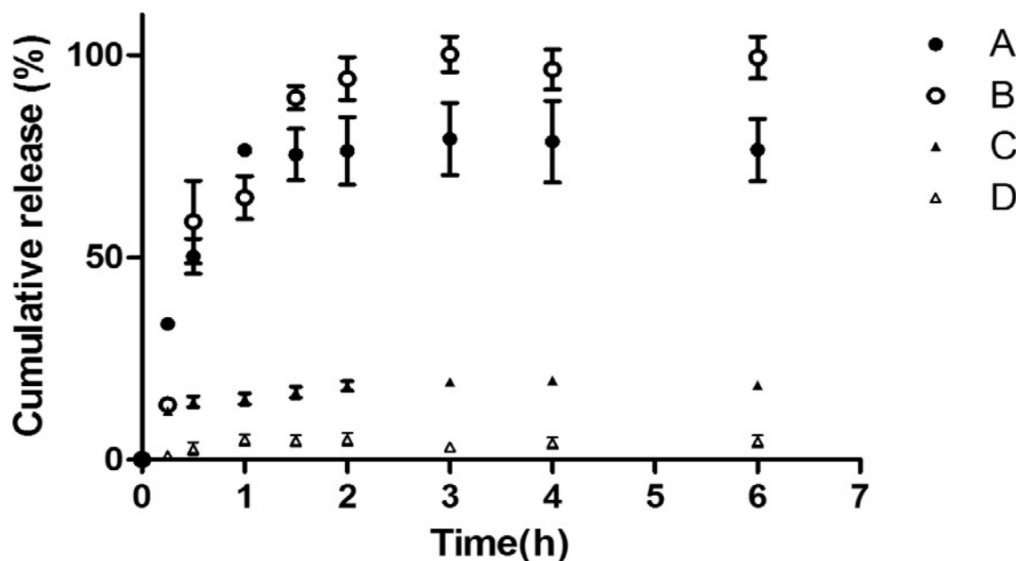


Eudragit Grade	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>
E	CH <sub>3</sub>	CH <sub>2</sub> CH <sub>2</sub> N(CH <sub>3</sub> ) <sub>2</sub>	CH <sub>3</sub>	CH <sub>3</sub> , C <sub>4</sub> H <sub>9</sub>
L and S	CH <sub>3</sub>	H	CH <sub>3</sub>	CH <sub>3</sub>
RL and RS	H, CH <sub>3</sub>	CH <sub>3</sub> , C <sub>2</sub> H <sub>5</sub>	CH <sub>3</sub>	CH <sub>2</sub> CH <sub>2</sub> N(CH <sub>3</sub> ) <sub>3</sub> <sup>+</sup> Cl <sup>-</sup>
NE 30D	H, CH <sub>3</sub>	CH <sub>3</sub> , C <sub>2</sub> H <sub>5</sub>	H, CH <sub>3</sub>	CH <sub>3</sub> , C <sub>2</sub> H <sub>5</sub>
L 30 D-55 and L 100-55	H, CH <sub>3</sub>	H	H, CH <sub>3</sub>	CH <sub>3</sub> , C <sub>2</sub> H <sub>5</sub>

**Figure 13.** Basic chemical structure of Eudragit and different grades (Thakral et al., 2013)

A number of studies are about Eudragit® polymers for NO delivery. In a study by Wen et al, (Wu et al., 2015), GSNO was successfully encapsulated by Eudragit® RL polymer by a double emulsion method. The positively charged polymeric nanoparticles were having 54% of satisfactory encapsulation. From these nanoparticles, 100% of GSNO was released within 3 h

while after release the GSNO was stable up to 6 h in phosphate buffer pH 7.4, while the temperature was 37 °C (Figure 14).



**Figure 14.** Release kinetics of GSNO-NP in phosphate buffer pH 7.4 at 37 °C. GSNO release from free GSNO (A) or from GSNO-NP (B); Nitrite ions released from free GSNO (C) or from GSNO-NP (D). Mean  $\pm$  SD, n = 3 (Wu et al., 2015)

In another study by Ramia Safar et al, (Safar et al., 2015), GSNO was encapsulated in Eudragit® RL polymer by double emulsion/solvent evaporation method. The positively charged particles were having 49 % encapsulation efficiency. These particles were checked for cytotoxicity tests by WST-1 test and were observed that the particles were not toxic.

### **Eudragit® FS 30D**

Eudragit® FS 30D is a copolymer of methyl acrylate, methacrylic acid and methacrylate ester and is soluble at pH above 7.0. It is a commonly used pH responsive polymer for drug delivery systems to facilitate the drug release to ileo-colonic region. Eudragit® FS 30D is an anionic copolymer available as 30% aqueous dispersion in which the ratio of free carboxyl groups to the ester groups is 1:10 and its average molecular weight is approximately 220,000 (Dvorackova et al., 2011). It's a milky white liquid of low viscosity with a faint characteristic odor. It is a very flexible polymer with a glass transition temperature of 48 °C (EvonikIndustries). Some studies

suggest the use of Eudragit® FS 30D as a coating material but to our knowledge it is not used for direct encapsulation of drugs. In a study (Dvorackova et al., 2011), hard capsules of gelatin were coated with hydroxypropyl cellulose, hypromellose and Eudragit® FS 30D and were evaluated for ileo-colonic delivery. The drug release was tested using a paddle dissolution method with continual pH change (i.e. pH 1.2- 2h, 6.8- 4h and 7.5- 2h). The release of drug after the pH changed to 7.5 suggested that Eudragit® FS 30D could be used as a coating material for desired colon delivery. In another study (Cole et al., 2002), HPMC capsules containing paracetamol was separately coated with two polymers, Eudragit® L 30 D-55 and Eudragit® FS 30D. It was demonstrated that the capsules coated with Eudragit® L 30 D-55 were gastro resistant for 2 h at pH 1.2 and the capsules coated with Eudragit® FS 30D were resistant for a further 1h at pH 6.8. for the Eudragit® FS 30D polymer, the complete disintegration did not occur until the distilled small intestine and proximal colon in an average time of 6.9h post dose.

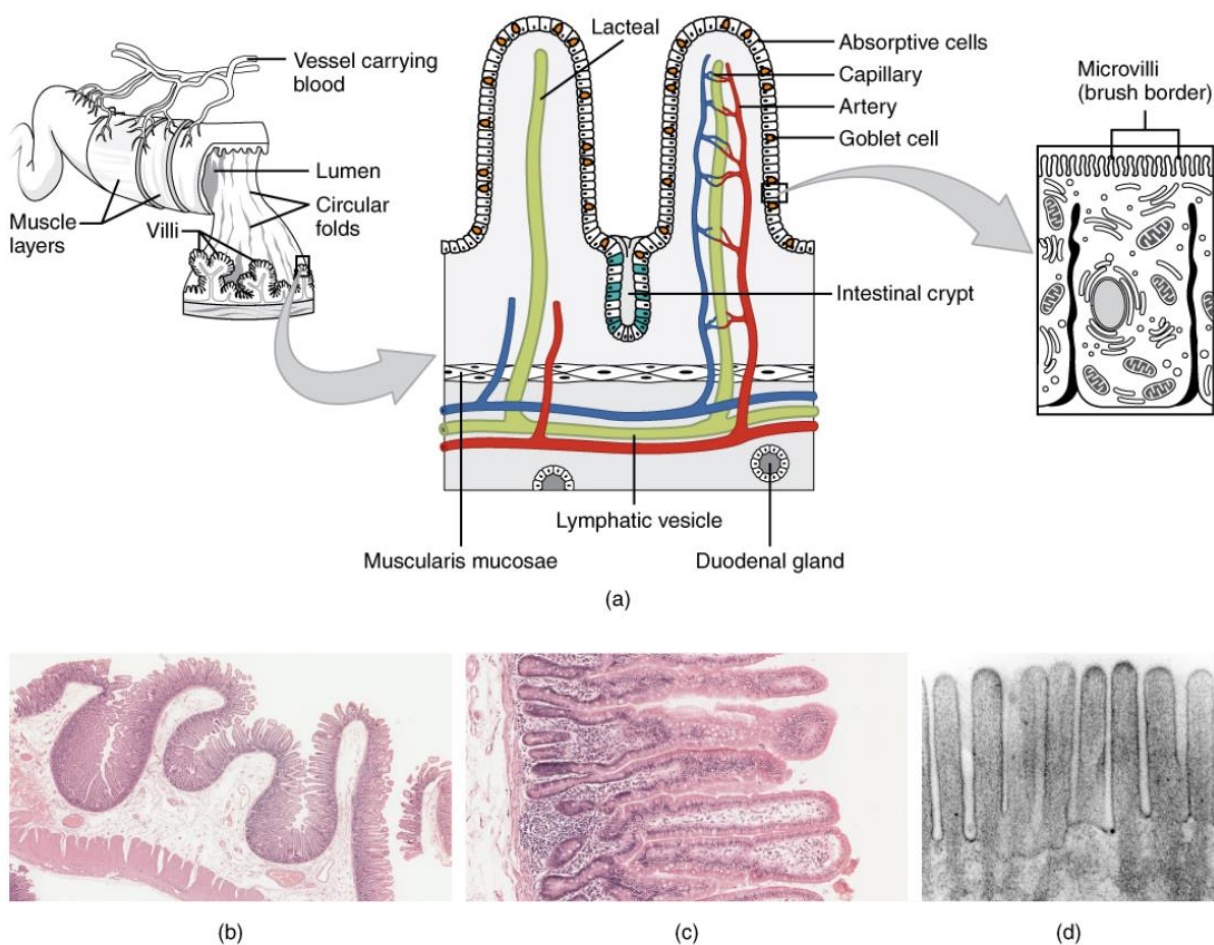
#### **4. Intestine and management of Crohn's disease**

The small and large intestines occupies most of the abdominal cavity. They are responsible for digestion, secretion, absorption, excretion and physical and immunological defense system. The large mucosal surface is adapted for the absorption of nutrients and electrolytes, and provides massive interface for possible antigenic interactions with the environment (Kishore and Chong, 2012).

##### **4.1. Small intestine**

Small intestine in humans begins at the pyloric sphincter and coils its way through the lower part of the abdominal cavity and joins the large intestine at the ileocaecal valve (Tortora and Derrickson, 2011). Its average length is 250 and 300 cm in newborns while increases to 600-800 cm in adults (Kishore and Chong, 2012). It is divided into three parts: the duodenum, jejunum and ileum. It has both sympathetic and parasympathetic nerve supply. The approximate length is 6.5 m with a diameter of 2.5 cm. Small intestine wall is consisted of 4 layers like other gastrointestinal (GI) tract parts (i.e. adventitia, muscularis, submucosa and mucosa); however, both the mucosal and submucosal layers are specific. The mucosal layer of intestine consists of

different glands known as intestinal glands, which are lined with glandular epithelium and secrete intestinal juices. Some of the glands secrete mucous, which is alkaline in nature and protect small intestine walls from acids and enzymes. The intestine throughout its length consists of columnar epithelium, which is having absorptive and goblet cells. The absorptive cells contain projections described as 'figure like' known as microvilli. There are approximately 20 to 40 villi per square millimeter of 0.5 to 1mm long. These microvilli are composed of columnar epithelial cells, which contain more tiny villi. It encloses a bunch of blood and lymphatic capillaries where the transportation and absorption occurs. The small intestine is having circular folds each about 10 mm in height and assists digestive and absorptive process when the chime passes through it (McGrath et al., 2005; Tortora and Derrickson, 2011).

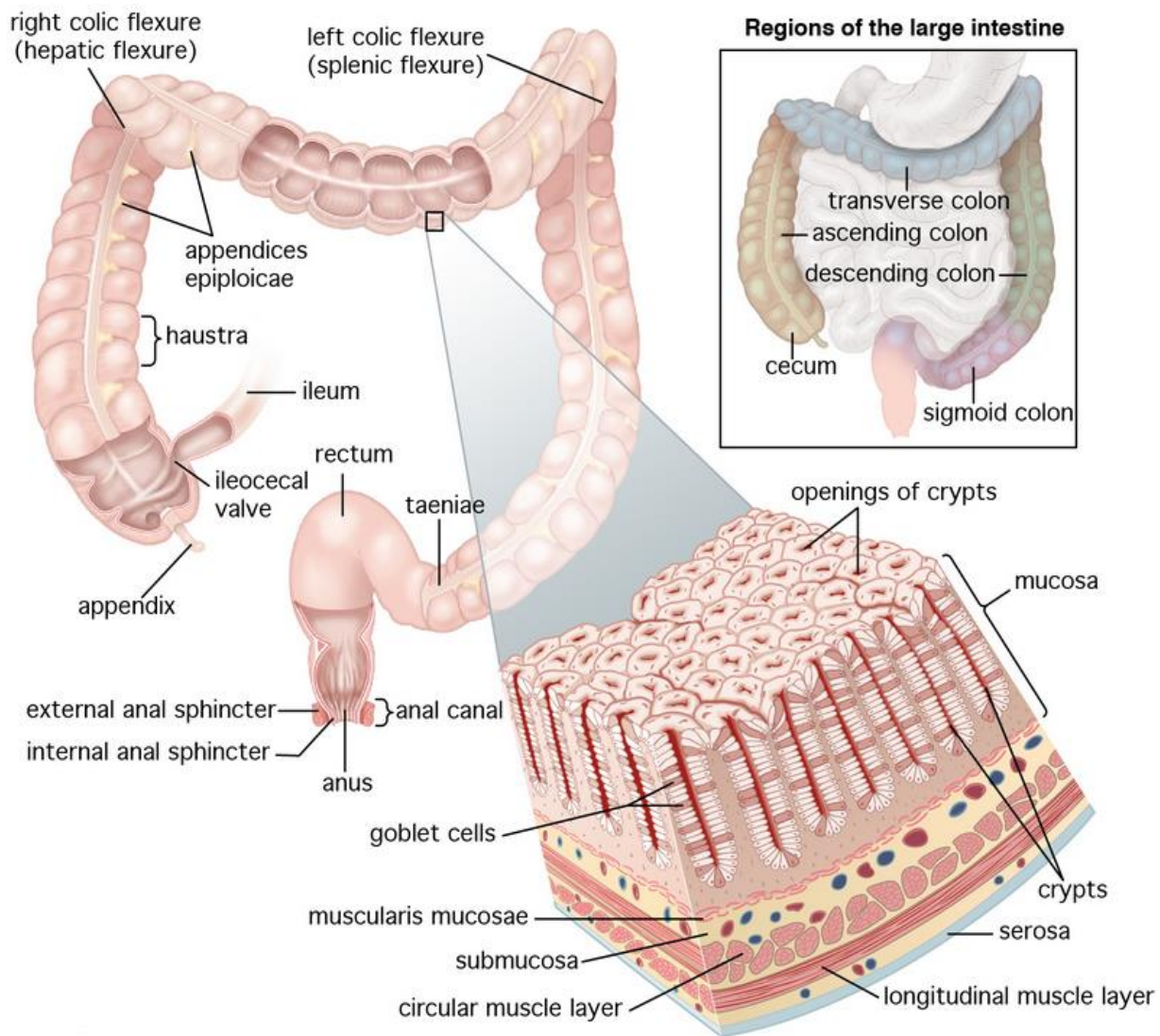


**Figure 15.** a) Absorptive part of the small intestine b) micrograph of circular folds c) electron micrograph of villi d) electron micrograph of microvilli (RiceUniversity)

## **4.2. Large intestine**

The large intestine also known as colon has the ability to distend. It surrounds the small intestine from three sides while leaving its inferior area open to pelvis. It is approximately 1.5 m long and extends from the ileum to the anus. The ascending colon starting from the caecum is approximately 7 cm in diameter and decreases gradually until it reaches a diameter of 2.5 cm near sigmoid portion. The large intestine is composed of 4 segments; caecum, colon, rectum and anal canal. It is divided into 4 parts; ascending colon (15 cm long), transverse colon (45 cm long), descending colon (25 cm long) and sigmoid colon (36 cm long). The large intestine consists of a variety of bacteria, which are known as beneficial bacteria causing no harmful effects but plays an important role in the digestion process. Their main activities are fermentation of carbohydrates and release hydrogen, carbon dioxide and methane gas. They also synthesize a variety of vitamins such as Vit K and B. the main blood supplies to the large intestine is through superior and inferior mesenteric arteries. The nerve supply is through sympathetic and parasympathetic nerves. The external anal sphincter is under voluntary control and received nerves from the spinal cord (McGrath et al., 2005; Norris and Siegfried, 2011).



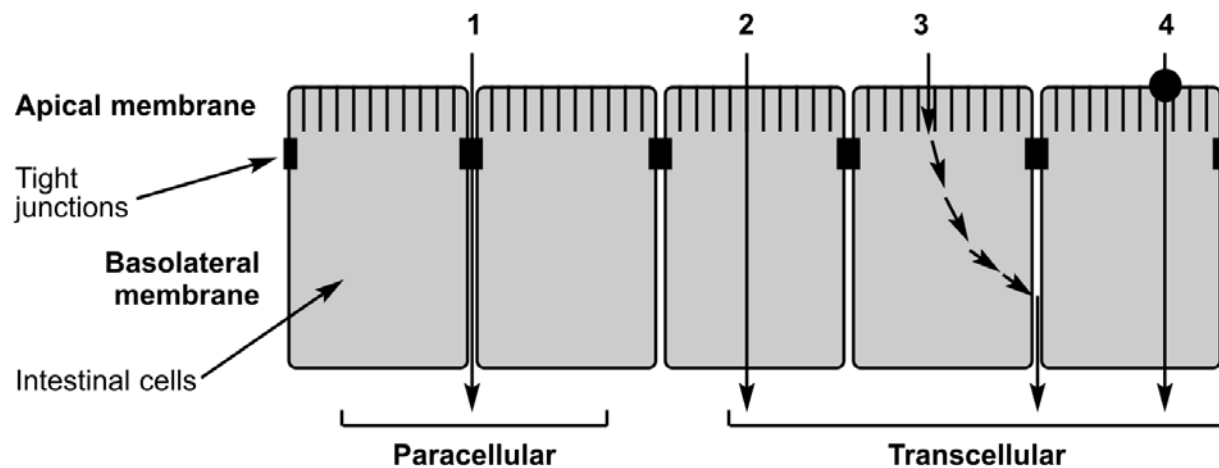


**Figure 16.** Anatomy of large intestine (Britannica, 2015)

#### 4.3. Intestinal barriers to the absorption

The alimentary canal from mouth to anus is covered with a mucous membrane called as mucosa, which serves as a first barrier for materials to enter into the body. The mucosa is composed of a thin epithelial layer having loose connective tissues (*lamina propria*) that contains blood and lymphatic capillaries. Absorption of a substance from lumen of the elementary canal occurs when the substance passes through the epithelium, a part of *lamina propria* and the walls of blood and lymph capillaries. Substances can also pass through the junctions between epithelial cells. Moreover, the absorption of orally ingested materials takes

place mainly in the small and large intestines (DeSesso and Jacobson, 2001). However, to form an intact epithelial barrier, the paracellular space should also be sealed. This paracellular seal is provided by the tight junction. Tight junctions cannot be seen well by light microscopes but they can be localized on hematoxylin and eosin-stained slides. However, transmission electron microscopy shows the tight junctions to be discrete rejoin of membrane apposition between adjacent epithelial cells at the luminal aspect of the apical junction aspect. The adjacent plasma membrane appears to fuse at this site which leads to the initial misinterpretation of the light junction as impermeable membrane (Clayburgh et al., 2004).



**Figure 17.** Routes and mechanism of transport of molecules across the intestinal epithelium (Le Ferrec et al., 2001)

Tight junctions were earlier thought to be secreted extracellular cement forming an absolute and unregulated barrier within the paracellular space. But this concept was changed latter on in researches carried in last decades and demonstrated that tight junctions are made up of a complex network of proteins, the interaction of which dictates their competency. Nowadays, it is apparent that tight junctions are dynamic structures that are involved in developmental, physiological and pathological process (Fasano and Shea-Donohue, 2005). The paracellular passage of macromolecules under certain circumstances (i.e. physiological or pathological) is safeguarded by Gut-Associated-Lymphoid-Tissue (GALT), which serves as a containment system that prevents potentially harmful intestinal antigens from reaching the systemic circulation. It also induces systemic tolerance against luminal antigens by a process that involves polymeric IgA secretion and induction of T-regulatory-cell activity. The GALT is made up of immune

inductive sites (Peyer's patches) and isolated lymphoid follicles (ILF). ILF are tertiary lymphoid structures that are formed in autoimmune diseases as well as in several inflammatory pathologies. Mature ILF resembles Peyer's patches in cellular composition and localization in the distal intestine (Fasano and Shea-Donohue, 2005). Upon oral administration of nanoparticles, they will disperse in the liquid GIT environment and come to a direct contact with the mucosa of GIT membrane. These nanoparticles might be immobilized at the mucosal layer (mucoadhesion) due to their size and mucoadhesive properties. In fact, the adhesion properties do not last for long time but this behavior could prolong the residence time with a reasonable drug concentration gradient from nano-carriers to the gut mucosa, which is the first step for drug transport through the epithelial layer known as translocation. Translocation possible takes place by three routes (i.e. paracellular for particles less than 50 nm, endocytosis for particles less than 200 nm and lymphatic uptake across the M- cells of the Peyer's patches for particles smaller than 5  $\mu$ m (Shahbazi and A Santos, 2013).

A proper balance is required between the immunity and tolerance for a healthy intestine. Any improper or abnormal immune response can result in inflammatory diseases. The barrier dysfunction which occurs in patients with intestinal diseases suggests that pathological factors could hijack the normal physiological ways that regulates the tight junction permeability. Increased intestinal permeability is caused by exogenous factors like infections; however, it is also suggested to be caused by the immune system. Two cytokines, interferon- $\gamma$  (IFN $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) were found in high levels in intestinal mucosa with inflammatory bowel disease (Clayburgh et al., 2004; MacDonald et al., 1990). These two cytokines leads to reorganization of many tight junction proteins and these changes are associated with marked increase in myosin light chain phosphorylation, which could be reversed by a membrane permeant inhibitor. It also indicates that both cytokines utilize the myosin light chain kinase driven pathway to increase tight junction permeability. Therefore, a major step in the pathogenesis of inflammatory bowel diseases could be myosin light chain kinase activation by IFN  $\gamma$  and TNF $\alpha$  leading to intestinal barrier dysfunction (Clayburgh et al., 2004).

There are three main components which are necessary in the progression of inflammatory bowel disease (a collective name of two chronic intestinal disorders i.e. Crohn's disease and

ulcerative colitis disease; 1) disruption of the epithelial barrier, 2) access of luminal contents to the *lamina propria* (immune cells), 3) an abnormal immune response. A defect in intestinal barrier leads to the freely mixing of luminal contents with lamina propria. These luminal contents include bacteria, bacterial products, food antigens and other immuno-stimulatory antigens. Antigen presenting cells in the *lamina propria* process and present these antigens to T cells, which in response initiate T<sub>H</sub>1 immune response. For the T<sub>H</sub>1 response, it is essential to secrete IFN $\gamma$  from T cells, which activates the macrophages to respond to the stimuli. These regulatory mechanisms might be disrupted in the inflammatory bowel disease. Another event in this immune torrent is the secretion of TNF $\alpha$  from the activated macrophages, which along with IFN $\gamma$  acts on epithelium and further disrupts the barrier and increases its permeability. This barrier dysfunction allows more leakage of luminal contents thus triggering more immune response, which feeds back the intestinal barrier to promote further leakage and a cycle is initiated. This occurrence might be as a result of genetic predisposition to a leaky barrier, an abnormal immune response, or other environmental factors like infections, which cause immune stimulation. Since, human inflammatory bowel disease shows abnormalities of all these three components, the therapies that effectively blocks this cycle at one or more points might be effective in allowing the intestine to return to its normal condition (Clayburgh et al., 2004). The occurrence of epithelial dysfunction and increased paracellular permeability was reported by Daniel Hollander in 1986 (Hollander et al., 1986), while the increased paracellular permeability in patients with Crohn's disease was reported around 33 years ago (Pearson et al., 1982).

NO-donors can be used in the treatment of Crohn's disease. In recent studies, GSNO has been proposed as a potential treatment to prevent mucosal barrier failure in Crohn's disease. It was observed that this effect was only in patients with Crohn's disease but not in patients having no inflammatory bowel diseases. There is a possibility that this tissue specificity may be related to the observations that the enteric glial cell network is specifically disrupted in the intestinal mucosa affected by Crohn's disease (Savidge et al., 2007).

## 5. Crohn's disease

Crohn's disease (CD) is a type of inflammatory bowel disease which is thought to be a result of inappropriate inflammatory response to intestinal microbes in a genetically susceptible host (Abraham and Cho, 2009), but the main cause is unknown (Thomsen et al., 1998). It is an immune related disease in which the immune system attacks the GI tract. It is characterized by focal, asymmetric, transmural and occasionally granulomatous inflammation, which primarily affects the GI tract (Thomsen et al., 1998). Crohn's disease related inflammation is generally segmental and transmural which results in various degrees of tissue damage (Monteleone et al., 2015). In CD, the most frequently effected parts are ileum and colon which are worsened by intestinal obstruction, inflammatory mass or abscess (Farmer et al., 1985). The genetic and environmental factors are believed to play a special role in its etiology and pathogenesis. It has a heterogeneous clinical expression and studies showed its female predominance, occasional familial nature, high rate of stricture formation and penetrating disease. It can appear in early childhood to late adulthood and 80% cases are diagnosed before the age of 40 years. In maximum cases the diagnosis reports in colonic and terminal ileal involvement. It is also suggested that it can turned into a more complex disease with stricture formation and penetrating disease complications [e.g. fistula and abscess (Freeman, 2009)]. Characteristic symptoms of CD include chronic or nocturnal diarrhea, abdominal pain, weight loss, fever and rectal bleeding. Extra-intestinal symptoms can include inflammation of the eyes, skin or joints and in children's anemia, fever, failure of growth. The potential complications of this multisystem disorder can affect patients of any age but the onset is more common in teenagers and young adults. Its clinical course is made complicated by other inflammatory bowel diseases and is neither medically nor surgically curable but requires therapeutic treatments to induce and maintain symptomatic control, life quality, minimize short and long term toxicity and complications (Thomsen et al., 1998). In CD, the mucosal healing can be promoted by using immune-suppressive drugs and anti-tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) antibodies but unfortunately more than one third of the patients do not show any response to these treatments (Monteleone et al., 2015). Novel therapies include the induction and maintenance of mucosal and histological healing (Lichtenstein et al., 2009). NO-donors can be suggested in the treatment of Crohn's disease (Savidge et al., 2007). In the recent years, GSNO has been

proposed in Crohn's disease to prevent the mucosal barrier failure. GSNO was found to significantly promote human intestinal mucosal barrier function while this effect was lacking in patients having no inflammatory bowel disease conditions. This may be due to the observations that enteric glial cell network is disrupted in non-inflamed Crohn's disease intestinal mucosa; hence, the tissue GSNO concentration levels might be lower in these patients (Savidge et al., 2007; Shah et al., 2015b). Another study (Laszlo et al., 1995) showed that, the concurrent infusion of NO-donors (GSNO or SNAP) dose-dependently reduce ileo-colonic microvascular leakage over 1h of investigation period. In the control studies, the infusion of carrier molecules of the corresponding NO-donors devoid of NO (GSH or *N*-acetyl-penicillamine) did not affect the microvascular plasma leakage. NO may prevent the ileo-colonic injury and microvascular leakage by counteracting the vasoactive or tissue damaging actions of pro-inflammatory mediators such as thromboxanes. It was showed that the intravenous administration of GSNO and SNAP in lowest doses (1 µg/kg body weight) significantly inhibited microvascular leakage without affecting the increase in BP of the rats; however, the authors could not eradicate the changes in intestinal blood flow.

### **5.1. Treatment of Crohn's disease**

All the drugs that can be used are presented on Table 3.

#### **5.1.1. Immunosuppressive drugs**

Patients with moderate to severe activity of CD are treated with traditional drugs like corticosteroids, which is the main therapy for such type of diseases. Corticosteroids induce the remission of symptoms in a high percentage of patients. Due to the different side effects produced by systemic steroids like Cushing syndrome, osteoporosis, diabetes and infection (Lichtenstein et al., 2006), a glucocorticoid derivative known as budesonide has been introduced few years ago. It is supplied as sustained-release microgranules and it acts on local mucosa and has positive results. Intravenous immunoglobulin (IVIG) has also been used in the management of aminosalicylate and steroid resistant CD for more than 20 years (Rogosnitzky et al., 2012). The use of immunomodulators is complicated because of delayed onset of action and having serious side effects like bone marrow suppression, hepatotoxicity and pancreatitis. The

use of both immunomodulators and corticosteroids also have a high effect on immune system and can increase the risk of serious and fatal infections in patients been treated with these drugs (Lichtenstein et al., 2006).

An immunosuppressive drug, methotrexate, is also used to treat CD. It is having side effects more common when used in high doses; however, no serious side effects have been observed. Some of the common side effects include nausea, vomiting, abdominal pain, diarrhea, skin rash and headache (McDonald et al., 2014).

Anti-tumor necrosis factor therapy (TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ) is an important therapy used in the treatment of patients with CD (Colombel et al., 2004). The chimeric anti-TNF- $\alpha$ , infliximab, is also an effective drug in closing fistulas in CD's patients (Lichtenstein et al., 2005). TNF- $\alpha$  is a pro-inflammatory cytokine, which is elevated in the stool, mucosa and blood of patients with CD (Hanauer et al., 2006), is having an important role in the pathogenesis of CD. Infliximab binds to TNF- $\alpha$  and neutralizes its biological activities; when given as a 5 mg/kg (IV infusion), infliximab induces remission in patients with moderate to severe CD (Rutgeerts et al., 2004). It reduces corticosteroids requirements (Rutgeerts et al., 2004) and facilitates closure of symptomatic fistulas in CD patient's, which results from inflammation penetrating from effected bowel into adjacent organs, tissues or skin. It is estimated that about 20 – 40% of individuals with CD will develop it over the life-time of the disease. Fistulas closure was observed in patients when a 3-dose regimen of infliximab was given at weeks 0, 2 and 6 (Lichtenstein et al., 2005).

For example, Adalimumab is a human immunoglobulin G1 (IgG1) antibody and binds with high affinity and specificity to human soluble TNF (Hanauer et al., 2006). It is effective for inducing of remission and maintaining clinical response in patients with moderate to severe CD. It is approved for the treatment of CD in North America and Europe (Feagan et al., 2008). In the induction therapy of Adalimumab, it was administered subcutaneously as a loading dose at week 0 followed by a second dose at week 2 to CD patients. It resulted in the induction of response and remission in infliximab-native patients with moderate to severe disease activity. The evaluation in its safety and efficacy in long term management of CD patients are currently in clinical trials (Hanauer et al., 2006).

### 5.1.2. Antibiotics/Fecal transplantation

It is also suggested that the bacteria's present in intestinal flora also plays an important role in the causing of CD; therefore, antibiotics including metronidazole and ciprofloxacin have been also recommended as an alternative or association with steroids (Scribano and Prantera, 2003). Drugs like sulfasalazine, azathioprine and prednisone have also been used in CD but are having only marginal role in the therapy of acute flare-up of Crohn's disease (Summers et al., 1979). This strategy is not used anymore.

Fecal microbiota transplantation (FMT) has gained a tremendous interest as a new option for treating IBS. In a study (Colman and Rubin, 2014), 122 patients described as (79 UC, 39 CD and 4 IBD) were treated with FMT. Overall, 45% patients achieved clinical remission from the disease after follow-up. It was suggested that FMT is safe but variably efficacious treatment for IBD.

Table 3. Principle medicines for the treatment of ulcerative colitis and Crohn's disease (Jean Calop, 2012)

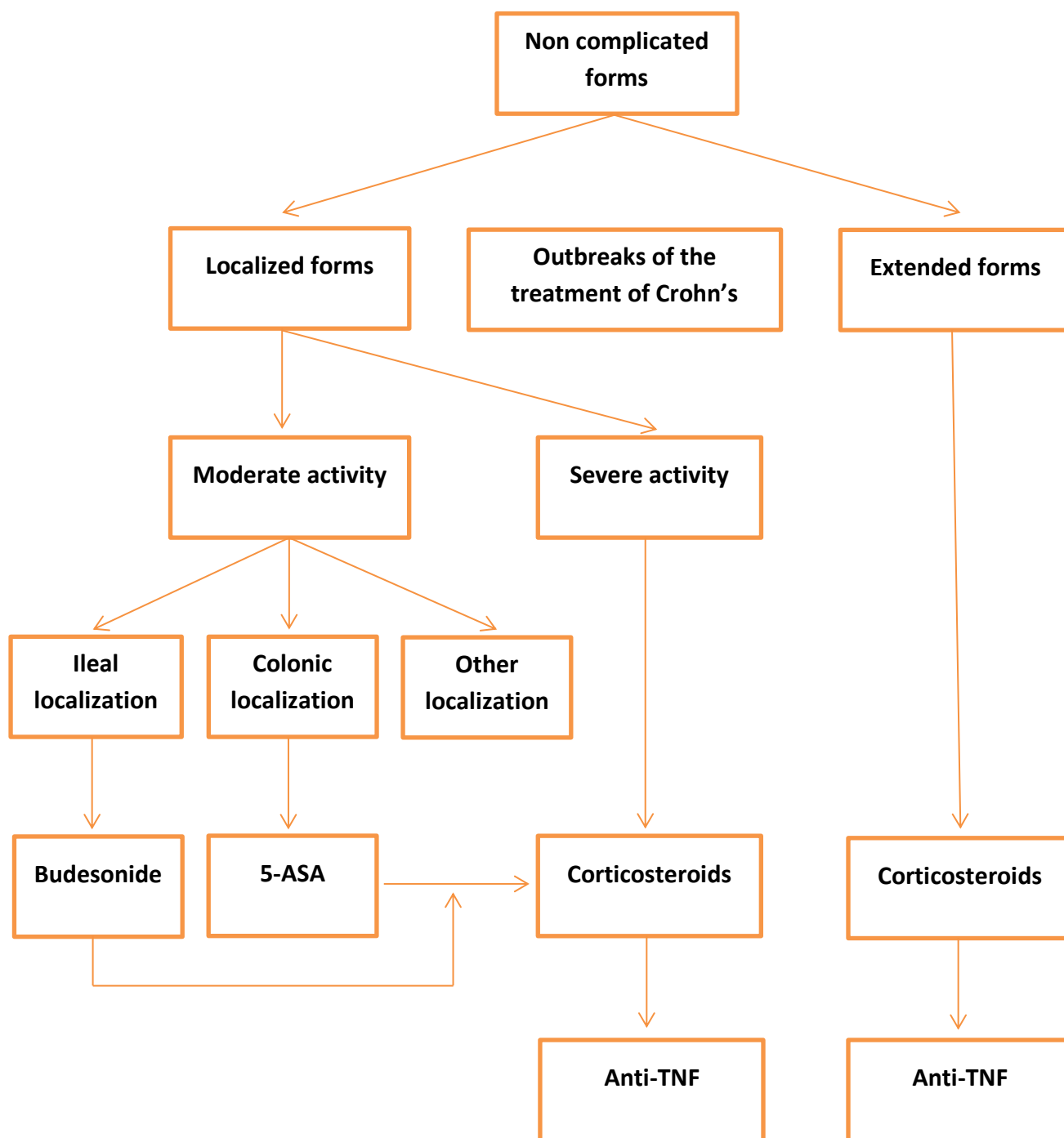
DCI	Trade name	Dosage form	Presentation and daily dosage
<b>Corticosteroids</b>			
Betamethasone phosphate	Betnesol®	Rectal solution	Pocket enema 5 mg/100 ml
Prednisolone acetate	Hydrocortancyl®	Injectable	125 mg/5 ml-3 ml (75 mg) to be prepared in 20-120 ml of NaCl solution
Prednisolone methanesulfonate	Solupred®	Effervescent and oral dispersibles	5,20 mg: 10-30 mg/day, low dose, one single dose in



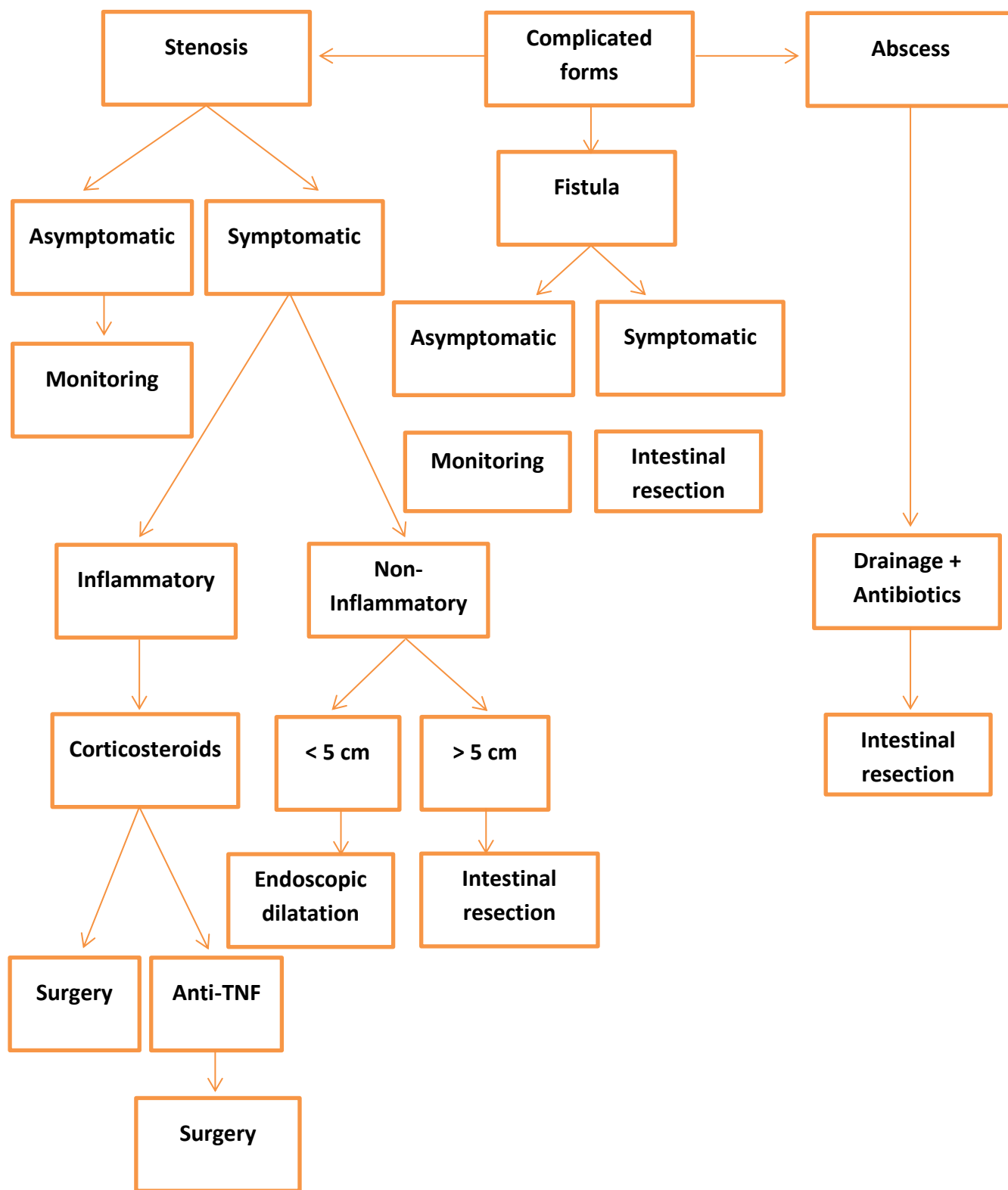
			the morning
Prednisolone	Cortancyl®	Tablets	
Dexamethasone	Dectancyl®	Tablets	
Methylprednisolone	Medrol®	Tablets	
Bethamethasone	Celestène®	Tablets	
Hydrocortisone acetate	Colofoam®	Rectal foam 10%	Ppressurized bottle 20 g (minimum 14 doses) and a dosing applicator, 1 application per day
Budesonide	Entocort®, Rafton®, Mikicort®	Tablets	3 mg - 9 mg/day, a single dose in the morning for 8 weeks
<b>Locals anti-inflammatory</b>			
Mesalazine	Pentasa®		
		Sachets granules	1, 2 g, 2-4 g/day
		Suppositories	1 g, 1 g/day for 2 weeks
		Rectal suspension	1 g/100 ml pocket,
	Rowasa®	Gastro-resistant tablets	250 mg, 500 mg, 1.5- 2 g/day
		suppositories	500 mg, 1-1.5 g/day for 4 weeks
	Fivasa®	Gastro-resistant tablets	400, 800 mg, 1.6 – 3.2 g/day, 2-4 g/day

		Ssuppositories	500 mg, 1-1.5 g/day for 4 weeks
Sodium para-aminosalicylate (4-ASA)	Quadrasa®	Powder for rectal solution	2 g bottle, to be prepared in 20-120 ml of NaCl
Sulfasalazine	Salazopyrine®	Gastro-resistant tablets	500 mg, 4-6 g/day, infants 100-150 mg/kg/day
Olsalazine	Dipentum®	Capsules	250 mg, 1.5-2 g/day
		Tablets	500 mg, 1.5 -2 g/day
<b>Immuno-suppressors</b>			
Azathioprine	Imurel®, Aazathioprine®	Tablets	
		Injectable	50 mg, 1 - 3 mg/kg/day
6-mercaptopurine (6-MP)	Purinéthol®	Tablets	50 mg, 1 - 3 mg/kg/day
Tioguanine	Lanvis®	Tablets	40 mg, 20 mg/day
Methotrexate	Novatrex®, Mmethotrexate®	Tablets	2.5 mg, 15 – 30 mg per week
	Methotrexate Bbellon®	Injectable bottle	25 mg/1ml, 15-25 mg/ week
	Ledertrexate®	Injectable bottle	25 mg/1ml, 15-25 mg/ week
	Metobject®	Prefilled syringe	10 mg/ml (7.5, 10, 15, 20, 25 mg) 15-25 mg/ week

Thalidomide	Thalidomide®	Capsules	50 mg, 50-100 mg/day
Ciclosporine	Sandimmun®, Neoral®	Soft capsules	10, 25, 50, 100 mg, 5 mg/kg/day, adapted according to the ciclosporinémie (150-250 ng/ml)
		Injectable	2 mg/kg/day, adapted according to the ciclosporinémie (150-250 ng/ml)
<b>Anticorps anti-TNF</b>			
Infliximab	Remicade®	Injectable bottle	160 mg SC (allocate site of injection) followed by 80 mg two weeks, then 40 mg every 2 weeks
Adalimumab	Humira®	Prefilled syringe, prefilled pen	160 mg SC (allocate site of injection) followed by 80 mg two weeks, then 40 mg every 2 weeks
Védolizumab	Entyvio®		



**Figure 18.** The algorithmic management of non-complicated form of Crohn's disease (Jean Calop, 2012)



**Figure 19.** The algorithmic management of complicated form of Crohn's disease (Jean Calop, 2012)

# Objectifs

L'objectif principal de ma thèse était de mettre au point des formulations qui puissent libérer du NO de façon prolongée au niveau intestinal. Ces formulations pourront dans un premier temps permettre d'étudier les effets du NO sur la maladie de Crohn.

Compte tenu des avantages des nitrosothiols par rapport aux dérivés nitrés classiques, nous avons choisi comme principe actif de base le GSNO.

Plusieurs objectifs ont ensuite pu être définis :

1. Synthétiser un nouveau donneur de NO par liaison du GSH avec le chitosan suivie d'une post-nitrosation pour obtenir un « support » de délivrance du NO nommée *S*-nitrosoglutathion-Chitosan-oligosaccharide (SNOC). Ce système de délivrance vise à être une forme plus stable que les autres *S*-nitrosothiols et qui pourrait ensuite permettre de fabriquer des systèmes microparticulaires. Le but final est d'améliorer la pharmacocinétique orale du NO.
2. Synthétiser un donneur de NO par liaison du GSH avec de l'alginate suivie d'une étape de post-nitrosation. Dans ce cas, le polymère sera appelé *S*-nitrosoglutathion-Alginate (SNA). Comme dans le cas précédent, le but recherché est d'obtenir une formulation plus stable.
3. De préparer des microparticules de *S*-nitrosoglutathion (GSNO) gastro-résistantes par spray-drying à base d'Eudragit® FS 30D.. Ces particules devraient résister aux conditions extrêmes d'acidité de l'estomac pour exercer leur action au niveau intestinal. A notre connaissance, aucune formulation de microparticules de *S*-nitrosothiols à base d'Eudragit FS 30D, n'a été rapportée dans la littérature.

# **Results and Discussions**



# **Chapter 1: Synthesis and characterization of S-nitrosoglutathione-oligosaccharide-chitosan as a nitric oxide donor**

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# Synthèse et caractérisation du chitosan-oligosaccharide-S-nitrosoglutathion comme donneur de monoxyde d'azote

## Résumé

L'objectif principal de ce travail était de mettre au point un polymère à base de chitosan qui puisse libérer du NO de façon prolongée au niveau intestinal : le S-nitrosoglutathion-chitosan-oligosaccharide (SNOC). Le couplage du chitosan et du GSH a été obtenu par l'utilisation d'un carbodiimide comme agent de couplage ; la nitrosation a été réalisée dans un second temps.

Ce polymère a été développé comme un « support donneur de NO » et devrait permettre d'étudier les effets du NO par voie orale dans le traitement de la maladie de Crohn.

Ce nouveau polymère a été caractérisé par RMN, spectrométrie infrarouge à transformée de Fourier et méthode d'Ellman (dosage des groupements thiols). Quant à la quantité de NO finale, elle a été déterminée par la méthode de Griess-Saville.

Des polymères avec un nombre différent de groupements NO ont été obtenus (concentrations :  $159,04 \pm 64,16 \mu\text{mol/g}$  de polymère pour SNOC G1 et  $525,08 \pm 151,35 \mu\text{mol/g}$  de polymère pour SNOC G2) selon la méthode de production. Quand ils ont été testés en chambre de Ussing, le SNOC G2 montre une libération retardée de NO/nitrites pendant environ 6h (20% restant après 6h).

Nous pensons que ce type de polymère est adapté pour le développement de diverses formulations, dont les microparticules. Ceci sera discuté dans la discussion générale de la thèse.

Dans cette publication, nous utilisons le chitosan comme polymère à libération contrôlée. Il a permis d'obtenir une forme stable de « donneur de NO » en comparaison au GSNO. Il est ainsi susceptible de transporter une grande quantité de NO jusqu'à son site d'action au niveau intestinal.

## EXPERT OPINION

1. Introduction
2. Materials and methods
3. Results and discussion
4. Conclusions

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## Original Research

# Synthesis and characterization of S-nitrosoglutathione-oligosaccharide-chitosan as a nitric oxide donor

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**Objectives:** The aim of this work is to synthesize a novel stable and biodegradable nitric oxide (NO) donor polymer based on a chitosan backbone. This polymer needed to be linked to glutathione (GSH), which was nitrosated in a second step. This polymer has been developed as an NO delivery platform that could be further evaluated for an oral delivery in Crohn's disease.

**Methods:** The new polymer (named S-nitrosoglutathione-oligosaccharide-chitosan or SNOC) was obtained using a two-step procedure involving the linkage of GSH to chitosan via an amidine reaction followed by a post-nitrosation with NaNO<sub>2</sub>. The GSH linkage was assessed using NMR, FTIR and an Ellman's test, whereas the final NO amount was determined by the Griess and Saville method.

**Results:** Polymers with different numbers of NO groups were obtained (159.04 ± 64.16 μmol/g of polymer for SNOC G1 and 525.08 ± 151.35 μmol/g of polymer for SNOC G2) depending on the procedure used for production. When tested in an Ussing chamber, SNOC G2 had a sustained release of NO and nitrites for at least 6 h.

**Conclusion:** We believe that this type of polymer is adapted for the development of various formulations, including microparticles.

**Keywords:** chitosan, Crohn's disease, glutathione, nitric oxide, Ussing chamber

Expert Opin. Drug Deliv. [Early Online]

## 1. Introduction

Nitric oxide (NO) is an endogenous free-radical that plays a key role in the bioregulation of several physiological intra- and extracellular pathways including control of vascular tone and platelet interactions [1,2].

For several years, organic nitrites have been used as NO donors for the treatment of angina pectoris [3], but the long-term use of these compounds is limited by a tolerance to [4] their hemodynamic effect.

In addition to these drugs, endogenous molecules, such as S-nitroso compounds (e.g., S-nitrosoglutathione [GSNO] and S-nitrosoalbumin), are still in the early research phase. These drugs have the very specific property of storing NO endogenously by coupling it to a thiol moiety [1,5] and offer advantages over existing drugs (e.g., patients do not develop tolerance). Small clinical studies have suggested that these drugs may be beneficial in various cardiovascular disorders [6]. S-nitrosothiols (RSNOs) may also be used for the treatment of brain ischemia [7], cancer [8], cystic fibrosis [9] or wound healing [10]. Recently, GSNO has also been proposed as a potential treatment to prevent mucosal barrier failure in Crohn's disease [11]. It has been observed that GSNO significantly promotes human intestinal mucosal barrier function in Crohn's disease patients, but not in intestinal tissues from

individuals without inflammatory bowel disease (IBD). Consequently, the authors have speculated that this tissue specificity may be related to the observation that the enteric glial cell network is particularly disrupted in non-inflamed Crohn's disease intestinal mucosa. Therefore, in these patients, tissue GSNO concentration levels may be lower [11], and we can suppose that an oral formulation of GSNO need to be tested as a potential treatment. Nevertheless, the use of conventional oral formulation (i.e., solution, tablets) is prevented by the lack of stability of the S-NO bound mainly due to enzymes, metallic cations, light and reductants [12]. Moreover, a specific targeting at the intestinal level would be an advantage.

Binding chitosan to GSNO has been envisioned as a method to increase the storage and delivery of NO in a controlled manner or to act as a platform for other technologies, such as coatings or the formation of nanoparticle/gel matrices.

Chitosan encompasses a series of polymers, extracted from chitin, that vary in molecular weight (MW) (from 1 kDa to > 5000 kDa) and degree of acetylation. Chitosan was also chosen due to its easy linkage to glutathione (GSH) and known biocompatibility and biodegradability properties. Due to its overall positive charge, it can interact with charged cell membranes (i.e., charged glysoaminoglycans) and with sialic groups of mucus (mucoadhesion properties) [13]. It has potential as enhancers of mucosal drug absorption [14,15]. We chose a low-molecular-weight chitosan (5 kDa MW) because it has demonstrated good stability, permeation and bioavailability when orally administered.

Our goal was to synthesize a novel stable and biodegradable NO donor based on a chitosan backbone. This polymer needed to be linked to GSNO moieties and adapted for oral delivery to ensure that it could be further evaluated in Crohn's disease.

There has been a tremendous increase in the development of new NO-carrier molecules. However, problems with biocompatibility and toxicity are commonly observed for these molecules [16]. Additional problems include poor NO storage and unstable release. Therefore, a new NO-carrier molecule is required to overcome the common problems observed for this class of compounds. Consequently, polymers and polymeric particles have emerged as an interesting alternative for incorporating S-NO molecules. These compounds have shown increased stability of the NO group and are able to provide higher storage capacity. There are currently numerous successful examples of this approach. Such examples include a polyethylene glycol-conjugated S-nitrosoalbumin [17] that has shown improved biodistribution and a controlled NO release profile, a hydrogel matrix with poly(ethylene glycol)-poly-L-Lysine-NO [18] and the inclusion of NO donors in Poly(lactic-co-glycolic acid), polystyrene-b-PEG and poly-L-lactide-b-poly (ethylene glycol) particles [19,20].

## 2. Materials and methods

### 2.1 Materials

Chitosan oligosaccharide lactate (low MW, 5 kDa), reduced L-GSH, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

hydrochloride (EDAC), N-hydroxysuccinimide (NHS), sulfanilamide, N-(1-naphthyl) ethylenediamine (NED), Ellman's reagent (5,5'-dithiobis(2-nitro benzoic acid) and 2,3-diaminonaphthalene (DAN) were obtained from Sigma-Aldrich (Saint-Quentin Fallavier France). All other chemicals were of analytical or chromatographic grade.

### 2.2 Synthesis of GSNO

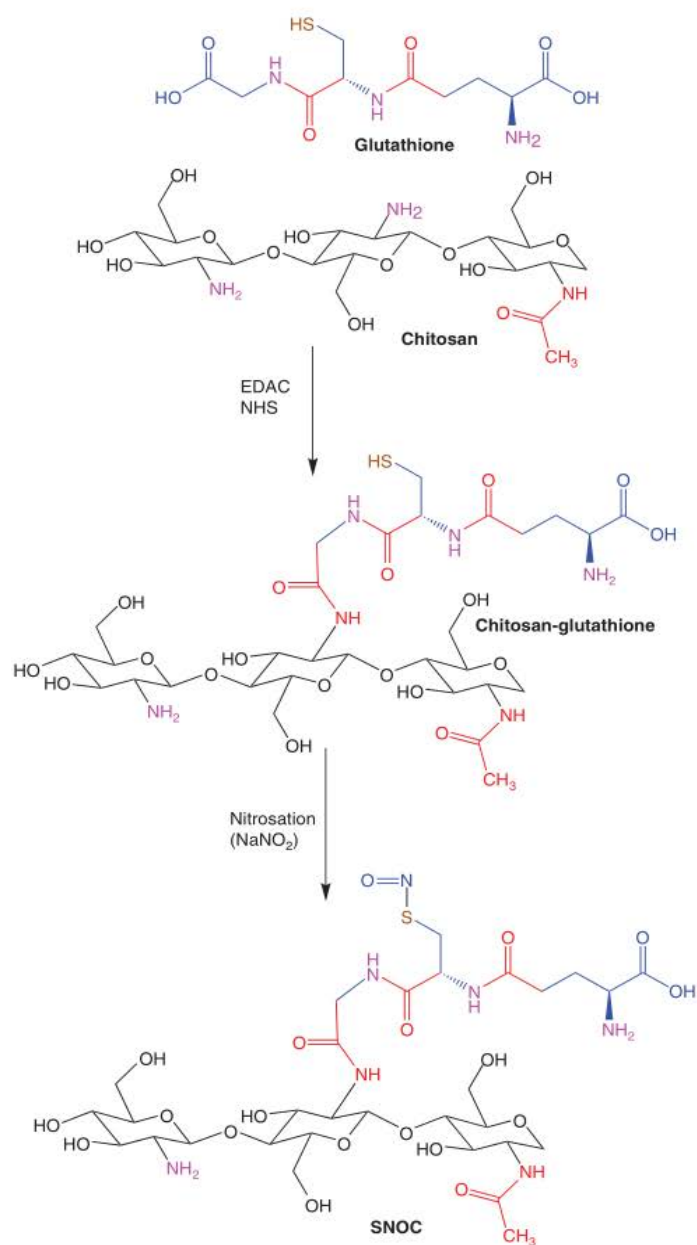
GSNO was synthesized in solution and used without further purification. Briefly, 20 mM thiol (GSH) was incubated with 20 mM NaNO<sub>2</sub> in a solution of HCl (0.5 M) at 4°C in the dark. After 2 h of incubation, the reaction was stopped by neutralization with NaOH (40%) and was further diluted (1:2) with phosphate buffer, pH 7.4. The formation and concentration of S-nitrosothiol was determined by UV-Vis spectrophotometry at a wavelength of 334 nm.

### 2.3 Synthesis of S-nitrosogluthathione-oligosaccharide chitosan with different NO concentrations (SNOC G1 and SNOC G2)

The synthesis of S-nitrosogluthathione-oligosaccharide chitosan (SNOC) was achieved by using two reaction steps (Figure 1): i) thiolation of chitosan via EDAC/NHS and ii) nitrosation of thiol content by sodium nitrite. For SNOC G2, the reduction of the disulfide bonds by NaBH<sub>4</sub> was performed before the nitrosation step.

#### 2.3.1 First step: synthesis of a chitosan-GSH conjugate

In the first step, GSH was covalently attached to low MW chitosan [21,22]. Briefly, exactly 1 g of low MW chitosan was weighed and dissolved in 8 ml HCl (1 M) solution and the final volume was adjusted to 100 ml by the addition of ultrapure water. The pH of the chitosan solution was adjusted to 6.0 with NaOH (5 M) solution. Then, 5 g of reduced GSH was dissolved in 10-ml ultrapure water and added to the above solution with continuous stirring at a speed of 200 r.p.m. EDAC (4.6 g in 5-ml ultrapure water) and NHS (2.8 g in 5-ml ultrapure water) were added to the above solution, and the pH was again readjusted to 6.0 with NaOH (5 M). The reaction mixture was allowed to continue for 18 h at room temperature with continuous stirring. Afterward, the unbound reagents from the chitosan-GSH conjugate were removed by dialysis in tubing (MW cutoff 12 kDa), first against HCl (5 mM), twice against HCl (5 mM) dissolved in NaCl (1% w/v) and twice against HCl (1 mM). The same methodology was used to prepare controls without using EDAC and NHS during the coupling reaction. The samples were frozen overnight at 20°C and freeze-dried in an SMH15 freeze-dryer (Usifroid, Maurepas, France): The temperature of the sample, coming from the cold chamber, was first equilibrated with a cooling plate at 56°C for 2 h. Then, the total pressure was maintained at 5 Pa at 12°C for 24 h. The samples were stored until further use.



**Figure 1. Reaction scheme for the synthesis of SNOG.** First step: synthesis of a chitosan-glutathione conjugate. Second step: nitrosation of chitosan-glutathione conjugate.  
SNOG: S-nitrosoglutathione-oligosaccharide chitosan.



### 2.3.2 Second step: nitrosation

The nitrosation of the chitosan-GSH conjugate was performed using two methods:

#### 2.3.2.1 5-nitrosoglutathione-oligosaccharide chitosan G1

Chitosan-GSH polymer weighing 100 mg was wetted in 45 ml HCl (0.5 M) for 30 min until complete hydration. Afterward, an equimolar concentration of NaNO<sub>2</sub> (equimolar to the total number of reduced thiols in the chitosan-GSH conjugate) in 1-ml ultrapure water was added to the above polymer conjugate. This reaction mixture was incubated for 2 h at 4°C in the dark. Afterward, the reaction was stopped by neutralizing the mixture with NaOH (40% w/v). The amount of free and linked nitrites was calculated by the methods described by Griess and Saville [23-26].

#### 2.3.2.2 5-nitrosoglutathione-oligosaccharide chitosan G2

In this method, prior to the nitrosation step, the GSH was reduced with NaBH<sub>4</sub> by breaking the disulfide bonds between the thiol groups. Briefly, 100 mg of chitosan-GSH was hydrated in 8.75-ml ultrapure water and 16.25 ml Tris-HCl for 30 min. Afterward, 5 ml freshly prepared NaBH<sub>4</sub> (4% w/v) was added and incubated for 1 h at 37°C in a shaking water bath. After 1 h, the remaining NaBH<sub>4</sub> was eliminated by adding 4 ml HCl (5 M) and was left for additional 15 min in the shaking water bath. Then, this solution was cooled by the addition of 25 ml HCl (0.5 M). The solution was sonicated for 10 s, and an equimolar concentration of NaNO<sub>2</sub> (equimolar to the total number of thiols in the polymer) was added and was kept at 4°C for 2 h while protected from light. Afterward, the solution was neutralized by the addition of NaOH (40% w/v). Finally, the solution was dialyzed in tubing (MW cutoff 12 kDa) in ultrapure water to remove the unbound reagents.

## 2.4 Analytical methods

### 2.4.1 <sup>1</sup>H-NMR measurements

All experiments were performed on a Bruker Avance DRX-NMR spectrometer operating at 9.4 T (proton frequency: 400.133 MHz) and at a sample temperature of 300 K.

The GSNO, chitosan and chitosan GSH were dissolved in D<sub>2</sub>O:DMSO-d<sub>6</sub> (90:10). Chemical shifts were given in parts per million (p.p.m.) relative to the solvent signal (HOD at 4.70 p.p.m.).

### 2.4.2 FTIR analysis

For the fourier transform infrared (FTIR) studies of the chitosan-GSH polymer, exactly 6 mg of the polymer was mixed with 300 mg KBr. A small amount of this mixed powder was compressed in a die to produce a thin film. The film was placed in the IR spectrophotometer (Vector 22 FT-IR spectrophotometer, Bruker) and the spectra were recorded.

### 2.4.3 UV-Vis spectrophotometry: determination of thiol concentration (Ellman's method)

To determine the thiol (-SH) content of chitosan-GSH conjugates, a modified version of the Ellman's test was performed. Briefly, a known amount of chitosan-GSH conjugate was hydrated in Tris-HCl, and subsequent dilutions were made in water. This solution was incubated with freshly prepared (0.3 mg/ml) Ellman's reagent, 5, 5'-dithiobis-(2-nitrobenzoic acid), and kept in the dark for 15 min before analysis by UV-Vis spectrophotometry at a wavelength of 412 nm. The amount of reduced thiol moieties present in the sample was calculated by a GSH standard calibration curve. In these analytical conditions, the limit of detection was 0.822 µmol/l and the limit of quantification was 2.49 µmol/l (correlation coefficient > 0.999).

### 2.4.4 UV-Vis spectrophotometry: determination of NO concentration (Griess-Saville method)

This method is an indirect determination of NO based on the spectroscopic measurement of its stable decomposition products NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup>. It has been previously described by Griess [23,24]. The reaction is a two-step diazotization reaction in which the NO-derived agent, dinitrogen trioxide (N<sub>2</sub>O<sub>3</sub>), generated from the acid-catalyzed formation of nitrous acid from nitrite (or autooxidation of NO) reacts with sulfanilamide to produce a diazonium ion, which is then coupled to N-(1-naphthyl)ethylenediamine to form a chromophoric azo product that strongly absorbs at 540 nm. The standard curves were obtained using NaNO<sub>2</sub>. In these analytical conditions, the limit of detection was 0.211 µmol/l and the limit of quantification was 0.64 µmol/l (correlation coefficient > 0.999).

The Saville assay is based on the mercuric chloride HgCl<sub>2</sub>-induced cleavage of RSNOs to form nitrite [26] and the quantification of the latter by the classical Griess reaction (limit of detection 0.061 µmol, limit of quantification 0.118 µmol, correlation coefficient 0.999).

For each sample, 20 µl sample was diluted with acetic acid buffer, pH 2.5 to a final volume of 200 µl. To this solution, 40 µl sulfanilamide (free nitrites quantification) or sulfanilamide + HgCl<sub>2</sub> (total nitrites quantification) was added, and this solution was incubated in the dark. After 3 min, 10 µl NED was added to each sample and the samples were kept in the dark for 5 min before analysis in the plate reader (ELx800 Absorbance Microplate Reader, BioTek, Colmar, France) at 570 nm or in a spectrophotometer (Cary® 50 UV-Vis, Varian, Les Ulis, France). The RSNO groups (i.e., GSNO or nitrated polymers) were calculated using the formula [Total nitrites] - [Free nitrites] = [RSNO].

### 2.4.5 Spectrofluorometry: determination of NO concentration (DAN method)

The DAN method allows the detection of lower nitrite amounts and, as a result, is more reliable for low nitrite concentrations [27]. This method is based on the reaction of nitrite

with DAN (slightly fluorescent) to give 1-[H]-naphthotriazole (highly fluorescent). For the fluorimetry method, 50 or 200  $\mu$ l samples were diluted to a final volume of 3 ml with water. Afterward, 300  $\mu$ l DAN (105  $\mu$ M) or DAN/HgCl<sub>2</sub> (105  $\mu$ M and 1.05 mM, respectively) was added and the samples were incubated at 37°C for 10 min. Thereafter, 200  $\mu$ l NaOH (1.2 M) was added and the samples were analyzed by spectrofluorimetry ( $\lambda_{\text{ex}}$  = 375 nm,  $\lambda_{\text{em}}$  = 415 nm).

## 2.5 Characterization of the chitosan-GSH polymer

The reaction described in Figure 1 can lead to different types of linkages.

The first possibility (case 1, Figure 2, left) is a direct fixation of reduced GSH on the chitosan backbone. However, we can also have oxidized GSH (cases 2 and 3, Figure 2, left), which we need to account for in our analytical procedures.

### 2.5.1 Reduced GSH moiety

As a standard procedure, 0.5 mg thiolated polymer was dissolved in ultrapure water and diluted with 1 ml Tris-HCl (0.05 M).

Then, the reduced thiols were determined spectrophotometrically using Ellman's reagent.

### 2.5.2 Total GSH moiety

For the determination of all GSH moieties (cases 1 + 2 + 3), the disulfide bonds were reduced by the addition of 1 ml NaBH<sub>4</sub> (4% w/v in NaOH 0.05 M, Figure 2) to the dissolved thiol polymer and kept in an agitating bath at 37°C for 1 h. The NaBH<sub>4</sub> was then neutralized with HCl (5 M) prior to analysis using the Ellman's method.

### 2.5.3 GSH directly linked to the polymer

The same procedure was employed to determine the GSH moieties directly linked (GSH moieties are in bold in Figure 2) to the chitosan backbone. After reduction with NaBH<sub>4</sub> and neutralization, the GSH moieties that were not directly linked (in italics in Figure 2) could be removed; the sample was dialyzed (molecular cutoff 3.5 kDa) for 4 h in water. To avoid reoxidation, the recovered solution was then reduced again with 1 ml NaBH<sub>4</sub> (4% w/v in NaOH 0.05 M). The sample was analyzed using UV-Vis at 412 nm.

### 2.5.4 Determination of GSH release after NaBH<sub>4</sub> reduction

The amount of GSH released after NaBH<sub>4</sub> reduction (in italics in Figure 2) was quantified using HPLC. Each batch was prepared as follows: 5 mg thiolated polymer was dissolved in a mixture of 5 ml water/Tris-HCl to which 5 ml NaBH<sub>4</sub> (4% w/v) was added. After 1 h at 37°C in an agitating bath, the solution was neutralized with 1 ml HCl (5 M) and sonicated to remove any entrapped gas. The samples were collected and diluted with water before HPLC analysis.

## 2.6 Stability of chitosan in the presence of sodium nitrite

The nitrosation step of the thiol can be performed using different techniques. Because GSH nitrosation by NaNO<sub>2</sub> has been shown to be an efficient and easy method, this method was chosen as the nitrosation step. However, the use of NaNO<sub>2</sub> has been reported to depolymerize high MW chitosan [28]. Therefore, the stability of chitosan in the presence of NaNO<sub>2</sub> was evaluated. Briefly, 3 mg chitosan was dissolved in 1 ml HCl (0.5 M) and diluted with another 1 ml HCl (0.5 M), with or without 1 mg GSH. Afterward, 17  $\mu$ l NaNO<sub>2</sub> (0.2 M) was added to each tube and were incubated for 1 h at 4°C. The theoretical concentration of NaNO<sub>2</sub> was 0.53 mmol/g chitosan, which is much higher than our operating conditions for SNOG preparation.

The reaction was then neutralized with NaOH (40% w/v) and 1 ml phosphate buffer saline was added. When NaBH<sub>4</sub> (4%) was used, the samples were dissolved in a basic solution of NaOH (0.05 M) and were subsequently neutralized. The total nitrites consumption was determined by the Griess and Saville method.

## 2.7 SNOG G1 and SNOG G2 characterization

### 2.7.1 Quantification of nitrosation efficiency

The nitrosation efficiency evaluation for SNOG G1 and SNOG G2 was also performed using the Griess-Saville method (plate reading at 570 nm) and UV spectrophotometry (334 nm).

### 2.7.2 Stability of SNOG G1 in aqueous media

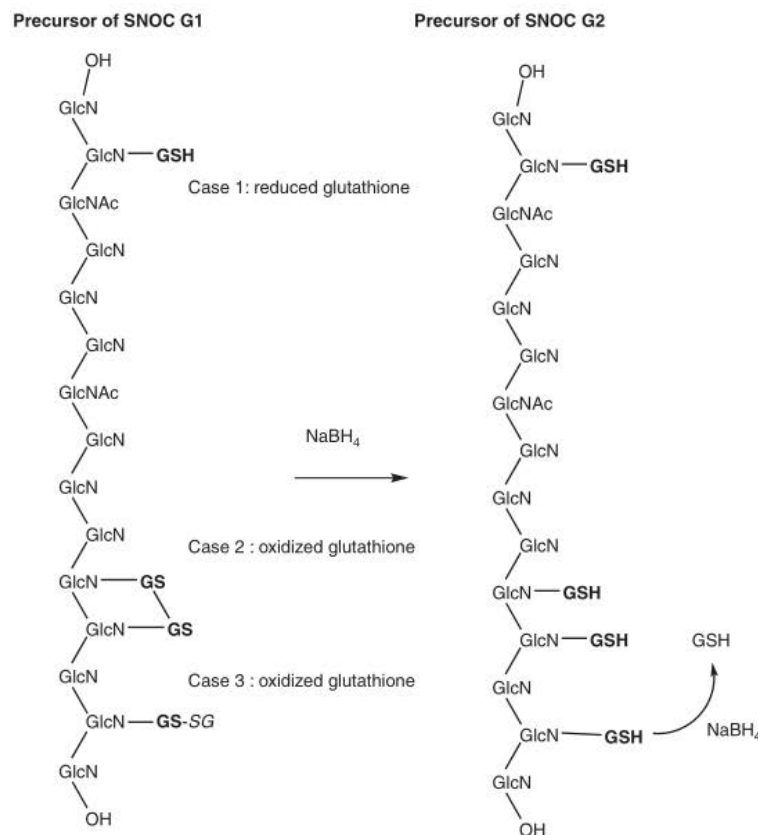
The stability of SNOG polymers was tested in different pH media. The quantification of total NO and free NO from GSNO and SNOG G1 at 4 and 37°C were measured at different time intervals to determine the stability kinetics of these compounds. GSNO and SNOG were prepared at a concentration of  $\sim 2 \times 10^{-5}$  M in different media (HCl-KCl 0.05 M, pH 1.2; phosphate buffer (0.05 M) pH 6.8; phosphate buffer (0.05 M) pH 7.4 (USP XXVIII)). At predetermined times, samples were withdrawn and the free and total nitrites were quantified.

### 2.7.3 Studies of intestinal drug permeation: Using chamber

Wistar rats (400 g, 8 – 10 weeks old, males, Charles River) were used for this experiment. The colon was rapidly surgically removed, washed with cold Krebs-bicarbonate Ringer's (KBR) solution (114 mM NaCl, 25 mM NaHCO<sub>3</sub>, 5 mM KCl, 1.1 mM MgCl<sub>2</sub>, 1.25 mM CaCl<sub>2</sub>, 1.65 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.3 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 10 mM glucose, pH 7.4) and placed in a beaker aerated with a mixture of O<sub>2</sub>/CO<sub>2</sub> (95:5).

The organ was immediately stripped under a dissection microscope and the serosal and muscular layers were removed.





**Figure 2. Various possibilities for GSH fixation to the chitosan backbone.** Case 1: GSH remains in the reduced form; Case 2: two GSH molecules bound on the polymer can form a disulfide bound; this can also occur between different polymer chains; Case 3: a linked GSH can form a disulfide bound with a free molecule. Reaction after  $\text{NaBH}_4$  treatment.

GSH: Glutathione; SNOC: S-nitrosoglutathione-oligosaccharide chitosan.

The mucosa was mounted between the two halves of an Ussing chamber (CHM8,  $0.5 \text{ cm}^2$  WPI, Stevenage, UK).

The two compartments of the Ussing chamber were filled with 8 ml Hank's Balanced Salt Solution (HBSS) in each compartment at  $37^\circ\text{C}$ . Current electrodes (Electrode kit for Ussing chamber, WPI) were placed on each side. The trans-mucosal electrical resistance (TER), which reflects the tissue integrity, was monitored during each experiment and tissue samples that had  $\text{TER} < 30 \Omega \text{ cm}^2$  were discarded.

The initial concentrations of GSNO and SNOC in the donor chamber were  $100 \mu\text{M}$ .

Samples ( $100 \mu\text{l}$ ) were taken from both chambers for analytical measurements (Ellman's method and Griess-Saville method) at 1, 2, 3, 4, 5 and 6 h.

## 2.8 Statistical analysis

All results are expressed as the means  $\pm$  standard deviation. The Student's t-test was used to determine statistically significant differences ( $p < 0.05$ ).

## 3. Results and discussion

### 3.1 Chitosan characterization

#### 3.1.1 $^1\text{H-NMR}$ spectroscopy

The  $^1\text{H-NMR}$  spectroscopy is the first approach for determining the degree of chitosan deacetylation. In our experiment, we used a method described by Shigemasa *et al.* [29]; the percentage of acetylation of chitosan oligosaccharide lactate was calculated from the area ratio between [H-7' methyl



protons of N-acetyl group] and [H-2, H-3, H-4, H-5, H-6 protons of GlcN residue + H-2', H-3', H-4', H-5', H-6' protons of GlcNAc residue] (Figure 3). The ratio (i.e., % N-acetylation) was 39% for chitosan oligosaccharide lactate.

### 3.1.2 FTIR analysis

FTIR analysis was first proposed to determine the percentage of acetylation by Moore and Roberts [30]. This method has several advantages: it is relatively quick and, unlike the various other spectroscopic methods, does not require a separate determination of sample purity [31].

Consequently, multiple methods for infrared spectroscopy have been studied. The method proposed by Baxter *et al.* [31] uses the following relationship:

$$\% \text{ N-acetylation} = \% \text{ amide} = (A_{1655}/A_{3450}) \times 115 \quad (1)$$

where  $A_{1655}$ , the absorbance at  $1655 \text{ cm}^{-1}$  of the amide I band (C=O stretching vibration) is determined using the baseline B (Figure 4A)

$A_{3450}$ , the N-H stretching vibration at  $3450 \text{ cm}^{-1}$ , is determined using the baseline A.

This method was applied to the spectra obtained from chitosan oligosaccharide lactate and resulted in a value of 36%.

Practically, the % N-acetylation can be written as follows:

$$\frac{n_{\text{GlcNAc}}}{n_{\text{GlcN}} + n_{\text{GlcNAc}}} = 36\% \quad (2)$$

where  $n_{\text{GlcNAc}}$  is the number of N-acetylglucosamine units per gram.

$n_{\text{GlcN}}$  is the number of glucosamine units per gram.

This value was in a good agreement with the value obtained from the  $^1\text{H-NMR}$  analysis (39%).

## 3.2 Chitosan-GSH polymer characterization

The obtained freeze-dried chitosan-GSH polymer was a white fibrous powder that adhered easily to any surface with moisture. In fact, the change in chitosan properties was easily noticeable due to the ease of polymer dissolution in aqueous mediums. The complete physicochemical characterization of the polymers is an important process because these properties will ultimately influence the polymer's ability to link to NO (i.e., nitrosation process: efficiency and stability) as well as its absorption, release kinetics and toxicity.

Initially, our objective was to verify the linkage of GSH to the chitosan polymer using two different methods: infrared spectroscopy (percentage of acetyl group and peptide bound) and NMR. However, the NMR spectra of chitosan-GSH did not allow us to determine GSH fixation on the chitosan backbone; therefore, we have only described the results of the FTIR experiments associated with Ellman's test.

### 3.2.1 FTIR analysis

In the chitosan-GSH spectra (Figure 4B), the amide I band ( $1655 \text{ cm}^{-1}$ ) corresponds to native acetyl groups and

## Synthesis and characterization of SNOC as a NO donor

additively to the three peptidic groups added by each GSH linkage (Figure 1, in red).

Consequently, the formula from Baxter *et al.* can be used to determine all the amide bonds of the structure:

$$\% \text{ amide} = (A_{1655}/A_{3450}) \times 115$$

The value from this formula was 53% in our experiments.

This ratio can also be written:

$$\frac{n_{\text{GlcNAc}} + 3n_{\text{GSH}}}{n_{\text{GlcN}} + n_{\text{GlcNAc}} + 3n_{\text{GSH}}} = 53\% \quad (3)$$

where  $n_{\text{GSH}}$  is the number of GSH units per gram.

From Equation 2 and Equation 3, we can obtain the substitution percentage by the GSH moieties:

$$\frac{n_{\text{GSH}}}{n_{\text{GlcN}} + n_{\text{GlcNAc}}} = 12\% \quad (4)$$

### 3.2.2 Structure determination using the Ellman's test

The free thiol concentration (case 1, before reduction, Figure 2) was directly determined after the dialysis of the polymer. Afterward, the amount of GSH linked to the thiol moiety of chitosan was evaluated after  $\text{NaBH}_4$  reduction. The reaction is displayed in Figure 2 and allowed the assessment of total thiols (case 1 + case 2 + case 3 after reduction, Figure 2). The resulting solution was either evaluated directly using HPLC or after an additional dialysis (removal of the released GSH in case 3). The amount of free thiol groups, disulfide bonds and total sulfhydryl groups immobilized on thiolated chitosan can be easily determined and are summarized in Table 1. In the following paragraphs, this polymer is considered as the precursor of SNOC G1.

Based on the chitosan NMR (Section 3.1.1.; % of acetylation: 39%) and chitosan FTIR results (Section 3.1.1.; % of acetylation: 39%), the acetylation percentage was arbitrarily fixed at 37.5% (median value of FTIR and NMR). During GSH linkage,  $n_{\text{GlcNAc}}$  cannot be modified and no matter the  $n_{\text{GSH}}/n_{\text{GlcN}}$  ratio (i.e., efficiency of the GSH linkage), we can write for chitosan-GSH:

$$n_{\text{GlcNAc}}/(n_{\text{GlcNAc}} + n_{\text{GlcN}} + n_{\text{GSH}}) = 37.5\%$$

Hence,

$$0.375 n_{\text{GlcNAc}} + 0.375 n_{\text{GlcN}} + 0.375 n_{\text{GSH}} = n_{\text{GlcNAc}}$$

$$0.375 n_{\text{GlcN}} + 0.375 n_{\text{GSH}} = 0.625 n_{\text{GlcNAc}}$$

From the results of the Ellman's test, we know that the amount of glucosamine-GSH units ( $n_{\text{GSH}}$ ) is  $558 \pm 2 \mu\text{mol/g}$  (Table 1), which corresponds to  $0.251 \text{ g/g}$  (25.1 % w/w).

Therefore, for 1 g of polymer,  $n_{\text{GSH}} = 558.10^{-6} \text{ mol}$  and:

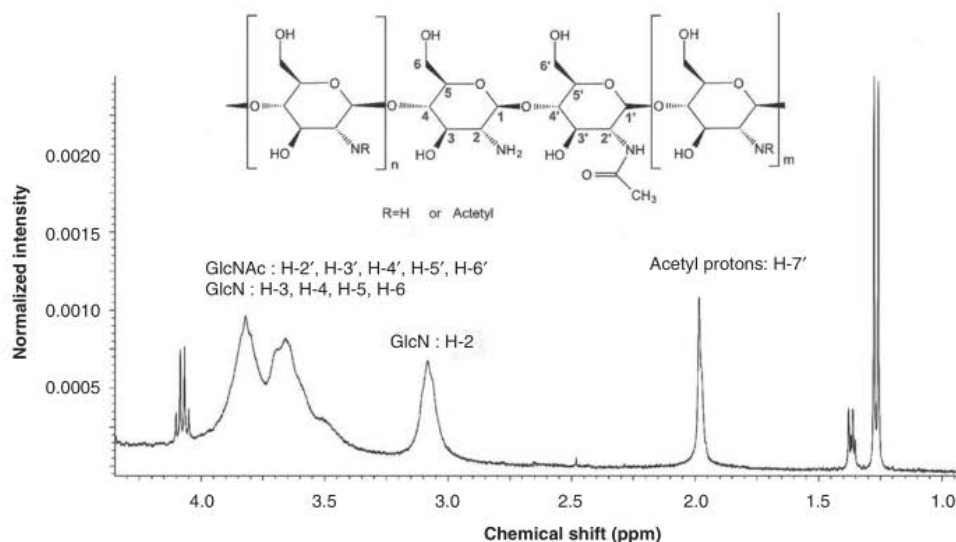
$$0.375 n_{\text{GlcN}} + 0.375 \times 558.10^{-6} = 0.625 n_{\text{GlcNAc}}$$

$$n_{\text{GlcN}} + 558.10^{-6} = 1.66 \times n_{\text{GlcNAc}}$$

$$n_{\text{GlcN}} = 1.66 \times n_{\text{GlcNAc}} - 558.10^{-6}$$

$$n_{\text{GlcNAc}} = (n_{\text{GlcN}} + 558.10^{-6})/1.66$$

$$n_{\text{GlcNAc}} = 0.60 n_{\text{GlcN}} + 334.10^{-6} \quad (5)$$



**Figure 3.**  $^1\text{H}$ -NMR spectra of chitosan-glutathione conjugate. The % acetylation is calculated from the area ratio between [H-7' methyl protons of the N-acetyl group] and [H-2', H-3', H-4', H-5', H-6' protons of GlcNAc residue + H-2, H-3, H-4, H-5, H-6 protons of the GlcN residue].

Based on the results from the Ellman's test, we can determine the weight of glucosamine-GSH units in 1 g of polymer:  $w_{\text{GSH}} = (558.10^{-6} \text{ mol/g} \times 450 \text{ g/mol}) = 0.251 \text{ g/g}$  (25.1% w/w). Consequently, expressed in weight, the remaining weight ( $w_{\text{GlcNAc}} + w_{\text{GSH}}$ ) corresponds to 74.9% w/w.

Therefore,

$$w_{\text{GlcNAc}} + w_{\text{GlcN}} = 0.749 \text{ g/g}$$

$$n_{\text{GlcNAc}} \times \text{mw}_{\text{GlcNAc}} + n_{\text{GlcN}} \times \text{mw}_{\text{GlcN}} = 0.749$$

where  $\text{mw}_{\text{GlcNAc}}$  (204 g/mol) and  $\text{mw}_{\text{GlcN}}$  (161 g/mol) are the MWs of the monomeric units.

$$n_{\text{GlcNAc}} \times 204 + n_{\text{GlcN}} \times 161 = 0.749$$

$$n_{\text{GlcN}} \times 161 = 0.749 - n_{\text{GlcNAc}} \times 204$$

$$n_{\text{GlcN}} = 0.749/161 - n_{\text{GlcNAc}} \times 204/161$$

$$n_{\text{GlcN}} = 4652.10^{-6} - 1.27 n_{\text{GlcNAc}} \quad (6)$$

From Equations 5 and 6, we can find, for 1 g of polymer, that  $n_{\text{GlcNAc}} = 1773.10^{-6} \text{ mol}$  and  $n_{\text{GlcN}} = 2401.10^{-6} \text{ mol}$ .

The final concentrations and percentages [% (n/n)] of this experiment are presented in Table 2. The percentages are also expressed in weight [g/g of polymer and % (w/w)]. Interestingly, the percentage of glucosamine-GSH units (11.8%) is very near the value obtained from FTIR (12%, Section 3.2.1), confirming that both methods can be used for these measurements.

The conjugated polymer (Table 1) had an amount of  $229 \pm 21 \mu\text{mol}$  immobilized as reduced thiols and  $708 \pm 60 \mu\text{mol}$  disulfide bonds per gram of polymer. Previous studies have demonstrated that the degree of substitution can be very high and depends on both the amount of conjugated

species [32] and the concentration of EDAC/NHS [33]. The efficiency of EDAC/NHS in the synthesis process and purification by dialysis was also confirmed by the small amount of thiol groups ( $62 \mu\text{mol/g}$  thiol groups) in the controls (i.e., without EDAC/NHS) compared with the samples that were generated with NHS and EDAC. The free thiol groups and total thiols had similar values as those described by previous studies ( $265 \mu\text{mol/g}$  [33] and  $271 \mu\text{mol/g}$  [34] for free thiol groups and  $397.9 \mu\text{mol/g}$  [33] and  $321.3 \mu\text{mol/g}$  [35] for disulfide bonds). The presence of oxidized disulfide bonds in the polymer may be due to either intra- or intermolecular reactions in the aqueous media. Consequently, during the coupling reaction, we can assume that different length polymers are formed because chitosan can cross-link between the polymeric chains through inter- and intramolecular reactions.

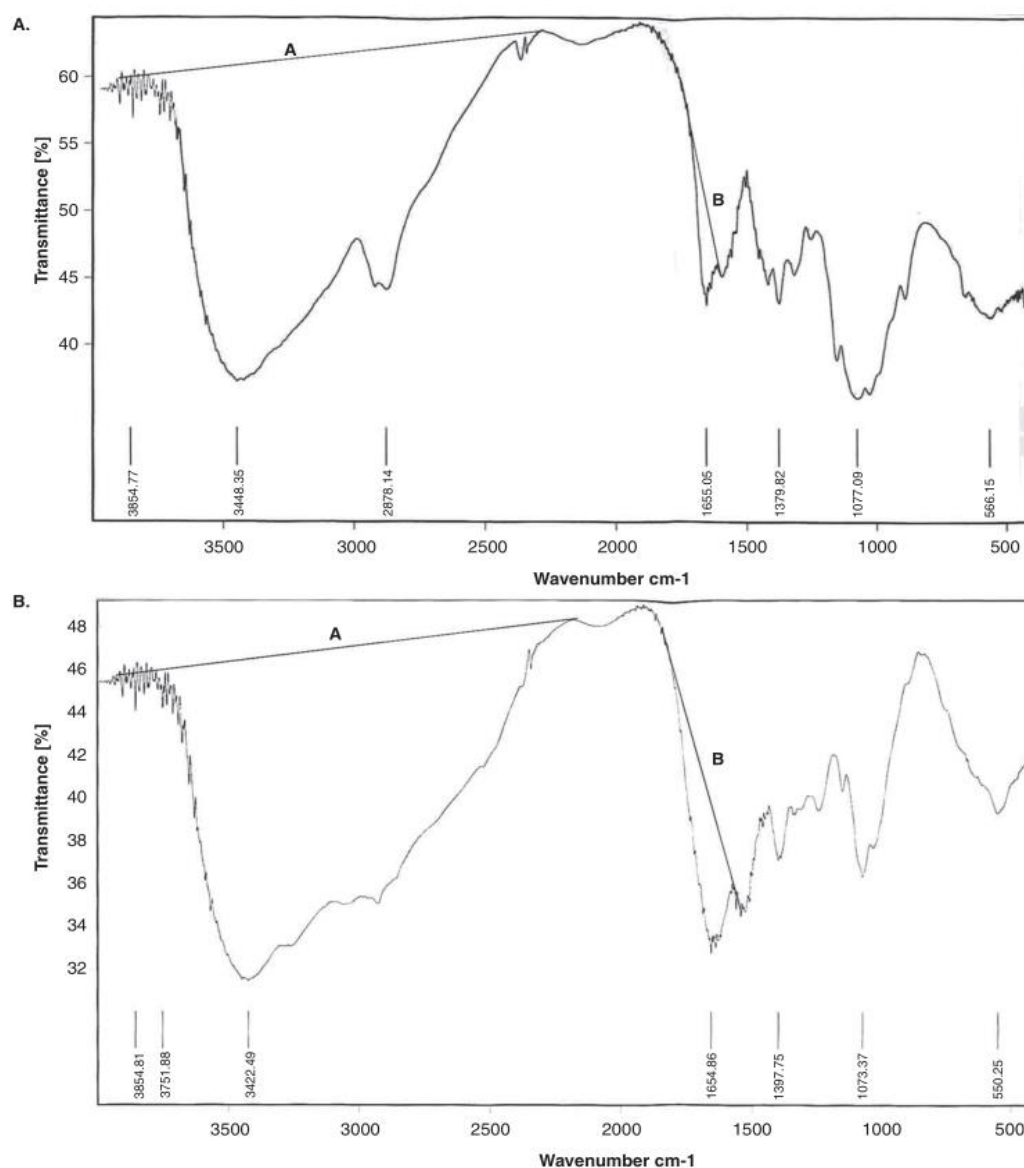
After  $\text{NaBH}_4$  reduction and dialysis, the disulfide bounds ( $709 \pm 60 \mu\text{mol/g}$ , Table 1) should be reduced in -SH groups and thiols that are not directly linked ( $319 \pm 72 \mu\text{mol/g}$ , Table 1) are removed by dialysis. The theoretical composition of the polymer is presented in Table 3.

In the following paragraphs, this polymer will be considered as the precursor of SNOC G2.

### 3.3 SNOC G1 and SNOC G2 characterization

#### 3.3.1 Stability of chitosan with $\text{NaNO}_2$

Before using sodium nitrite as a nitrosation agent, the effect of  $\text{NaNO}_2$  on chitosan depolymerization needed to be studied to prevent the degradation process. It is commonly reported that sodium nitrite induces chitosan degradation (depolymerization)



**Figure 4.** FTIR spectra used for % acetylation determinations. A. Chitosan; B. Chitosan GSH. GSH: Glutathione.

to form smaller polymer sizes by attacking the glucosamine moiety, but not the N-acetylglucosamine moieties, and cleave the anhydroglycosidic linkage with release of  $N_2$  [35-38]. In fact, a linear relationship between  $1/[NaNO_2]$  and the final polymer size has been established [35]. Moreover, it has been described

that depolymerization forms more homogenous polymer size populations. This has been attributed to the proportionality of the number of breaks to the length of the macromolecular chain; the longer the chitosan chain, the higher the probability of its oxidative breakdown by sodium nitrite [35]. As shown



**Table 1. Amount of glutathione (SH groups) determined by Ellman's test – before reduction (precursor of SNOC G1).**

Glutathione fraction (see Figure 2 for the different cases)	Ellman's method (μmol/g)
Reduced thiols ( <b>case I</b> )*	229 ± 21
Total thiols ( <b>cases I, II, III</b> )*	937 ± 72
Disulfide bounds ( <b>cases II,III</b> ) <sup>‡</sup>	708 ± 60
Directly linked thiols ( <b>in bold</b> )*	558 ± 2
Not directly linked thiols ( <b>in italic</b> ) <sup>‡</sup>	379 ± 72

\*Obtained experimentally.

<sup>‡</sup>Obtained by calculation.

SNOC: S-nitrosoglutathione-oligosaccharide chitosan.

**Table 2. Amounts and percentages of each glycosidic unit in the chitosan-GSH – from Ellman's test.**

Monomeric units in Chitosan-GSH	Amount (μmol/g of polymer)	Percentage % (n/n)	Amount (g/g of polymer)	Percentage % (w/w)
Glucosamine (GlcN)	2401	50.7%	0.387	38.7%
N-acetylglucosamine (GlcNAc)	1773	37.5%	0.362	36.2%
Glucosamine-glutathion (GSH)	558	11.8%	0.251	25.1%
Total	-	100.0%	1.000	100.0%

**Table 3. Amount of glutathione (SH groups) determined by Ellman's test – before reduction (precursor of SNOC G2).**

Glutathione fraction (see Figure 2 for the different cases)	Ellman's method (μmol/g)
Reduced thiols ( <b>case I</b> )	937 ± 72
Total thiols ( <b>cases I, II, III</b> )	937 ± 72
Disulfide bounds ( <b>cases II,III</b> )	-
Directly linked thiols ( <b>in bold</b> )	937 ± 72
Not directly linked thiols ( <b>in italic</b> )	-

SNOC: S-nitrosoglutathione-oligosaccharide chitosan.

in Figure 5, no significant loss in total nitrites was observed compared with controls composed of either GSH/NaNO<sub>2</sub> or NaNO<sub>2</sub> alone in the presence of chitosan and/or GSH with or without NaBH<sub>4</sub>. Additionally, NaNO<sub>2</sub> consumption was similar in the presence or absence of chitosan. Finally, it has also been reported that nitrosation preferably occurs in the thiol groups rather than the amine groups [35]. Consequently, any minor degradation that could occur would be prevented by the disposition of the thiol groups in the polymer. As a result, because no degradation was observed using this methodology, the nitrosation of the thiol polymer with sodium nitrite was pursued.

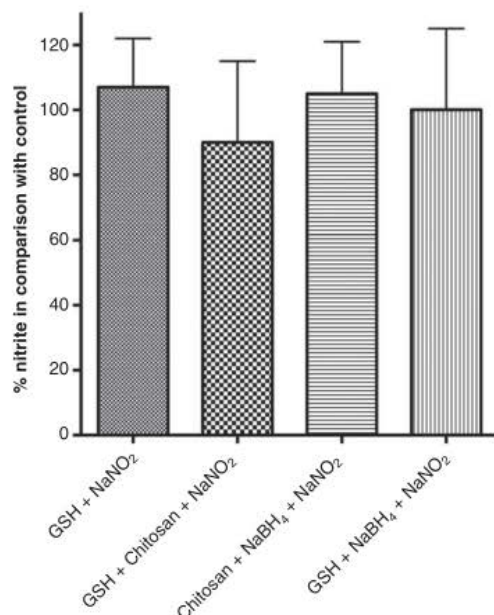
### 3.3.2 Quantification of nitrosation efficiency (SNOC G1 and SNOC G2)

The thiol nitrosation was performed using two techniques, depending on the degree of nitrosation. The direct thiol

nitrosation (freely reduced thiol groups on the chitosan backbone) resulted in SNOC G1. As shown in Table 4, the degree of thiol chitosan nitrosation achieved was 159.04 ± 64.16 μmol/g (by Griess-Saville method) of dried powder. However, the thiol chitosan nitrosation after sodium borohydride reduction resulted in SNOC G2. SNOC G2 had a higher degree of S-NO groups (525.08 ± 151.35 μmol/g by Griess-Saville) and no (expected) disulfide bonds between the polymer and GSH as observed in SNOC G1. Consequently, high NO storage was achieved with a nitrosation efficiency of 56% for SNOC G2.

### 3.3.3 SNOC stability

SNOC G1 stability at 37°C in pH 1.2, pH 6.8 and pH 7.4 was determined using fluorimetry (Figure 6A). To enhance the NO measurement sensitivity, the use of the aromatic diamino-compound (DAN) as a fluorescence indicator



**Figure 5. Effect of NaNO<sub>2</sub> on chitosan.** The total nitrites consumption was determined using the Griess and Saville method. The values are expressed as percentages compared with NaNO<sub>2</sub> solution ( $n = 5$ , mean  $\pm$  s.d.). None of the comparison reached the statistical significance ( $p > 0.05$ , Mann and Whitney).

GSH: Glutathione.

**Table 4. Amount of nitrites determined by the Griess and Saville method.**

	SNOC G1	SNOC G2
Nitrites ( $\mu\text{mol/g}$ )	$159 \pm 64$	$525 \pm 151$

provided more reliable results than the Griess-Saville method. Using this method, very dilute samples could be analyzed. The effect of pH on GSNO degradation has been reported to not induce differences until 5 days in neutral and acidic media [39], and our results are consistent with this observation.

As shown in Figure 6B, SNOC G1 was less stable than SNOC G2 at 4°C.

An explanation for this result may come from the ratio of [NO groups]/[SH groups] of each polymer. It has been demonstrated that when GSNO was allowed to react with GSH, the only product detected by HPLC was GSSG (dimer of GSH) after 40 h at pH 7.4 [40]. Therefore, GSNO decomposition can be increased by GSH addition, and generally, the

## Synthesis and characterization of SNOC as a NO donor

reaction of thiol radicals with molecules bearing -SNO moieties (RSNO) accelerates the decomposition reaction in concentrated solutions (Figure 7).

The oxidation of thiol groups to form disulfide was also observed in the immobilized thiol groups in the polymers [33,41]. Thiol groups that are closely located may form disulfide bonds more rapidly than the remaining isolated thiol groups [33].

RSNO decomposition occurs by the cleavage of S-N bonds to give NO and an alkyl thiol radical that may then react with another molecule of S-NO, generating the disulfide and an additional NO molecule [16]. Additionally, these free thiols could reduce the disulfide bonds in SNOC G1, which are not present in SNOC G2. Therefore, these free thiols could occasionally release free GSH, which would further attack the -SNO links, releasing additional NO and again forming disulfide bonds with the polymer.

### 3.3.4 Intestinal drug permeation studies: Ussing chamber

As previously stated, the oral absorption of NO or NO-derivatives compounds is important for further development of these types of polymers.

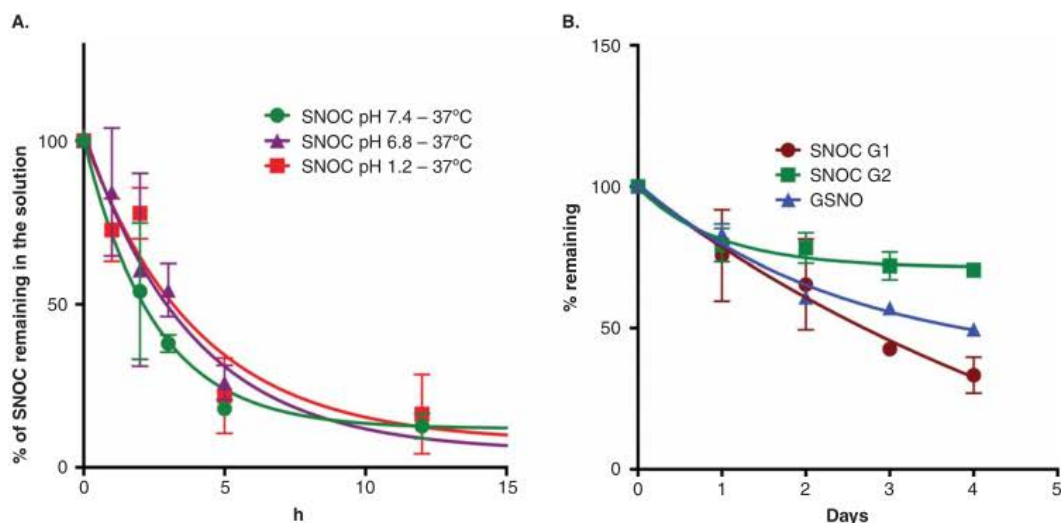
The primary indication that could be orally treated is IBD, particularly Crohn's, which affects the end of the small bowel (the ileum) and the beginning of the colon but may affect any part of the gastrointestinal tract from the mouth to the anus. For example, GSNO prevents epithelial barrier failure after injury and can improve the expression of tight junction proteins [42]. To date, *in vitro* assessments have been performed in epithelial monolayers [42], but it is important to develop an adapted formulation to facilitate *in vivo* stage research.

Our *ex vivo* experiment was first developed to assess the SNOC G2 polymer (Figure 8), which proved to have the best nitrosation rate. It was also interesting to assess GSNO, which was able to deliver small amounts of NO by the oral route. Khan *et al.* [43] have demonstrated that GSNO (50  $\mu\text{g/kg}$ ) administered orally to rats can increase the NO concentration in the brain.

Therefore, we used GSNO absorption as the reference in our Ussing chamber model. Figure 9A shows that the release kinetics in the donor compartment has a duration of 5 h. In the Ussing chamber, nitrites ( $\text{NO}_2^-$ ), which can be considered a marker of NO formation [44], are released in the first hours and remain in the compartment throughout the experiment.

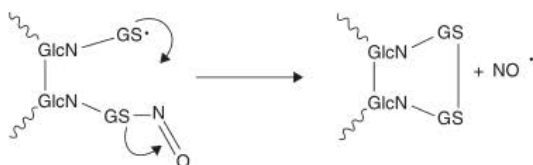
Because the acceptor and the donor chambers have the same volume (8 ml), an equal distribution would lead to a theoretical value of 50% for  $\text{NO}/\text{NO}_2^-$  and  $\text{NO}_3^-$  (corresponding to the horizontal dotted lines on Figure 9A). The concentration of nitrates ( $\text{NO}_3^-$ ), which can be considered the final product, increases throughout the experiment to a maximal value of 50% after 6 h (Figure 9A and B). In the acceptor compartment, we observed that all species ( $\text{NO}_2^-$ ,  $\text{NO}_3^-$  and GSNO) were able to cross the intestinal barrier (Figure 9B).





**Figure 6. Stability.** A) SNOC G1 at + 37°C and various pH values; B) GSNO, SNOC G1 and SNOC G2 at + 4°C and pH 7.4 (n = 3, mean ± s.d.).

GSNO: S-nitrosoglutathione; SNOC: S-nitrosoglutathione-oligosaccharide chitosan.



**Figure 7. Proposed scheme showing the influence of proximal thiols in the NO release process from nitrosothiols.**

In fact, the inorganic anions nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ) were previously thought to be inert end products of endogenous NO metabolism. These compounds are primarily known as undesired residues in the food chain that have potentially carcinogenic effects. However, recent studies have shown that these compounds can be recycled *in vivo* to form NO, representing an important alternative source of NO outside of the classical L-arginine–NO-synthase pathway, particularly in hypoxic states [45]. It is possible that  $\text{NO}_2^-$  and  $\text{NO}_3^-$  are a significant precursors and are related to the increase of NO observed by Khan in the brain [43].

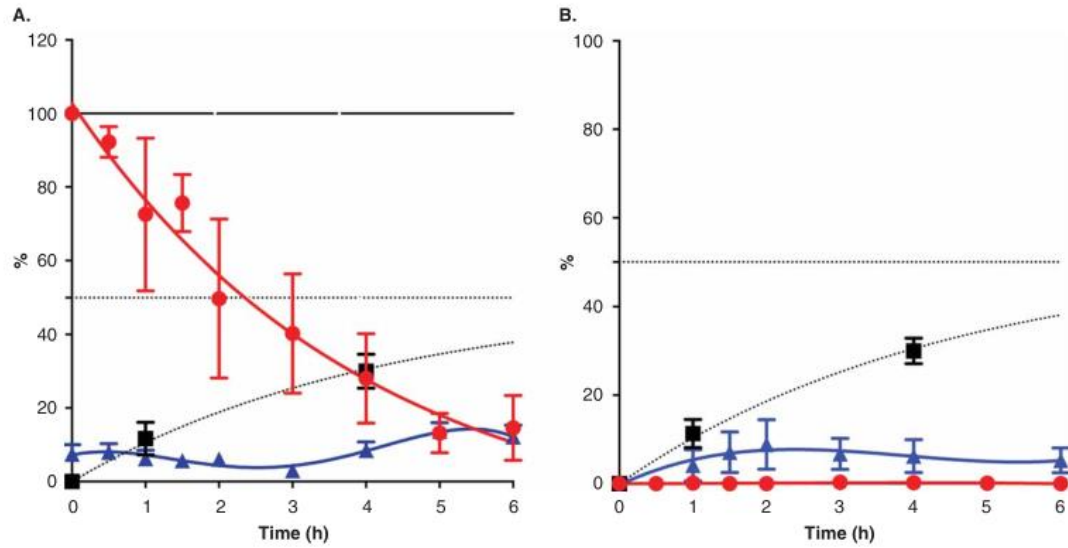
Compared to GSNO, SNOC G2 has several advantages. It could be easily formulated in polymeric microparticles (e.g., polyelectrolyte formation between chitosan and Eudragit®) [46] to produce sustained release formulations. Figure 8A shows that the SNOC G2 release kinetics in the donor compartment last for over 6 h (Figure 8A). These sustained release characteristics are slightly improved compared with GSNO;  $\text{NO}/\text{NO}_2^-$  remains high after 6 h in the acceptor chamber (Figure 8B).

Moreover, SNOC G2 is not able to cross the intestinal barrier (Figure 8B). However, after release,  $\text{NO}_2^-/\text{NO}$  and  $\text{NO}_3^-$  can be observed in the acceptor chamber, which is a potential, but unintended, source of NO.

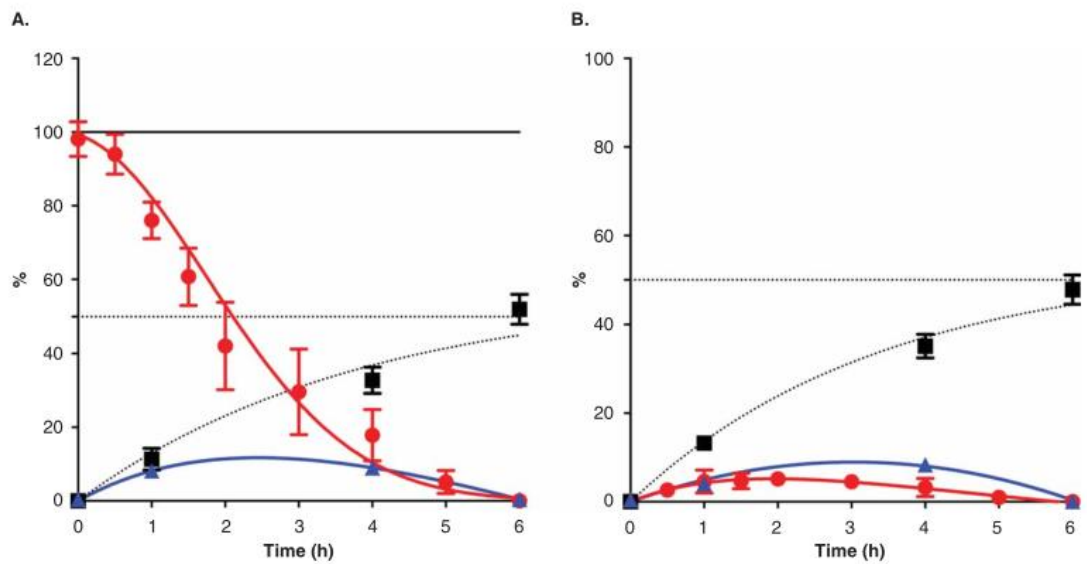
#### 4. Conclusions

The SNOC G2 polymer, as a delivery agent, was able to carry significant amounts of NO with an interesting sustained release effect. There was no direct absorption of GSNO (Figure 8B) but only a local delivery of NO. Chitosan allowed a high degree of substitution, which is able to be modulated.

SNOC G2 was chosen for *ex vivo* studies and produced an NO release for > 6 h (Figure 8A):  $20 \pm 10\%$  of SNOC was still remaining in the chamber after 6 h, whereas all GSNO was degraded within 6 h (Figure 9A). This difference was significant ( $p < 0.05$ , Mann and Whitney). We believe that this type of polymer is adapted for the development of various formulations, including microparticles [46]. After GSH linkage, the polymer becomes more hydrophilic and this property depends on the degree of GSH substitution. For example, chitosan could be acetylated or deacetylated before the thiolation to modulate hydrophobicity. These formulations cannot prevent the production of  $\text{NO}_3^-$  but can slow its production. The impact on blood pressure remains to be tested but this need usually a very high dosage of RSNO. Results obtained by Khan *et al.* [43] showed no alteration in mean arterial blood pressure and heart rate of rats at  $50 \mu\text{g}$  of GSNO/kg body weight.



**Figure 8.** Concentrations of SNOC G2 (●), NO<sub>2</sub>/NO (▲) and NO<sub>3</sub> (■) as the percentage of the initial concentration of the donor chamber (100 μM). A) Donor chamber; B) acceptor chamber (n = 3, mean value ± s.d.). SNOC: S-nitrosoglutathione-oligosaccharide chitosan.



**Figure 9.** Concentrations of GSNO (●), NO<sub>2</sub>/NO (▲) and NO<sub>3</sub> (■) as the percentage of the initial concentration of the donor chamber (100 μM). A) Donor chamber; B) acceptor chamber (n = 3, mean value ± s.d.). GSNO: S-nitrosoglutathione.

Organic nitrites, that is, trinitrine, are completely absorbed after oral administration and NO release occurs in the blood stream. This mechanism avoids direct contact with intestine.

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## Declaration of interest

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## **Chapter 2: Synthesis of S-nitrosoglutathione-alginate for prolonged delivery of nitric oxide in intestines**

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## Synthèse de l'alginate S-nitrosoglutathion pour la délivrance prolongée de monoxyde d'azote dans l'intestin

### Résumé

Dans cette étude, nous avons développé un nouveau donneur de NO (l'alginate-S-nitrosoglutathion, SNA) en couplant de l'alginate avec du S-nitrosoglutathion. Comme dans la méthode précédente, ce nouveau composé a été préparé en utilisant une procédure à deux étapes incluant (I) la liaison de GSH à l'alginate (II) et une post-nitrosation grâce au nitrite de sodium ( $\text{NaNO}_2$ ). La quantité de fonctions thiols (disponibles pour la nitrosation) a été calculée en utilisant la méthode d'Ellman, et la quantité de « NO fixés » sur le polymère a été calculée en utilisant la méthode de Griess-Saville. Un modèle *ex vivo* (intestin monté sur une chambre de Ussing) a été utilisé pour étudier la pénétration du NO et de ses dérivés à travers la barrière intestinale du rat.

Nous avons utilisé différentes procédures et la quantité de « NO fixés » était différente en fonction de la méthode ( $174 \pm 21 \mu\text{mol/g}$  pour la méthode « SNA F1 », sans réduction préalable des fonctions thiols et  $468 \pm 23 \mu\text{mol/g}$  pour la méthode « SNA F2 » avec réduction préalable des fonctions thiols).

Dans les études *ex vivo* sur chambre de Ussing, le SNA F2 montre une libération prolongée de plus de 10h. L'effet du pH sur la stabilité de ce nouveau composé a lui aussi été étudié, il s'est montré plus stable à pH légèrement basique (pH 8,4 environ) : 73% de « NO fixé » restaient après une semaine. Cependant, seulement 50% restaient après une semaine à pH acide (pH 1,2).

Dans les études de cytotoxicité (effets sur des cellules Caco-2 intestinales isolées d'adénocarcinome), ce composé n'était pas toxique à des concentrations inférieures à  $10 \mu\text{M}$  de NO  $\mu\text{M}$ . Ce résultat en fait un polymère particulièrement intéressant pour l'administration de NO par voie orale.

## Abstract

S-nitrosothiols are a class of NO-donors currently under investigation for the treatment of various diseases. These NO-donors are valuable due to their fast decomposition rates. However, poor distribution to the targeted site is a serious obstacle to their potential widespread therapeutic use. In this study, we developed a novel NO-donor (S-nitrosoglutathione-alginate, SNA) by cross-linking alginate with S-nitrosothiols, which can deliver NO in a sustained manner. This compound can be further evaluated for oral delivery to treat Crohn's disease. This new compound was prepared using a two-step procedure involving (I) linkage of reduced glutathione (GSH) to alginate and (II) post-nitrosation with sodium nitrite ( $\text{NaNO}_2$ ). The amount of linked thiol moieties for the possible nitrosation was calculated using Ellman's method, and the amount of NO abducted on the polymer was calculated using the Griess-Saville method. An *ex vivo* model (i.e., Ussing chamber) was used to investigate the permeation of this new NO-donor across the rat intestinal barrier. We obtained polymers with different numbers of abducted NOs ( $174 \pm 21 \mu\text{mol/g}$  for SNA F1 and  $468 \pm 23 \mu\text{mol/g}$  for SNA F2) depending on the procedure used for nitrosation. In the *ex vivo* studies in the Ussing chamber, SNA F2 exhibited a sustained release for at least 10 h. The effect of pH on the stability of the new compound was also investigated, and the new compound was more stable at a mildly basic pH of 8.4 where 73% remained after 1 week. However, only 50% remained after 1 week at an acidic pH of 1.2. In the cytotoxicity studies (Caco2), this compound was non-toxic at  $10 \mu\text{M}$  NO and exhibits 100% metabolic activity in the cells after 24 h of incubation.

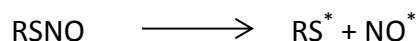
**Keywords:** nitric oxide, alginate, polymer, Crohn's disease, oral route

## 1. Introduction

Nitric oxide (NO) is the smallest endogenous molecule and a free radical gas (Saraiva et al., 2011). NO was first introduced in 1987 (Ignarro et al., 1981; Jerca et al.) and received medical and scientific interest in 1992 when it was nominated as molecule of the year (Friedman and Friedman, 2009). NO is produced by endothelial cells of the body and performs different

physiological functions. NO is released as NO\* or bound to –SH groups containing carrier molecules that stabilize its release (Kilbourn, 1996; Moncada et al., 1991).

To evaluate the biological and physiological functions of NO, exogenous sources of NO-donors are required that can be used both as a drug and a research tool (Saraiva et al., 2011). A variety of NO-donors are available and pharmacologically active as NO releasing drugs including S-nitrosothiols (RSNOs) (Saraiva et al., 2011). In fact, due to the ability to act as NO-donors, RSNOs have attracted considerable attention. RSNOs are involved in different reactions in the bioregulatory system based on NO transfer (Singh et al., 1996b). RSNOs are more stable than NO, which has a very short *in vivo* half-life (0.1-15 s). For example, S-nitrosogluthathione (GSNO) decomposes in hours (Josephy et al., 1984; Shin and George, 2001). Some RSNOs, such as GSNO and S-nitrosoalbumin, are endogenous NO carriers in mammals (Seabra et al., 2005). NO is covalently attached to a sulfur atom in the R-S-NO molecule and can be released via S-N bond cleavage to transfer NO to specific receptors for *in vivo* biological effects (Seabra et al., 2005).



Chemically, RSNOs have been synthesized by reacting NO with compounds possessing thiol groups, such as reduced L-glutathione (GSH) and albumin. Then, these compounds are transported to the reaction sites where its corresponding biological effect is achieved by release of NO (Singh et al., 1996a). The most commonly used RSNOs are GSNO and S-nitroso-N-acetyl-D,L-penicillamine (SNAP), which exhibit remarkable biological effects [e.g., GSNO offers significant protection to myocardial ischemia and SNAP is a powerful vasodilator (Ignarro et al., 1981)].

Several other NO-donors, such as nitroglycerine and isosorbide dinitrate, rapidly release NO but can cause oxidative stress and frequent tolerance. Therefore, to minimize these problems, research is now focused on innovative NO-donors such as RSNOs and especially GSNO. GSNO is considered a lead molecule due to its safety (NO released is accompanied by GSH production). Therefore, GSNO is used to investigate the biochemical and pharmacological mechanisms of NO and its therapeutic applications (Parent et al., 2013). S-nitroso compounds, such as GSNO, have a specific thiol coupling capability that allows it to store and carry NO and offers the advantage of preventing the development of tolerance in patients. These compounds can be used in

different disease states, such as cardiovascular disorders (Everett et al., 2014), brain ischaemia (Khan et al., 2009), cancer (Furuhashi et al., 2012), cystic fibrosis (Servetnyk et al., 2011), and wound healing (Georgii et al., 2011). In addition, these compounds may be beneficial in the treatment of Crohn's disease (Savidge et al., 2007). In the recent years, GSNO has been proposed for the treatment of Crohn's disease to prevent mucosal barrier failure. GSNO significantly promotes human intestinal mucosal barrier function, while this effect was lacking in patients with no inflammatory bowel disease conditions (Savidge et al., 2007). This result may be due to the enteric glial cell network being disrupted in non-inflamed Crohn's disease intestinal mucosa. Therefore, the tissue GSNO concentration levels might be lower in these patients (Shah et al., 2015b).

NO-donors can also be chemically linked to biopolymers, which can provide site specific delivery of NO (Saraiva et al., 2011). Therefore, the aim of our study was to synthesize a new stable polymer by linking GSNO to alginate, and this compound was evaluated for the treatment of Crohn's disease.

## **2. Materials and methods**

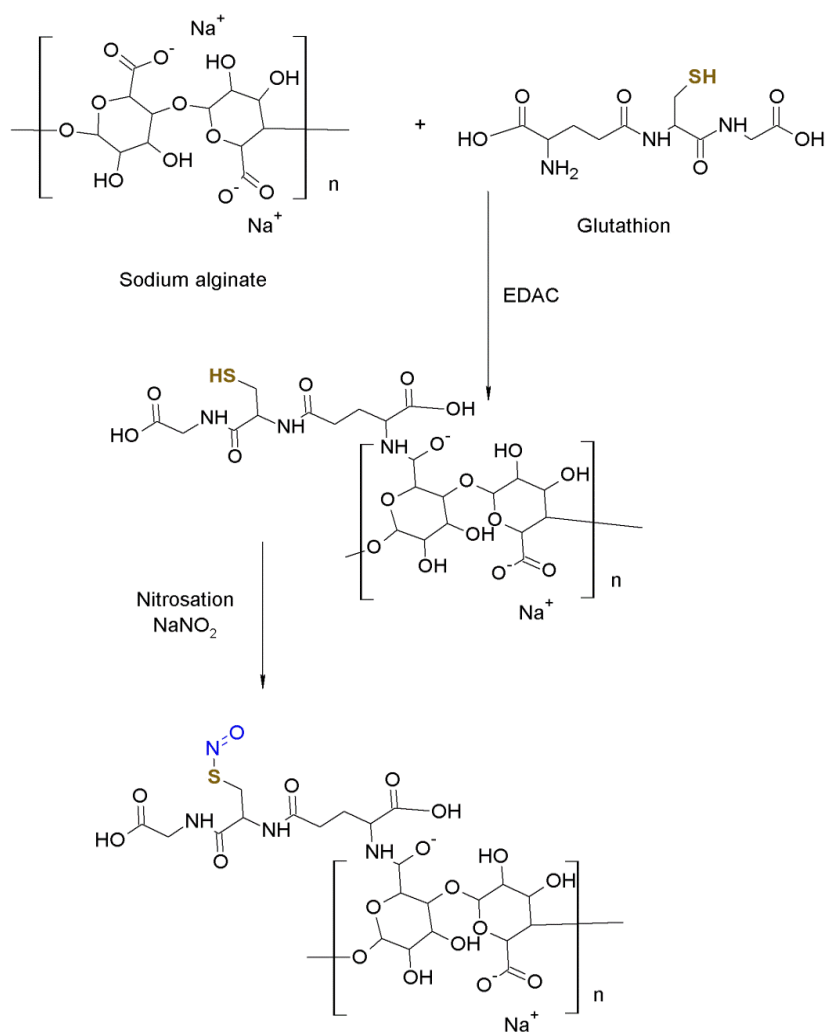
### **2.1. Materials**

GSH, sodium alginate, Ellman's reagent (5,5'-dithiobis (2-nitro benzoic acid), 2,3-diaminonaphthalene (DAN), *N*-1-(naphthyl)ethylenediamine (NED), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and sulfanilamide were obtained from Sigma-Aldrich (Quentin Fallavier, France). Sodium nitrite and potassium hydrogen phosphate were purchased from Merck (Germany), and "total nitric oxide and nitrate/nitrite kit" was obtained from R&D systems (Abingdon, UK). All of the other chemicals were of analytical or chromatographic grade.

### **2.2. Synthesis and purification of the alginate-GSH conjugate**

GSH was covalently attached to sodium alginate via amide bonds between the carboxylic acid groups of the polymer and the amine groups of GSH (Figure 1) according to a previously reported method (Bernkop-Schnurch et al., 2001). Briefly, 10 g of sodium alginate was hydrated

in 1000 ml of ultrapure demineralized water. For the amidation reaction, the carboxylic groups of the polymer were activated by addition of EDAC in a final concentration of 50 mM. The reaction was allowed to continue for 45 min at room temperature. Then, 5 g of GSH in a polymer to drug ratio of 2:1 was added, and the pH was adjusted to 4 with HCl (1 M). This mixture was continuously stirred at 200 rpm for 2 h at room temperature. The pH was readjusted to 6 with NaOH (1 M), and the reaction was allowed to continue for 1 h.



**Fig. 1: Reaction scheme for the synthesis of SNA. 1<sup>st</sup> step: synthesis of an alginate-glutathione conjugate. 2<sup>nd</sup> step: nitrosation of alginate-glutathione conjugate.**

The resulting conjugate was dialyzed in tubing (molecular weight cut-off 12 kDa) against 1 mM HCl, twice against HCl (1 mM) containing 1% NaCl and then against HCl (1 mM) for 5 h. The

controls were prepared in the same way except using EDAC for the coupling reactions. The resulting conjugate was freeze-dried at -50 °C and 0.01 mbar followed by storage at 4 °C prior to use.

### **2.3. Determination of the thiol group content of various fractions of thiols after synthesis (Ellman's method)**

The thiol group content was determined using previously reported methods with slight modifications (Atyabi et al., 2008; Yousefpour et al., 2011). Briefly, 0.5 mg of the polymer conjugate was hydrated in 0.35 ml of deionized ultrapure water for 30 min and diluted to a final volume of 1 ml by adding 0.65 ml of Tris-HCl. This solution was labelled solution-1.

#### **2.3.1. Reduced thiols**

To a 100 µl of solution-1, 900 µl of phosphate buffer (0.1 M, pH 7.5) and 200 µl of Ellman's reagent (0.3 mg/ml) were added and incubated for 15 min in the dark at room temperature. By using a UV-Visible spectrophotometer (Cary® 50 UV-Vis, Varian, Les Ulis, France), the absorbance was measured at 412 nm. The reduced thiol content was determined from a standard calibration curve of GSH with concentrations ranging from 3.25 to 32 µM.

#### **2.3.2. Total thiols**

For the determination of the total thiol groups either in their reduced form or disulfide bond form, 1 ml of NaBH<sub>4</sub> (4% solution w/v) was added to 1 ml of solution-1 followed by incubation at 37 °C for 1 h. Then, this solution was neutralized with 200 µl of HCl (5 M). After 10 min of treatment with HCl, 1 ml of phosphate buffer (0.1 M, pH 7.5) was added followed by the addition of 200 µl of Ellman's reagent. The solution was incubated in the dark for 15 min at room temperature, and the amount of total thiol groups was determined using a UV-Visible spectrophotometer.



### **2.3.3. Thiol groups directly linked to alginate**

The amount of thiol groups directly linked to alginate was determined using the same procedure that was mentioned above for the total thiol content. After a reducing pre-treatment (1 ml of solution-1 with 1 ml of  $\text{NaBH}_4$ ) and neutralization, the polymer was dialyzed in tubing (molecular weight cut-off 12 kDa) for 4 h in distilled water to remove the free thiol groups. The amount of free thiol groups were determined by subtracting the number of thiol groups directly linked to the polymer from the total number of thiol groups.

### **2.4. Nitrosation of alginate-GSH conjugates (SNA)**

NO was linked to the alginate-GSH conjugate via nitrosation with  $\text{NaNO}_2$  under acidic condition. Two methods were used to achieve nitrosation.

#### **2.4.1. SNA-F1**

Briefly, 100 mg of the alginate-GSH conjugates were hydrated in 45 ml of HCl (0.5 M) for 30 min. To this alginate-GSH conjugate solution, 1 ml of a  $\text{NaNO}_2$  solution (equimolar to the reduced thiols in alginate-GSH conjugates) in ultrapure water was added. The reaction was continued for 2 h at 4 °C in the dark and then neutralized with a NaOH solution (40% w/v).

#### **2.4.2. SNA-F2**

This method includes the nitrosation of all of the thiol groups (free and linked thiols) present in alginate-GSH conjugate. Prior to nitrosation of the polymer, the disulfide bonds were broken by the addition of  $\text{NaBH}_4$  followed by treatment with HCl (5 M) to remove the unwanted  $\text{NaBH}_4$ . Briefly, 100 mg of the alginate-GSH conjugate was wetted in 8.75 ml of ultrapure water and then diluted with 16.25 ml of Tris-HCl for 30 min to achieve complete hydration. To this solution, 5 ml of  $\text{NaBH}_4$  (4% w/v) was added and incubated for 1 h at 37 °C to break the disulfide bonds (R-S-S-R) present between the thiol groups in the conjugate solution.

After 1 h of incubation, the remaining  $\text{NaBH}_4$  was eliminated by addition of 4 ml of HCl (5 M) followed by incubation for an additional 15 min under the same conditions. The final solution was cooled by adding 25 ml of HCl (0.5 M, 4 °C). To this solution, an equimolar  $\text{NaNO}_2$  amount (equal to total amount of thiol groups present in the conjugate solution) was added, and this solution allowed standing in the dark at 4°C for 2 h. The reaction was terminated by neutralizing it by adding NaOH (40% w/v). This solution was dialyzed for 4 h in ultrapure water using dialysis tubing (molecular weight cut-off 12 kDa) followed by storage at 4 °C in the dark prior to use.

## **2.5. Determination of the $\text{NO}/\text{NO}_2^-$ concentration (Griess-Saville method)**

An indirect method was employed for the determination of  $\text{NO}/\text{NO}_2^-$ , as described by Griess and Saville in 1958 (Heines, 1958; Saville, 1958). In a two-step diazotation reaction, dinitrogen trioxide ( $\text{N}_2\text{O}_3$ , NO derived agent) generated from the acid-catalysed formation of nitrous acid from nitrite reacts with sulfanilamide to produce a diazonium ion. Then, this diazonium ion couples to N-(1-naphthyl) ethylenediamine to form a chromophoric azo product that can be detected at 540 nm. The concentration of the NO moieties was calculated from standard calibration curves of  $\text{NaNO}_2$ . The limit of detection for these analytical experiments was 0.25  $\mu\text{mol/l}$ , the limit of quantification was 0.77  $\mu\text{mol/L}$  and the correlation coefficient was 0.999. The method described by Saville (Saville, 1958) is based on a pre-treatment using mercuric chloride ( $\text{HgCl}_2$ ) that induces cleavage of RSNOs to form nitrite. This method allows for the determination of the total amount of  $\text{NO}/\text{NO}_2^-$ . For these experiments, the limit of detection was 0.11  $\mu\text{mol/l}$ , the limit of quantification was 0.35  $\mu\text{mol/l}$  and the correlation coefficient was 0.999.

In the experiment, 20  $\mu\text{l}$  of the sample was added to an acetic acid buffer (pH 2.5), and the final volume was adjusted to 200  $\mu\text{l}$ . To determine the nitrite concentration, 40  $\mu\text{l}$  of sulfanilamide (free nitrites) or sulfanilamide+  $\text{HgCl}_2$  (total nitrites) was added and incubated in the dark for 3 min. After incubation, 10  $\mu\text{l}$  of a NED solution was added and incubated in the dark for an additional 5 min. The nitrite concentration was determined using a microplate reader (Elx800 Absorbance Microplate Reader, BioTek, Colmar, France) or UV-Visible spectrophotometer (Cary® 50 UV-Vis, Varian, Les Ulis, France). The concentration of the RSNOs (GSNO and SNA) was calculated using the following formula:

$$\text{RSNO's} = (\text{total nitrites}) - (\text{free nitrites})$$

## **2.6. Stability of SNA F1 and SNA F2 in the Ussing chamber**

The effect of the experimental conditions (temperature and O<sub>2</sub>/CO<sub>2</sub>) on the stability of SNA-F2 was investigated in one compartment of the Ussing chamber. A 100 µM SNA solution in Hank's buffer was added to the donor compartment of the Ussing chamber that was separated from the acceptor compartment with a folded plastic film, consisting of several layers, to prevent flow to the other side. The temperature was adjusted to 37 °C, and the solution was circulated by a constant flow of O<sub>2</sub>/CO<sub>2</sub>. The samples were withdrawn at regular time intervals (i.e., 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9 and 10 h) and analysed using a UV-Visible spectrophotometer.

## **2.7. Effect of pH on the storage conditions of SNA F2**

The SNA F2 solution was stored at 3 different pH values (acidic pH 1.2, neutral pH 7.0 and basic pH 8.4) and 4 °C in the dark. The effect of pH was assessed every 2 days by calculating the amount of free and linked nitrites. The results were compared to the initial concentration of SNA F2 and are expressed as percentages.

## **2.8. Intestinal permeation studies in the Ussing Chamber**

### **2.8.1. Preparation of intestinal segments**

Male Wistar rats (RjHan: WI, 400 g, 8-10 weeks old, males, Charles Rivers) were killed with 5% halothane in oxygen gas. Segments from the distal portion of the ileum were excised and washed in a beaker containing Krebs's bicarbonate Ringer (KBR, 4 °C) bubbled with an O<sub>2</sub>/CO<sub>2</sub> mixture to maintain membrane viability. These segments were flushed internally with approximately 20 ml of KBR and slid onto glass rods with tapered points wetted with KBR. These rods with intestinal segments were placed in buffer chilled in an ice bath at 3-4 °C. The *serosa* and *muscularis externa* were removed with tweezers according to the method described by Wolfe et al. (1973). The segments were opened along the mesenteric border and mounted on the Ussing chamber (CHM8, 0.5 cm<sup>2</sup> WPI, Hitchin, UK) equipped with a water jacket (37 °C).

### **2.8.2. Permeability studies through the rat intestine (Ussing chamber)**

Eight millilitres of the Hank's BSS buffer without phenol red was added to both the donor and acceptor compartments of the Ussing chamber with the intestinal segment mounted between them. The temperature was maintained at 37 °C, and the flow in both chambers was controlled by O<sub>2</sub>/CO<sub>2</sub>. The trans-mucosal electrical resistance (TER) was monitored by current electrodes (WPI, Hitchin, UK) fixed in the two compartments. The tissues with TER < 30 Ωcm<sup>2</sup> were discarded. A 100 µL sample was withdrawn from the donor and acceptor compartments at appropriate time intervals (starting from 0 to 6 h) to calculate the SNA concentrations using the Griess-Saville method. Nitrite and nitrate ions, which are degradation products of SNA, were measured by Griess and Saville method and by "Total nitric oxide and nitrate/nitrite kit" (R&D systems), respectively.

### **2.9. Cytotoxicity study on the Caco2 cell line**

The cytotoxic activity of SNA F2 was tested on the Caco2 cell line. The Caco2 cell assay is based on the reduction of tetrazolium dye MTT by mitochondrial succinate dehydrogenase to its insoluble form (i.e., formazan), which has a purple colour and is quantified spectrophotometrically at 570 and 630 nm using a microplate reader. Briefly, the Caco2 cells at a concentration of 20.10<sup>3</sup> cells per well in 200 µl of medium were seeded into a 96-well culture plate. After 24 h, various concentrations of SNA F2 (1 to 200 µM) were added to the culture plates and incubated for an additional 24 h at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere. Afterwards, 50 µl of MTT (5 mg/ml stock solution in HBSS buffer) was added to each well in the culture plate and incubated for an additional 3 h at 37 °C. The medium was removed, and the formazan crystals were dissolved in 50 µl of DMSO. Then, the culture plate was agitated for 5 min at 500 rpm. The absorbance was measured by a microplate reader. The experiments were carried out in triplicate, and the results are expressed as a percentage of viable cells compared to the control group.

## **2.10. Statistical data analysis**

Statistical analyses were performed using the *U-test* with  $p < 0.05$  as the minimum level of significance (Mann and Whitney, 1947) or Kruskal-Wallis test.

## **3. Results and Discussion**

### **3.1. Synthesis of alginate-glutathione conjugates**

The lyophilized form of alginate-GSH consists of a creamy white powder with a fibrous structure. Alginate was the first anionic biodegradable polymer, and this polymer has been chemically modified by covalent attachment to thiol (-SH) groups by Bernkop-Schnurch (2001). Following this method, alginate was successfully attached to GSH, and the number of thiol groups attached to alginate was quantified using Ellman's method. The alginate-GSH conjugate contained  $711 \pm 24$   $\mu\text{mol}$  thiol groups per g of polymer, where  $481 \pm 22$   $\mu\text{mol}$  thiol groups were directly linked to the alginate polymer (Table I). GSH consists of three amino acids i.e. glutamate, cysteine and glycine. Due to the sulfhydryl (-SH) groups in cysteine, GSH is present in a reduced form and chemically active for a variety of reactions (e.g., antioxidant reactions). GSH is also involved in direct reactions with a variety of oxidants in a non-enzymatic manner that results in its conversion to its dimeric oxidized form (i.e., glutathione disulfide, GSSG) (Appenzeller-Herzog, 2011). By breaking the disulfide bonds with  $\text{NaBH}_4$ , all of the available thiol (-SH) groups can be converted to their reduced form (Table II), which provides an opportunity to link a large quantity of NO molecules (e.g., the nitrosation process) (Table III).

**Table I: Amount of glutathione (SH groups) determined by Ellman's test (precursor of SNA F1)**

Glutathione Fraction	Ellman's method ( $\mu\text{mol/g}$ )
Reduced thiols	$214 \pm 10$
Total thiols	$711 \pm 24$
Directly linked thiols	$481 \pm 22$

**Table II: Amount of glutathione (SH groups) determined by Ellman's test (precursor of SNA F2)**

Glutathione Fraction	Ellman's method ( $\mu\text{mol/g}$ )
Reduced thiols	$711 \pm 24$
Total thiols	$711 \pm 24$
Directly linked thiols	$481 \pm 22$

**Table III: Amount of nitrites determined by the Griess and Saville tests**

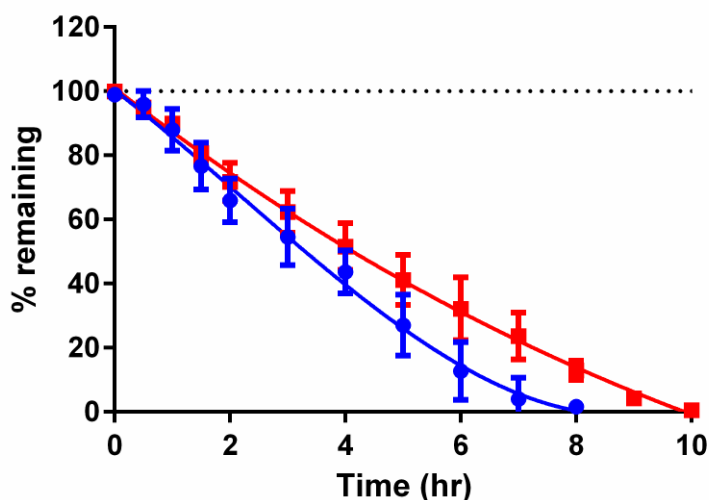
	SNA F1	SNA F2
Nitrites ( $\mu\text{mol/g}$ )	$174 \pm 21$	$468 \pm 23$

### **3.2. Nitrosation of the alginate-GSH conjugates**

A pale yellowish solution was obtained after reacting the alginate-GSH conjugates with sodium nitrite ( $\text{NaNO}_2$ ) under acidic conditions (pH 1.2). The nitrosation process was performed using two different methods, and the amount of NO adducts to thiol moieties using both methods are shown in Table III. SNA F2 has a greater number of linked NO groups compared with SNA F1 because SNA F1 is based on the nitrosation of only the reduced thiols present in the alginate-GSH conjugates, which are smaller in number compared with SNA F2 based on the nitrosation of all of the thiol groups present in the conjugates. In SNA F2, the disulfide bonds between the -SH groups were reduced by the addition of  $\text{NaBH}_4$  prior to nitrosation, which allows for the linkage of a larger number of thiol groups to NO. Comparatively, the amount of adducted NO to thiol groups was  $174 \pm 21$   $\mu\text{mol per g}$  in SNA F1. However, in SNA F2, this value was  $468 \pm 23$   $\mu\text{mol per g}$ , which suggests the suitability of SNA F2 for further experiments. Acidified  $\text{NO}_2^-$  can react with several functional groups on proteins, and two types of amino acid groups (i.e., cysteine and tryptophan) have been nitrosated in previous studies (Katsumi et al., 2004). The study also indicated that other amino acid residues including arginine, lysine, asparagine, glutamine and tyrosine can also be nitrosated but it can result in unstable products. The nitrosation of the cysteine thiol group in the alginate-GSH conjugate resulted in a high concentration of adducted NO and good stability under different experimental conditions (Figures 2 and 3).

### **3.3. Stability of SNA F1 and SNA F2 in the Ussing Chamber**

The stabilities of SNA F1 and SNA F2 were evaluated in the Ussing chamber without intestine at 37 °C in Hank's buffer with a constant  $\text{O}_2/\text{CO}_2$  flow. As shown in Figure 2, SNA F2 was more stable compared with SNA F1 and degraded into its degradation products after 10 h. This results may be due to the amount of adducted NO on each polymer because SNA F2 has a higher amount of NO, which makes it last longer. In our previous study (Shah et al., 2015b), the stability of GSNO, which was studied at a basic pH of 7.4, was comparatively less stable than our new SNA compound.



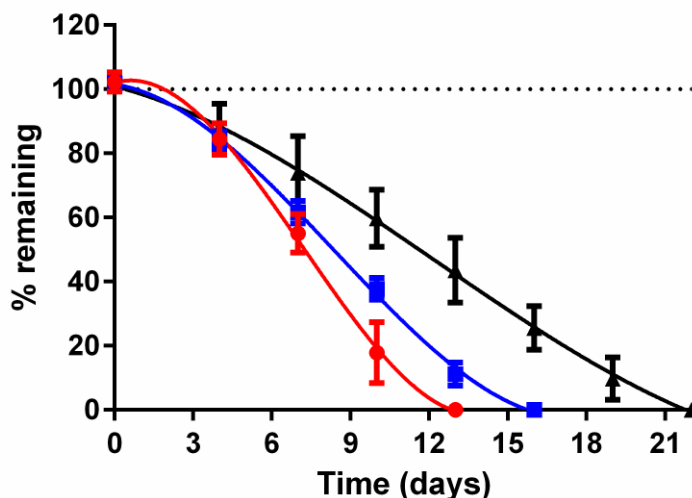
**Fig. 2: Stability of SNA F1 (-●-), SNA F2 (-■-) in the Ussing chamber without rat intestine at 37°C (n=3, mean ± S.D.)**

#### **3.4. Stability of SNA F2 under different storage conditions**

The stability of SNA F2 was studied under various pH conditions at 4 °C in the dark for several weeks. The acidic conditions consisted of acidic pH 1.2, a USP phosphate buffer at a neutral pH 7.0 and the mildly basic conditions consisted of USP phosphate buffer at pH 8.4. As shown in Figure 3, at an acidic pH of 1.2, only 55 ± 4.9% of SNA F2 remained after 1 week, and all of the SNA F2 degraded within 2 weeks. The stability of SNA F2 did not improve at a neutral pH of 7.0 where 61 ± 2.8% remained after 1 week and 11 ± 2.9% remained after 2 weeks. As shown in Figure 3, a mildly basic pH of 8.4 was more effective at decreasing the decomposition of RSNO where 73 ± 9.3% remained after 1 week and 43 ± 8.2% remained after 2 weeks. In addition, small amounts were still present until the 3<sup>rd</sup> week. The stability of RSNOs at various pH values was also evaluated in a previous study (Parent et al., 2013). In this study, the GSNO stability was evaluated at an acidic pH of 0.3 and a basic pH of 7.4 at a storage temperature of 4 °C. Based on the previous results, GSNO should be stored at a basic pH to ensure better storage conditions. A recent study conducted on the stability of RSNOs (i.e., GSNO, SNAC and SN3MPA) suggested that RSNOs were more stable at mildly basic pH values (i.e., 8.4 to 8.8) after the exclusion of light at 4 °C, which are similar to our results (Hornyak et al., 2012). This new compound (i.e.,



SNA F2) is more stable than our previous compound S-nitrosoglutathione-oligosaccharide-chitosan [SNOC, (Shah et al., 2015b)].

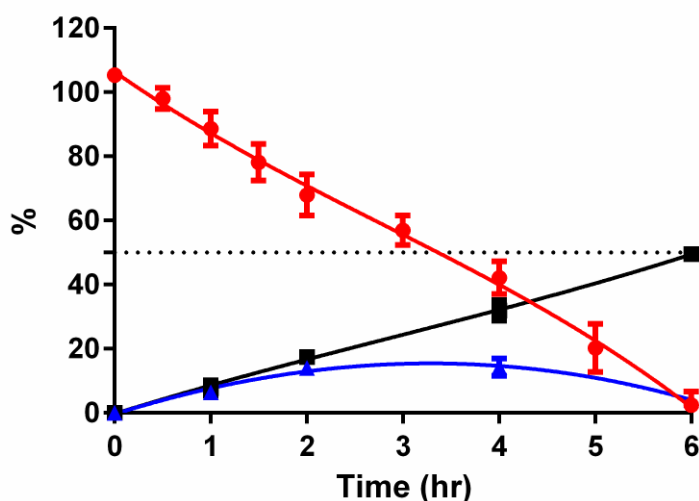


**Fig. 3: Stability of SNA F2 at different pH conditions at 4°C in dark pH 1.2 (-●-), pH 7.0 (-■-) and pH 8.4 (-▲-) (n=3, mean ± S.D.)**

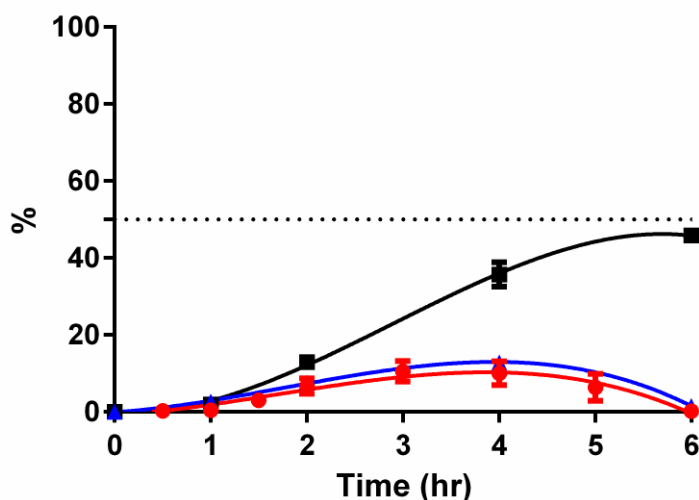
### 3.5. Permeability studies in the Ussing chamber

The *ex vivo* drug permeation studies were performed in an Ussing chamber across rat intestine. For the *ex vivo* study, only SNA F2 was selected due to its higher nitrosation values (Table III) and improved stability (Figure 2) compared with SNA F1. GSNO was also studied through the rat intestine for use as a control. The GSNO concentration remained for 5 h in the Ussing chamber (Figures 4 and 5) prior to being degraded into its two degradation products (i.e., nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ) ions). The concentrations of the two degradation products were also calculated in the acceptor compartment, because these products were able to permeate the intestinal barriers. Nitrite ions were released in the first hours of the experiment and found at the end of the experiments. These nitrite ions can be considered a sign of NO formation (Schulz et al., 1999; Shah et al., 2015b). Concentrations of nitrate ions, which can be considered the final products of RSNO degradation, increased throughout the experiments. Because equal amounts of medium were employed in both the donor and acceptor compartments of the Ussing chamber, an equal distribution of nitrite and nitrate ions should be present in both the

compartments, leading to a theoretical value of 50%. Nitrite and nitrate ions were believed to be inert end products of endogenous NO metabolism. However, recently reported results indicate that these products can be *in vivo* recycled and transformed back into NO, which represents an important unconventional source of NO outside of the classical L-arginine-NO synthase pathway (Lundberg et al., 2008). Based on the by Khan et al. (2011), both nitrite and nitrate ions may be significant precursors of NO and can increase the NO concentration in brain.



**Fig. 4: Concentrations of GSNO (-●-), NO<sub>2</sub>/NO (-▲-) and NO<sub>3</sub> (-■-) as the percentage of the initial concentration of the donor chamber (100 μM). Donor chamber; (n=3, mean value ± S.D)**



**Fig. 5: Concentrations of GSNO (-●-), NO<sub>2</sub>/NO (-▲-) and NO<sub>3</sub> (-■-) as the percentage of the initial concentration of the donor chamber (100 μM). Acceptor chamber; (n=3, mean value ± S.D)**

SNA F2 was comparatively more stable than GSNO and finally degraded to nitrite and nitrate ions after 10 h (Figure 2). These results indicate that the conjugation of alginate to RSNOs can prolong the release of NO from RSNOs. This prolonged release of NO could have potential benefits for the treatment of various diseases. A number of studies have been performed to study the therapeutic benefits of RSNOs in different clinical settings. However, their therapeutic potential is limited due to the poor distribution to the targeted area and rapid release of NO. Therefore, for the development of a more effective NO delivery system, the release rate of NO from RSNOs and its tissue distribution must be properly controlled (Katsumi et al., 2005). SNA F2 cannot cross the intestinal barriers but can deliver nitrite and nitrate ions to systemic circulation (Figures 6 and 7).

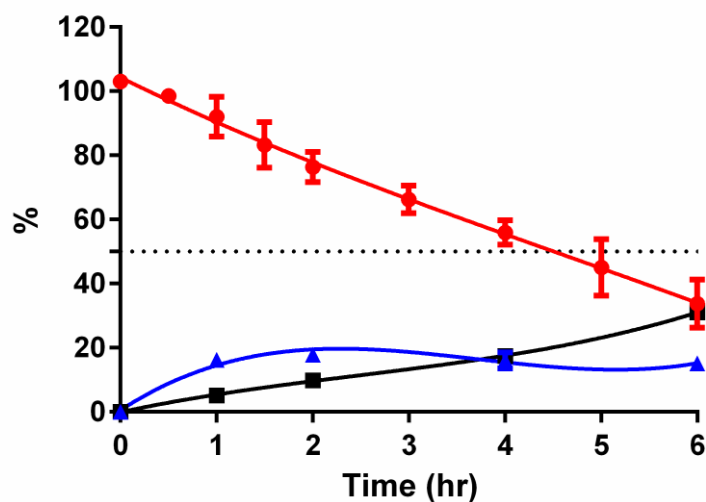


Fig. 6: Concentrations of SNA F2 (-●-), NO<sub>2</sub>/NO (-▲-) and NO<sub>3</sub> (-■-) as the percentage of the initial concentration of the donor chamber (100 μM). Donor chamber; (n=3, mean value ± S.D)

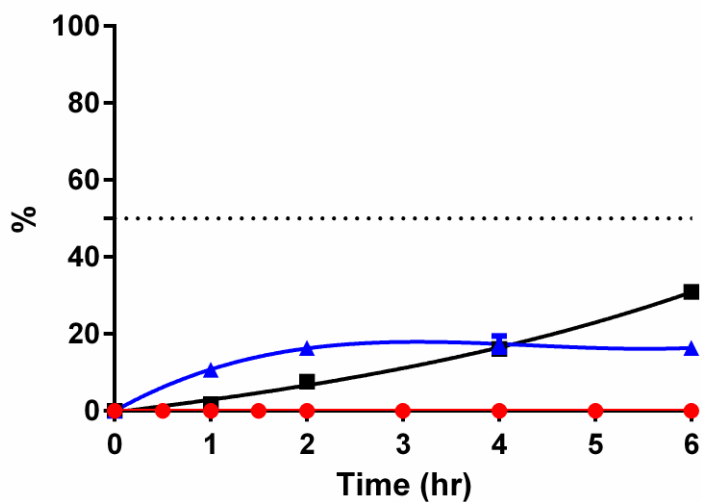
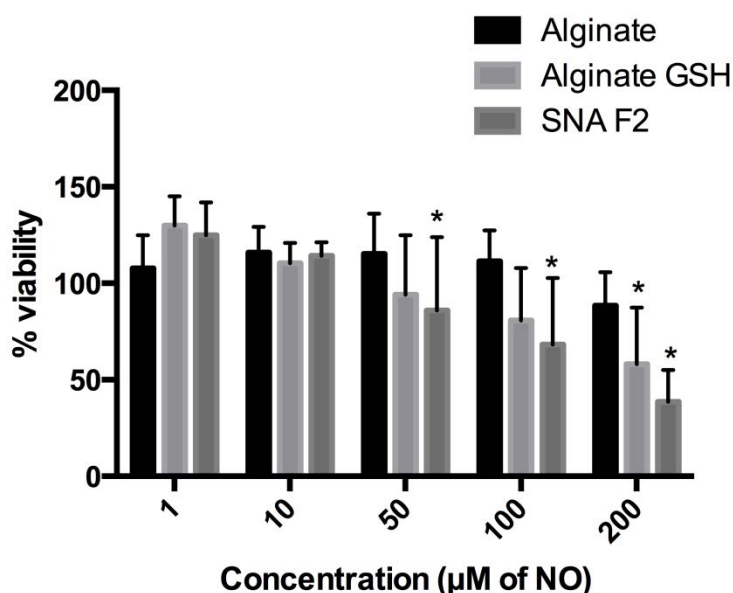


Fig. 7: Concentrations of SNA F2 (-●-), NO<sub>2</sub>/NO (-▲-) and NO<sub>3</sub> (-■-) as the percentage of the initial concentration of the donor chamber (100 μM). Acceptor chamber; (n=3, mean value ± S.D)

In the treatment of Crohn's disease, a sustained release of NO is required in the colon, and the systematic absorption is considered a risk for cardiovascular side effects (e.g., hypotension).

### 3.6. Cytotoxicity study using the Caco2 cell line

Caco2 cells were incubated with specified concentrations of SNA F2 (Figure 8) under suitable conditions. The cells were observed before and after incubation with the drug. The MTT test indicated that SNA F2 with less than 50  $\mu\text{M}$  NO exhibited a viability of more than 100% and 100  $\mu\text{M}$  SNA F2 exhibited 86% viability. However, SNA F2 with 200  $\mu\text{M}$  NO was cytotoxic, and the viability of cells decreases to 38%.



**Fig. 8: Cell viability of Caco2 cell line after exposure to different concentrations of alginate, alginate-GSH conjugate and SNA F2 (n=3, mean  $\pm$  S.D.). For SNA F2, the concentrations are expressed in  $\mu\text{M}$  of NO; for the other polymers, corresponding concentration are used. \* Kruskal Wallis test (comparison with alginate) :  $p < 0.05$**

Based on our results, alginate alone increased the viability of the cells even in the column "100  $\mu\text{M}$  of NO". In addition, the alginate-GSH conjugate exhibits 100% viability in the column "50  $\mu\text{M}$  of NO". However, its viability decreased at higher concentrations. Therefore, based on this MTT assay, our polymer was not toxic at 10  $\mu\text{M}$  NO and exhibits 100% metabolic activity in the cells after 24 h of incubation. Similar results were obtained in a study performed by Ramia Safar

et al. and Ronzani et al. (Ronzani et al., 2014; Safar et al., 2015), where empty nanoparticles consisting of Eudragit RL were seeded into the human THP-1 monocytic cell line, and a significant increase in the viability of the cells up to 40% was observed compared with the control. The cell viability increased due to an increase in the metabolic activity and growth of the cells after contact with the nanoparticles, and this phenomenon was related to an augmentation of the mitochondrial volume and activity. The mitochondrial activity may be related to cell proliferation, which leads to an increase in the mitochondrion number. The up regulation of genes involved in metabolism and proliferation may be related to this increased mitochondrial activity, which belongs to “transcription”, “nucleus”, “chromosomal part”, “metabolic process” and “proliferation” (Safar et al., 2015).

#### **4. Conclusions**

Based on our results, a novel polymeric NO-donor was successfully synthesized by linking alginate to RSNOs, which can release pharmacologically active NO in a sustained manner. A stable NO-donor under physiologically acceptable pH conditions was achieved, and this donor has the potential for long-term therapeutic effects. Alginate is a good candidate for the long-term stability and controlled delivery of NO. The low toxicity of this new compound suggests future *in vivo* studies in animal models should be performed. This type of compound would be beneficial for the treatment of various types of diseases including Crohn’s disease.

The release of NO may differ under physiological conditions, especially due to the concentration of O<sub>2</sub>, which is necessary for tissue viability. This concentration may be lower in the colonic lumen.

We believe that our compound can be encapsulated in gastro-resistant soft gelatine capsules for oral delivery. Microparticles of SNA with chitosan can also be prepared (D Marcato et al., 2013a). In addition, a polyelectrolyte complex of alginate with chitosan and Eudragit® L100-55 can also be prepared (Calija et al., 2013).

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# **Chapter 3: Spray dried microparticles of glutathione and S-nitrosoglutathione based on Eudragit® FS 30D polymer**

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## **Spray-dried microparticles of glutathione and S-nitrosoglutathione based on Eudragit® FS 30D polymer**

### **Résumé**

Dans cette dernière étude, nous avons réalisé une formulation par spray drying (séchage par atomisation) de S-nitrosoglutathion à base de polymère d'Eudragit® FS 30D (ce polymère permet de libérer spécifiquement les principes actifs au niveau du colon).

L'influence de certains paramètres de fabrication (température d'entrée de l'air : 80-150°C, flux de solvant : 5 à 10 ml/min) a été étudiée. Les paramètres qui permettent d'obtenir la meilleure formulation (température d'entrée 120°C, température de sortie 47°C, flux de solvant 5 ml/min et flux d'air 100%) ont été sélectionnés pour des études supplémentaires.

Des microparticules de GSH « témoins » ont été préparées exactement selon les mêmes conditions

La microscopie électronique à balayage a révélé que les microparticules résultantes étaient généralement sphériques. La diffraction par rayons X de la poudre de GSH et GSNO purs a montré des pics qui n'étaient pas présents dans les microparticules, confirmant la dispersion moléculaire et l'encapsulation complète de ces principes actifs par le polymère.

La stabilité de ces principes actifs dans les microparticules a été confirmée par spectroscopie infrarouge à transformée de Fourier.

Des études de libération en tampon phosphate ont montré une libération rapide à pH basique 7,4, une libération retardée à pH 6,8, mais quasiment aucune libération aux pH acides de 1,2, 3 et 6 pour les microparticules sensibles au pH. Les particules préviennent la libération des médicaments au pH acide mais démontrent une libération rapide à un pH basique de 7,4 ; cela suggère l'adéquation de ces particules pour être évaluées pour un ciblage spécifique du colon.

En utilisant l'Eudragit® FS 30D comme polymère de libération dépendant du pH, le principe actif pourrait cibler le colon pour le traitement de la maladie de Crohn.

## **Abstract**

In this study, we report the formulation of spray dried microparticles of glutathione (GSH) and S-nitrosoglutathione (GSNO) based on Eudragit® FS 30D polymer. Eudragit® FS 30D is a pH sensitive polymer and the aim was to develop colon specific delivery system avoiding the release of drug in upper gastro intestinal tract (the administration of GSNO was especially developed for the treatment of Crohn's disease). Influence of several spray drying parameters (Inlet temperature 80–150 °C, solvent flow 5-10 ml/min and) on particle size, shape and production yield were studied. Scanning Electron Microscopy (SEM) revealed that the resulting microparticles were spherical in shape. Powder X-ray diffraction (PXRD) patterns of pure GSH and GSNO show sharp peaks that were disappeared in microparticles confirming molecular dispersion and complete encapsulation of the drugs by the polymer. The stability of drugs in microparticles was confirmed by Fourier Transform Infrared (FTIR), which showed no significant difference in the characteristic peaks of pure and encapsulated drugs. Release studies in phosphate buffer solution showed fast release at basic pH 7.4, sustained release at pH 6.8 while practically no release at acidic pH 1.2, 3 and 6 for pH sensitive microparticles. The particles prevented the release of drugs at acidic pH, while demonstrating fast release at basic pH 7.4; this suggests the suitability of these particles to be evaluated for specific colon targeting.

**Keywords:** Microparticles; Spray-Drying; Nitrosothiols; Glutathione;

## 1. Introduction

S-nitrosothiols (RSNO's) is a class of NO donors, which exercise a variety of physiological effects in human body including inhibition of platelet aggregation and vasorelaxation. RSNO's are proposed to be the indigenous species that mediates the physiological actions of NO (Tullett et al., 2001). RSNO's have been given specific attention not only because they store, transport and release NO but they are also being used in the treatment of angina and circulatory diseases(Wang et al., 1999). In recent years, S-nitrosoglutathione (GSNO) has also been proposed in the treatment of Crohn's disease to prevent mucosal barrier failure. It has been observed that GSNO improves human intestinal mucosal barrier functions in patients with inflammatory bowel disease(Shah et al.). Nevertheless, higher doses of S-nitrosothiols are required to obtain therapeutic effects because they release NO very quickly and are rapidly cleared from systemic circulation(Katsumi et al., 2009). Therefore, certain delivery carriers are needed that could slower the release of NO and make it available at the site of action.

Polymeric systems have been widely investigated because they can modulate drug activity by passive or active targeting to different tissues. Several polymeric systems have been efficiently used for the delivery of NO in which the NO donors were incorporated into polymer microparticles. These polymeric systems demonstrated stabilization and improved therapeutic effects. However, the drug release rates can be different according to the polymer used, while experimental conditions and methods used for encapsulation can effect particle size, morphology and encapsulation efficiency(Saraiva et al., 2011).

Spray drying is the technique of transformation of liquid form (suspension, emulsion or dispersion) into solid form by passing it through a hot gas. It is a continuous process of microencapsulation feasible for a variety of materials. It is widely used by food and cosmetic industries and also being used by pharmaceutical industries for a variety of drug products in dry form(Patel et al.). This technique represents a promising method to control the release of hydrophilic drugs such as citrulline(Bahri et al., 2014) and to improve their physiochemical stability(Guterres et al., 2009). Spray drying technique is described by a number of publications(Durriegl et al., 2011; Esposito et al., 2000; Kristmundsdóttir et al., 1996; Pignatello et al., 1997) for making controlled release microparticles by using water insoluble and low

permeability acrylic polymers including ammonium methacrylate copolymers (i.e. Eudragit®). It was reported (Esposito et al., 2000; Rassa et al., 2008) that the increase in inlet temperature was indirectly proportion of the particle size while no correlation was found to drug release rates. Spray drying is a one-step complex technological process which can be adjusted by different parameters which affect the physiochemical properties and performance of the particles, however, the output is not controlled by an individual parameter but a combination of all factors (Durrigl et al., 2011). These parameters include inlet temperature, outlet temperature, inlet air and content of feed (Mishra and Mishra, 2011).

The objective of this study was to develop spray dried microparticles of glutathione (GSH) and S-nitroso-glutathione (GSNO) by using Eudragit® FS 30D polymer for an effective colon specific drug delivery. To the best of our knowledge, spray dried microparticles of S-nitrosothiols by using Eudragit® FS 30D as a pH dependent rate controlling polymer has not been reported in any literature earlier.

## **2. Materials and Methods**

Reduced GSH (Sigma Aldrich), GSNO (synthesized in dry form by our lab), Eudragit® FS 30D (copolymer based on methyl acrylate, methyl methacrylate and methacrylic acid that dissolves by salt formation above pH 7.0, Evonik, Germany), Ammonia (25% v/v), dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>), dialysis tubing (Spectra/Por® molecularporous membrane tubing, MWCO 3500), Syringe filters 0.5µm (Millipore, France) and ultrapure water was used in all experiments. All chemicals were of analytical grades.

### **2.1. Preparation of microparticles**

The GSH and GSNO loaded Eudragit® FS 30D microparticles were prepared by a spray drying technique. In this technique, freely water-soluble drugs and a pH dependent polymer (i.e. Eudragit® FS 30D) was used. Spray-dried Eudragit® FS 30D-GSH particles were prepared by taking 20 ml of Eudragit® FS 30D suspension 30% w/v (i.e. 6 g dry weight) and mixed with 80 g of water. After, 1 ml of ammonia (25% v/v) was added dropwise to the above mixture at continuous stirring up till the colour of the mixture changed from milky white to transparent.

The GSH 1.8 g (23% of polymer) was dissolved in 1.5 ml of water and 0.5 ml of ammonia and was added dropwise to above Eudragit® FS 30D solution with continuous stirring. The final solution was spray dried by a Mini Spray Dryer Büchi 190 (Büchi, Rungis, France) at 100 % airflow and using various parameters. At the end, microparticles (Eudragit® FS 30D-GSH) were harvested and closed in an airtight container with silica gel to prevent from moisture. An identical procedure and same amounts were used to make GSNO microparticles (Eudragit® FS 30D-GSNO).

Several batches of Eudragit® FS 30D-GSH and Eudragit® FS 30D-GSNO microparticles were prepared by using optimized process variables (Inlet temperature 80–150 °C, solvent flow 5-10 ml/min and air flow 100%) in order to select the best. The outlet temperature, which was related to the inlet temperature, was recorded. All batches were prepared in triplicate and were stored until characterization studies.

## **2.2. Encapsulation efficiency**

Encapsulation efficiency of GSH and GSNO microparticles was determined by liquid-liquid extraction process. GSH and GSNO microparticles were first dissolved in dichloromethane and after diluted with phosphate buffer (pH 7.4, 0.15 M). After dilution, the solution was filtered through a membrane filter (0.5µm) and the filtrate was spectrophotometrically analyzed for drug concentrations. GSNO concentrations were calculated by Griess and Saville methods (Heines, 1958; Saville, 1958) and GSH concentrations were calculated by Ellman's method (Ellman, 1959).

Percentage encapsulation efficiency can be calculated by the following formula (Ranjha et al., 2009):

$$\text{Encapsulation efficiency \%} = \frac{\text{entrapped amount of drug per gram of particles}}{\text{theoretical amount of drug per gram of particles}} \times 100$$



### **2.3. Zeta potential and particle size**

The zeta potential and particle size of Eudragit® FS 30 D-GSH and Eudragit® FS 30 D-GSNO microparticles were checked by Zeta sizer (Zetasizer, Malvern, Orsay, France). All experiments were done in triplicate.

### **2.4. Fourier transformed Infrared spectroscopy**

Fourier transformed Infrared (FTIR) spectroscopy technique was used to observe different functional groups and to check any interaction between drugs and polymer. The frequency shifts and splitting in absorption peaks are resulted due to spectral variations, which are attributed to alteration in bonds that divulge characteristic vibrational frequencies. Briefly, 6 mg of each drug and polymer was taken separately and mixed with 300 mg of Kbr. A small amount of this mixed powder was compressed in a die to make a thin film. The film was kept in IR spectrophotometer (Vector 22 FT-IR spectrophotometer, Bruker, Wissembourg, France) and the spectra were recorded. The spectra were recorded for pure drugs, polymer and microparticles of polymer with drugs (Khan et al., 2013).

### **2.5. Powder X-ray diffraction analysis**

The powder X-ray diffraction (PXRD) measurements were performed to check the physical state of drugs after encapsulation by the polymer. The PXRD patterns were obtained by using a Panalytical X'Pert pro diffractometer (Limeil – Brévannes, France) equipped with a Cu tube, a Ge (III) incident-beam monochromator ( $\lambda = 1.5406 \text{ \AA}$ ) and an X'Celerator detector. Data collection was carried out in the scattering angle range  $3\text{--}70^\circ$  with a  $0.0167^\circ$  step over 90 min.

### **2.6. Scanning electron microscopy**

Scanning electron microscope (Hitachi FEG S4800 high-resolution electron microscope) was used to investigate the surface topography and shape of the microparticles. The powder sample was placed on a stub with help of adhesives and coated for 90 seconds with gold-palladium alloy and was putted in a vacuum chamber of microscope for imaging.

## 2.7. *In vitro* drug release studies

A classical method was used to perform drug release studies for microparticles. Briefly, 42.6 mg Eudragit® FS 30D-GSH microparticles and 62.5 mg of Eudragit® FS 30D -GSNO microparticles were weighted in 1 inch long dialysis tubing (Spectra/Por® molecularporous membrane tubing, molecular weight cut off: 3500 daltons) containing 2 ml of suspending buffer and closing the ends with threads. The tube was suspended in 500 ml of release medium [Phosphate buffer pH 6, 6.8 and 7.4] and acidic medium pH 1.2 and 3 in a beaker, stirred at 100 rpm while maintaining the temperature at 37 °C. At predetermined time intervals, 1 ml of the release medium was withdrawn and replaced with 1 ml of the same release medium incubated at 37 °C. The amount of GSH released from Eudragit® FS 30D-GSH microparticles was calculated by Ellman's method (Ellman, 1959) and amount of GSNO released from Eudragit® FS 30D-GSNO microparticles was calculated by Griess and Saville methods (Heines, 1958; Saville, 1958) through a UV visible spectrophotometer (Cary 50 UV-Vis, Varian, Les Ulis, France). All the experiments were done in triplicate.

Experimental points were fitted using the Weibull model (Papadopoulou et al., 2006):

$$\frac{M_t}{M_\infty} = 1 - \exp(-at^b) \quad \text{Eq.1}$$

Where,  $M_t$  is the amount of drug dissolved as a function of time  $t$ ,  $M_\infty$  is total amount of drug being released. 'T' is lag time measured as a result of dissolution process, parameter 'a' denotes a scale parameter that describes the time dependence, whereas 'b' describes the shape of the dissolution curve progression.

## 3. Results and discussion

The aim of our work was to develop and characterize new microparticles delivery system for S-nitrosothiols. We successfully prepared microparticles of GSH and GSNO by using Eudragit® FS 30D with high encapsulation efficiency (Table 1 and 2). Several batches were prepared by spray drying technique with varying physical parameters out of which the best formulations with parameters (Inlet temperature 120 °C, outlet temperature 47 °C, solvent flow 5 ml/min and air flow 100%) were selected for further studies (Table 1, 2). Spray dried microparticles were

negatively charged with mean particle size ranging from 5 to 7  $\mu\text{m}$ . We can see a variation in size with respect to spray drying parameters. Process parameters like temperature and solvent flow have a significant effect on percentage yield and particle size distribution. The size of the particles with high solvent flow (10 ml/min) was larger as compared to particles with less solvent flow (5 ml/min) which could be due to the fact that at high solvent flow rates, more volume of solvent reaches the nozzle and allowing very less time for optimization leading to variation in particle size (Anish et al., 2014; Patel et al., 2011). The solvent flow has also a significant effect on production yield, the higher the solvent flow rate the less the production yield. It could be due to the reason that at high solvent flow rates the spray pattern is non-uniform and the solvent is sprayed at the walls of the drying chamber (Anish et al., 2014) leading to less production yield. The low production yield values are frequent in spray drying methods as studied earlier (Al-Zoubi et al., 2008). In the batch 4, we were unable to get any particles because of the high solvent flow and low inlet and outlet temperatures, which could not readily convert the solvent to microparticles. Inlet temperature has a significant role in drying and influences the yield of particles. At lower inlet temperatures, solvent is not properly evaporated from the finely atomized droplets, leading to formation of polymer films on the walls of drying chamber and reduces the production yield as could it be seen in case of batch 4.

**Table 1. Eudragit® FS 30D-GSH microparticles with different physical parameters**

Parameters	Batch 1	Batch 2	<b>Batch 3</b>	Batch 4	Batch 5	Batch 6	Batch 7
Inlet temperature ( $^{\circ}\text{C}$ )	80	100	<b>120</b>	80	100	120	150
Outlet temperature ( $^{\circ}\text{C}$ )	37	41	<b>47</b>	37	41	47	52
Solvent flow (ml/min)	5	5	<b>5</b>	10	10	10	10
Production Yield (%)	29 $\pm$ 0.81	29 $\pm$ 0.93	<b>31<math>\pm</math>0.91</b>	0	20 $\pm$ 1.6	20 $\pm$ 1.6	16 $\pm$ 1.5
Particle size ( $\mu\text{m}$ )	5 $\pm$ 0.13	5 $\pm$ 0.11	<b>5<math>\pm</math>0.14</b>	0	7 $\pm$ 1.2	7 $\pm$ 1.2	7 $\pm$ 1.7
Zeta potential (mV)	-36 $\pm$ 7.1	-39 $\pm$ 6.2	<b>-39<math>\pm</math>6.7</b>	0	-40 $\pm$ 7.1	-40 $\pm$ 6.9	-39 $\pm$ 6.9
Encapsulation (%)	79 $\pm$ 2.1	82 $\pm$ 1.9	<b>82<math>\pm</math>1.3</b>	0	80 $\pm$ 2.1	74 $\pm$ 3.1	76 $\pm$ 2.1

**Table 2. Eudragit® FS 30D-GSNO microparticles with different physical parameters**

Parameters	Batch 1	Batch 2	<b>Batch 3</b>	Batch 4	Batch 5	Batch 6	Batch 7
Inlet temperature (°C)	80	100	<b>120</b>	80	100	120	150
Outlet temperature (°C)	37	41	<b>47</b>	37	41	47	52
Solvent flow (ml/min)	5	5	<b>5</b>	10	10	10	10
Production Yield (%)	28±0.88	27±1.22	<b>29±0.84</b>	0	22±0.97	20±1.7	17±0.93
Particle size (µm)	5±0.09	5±0.16	<b>5±0.12</b>	0	7±1.11	7±0.7	7±1.3
Zeta potential (mV)	-38±6.4	-38±6.5	<b>-39±4.7</b>	0	-47±7.5	-38±4.2	-41±2.4
Encapsulation (%)	84±3.2	79±2.7	<b>83±1.6</b>	0	76±3.4	80±1.9	78±3.1

The possible degradation of GSNO in nitrites/nitrates is also a key point of the process. In fact, a preliminary experiment had been realized with a pure solution of GSNO that has been spray dried under the same condition. No variation of the GSNO amount was observed in the spray dried powder compared to the initial powder. We concluded that the degradation of GSNO was negligible and this was probably due to a very short time of contact between the GSNO and the oxygen of the air.

### **3.1. Fourier transformed Infrared spectroscopy**

The FTIR spectral analysis for GSH, GSNO and their polymeric microparticles (Eudragit® FS 30D-GSH and Eudragit® FS 30D-GSNO) were done in order to check interaction of drugs with the polymer and to confirm the linkage of NO to (-SH) groups of GSH (Figure 1). Each molecule gives a characteristic spectral bending and stretching vibrations due to the influence of IR radiations, which are due to specific functional groups. These specific functional groups can be used as identification markers(Khan et al., 2015).

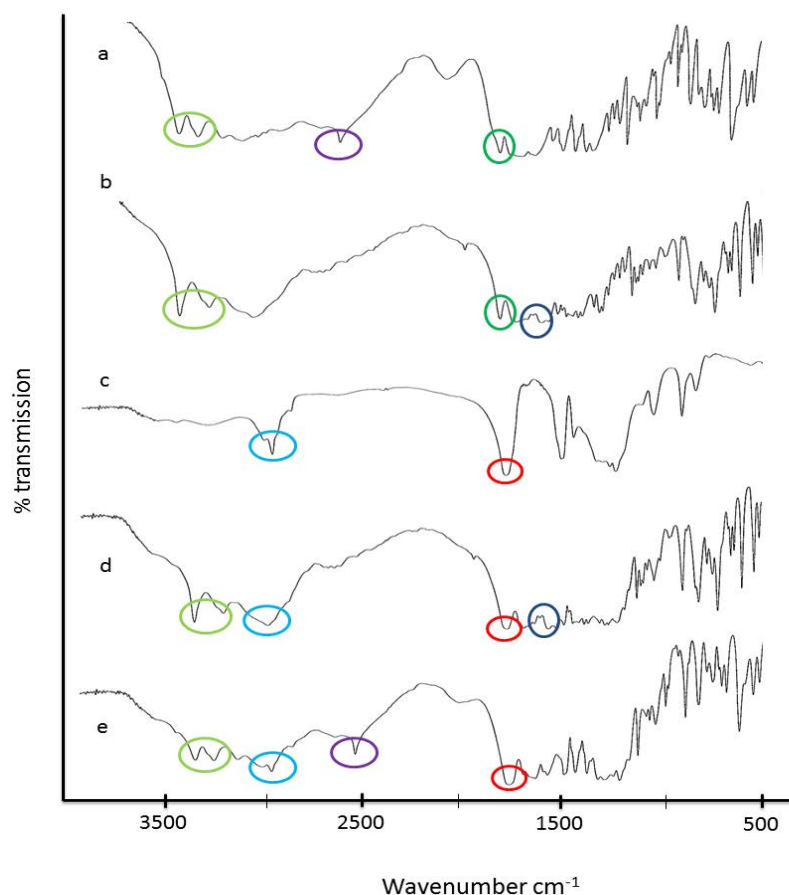


Figure 1. FTIR spectra of a) GSH, b) GSNO, c) Eudragit® FS 30D, d) Eudragit® FS 30D-GSNO, e) Eudragit® FS 30D-GSH; ( — ) vibration of specific thiol (—SH) group for glutathione at  $2524\text{ cm}^{-1}$ , ( — )  $\text{NH}_2$  stretch at  $3250$  and  $3346\text{ cm}^{-1}$  and ( — ) C=O stretch at  $1714\text{ cm}^{-1}$ , ( — ) N=O stretching vibrations at  $1520\text{ cm}^{-1}$  for GSNO, ( — ) characteristic band of the C=O vibration of the esterified carboxyl groups at  $1742\text{ cm}^{-1}$  for Eudragit® FS 30D and a wide absorption range of associated OH groups between  $2500$  and  $3500\text{ cm}^{-1}$  superimposed by CH group ( — ) at  $2954\text{ cm}^{-1}$ .

Comparing the FTIR spectra of pure GSH and GSNO, we can see the characteristics absorption peaks for N-H stretching bands were at  $3249$  and  $3346\text{ cm}^{-1}$  (Balavandy et al., 2014) while for GSNO they were at  $3168$  and  $3333\text{ cm}^{-1}$ . The vibration band at  $3020\text{ cm}^{-1}$  for GSH was due to C-H stretching while the characteristic thiol (—SH) stretching was observed at  $2524\text{ cm}^{-1}$  (Ahmed, 2014; Balavandy et al., 2014) which was disappeared in GSNO which could be due to the

formation of new nitroso bonds between NO and thiol groups of GSH. Band vibration at 1714  $\text{cm}^{-1}$  and 1725  $\text{cm}^{-1}$  in GSH and GSNO represents the carboxylic groups ( $-\text{C}=\text{O}$ ) respectively. However, the carboxylic groups of GSH and GSNO cannot be seen in their respective microparticles, which could be due to the reason that they were overlapped by the strong ester vibration of the polymer at 1734  $\text{cm}^{-1}$ . The formation of new small peaks at 1530  $\text{cm}^{-1}$  represents the nitroso bonds ( $\text{N}=\text{O}$ ) in GSNO which seems to be closer to 1486  $\text{cm}^{-1}$  as reported earlier by M. Piskorz and T. Urbanski (Piskorz and Urbanski, 1963). From our results we can see that Eudragit® FS 30D shows a strong characteristic  $\text{C}=\text{O}$  vibration of the esterified carboxyl group at 1734  $\text{cm}^{-1}$  (Khan et al., 2015) and 1165  $\text{cm}^{-1}$ . A strong CH vibration in Eudragit® FS 30D can be observed at 2954  $\text{cm}^{-1}$ . We observed strong N-H bands of GSH and GSNO and  $-\text{SH}$  group of GSH in their polymeric form with Eudragit® FS 30D which means that there was no interaction between the two drugs and polymer Eudragit® FS 30D.

### **3.2. Powder X-ray diffraction analysis**

Being the most sensitive technique, PXRD acquire information about the arrangement of molecules within the crystal (Khan et al., 2010). In this study, we obtained diffraction patterns of pure GSH, GSNO, Eudragit® FS 30D and their microparticles (Figure 2).

The pure drugs GSH and GSNO demonstrated crystalline characteristics while Eudragit® FS30D polymer demonstrates amorphous characteristics. The GSH was having a high intensity peak at 22.5° (Lopedota et al., 2009) and GSNO was having two high intensity peaks at 19° and 30°. However, the high intensity crystalline peaks of GSH and GSNO were dramatically disappeared in their polymeric microparticles with Eudragit® FS 30D revealing the amorphous form of the microparticles. The amorphous nature of microparticles is helpful to improve *in vitro* dissolution properties and bioavailability.

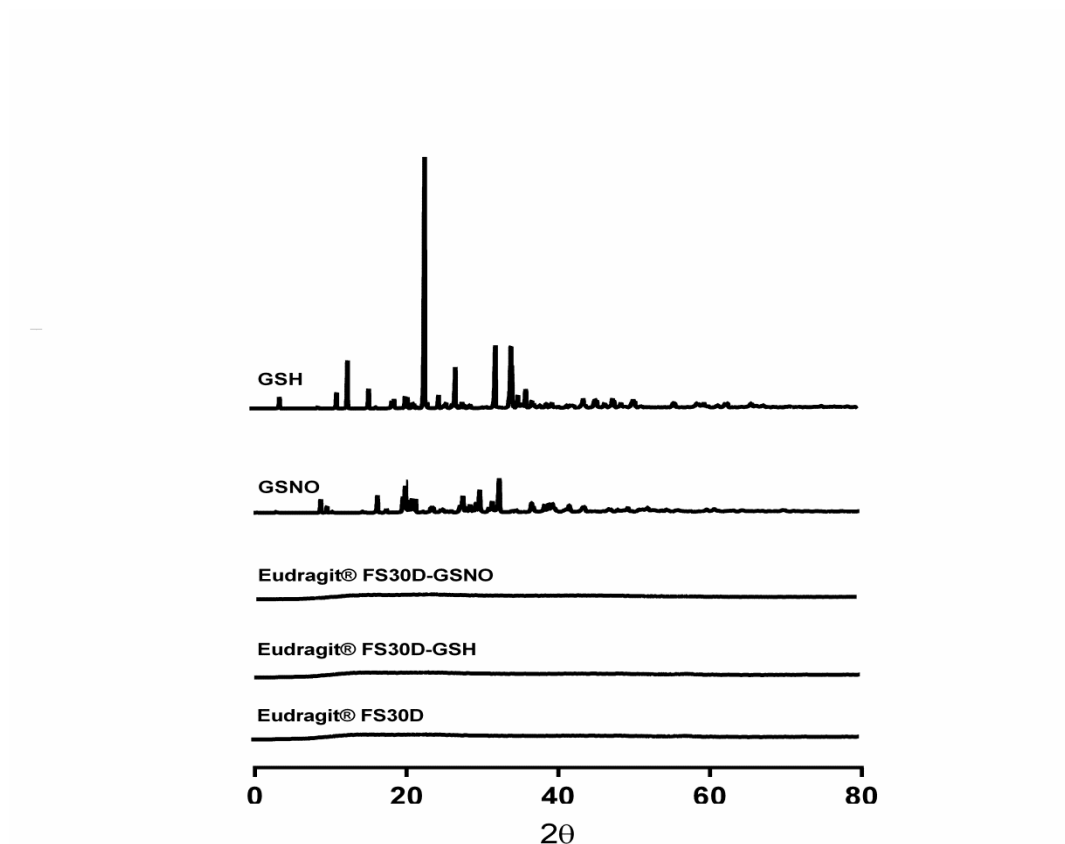
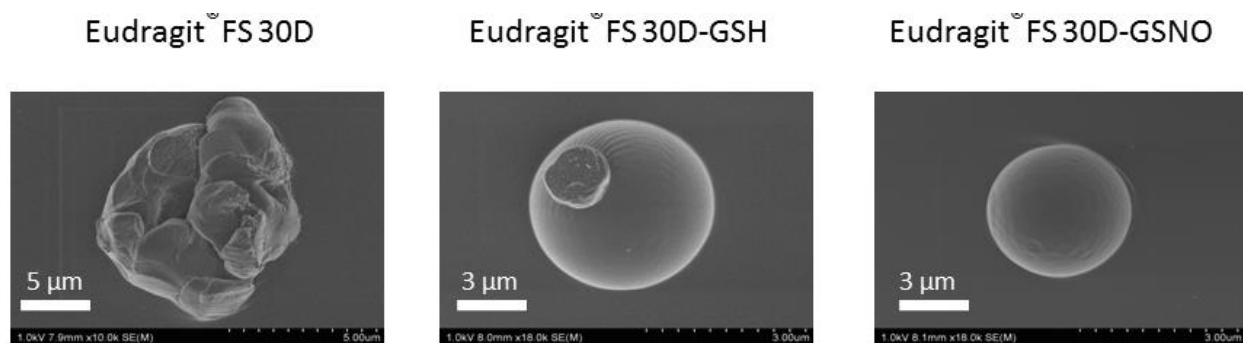


Figure 2. Powder X-ray diffraction (PXRD) pattern of GSH, GSNO and their microparticles

### 3.3. Scanning electron microscopy

Spherical shape and smooth surface are two important factors that can ensure good flow properties of microparticles (Anish et al., 2014). In our experiment, Figure 3 represents the SEM images of Eudragit® FS 30D polymer, Eudragit® FS 30D-GSH and Eudragit® FS 30D-GSNO microparticles from left to right respectively. These images were taken at different magnifications to facilitate visual comparison. Spray dried Eudragit® FS 30D-GSH and Eudragit® FS 30D-GSNO microparticles were mostly spherical with smooth surfaces and most having no visible pores on their surfaces (Durrigl et al., 2011; Kristmundsdóttir et al., 1996).



**Figure 3. Scanning electron microscopy (SEM) pictures of microparticles at different magnification.**

From all these images, a difference in shape and surface morphology can be observed between loaded microparticles (i.e. Eudragit® FS 30D-GSH and Eudragit® FS 30D-GSNO) as compared to simple Eudragit® FS 30D microparticles; this could be due to the influence of drug loading (Durrigl et al., 2011). Blank Eudragit® FS 30D microparticles were wrinkled in structure which could be due to the higher polymer percentage (i.e. 100% polymer) and might be formed during early drying process when the polymer soft surface folds to form wrinkled particles as investigated earlier (Vehring, 2008) where wrinkled surfaces was formed when polymer was used in higher percentage. Another possible reason of the wrinkled surface of pure Eudragit® FS 30D could be that it is hydrophobic in nature resulting in hydration of its outer surface which makes its surface wrinkled but after encapsulation of the drug the surface hydration don't take place because of the highly hydrophilic nature of drugs (GSH and GSNO). We could not see any visible drug crystals on the surface of the microparticles or among the particles, which means that the drug was properly embedded in the microparticles.

### **3.4. Drug release:**

Drug release studies were carried out in phosphate buffer solution at different pH values (6, 6.8 and 7.4) and acidic medium pH 1.2 and 3. Two formulations (Eudragit® FS 30D-GSH batch 3 and Eudragit® FS 30D-GSNO batch 3, (Table 1, 2) were selected. As it can be seen in Figure 4 and 5, the formulations do not released the drug at acidic pH 1.2, 3 and 6. Only 20% of initial burst release was observed in the three formulations (Table 3), which could be due to the loss-bounded drug at the surface of microparticles, which readily diffuses into the dissolution



medium. The drug levels as a result of this initial burst release mechanism were constant throughout the experiment.

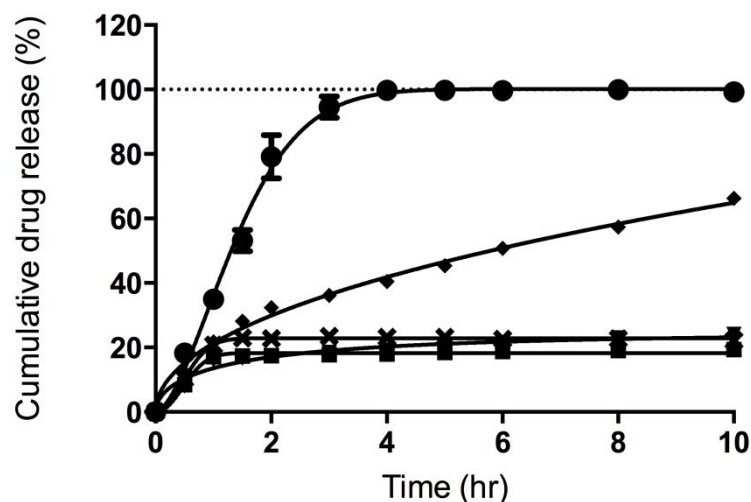


Figure 4. Drug release of GSH (GSH + GSSG) from microparticles at different pH values, - X - pH 1.2, - ■ - pH 3, - ▼ - pH 6, - ♦ - pH 6.8, - ● - pH 7.4, (n = 3, mean value  $\pm$  S.D).

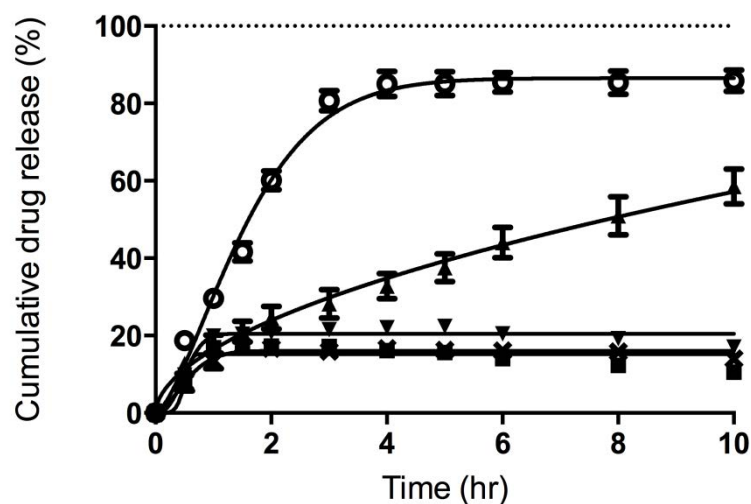


Figure 5. Drug release of GSNO from microparticles at different pH values, - X - pH 1.2, - ■ - pH 3, - ▼ - pH 6, - ▲ - pH 6.8, - ○ - pH 7.4, (n = 3, mean value  $\pm$  S.D).

At pH 6.8 the microparticles exhibited a sustained release pattern during more than 10 h. Due to the complex mechanism process, this curves did not fit well with the Weibull model. This pH

is near the pH that is observed in the small intestine (pH 6 in the duodenum and it increases to pH 7.4 in the terminal ileum) and we can suppose that our formulation can deliver the drug in this portion and in the colon where Crohn's inflammations can occur. Seventy five percent of the drug was released within 2 h at pH 7.4 from both formulations (Figure 4, 5, Table 3). The Eudragit® FS 30D-GSNO microparticles released only 86.5% of the initial concentrations of GSNO while the remaining 13.5% was in the form of its degradation product nitrites (data not shown).

Sustained release properties of these microparticles in acidic conditions could ensure protection in the upper gastro-intestinal tract (GIT) and thus can be used as specific intestinal targeting drug delivery systems. Several studies showed that GSNO can be used as a potential cure to prevent mucosal barrier failure in Crohn's disease (Savidge et al., 2007), thus the colon specific targeted drug delivery of GSNO via Eudragit® FS 30D based polymeric microparticles could be proposed for the experiments in the treatment of Crohn's disease. In order to achieve therapeutic effects of NO donors, usually a very high dose is required because it not only release the NO very quickly but also it is rapidly transformed in nitrites and nitrates (Wu et al., 2015). Therefore, a delivery system is required to slower the release of NO. To overcome these problems, we used Eudragit® FS 30D to directly encapsulate NO donors (GSNO) which gives pH dependent sustained release profiles at basic conditions specifically targeting colon. There are no studies so far in our knowledge dealing with direct encapsulating of a drug into Eudragit® FS 30D polymer by spray drying technique, however, there are some articles describing the application of Eudragit® FS 30D on hydroxypropyl methylcellulose (HPMC) and gelatin capsules (Cole et al., 2002; Dvorackova et al., 2011). In a set of experiments aimed at colonic release of paracetamol and to achieve enteric properties, the drug was enclosed in HPMC capsules and coated with Eudragit® FS 30D polymers. The study demonstrated that the capsules coated with Eudragit® FS 30D were gastro resistant to acidic pH 1.2 for 2h and another 1h to pH 6.8 and did not release the complete drug until the distilled small intestine and proximal colon in an average time of 6.9h post dose (Cole et al., 2002). In a further study (Dvorackova et al., 2011), the authors showed that the gelatin capsules coated with Eudragit® FS 30D released the drug after the pH was changed to 7.5 from pH 1.2 and 6.8 and were considered as a promising transport system to ileo-colonic area. They also showed that the formulation with 7h lag time could be used for colonic delivery. Several other studies showed that Eudragit® FS 30D could be

successfully used as a pH dependent polymer for drugs intended to be carried to ileocecal region for the treatment of mid to distal ulcerative colitis (Bott et al., 2004). The release profiles of GSH and GSNO (Figure 4, 5) showed that the drug levels were constant till the end of experiment after the complete release of drug at pH 7.4, which show that Eudragit® FS 30D polymer could be used successfully as polymeric carriers for unstable NO donors.

**Table 3. Release parameters of Eudragit® FS 30D-GSH and Eudragit® FS 30D-GSNO microparticles using Weibull model.**

pH	Eudragit® FS 30D-GSH		Eudragit® FS 30D-GSNO	
	$Q_{\infty}$ (%)	$T_{75\%}$	$Q_{\infty}$ (%)	$T_{75\%}$
1.2	22.8 ± 0.3	45 min	16.0 ± 0.5	46 min
3	18.3 ± 0.2	44 min	15.2 ± 0.5	34 min
6	24.4 ± 2.8	1 h 35 min	20.5 ± 0.4	41 min
6.8	n.d.	n.d.	n.d.	n.d.
7.4	100.2 ± 1.2	1 h 59 min	86.5 ± 1.4	1h 13 min

n.d. : not determined by the Weibull model

#### 4. Conclusions:

Spray dried microparticles were developed to provide an easy approach for the delivery of S-nitrosothiols to lower GIT specially colon area. The spray drying technique allowed us to microparticles in the size range of 5 to 7 µm with high encapsulation efficiency. FTIR studies confirm the stability of drugs in microparticles and PXRD confirms the mono-dispersed amorphous form of the microparticles. SEM confirms the smooth surface and spherical shape of the particles allowing good flow properties. By using Eudragit® FS 30D as a pH resistant rate controlling polymer, the drug could be targeted to colon area for the treatment of certain

disease conditions like Crohn's disease. Moreover, the sustained release enteric nature of microparticles could reduce gastric irritation.

### **Acknowledgment**

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**Discussion générale  
(anglais)**

**Conclusion et  
perspectives (français)**

# Discussion générale (anglais)

## 1. Polymer formulations

Since the aim of this thesis was to find out the best formulation for an intestinal delivery of NO, we compare various polymers.

Both the intermediate conjugates (i.e. chitosan-GSH and alginate-GSH) were freeze-dried after synthesis for further experiments. The freeze-dried chitosan-GSH conjugate was white fibrous powder with adhesive properties to any surface (Atyabi et al., 2008; Kafedjiiski et al., 2007). The covalent attachment of chitosan to GSH was previously done by Atyabi F and Kafedjiiski K (Atyabi et al., 2008; Kafedjiiski et al., 2007). Pure chitosan is soluble at acidic pH but the calm solubility of its conjugates with GSH in aqueous mediums (both acidic and neutral) characterizes its changed physical properties. It is important to completely characterize the physiochemical properties of polymer in order to understand the polymer ability to link to NO groups, stability, release kinetics and toxicity.

Similarly, the alginate-GSH conjugate was a creamy white powder with a fibrous structure. The chemical modification and covalent attachment of alginate to GSH was also done by Bernkop Schnurch in 2001 (Bernkop-Schnurch et al., 2001). The amount of thiol groups in both the conjugates were determined by using the Ellman's method (Ellman, 1959).

GSH is consisted of three amino acids (glutamate, cysteine and glycine). In cysteine, the sulfhydryl group (-SH) is present in reduced form and is involved in a variety of chemical reactions including antioxidants reactions. The direct reaction of GSH with several oxidants in a non-enzymatic manner results its conversion to dimeric oxidized form [i.e. glutathione disulfide, GSSG (Appenzeller-Herzog, 2011)]. These disulfide (GSSG) bonds can be break by  $\text{NaBH}_4$  to convert them to reduce form, which can be linked to higher amount of NO molecules.

### 1.1. Nitrosation of conjugates

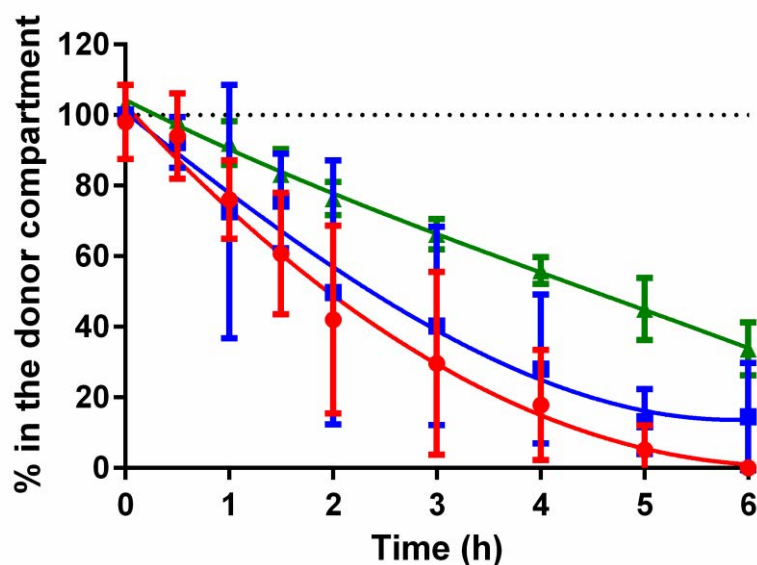
The two intermediate conjugates (i.e. chitosan-GSH and alginate-GSH) act as precursors for the synthesis of S-nitrosothiols. By the nitrosation of chitosan-GSH conjugates, we obtained SNOG

(S-Nitrosoglutathione-oligosaccharide-chitosan) and the nitrosation of alginate-GSH conjugates leads to the formation of SNA (S-Nitrosoglutathione-alginate), both acting as NO-donors. In this study, we use two methods for the nitrosation of both the conjugates i.e. a) the nitrosation of available reduced thiols in the conjugates (i.e. SNOC G1 and SNA F1), b) the nitrosation of all the thiol groups available in the conjugates (i.e. SNOC G2 and SNA F2).

## **1.2. Stability studies**

The stability testing of pharmaceutical products is a set of procedure ensuring the maintenance of product quality, safety and efficacy throughout the shelf life. It is considered as a prerequisite for the acceptance and approval of pharmaceutical products. The pharmaceutical analysis and stability are the two important steps in the development of a pharmaceutical product that assures the identity, purity and potency of ingredients as well as their formulated product. Stability could be defined as the capacity of a particular formulation in a specific container/system to remain within its physical, chemical, microbiological, toxicological, protective and informational specifications. Hence, it is an extent to which a formulation remains within its specified limits, throughout its period of storage and use, the same properties possessed at the time of packaging. It thus evaluates the environmental factors affecting the quality of a formulation which is utilized for prediction of its shelf life and determination of proper storage conditions (Bajaj et al., 2012).

From our results, we can compare the stabilities of each formulation in the donor compartment of the Ussing chamber (Figure 20).

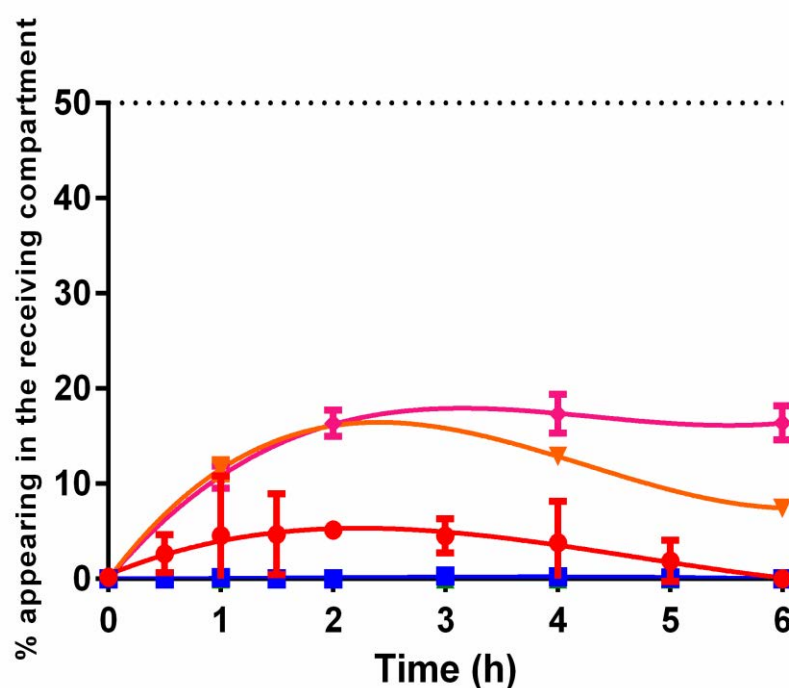


**Figure 20.** Concentrations of GSNO (- ● -), SNOC G2 (- ■ -) and SNA F2 (- ▲ -) as a function of percentage of the initial concentration of the donor compartment (100  $\mu$ M). Donor compartment; (n=3, mean value  $\pm$  S.D)

The temperature for the experiments was kept constant at 37 °C and Hank's BBS buffer was used as medium for all the experiments. As we can see from the Figure 20, SNA F2 was more stable. It remained approximately 40% after 6 h. All the GSNO was degraded within 6h whereas approximately 20% SNOC G2 remained after 6h, hence, we can say that SNA F2 was more stable as compared to GSNO and SNOC G2. Moreover, the stability studies of SNA F2 at different pH storage conditions showed that it was more stable at mildly basic pH conditions. However, in our experiments SNOC G1 and SNA F1 were less stable as compared to SNOC G2 and SNA F2. It is suggested that the decomposition of RSNO's occurs by the cleavage of S-N bonds liberating NO and an alkyl thiyl radical which could react with other molecules of S-NO, generating disulfide and an additional NO molecule (Gao et al.). Some recent studies showed that RSNO's are more stable at mildly basic pH conditions (8.4 to 8.8) in dark (Hornyak et al., 2012). The stability of both formulations showed that conjugation of chitosan or alginate to GSH and ultimately NO can increase their *in vitro/ex vivo* stability.

### 1.3. Permeability studies in the Ussing chamber

The *ex vivo* permeation studies of GSNO, SNOC G2 and SNA F2 were performed in an Ussing chamber across rat intestine. SNOC G2 and SNA F2 were selected for *ex vivo* studies because of their higher NO concentrations and better stabilities as compared to SNOC G1 and SNA F1 with smaller amounts of NO and least stabilities (Figure 20). In these experiments we use GSNO as a control. GSNO was able to cross the rat intestinal barriers and penetrated into the acceptor compartment of the Ussing chamber, but SNOC G2 and SNA F2 were unable to penetrate the intestinal walls and enter into the acceptor compartment. However, both formulations were able to deliver NO/nitrites in a sustained manner into the acceptor compartment (Figure 21).



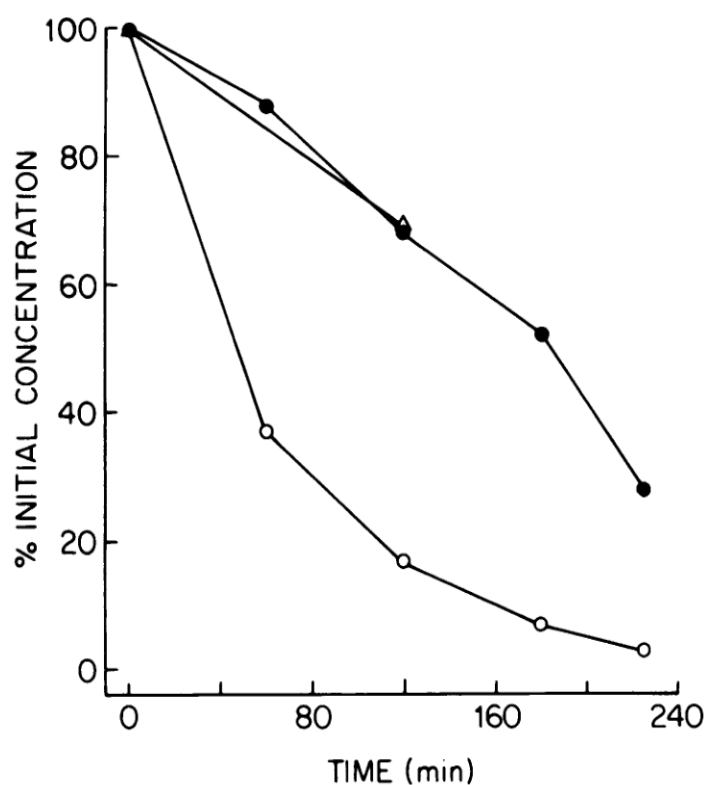
**Figure 21.** Concentrations of GSNO (●), SNOC G2 (■) and SNA F2 (▲), NO/nitrites SNOC G2 (▼) and nitrites SNA F2 (◆), as the percentage of the initial concentration of the donor compartment (100  $\mu$ M). Acceptor compartment; (n=3, mean value  $\pm$  S.D)

Finally all the formulations degraded into their final degradation products [i.e. nitrites ( $\text{NO}_2^-$ ) and nitrates ( $\text{NO}_3^-$ )] in their respective times. In the degradation products, nitrites were released in the first hours of the experiments and remained in certain concentrations at the end of the experiments. However, a part of nitrites was readily converted to nitrates, which

were found at maximum concentrations at the end of the experiments. It is suggested that nitrites can be considered as a sign of NO formation (Schulz et al., 1999). As an equal amount of medium was added to both compartments of the Ussing chamber, an equal distribution of nitrites and nitrates should be present in both the compartments of the Ussing chamber with a theoretical value of 50%. In previous decades, it was thought that both the nitrites and nitrates were inert end products of endogenous NO metabolism, but recent studies showed that these products can be recycled *in vivo* and could be transformed back into NO, which represents an important unconventional source of endogenous NO beside the classical L-arginine-NO-synthase pathway (Lundberg et al., 2008). In a study published by Khan et al (Khan et al., 2011), it was shown that both nitrites and nitrates ions might be significant precursors of NO and could increase the NO concentrations in brain.

It was observed from our experiments that chitosan or alginate could enhance the sustained release properties of S-nitrosothiols which might be of potential benefits for the treatment of certain diseases like Crohn's disease. In Crohn's disease, a sustained release of NO is required in the colon for proper treatment, while the systemic absorption is considered as a risk for cardiovascular side effects.

The proper supply of oxygen is necessary to the Ussing chamber because it not only maintains the viability of the intestine but also properly circulate the drug in the medium for a constant contact with the intestine. However, it has a direct impact on the degradation of S-nitrosothiols. In a study published by Gaston et al, (Gaston et al., 1993) showed that the degradation of GSNO in human Bronchoalveolar lavage fluid (BALF) was accelerated when the oxygen concentration was increased from 21% to 95% as shown in (Figure 22). Since the concentration of O<sub>2</sub> is very low in the intestine lumen, the sustained release property of the polymer should be improved.



**Figure 22.** Stability of GSNO in human BALF. Under identical conditions (pH 7.30, 21% O<sub>2</sub>, 25 °C) endogenous GSNO in BALF with a patient with cystic fibrosis and pneumonia (▲) has stability identical to that of exogenous GSNO (●). The decomposition of exogenous GSNO is accelerated in the presence of 95% O<sub>2</sub> (○) (Gaston et al., 1993)

#### 1.4. Cytotoxicity studies in the Caco2 cell line

Toxicity studies were performed for SNA F2 with Caco2 cells under suitable conditions. The cells were carefully observed before and after the incubation with SNA F2. We observed that SNA F2 in concentrations of more than 100µM exhibited 86% viability, while the viability of cells was more than 100 % when incubated with SNA F2 in less than 50µM concentrations. Moreover, SNA F2 was cytotoxic in concentrations of 200µM and exhibited 38% cells viability. It was observed from our experiments that alginate alone increased the viability of the cells. The cells viability increases because of the increased metabolic activity and growth of the cells when they stay in contact with microparticles as mentioned by Ramia Safar et al, and Ronzani et al, in their studies (Ronzani et al., 2014; Safar et al., 2015). Eudragit® RL was seeded into human THP-

1 monocytic cell line and an increase of 40% in cell viability was observed. The increase in metabolic activity and cell growth increment was related to augmentation of the cell mitochondrial volume and activity. This mitochondrial activity could be related to cell proliferation which increases its number (Ronzani et al., 2014; Safar et al., 2015).

## **2. GSH and GSNO spray dried microparticles based on Eudragit® FS 30D polymer**

In the previous two publications, we prepared direct NO-donors (SNOC and SNA) which were able to carry a sufficient amount of NO to be tested for *in vitro/ex vivo* experiments. The *in vitro/ex vivo* studies showed that they were stable for up to 10h in mildly basic medium. However, these two compounds were not obtained in drug dosage forms because they were in liquid form which can't be taken orally like syrups; therefore, we tried to develop orally administered polymeric microparticulate delivery system for S-nitrosothiols. For this purpose, we use Eudragit® FS 30D (pH resistant polymer; soluble at pH > 7) to directly encapsulate S-nitrosothiols for colonic delivery.

Polymeric systems are widely used and they can modulate the drug activity by active and/or passive targeting to different sites in the body. Several polymers have been successfully used for the delivery of NO, which showed stabilities and improved therapeutic effects. However, depending upon the polymer, used the drug release rates can vary from formulation to formulation, whereas experimental conditions and methods used for encapsulation can affect the particle size, morphology and encapsulation efficacy (Saraiva et al., 2011).

In this study, we used spray drying technique for the preparation of microparticles. These spray dried microparticles were developed at several physical parameters including inlet temperature, outlet temperature and solvent flow. The parameters that yielded the best formulations (i.e. inlet temperature of 120 °C, outlet temperature of 47 °C and solvent flow 5 ml/min) were selected for further studies. These spray dried particles were negatively charged with particle size ranging from 5 to 7 µm. It was observed that the physical parameters including temperature and solvent flow had a significant role on the particle size and production yield. High solvent flow produced larger size particles as compared to low solvent



flow. It might be due to the reason that high volume of solvent reaches the nozzle of the spray thus allowing very less time for optimization leading to variation in particle size (Anish et al., 2014). Solvent flow has also a significant effect on the particle size and production yield, higher the solvent flow lower will be the production yield. This might be due to the fact that at high solvent flows, the spray pattern is not uniform and maximum of the solvent is sprayed on the walls of the spraying chamber which yields to a low production yield (Anish et al., 2014). The inlet and outlet temperatures had also a significant role and it influences the particles yield. At low inlet temperatures, the solvent is not evaporated from the droplets, which when increases in mass attach to the walls of the chamber and drops in form of liquids to the bottom of the chamber, thus reduces the production yield. Meanwhile in the spray drying process, the possible degradation of GSNO into nitrites/nitrates is also a key point. Prior to the preliminary experiments, pure GSNO solution was spray dried under the same physical conditions and no variation in the GSNO concentration was observed. There was a very negligible degradation in the GSNO because of the very short time of GSNO contact with the oxygen present in the air.

### **2.1. Physical characterization**

Spray dried microparticles were characterized for their physical behaviour. Different techniques were used for this purpose.

In the FTIR studies, each molecule in the polymer matrix demonstrates a unique characteristic spectral bending and stretching vibrations due to specific functional groups under the influence of IR radiations, which can be used as identification markers. The characteristic vibrations of all the functional groups were observed in our experiments at specific points and it was concluded that there was no interaction between the drug (GSH and GSNO) and the polymer. Moreover, the drug was stable in the polymer matrix.

PXRD (Powder X-ray diffraction analysis) is a sensitive method which can provide information about the arrangement of molecules with in the crystal. In our experiments, we obtained the characteristic peaks of drugs and polymers. We observed that pure GSH and GSNO demonstrates crystalline structure, whereas, their polymeric forms and pure Eudragit® FS 30D demonstrated amorphous form means that the drugs were properly embedded into the

polymer matrixes. The amorphous form of drugs can improve its *in vitro/in vivo* dissolution properties.

In the SEM (scanning electron microscopy), we observed the physical structure or shape of the micro particles. Spherical shape and a smooth surface are two important factors in pharmaceutical ingredients that can contribute to the flow properties of microparticles (Anish et al., 2014). We took SEM images of our particles at different resolutions. It was observed that simple Eudragit® FS 30D particles were wrinkled in shape, while the microparticles of GSH and GSNO with Eudragit® FS 30D were rounded with smooth surfaces. This might be due to the higher percentage of polymer in the blank particles. The wrinkled shell could be originated during the early drying process, where the polymer soft surface folds to form wrinkled structure as was described in some previous studies (Vehring, 2008). Moreover, the amount of proteins or peptides in the polymeric structure can increase the hydrophilicity of the particles which delays the formation of shell and then change the surface morphology of the microparticles. Our results were confirmed in a study where albumin was encapsulated in a polymer and spherical dense particles were collected (Bosquillon et al., 2001; Vanbever et al., 1999).

## **2.2. *In vitro* drug release behaviour**

The *in vitro* drug release studies are performed to observe the drug release behaviour and mechanism in the dissolution medium. We use different basic and acidic solutions with pH values 1.2, 3, 6, 6.8 and 7.4 respectively for the dissolution studies. An initial burst release mechanism was observed for formulations, which could be due to the drug bounded to the surface of the particles, which are then released when in contact with the dissolution medium. The amount of drug released as a result of initial burst release mechanism was constant throughout the experiment in the formulations, which do not released the drug in acidic conditions. The formulations do not released drug at acidic conditions (i.e. pH 1.2, 3 and 6), slow sustained release at pH 6.8 and faster release profiles at pH 7.4.

Terminal ileum and proximal colon could be primarily affected by several types of colonic diseases mainly including chronic irritable bowel syndrome (IBS), Crohn's disease, ulcerative colitis with unknown origin, and colon cancer (Dvorackova et al., 2011). Colonic delivery can be

used for the improvement of oral bioavailability of drugs used for the treatment of such types of diseases, whereas the long colonic residence time provides a significant opportunity for the drug absorption. Table 6 shows the pH values of the different parts of the GIT. The physiological pH in small intestine (duodenum) is 6, which increase to 7.8 in the terminal ileum; hence, we can propose that our formulation can deliver drug to this region and release for its specific action for the treatment of Crohn's disease. The colon is an advantageous site for drug delivery because of its long transit time and low proteolytic enzyme activity. The colon offers advantage not only for the treatment of localized diseases but is also a suitable site for systemic treatments. The advantage and necessity of specific colon targeting drug delivery systems has been well recognized recently. Colon specific delivery has the potential to address important therapeutic needs for the effective treatment of colon related disease including Crohn's disease. Continuous efforts have been done in developing colon specific drug delivery systems with improved site specificity and versatile drug release kinetics to accomplish different therapeutic needs (Yang et al., 2002).

Table 4. Human intestine physiology (Dvorackova et al., 2011)

Small intestine region	pH range	Transit time (h)
Duodenum	6.0–6.1	1
Jejunum	6.1–7.2	1
Ileum	7.0–7.8	2
Proximal colon	6.4	6–48
Distal colon	7.0	

However, the big obstacle for a specific colon delivery is the protection of drug from harsh acidic environments of the stomach. Our formulations didn't release the drug in acidic conditions which ensures the protection of drug in harsh acidic environment of stomach and could be suggested as a suitable colon specific drug delivery system. Several researchers have studied the potential use of GSNO in the treatment of mucosal barrier failure in Crohn's disease (Savidge et al., 2007); hence, our formulation based on pH sensitive Eudragit® FS 30D polymer could be suggested for further experiments in the treatment of Crohn's disease.

A very high dose of NO is required in order to achieve therapeutic effects of NO-donors because not only NO is released very quickly from the formulations but is also readily transformed into

its degradation products nitrites and nitrates (Wu et al., 2015). Eudragit® FS 30D is a pH dependant rate controlling polymer, which can inhibit the drug release profiles in acidic conditions, while slows down the release rates at basic conditions, hence, specifically targeting the intestines. In a study (Cole et al., 2002) , paracetamol was delivered to intestines by encapsulating in HPMC capsules coated with Eudragit® FS 30D. These formulations were resistant to acidic conditions in upper GIT at pH 1.2 for 2 h followed by 1h at pH 6.8 and did not release the complete drug until it was reached to the small intestine and proximal colon in an average time of 6.9 h post dose. In a second study, gelatine capsules were coated with Eudragit® FS 30D polymer released the drug when the pH of the medium was changed from 1.2 or 6.8 to 7.5, and was found to be an effective drug delivery carrier for colon targeting (Dvorackova et al., 2011). In several more studies, Eudragit® FS 30D was used as a pH dependant rate controlling polymer, and drugs were carried to ileocecal regions for the treatment of mild to moderate ulcerative colitis (Bott et al., 2004).

### **2.3. Advantages of spray drying**

Spray dried microparticles are in suitable dosage form and can be taken orally. The use of different polymers can make the drug to target specific sites in the body just like pH resistant polymers. The polymeric spray dried microparticles gives a sustained drug release profiles *in vitro*.

## **3. Perspectives**

Both the formulations (i.e. SNOC and SNA) were in liquid form and were not suitable for oral delivery. However, we can propose various drug delivery systems which can make it suitable for oral delivery. This thesis include spray drying technique used for direct encapsulation of GSNO and GSH with Eudragit® FS 30D polymer suitable for oral delivery, which gave a sustained drug release at basic pH while resistant to acidic pH. We can use the same technique to develop spray dried microparticles of SNOC and SNA. We believe that our compound can be encapsulated in gastro-resistant soft gelatine capsules for oral delivery. Microparticles of SNA with chitosan can also be prepared (D Marcato et al., 2013a). In addition, a polyelectrolyte

complex of alginate with chitosan and Eudragit® L100-55 can also be prepared (Calija et al., 2013).

Currently, we are working on polyelectrolyte complex of chitosan with Eudragit® L100-55. This complex would be further used to make particles with GSH, GSNO, SNOC and SNA to make them suitable for oral delivery. These particles will be further evaluated for their physical structure, size, zeta potential and *in vitro* dissolution studies. Meanwhile, we will start research work on hydrogels to provide a suitable oral delivery vehicle for S-nitrosothiols.

Animal models will be used in future to evaluate the *in vivo* potential of the new compound and to study its pharmacokinetics. Furthermore, this type of conjugation will be tried for other biodegradable polymers.

## Conclusion et perspectives

Le chitosan et l'alginate permettent de fixer des quantités importantes de NO. Les polymères SNOC G2 et SNA F2 sont ainsi susceptibles de transporter des quantités significatives de NO avec un effet de « libération prolongée » intéressant. Nous n'avons pas observé d'absorption directe de GSNO mais uniquement une libération locale de NO. Nous pensons que ce type de polymère est adapté pour le développement de formulations variées comme des microparticules (Moustafine, Margulis et al. 2008).

Après liaison du GSH, le chitosan devient plus hydrophile et cette propriété dépend du degré de substitution en GSH. Il peut par exemple être désacétylé avant la thiolation pour moduler son hydrophobicité.

Les polymères finaux (SNOC G2 et SNA F2) ne peuvent pas prévenir la formation du NO<sub>3</sub> mais peuvent ralentir sa production.

L'impact sur la pression artérielle reste à évaluer mais cela nécessite des doses élevées de RSNO. Les résultats obtenus par Khan et al. (Khan, Sakakima et al. 2011) ne montraient aucune altération de la pression sanguine artérielle ni du rythme cardiaque des rats à une dose de 50 µg de GSNO/kg poids corporel.

Il est également possible de comparer le comportement de nos polymères aux médicaments actuellement sur le marché : les nitrites organiques comme la trinitrine sont complètement absorbés après une administration par voie orale et la libération de NO se produit essentiellement dans la circulation sanguine. Ce mécanisme limite l'effet local du NO sur l'intestin.

L'alginate permet d'obtenir un donneur de NO particulièrement stable entre pH 1,2 et 8,4. L'alginate est donc un bon candidat s'il on veut obtenir une libération contrôlée de NO. La faible toxicité de ce nouveau composé suggère également que des études *in vivo* sur des modèles animaux pourraient être développées.

Nous pensons que notre composé peut être encapsulé dans des capsules gastro-résistantes de gélatine destinées à la voie orale. De plus, un complexe polyélectrolytique d'alginate avec chitosan et Eudragit L100-55 pourra être proposé (Calija, Cekic et al. 2013). Actuellement, nous travaillons sur un complexe polyélectrolytique de chitosan avec l'Eudragit® L100-55. Ce complexe sera utilisé pour fabriquer des particules avec du GSH, du GSNO, du SNO et du SNA afin de mettre au point une forme orale adéquate. Nous avons également commencé à mettre au point des hydrogels de SNA pour fournir des vecteurs adaptés à la voie orale.

Les microparticules séchées par *spray drying* ont été développées pour fournir une approche simple de délivrance spécifique au niveau colon. La technique de *spray drying* permet d'obtenir des microparticules qui ont une taille comprise entre 5 et 7  $\mu\text{m}$  avec un bon taux d'encapsulation (de 74% à 84%). Les études de spectroscopie infrarouge (IRTF) confirment la stabilité des principes actifs dans les microparticules et les études par rayons X (PXRD) confirment la forme amorphe de ces microparticules. La microscopie électronique à balayage montre que les microparticules ont une surface lisse et une forme sphérique ce qui devrait conférer à la formulation de bonnes propriétés d'écoulement. En utilisant l'Eudragit® FS 30D comme un polymère de « libération contrôlée dépendante du pH », le principe actif peut cibler le colon pour le traitement de certaines maladies comme la maladie de Crohn. De plus, la libération retardée entérique des microparticules peut réduire l'irritation gastrique.

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## Summary

Nitric oxide (NO) is an endogenous molecule produced by the endothelial cells and many other cells of the body. It performs different physiological functions (vasodilatation, platelet aggregation/vascular myocyte proliferation, intestinal barrier function regulation,...). Several pharmacologically active S-nitrosothiols are available; they have the ability to release NO.

The aim of the thesis was to develop novel and stable NO-donors by linking S-nitrosoglutathione (GSNO) to a polymer (i.e. chitosan and alginate) backbone. This new polymer could be used in the treatment of Crohn's disease. In the first step, chitosan-GSH and alginate-GSH conjugates were prepared by a carbodiimide reaction and in the second step SNO (S-nitrosoglutathione-oligosaccharide-chitosan) and SNA (S-nitrosoglutathione-alginate) were prepared by the nitrosation of both conjugates respectively. The amount of NO was determined by Griess and Saville methods. Stability and *ex vivo* experiments of SNO and SNA were performed in an Ussing chamber through rat intestine [37 °C temperature and constant O<sub>2</sub> pressure].

We obtained polymers with different amount of NO (i.e. 159 µmol of NO/g to 525 µmol of NO/g for SNO; 174 µmol of NO/g to 468 µmol of NO/g for SNA) depending upon the procedure of nitrosation. In the Ussing chamber, SNO was stable for at least 6h and SNA for at least 10h. Both were able to carry significant amounts of NO with a sustained manner.

Also, we aimed to develop spray dried microparticles of GSNO based on Eudragit® FS 30D polymer. The microparticles characterization was done by scanning electron microscopy (SEM), X-ray diffraction (PXRD), infrared spectroscopy (FTIR) and *in vitro* release studies were performed in a phosphate buffer (pH 1.2, 3, 6, 6.8 and 7.4). The microparticles were negatively charged with mean particle size ranging from 5 to 7 µm. The formulation was stable and was resistant to acidic pH but showed rapid release in basic pH. The polymeric microparticles were acid resistant; hence, they can be used as colon specific drug delivery systems for the treatment of Crohn's disease.

We think that these formulations could be used on animal models in the treatment of Crohn's disease.

**Keywords:** chitosan, alginate, glutathione, nitric oxide, Ussing chamber, Crohn's disease

## Résumé

L'oxyde nitrique (NO) est une molécule endogène produite par les cellules endothéliales et de nombreuses autres cellules de l'organisme. Il exerce des fonctions physiologiques variées (vasodilatation, inhibition de la prolifération des cellules musculaires lisses, inhibition de l'agrégation plaquettaire, régulation de la barrière intestinale,...). Les S-nitrosothiols sont des substances pharmacologiquement actives qui ont la capacité de libérer du NO.

L'objectif de cette thèse était de développer de nouveaux « donneurs de NO » stables en liant du S-nitrosoglutathion (GSNO) à une structure polymérique (chitosan ou alginate). Ce nouveau polymère pourrait être utilisé dans le traitement de la maladie de Crohn. Dans une première étape, les polymères ont été liés au glutathion (GSH) : le chitosan-GSH et l'alginate-GSH ont ainsi été préparés par la « méthode des carbodiimides » et dans une deuxième étape, les polymères finaux [SNO (S-nitrosoglutathione-oligosaccharide-chitosan) et SNA (S-nitrosoglutathione-alginate)] ont été préparés par nitrosation des deux conjugués précédent. La quantité de NO fixée a été déterminée par les méthodes Griess et Saville. L'aptitude des polymères à libérer du NO et à passer la barrière intestinale [SNO et SNA] a été évaluée dans une chambre d'Ussing [température de 37°C avec une pression constante O<sub>2</sub>].

Nous avons obtenu des polymères avec des quantités variables de NO en fonction de la méthode utilisée (159 µmol de NO/g à 525 µmol de NO/g pour le SNO ; 174 µmol de NO/g à 468 µmol de NO/g pour le SNA). Dans la chambre d'Ussing, le SNO était stable pendant au moins 6h et le SNA pendant au moins 10h. Les deux polymères étaient en mesure de transporter une quantité importante de NO d'une manière durable.

Enfin, nous avons essayé de mettre au point des microparticules de S-nitrosoglutathion (GSNO) par spray drying. L'Eudragit® FS 30D, qui est gastro-résistant, a été choisi comme polymère. La caractérisation de microparticules a été réalisée par microscopie électronique à balayage (SEM), par diffraction X (PXRD), par spectroscopie infrarouge à transformée de Fourier (FTIR). Les essais de libération *in vitro* ont été réalisés dans un tampon phosphate (pH 1,2, 3, 6, 6,8 et 7,4). Les microparticules étaient chargées négativement avec une taille moyenne allant de 5 à 7 µm. La formulation était stable à pH acide mais a montré une libération rapide à pH basique. Ces microparticules polymériques sont donc résistantes à l'acide ; elles pourraient donc servir de systèmes de délivrance du NO au niveau intestinal.

Nous pensons que ces différentes formulations pourraient être testées sur des modèles animaux dans le traitement de la maladie de Crohn.

**Mots clés :** chitosan, alginate, glutathion, oxyde nitrique, chambre de Ussing, maladie de Crohn