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PREPARATION AND CHARACTERIZATION OF NITROSOTHIOLS AS NITRIC OXIDE DONORS

PRIPRAVA IN KARAKTERIZACIJA NITROZOTIOLOV KOT DONORJEV DUŠIKOVEGA OKSIDA

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Kaja Antlej

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Abstract

NO-donors represent numerous pharmacologically and physiologically various molecules that act as precursors of NO in human body. NO is a free endogen radical, which plays the main role in important physiological functions in a complex and diversified system of chemical reactions, mostly in cardiovascular system. Among the most known NO impacts are its inhibitory effect on adhesion and aggregation of circulating platelets, anti-inflammatory activity, protective effect in endothelial dysfunction and its effect on the systemic vasodilatation. As a consequence to written above, delivery of exogenous NO would be an attractive therapeutic option, thus this work will describe our initial research in developing potential novel polymer-based NO conjugates. In the framework of this study we wanted to develop *S*-nitrosothiols, as a main goal from scientific view the most interesting subgroup of NO donors of the new generation with less side effects and more significant targeting properties. In our laboratory work over coupling polysaccharides with naturally occurring molecule glutathione we synthetized polymeric solutions of Methocel™ and alginate conjugates that were instead lyophilised. The obtained polymeric particles were evaluated for their loading of free thiol groups with Ellman's method. As the next step it followed the nitrosation and characterization of obtained conjugates with Griess-Saville method. Particles of SNOA were grafted with thiol groups representing a fairly low capacity to store NO with 57.36 μmol/g of NO respectively. We evaluated the particle size and their molecular weight with Zetasizer. Characterized with high polydispersity index their size varied from very small to very big, refusing nanoparticle size that was desired as one of main goals. The preparation of GSNO and SNOA was found to be difficult because of its sensitivity to various parameters, which caused instability of our solution when stored longer than 1 day (GSNO) or 4 days (SNOA). Many inconveniences were caused by the mucoadhesive nature of alginate and its tendency to self-assembly. The variables having the biggest impact on the results described below in the presented work should be examined in addition in future experiments.

Povzetek

NO donorji predstavljajo številne farmakološko in fiziološko različne molekule, ki v človeškem telesu delujejo kot prekurzorji NO. NO je endogen radikal, ki igra glavno vlogo v mnogih pomembnih fizioloških funkcijah v prepletenem in razvejanem sistemu kemijskih reakcij, pretežno v kardiovaskularnem sistemu. Med najbolj raziskane učinke NO sodijo predvsem inhibicija adhezije in agregacije krožečih krvnih ploščic, njegovo protivnetno delovanje in zaščitna vloga v endotelijski disfunkciji ter sistemska vazodilatacija. Glede na njegovo večplastno vlogo se torej postavlja vprašanje ali bi dostavo eksogenega NO lahko izkoriščali v terapevtske namene, zato to delo opisuje naše začetne raziskave v razvoju potencialnih inovativnih polimernih NO konjugatov. V okviru magistrske naloge smo želeli razviti donorje dušikovega monoksida, in sicer iz znanstvenega vidika najzanimivejšo podskupino molekul *S*-nitrozotiole, ki spadajo med NO donorje nove generacije z manj neželenimi učinki in učinkovitejšim tarčnim delovanjem. V sklopu raziskovalnega dela smo preko sklapljanja polisaharidov z naravno prisotno molekulo glutation pridobili polimerne raztopine konjugatov Methocel™-a in alginata, ki smo jih liofilizirali. Dobljene polimerne delce smo okarakterizirali glede na prisotnost prostih tiolnih skupin s pomočjo Elmanove metode. Sledilo je nitroziranje in vrednotenje dobljenih konjugatov z metodo Griess-Saville. Sintetizirani delci SNOA so imeli precej nizko vsebnost NO, in sicer 57.36 μmol/g NO. Velikost delcev in molekulsko maso smo preverili na napravi Zetasizer. Glede na velik izmerjen polidisperzni indeks je velikost delcev variirala od zelo majhnih do zelo velikih, kar je bilo v nasprotju z našim ciljem sintetizirati delce nanometerskih velikosti. Priprava GSNO in SNOA je bila težavna zaradi njune občutljivosti na številne parametre, ki so povzročili nestabilnost raztopine, če je bila le-ta shranjena več kot 1 dan (GSNO) ali 4 dni (SNOA). Veliko nevšečnosti nam je povzročila mukoadhezivna narava polisaharida alginata in njegova težnja k samozdruževanju. Spremenljivke z največjim vplivom na rezultate v spodaj opisanem delu bi bilo potrebno dodobra preučiti v prihodnjih poskusih.

List of abbreviations

AlbNO – *S*-nitrosoalbumin

CysNO – *S*- nitrosocysteine

DTNB – 5,5′-dithio-bis(2-nitrobenzoic acid)

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

GSH – glutathione

GSNO – *S*-nitrosoglutathion

GC – guanylate cyclase

cGMP – cyclic guanosine-3’5’-monophosphate

MC – methylcellulose

MWCO – molecular weight cutoff

NACNO – *S*-nitroso-*N*-acetylcysteine

NED – *N*-(1-naphthyl)ethylenediamine

NHS – *N*-hydroxysuccinimide

NO – nitrogen monoxide

NOS – nitric oxide synthase

eNOS – endothelial nitric oxide synthase

iNOS – inducible nitric oxide synthase

nNOS – neuronal nitric oxide synthase

SNAP – *S*-nitroso-*N*-acetylpenicillamine

SNOA – *S*-nitrosoalginate

VSMC(s) – vascular smooth muscle cell(s)

RSNO – radical nitrosothiol

1. INTRODUCTION

1.1 Nitric oxide

Nitric oxide (also nitrogen monoxide, NO radical, formula ·N=O, abbreviated to ·NO, from now on NO), first called "endothelium derived relaxation factor", was only in 1979 identified as NO and in 1992 the journal "Science" rewarded it with the title "Molecule of the year", remaining under unabated investigation throughout its long history from the beginning of nineteenth century. This small diatomic molecule has despite its structural simplicity a complex chemistry, which endows the free radical with diverse and wide biological actions, influencing in particularly cells of the vascular endothelium, immune and neural system. It is a highly regulated paracrine and autocrine signaling molecule, constitutively synthesized by a large number of cells, with a sphere of influence diffusing from 40 to 200 μm (1) of its origin on account of its reactivity (2). The latter produces important consequences, giving it both, the greatest weaknesses and strengths. Firstly, NO has to be rapidly synthesized on demand in response to stimuli. And secondly, it makes it a real local mediator, not requiring complex metabolism for clearance – it is diluted and oxidized to nitrite and nitrate, as it diffuses away from the active site. Therefore, *in vivo* NOs fate is either reduction or oxidation – it depends of the redox status of the surrounding micro-environment. In physiological conditions its stable conjugates are found, resulting from the formation of covalent S-nitroso links (RSNOs): *S*-nitrosoalbumin (AlbSNO), *S*-nitrocysteine (CysSNO) and *S*-nitrosoglutathione (GSNO) most commonly. Other forms of NO include iron-nitrosyl complexes and nitrites.

Because of its continuous production in the blood vessels, the most studied actions of NO are in the **cardiovascular system.** NO is produced by the endothelial cells that line the lumen of blood vessels: L-arginine is converted by nitric oxide synthase (NOS) into NO as a response to chemical and mechanical stimuli mobilizing intracellular calcium. There are three mayor isoforms of NOS: neuronal NOS (nNOS, has important role in the neurotransmission), inducible NOS (iNOS, also macrophage-inducible NOS, associated with endotoxin stimulation) and endothelial NOS (eNOS), therefore producing three different types of nitrogen monoxide. They are expressed in different cell types and also under different conditions, guaranteeing among

other that the vascular tone is maintained by steady release of NO and that inflammation or pathogens remain under control (2).

NO plays a key role in the bioregulation of several physiological intra- and extracellular pathways and it is known for his **impact on the circulating platelets**. In response to endothelium and platelet derived NO, cGMP-dependent and -independent mechanisms produce a strong inhibitory effect on aggregation and adhesion of platelets. Correspondingly, endothelium-derived NO is a powerful inhibitor of the cell **inflammation** activity and also known to be an important inhibitor of **monocyte activity**. In the contrary to other endothelium-derived vasodilators (e.g. prostacyclins in resistance vessels), NO generally predominates in large conduits, having little impact on blood pressure, which might suggest that its primary role lies in its **antiatherothrombotic properties** rather than its vasodilator effects (2).

In vascular smooth muscle cells (VSMC), acting via the enzyme guanylate cyclase (sGC), NO stimulates the production of cyclic guanosine-3'5'-monophosphate (cGMP), evoking **vasodilatation** via cGMP-dependent protein kinases that decrease calcium concentration. Throughout this mechanism, it is known that it inhibits **VSMC proliferation** (2).

Concentrations of NO providing those primarily protective effects are extremely low – picomolar to nanomolar pointedly. Therefore, in higher concentrations we can expect different properties and cellular targets, particularly under condition of cellular stress where NO is highly cytotoxic, reacting with superoxide to form peroxynitrite ($ONOO^-$) mostly known in **radical reactions**. As innate response during pathogen invasion, NO is **formed by inflammatory cells** macrophages expressing an inducible form of NOS (iNOS) along with activation of NAD(P)H oxidase to produce NO and superoxide, forming highly cytostatic and cytotoxic $ONOO^-$. Pursuant to this, at high concentrations additional chemical reactions become more plausible, especially those with molecular oxygen which generate nitrosating species, capable of stimulating receptors, activating nuclear regulatory proteins and altering cell functions leading to oxidation of functional groups of nucleic acid and proteins (rapid nitration of aromatic residues of amino acids, deamination of bases) (9). Their probability is proportional with oxygen availability in the environment. Of particular importance is also the inhibitory impact of NO on **cellular respiration** interacting with the respirational chain (along with other endogenous *S*-nitrosothiols and $ONOO^-$) and its mediation of apoptosis in inflammatory cells (2).

We must not forget to mention the role of NO in the **endothelial dysfunction**, where the depression of the NO-sGC pathway is a key future of the mentioned systemic pathological state of the endothelium. Alterations in endogenous NO activity (impaired NO production and/or its increased inactivation) and therefore a decrease in the NO bioavailability makes the arteries and arterioles unable to dilate fully in response to vasodilator(s), resulting in vasoconstriction, increased smooth muscle cell proliferation, along with increased activity and adherence of platelets and inflammatory cells at sites of endothelial damage (2).

As a consequence to written above, delivery of exogenous NO would be an interesting therapeutic option when it comes to blood pressure regulation, atherosclerosis, thromboembolic disease (endothelial dysfunction), hypoxia and related pulmonary diseases, inflammation, heart failure and angina, seen that it's altered levels (caused by a decrease of either bioavailability or NO synthesis) are commonly implicated into a vast number of pathological conditions. Unfortunately, its benefits do not appear to be realizable with organic nitrates – they remain effective, rather than agents that might slow disease progression, only as a symptomatic treatment. The greatness of NO versatile effects is apparently also its limitation regarding systemic NO delivery, undoubtedly causing unwanted side effects outside the target tissue. This nonpolar gaseous molecule is reasonably produced in controlled amounts when demanded and is used effectively without exerting any noticeable toxicity in mammalian tissues. Therefore, novel molecules are taking the main stage in order to improve NO alterations and by mimicking its normal physiological storage try to ameliorate or heal different pathological conditions.

1.2 NO donor drugs

The astonishing fact when keeping in mind the enormous potential of NO is that only few types of NO donor drugs are being currently clinically used. Because of its extremely short half-life *in vivo* (ca. 5 s, (1)) followed by a quick oxidation to nitrogen dioxide, NO gas is particularly troublesome to handle and in administered *per se* it can be used therapeutically in pulmonary hypertension and in neonates, delivered to lungs via inhalation. The need to overcome such limitations has been pushing the researches into the development of NO donor drugs in form of molecular carriers that are supposed to chemically store and stabilize the radical until its release is required in controlled and targeted matter. NO donors are pro-drugs that decompose in the

body releasing NO in different ways (depending of their structure and pathology targeted) and can be classified into following classes:

- **Organic nitrates and nitroso compounds:**
    - nitroglycerin (glyceryl trinitrate, GTN) used in acute relief of pain in angine, also in ointments (anal fissures) and nebulizers (pulmonary hypertension),
    - isosorbide mononitrate (ISMN, treatment of chronic angina),
    - sodium nitroprusside (rapid lowering of blood pressure in hypertensive crisis, gold standard vasodilator in clinical trials)

Organic nitrates are already for decades the most commonly used NO donor drugs, although having many inconveniences. The main limitations are a rather poor distribution to target cells (vascular smooth muscle), fast release, development of **tolerance** and with a long time use increasing the risk of cardiac events through the induction of oxidative stress.

- **Diazeniumdiolates (NONOlates):**
    - Diethylamine NONOate, DEA/NO

This class of compounds consists of diolate group (N(O-)N=O) bound to a nucleophile adduct (primary-/secondary-/poly-amine) via a nitrogen atom. The release of NO can be precisely predicted because NO release follows the simple first order kinetics (decomposition rate depends on the structure). Clinical trials show they could be used in the treatment of erectile dysfunction, prevention of thrombosis or targeted delivery of high concentrations of NO to tumor cells without harming healthy cells, but all in all their toxicity needs to be more examined.

- ***S*-nitrosothiols:**
    - *S*-nitroso-*N*-acetylcysteine (NACNO)
    - *S*-nitroso-*N*-acetyl-penicillamine (SNAP)
    - *S*-nitroso-*N*-valerylpenicillamine
    - *S*-nitrosoalbumine (AlbNO)
    - *S*-nitroso-cysteine (CysNO)
    - *S*-nitrosoglutathione (GSNO)

S-nitrosothiols represent compounds consisting of a single chemical bond between a thiol (sulphydryl) group (R-SH) and the nitroso group. They have a promising future and will be further described below.

- **NO Hybrid drugs:**
    - NO-NSAIDs (derivatives of aspirin or "nitroaspirins")
    - Nicotinamide derivatives (e.g. nicorandil)
    - β-receptor antagonist containing a nitrate group (e.g. nipradilol)
    - Nitrate derivatives of statins (e.g. NCX6550)
    - Nitrosated ACE inhibitors (e.g. *S*-nitrosocaptopril or SNO-Cap)
    - Dihydropyridins with linked furoxan group (calcium antagonists + vasodilators)
    - Furoxan and nitrated phenols (furoxans linked with Histamine receptor antagonists, in form of protein pump inhibitors, linked to metronidazole etc.)

They represent a range of already established drugs structurally changed by incorporating NO-containing molecules, with the aim of preserving the pharmacological activity of the parent compound and "boosting" it with the biological actions of NO. For instance, associating aspirin with the NO (can promote healing of gastric ulcers by increasing the secretion of protective gastric mucus and promoting reparation and removal of toxins) could minimize the gastric side effects while having comparable or greater antiplatelet action.

- **Zeolites**

Ion-exchange zeolites are microporous insoluble materials that form a framework containing metal ions capable of binding NO. With the ability to modulate the rate of NO release they can be used for different purposes: as fast-acting antimicrobial coatings for urinary catheters and wound dressings or as slow acting antithrombotic coatings for stents, bypass tubings, cannulae and catheters (2,3).

RSNOs are potent vasodilators, involved in bronchodilation and neuroprotection. They exhibit anticancer activity and are mostly known for being strong inhibitors of leukocyte binding to vascular endothelium and for platelet aggregation. Commercially most commonly used NO-donors are organic nitrates, used mainly as vasodilators in cardiovascular therapies for congestive heart failure and coronary artery disease, but they are known for the development of tolerance and for their toxic metabolites (e.g. cyanide toxicity with sodium nitroprusside) (4). In the search of constant improvement, innovative NO-donors submerged and remarkable progress has been shown in the area of *S*-nitrosothiols. Some of the mentioned molecules are presented on the picture below (Figure 1).

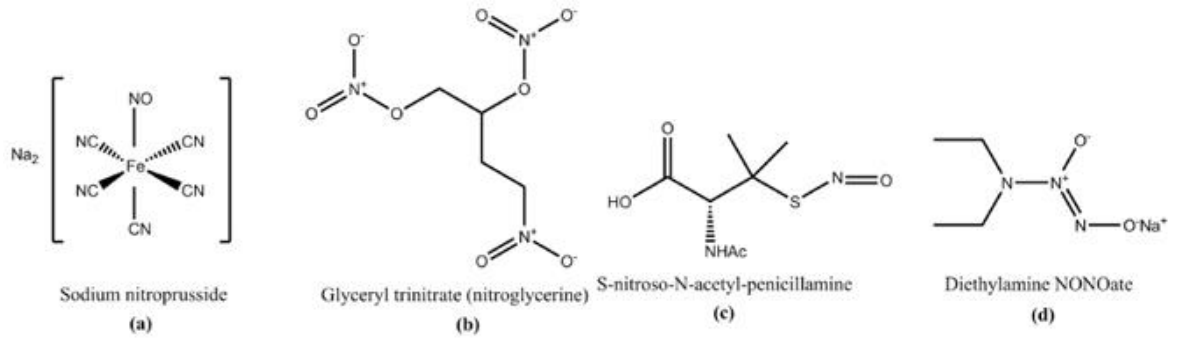


Figure 1. Some representatives of NO-donor drugs (5).

1.3 *S*-nitrosothiols

S-nitrosothiols (also thionitrites, RSNOs), NO carriers and donors, **represent** intermediates in signal transduction and have many highly important biological functions in the form of potentiating NOs biological effects. These organic compounds have a generic **structure** of R-SNO (R denoting an organic group), containing a nitroso group attached to a thiol group. The latter are easily nitrosated in *S*-nitrosation reactions, recently defined being as important as glycosylation and phosphorylation (1). According to this, *S*-nitrosothiols are synthesized chemically in the reaction of highly reactive NO with thiol group and serve as **storage or transport of NO**. After their transport to the site of action, their decomposition by hemolytic cleavage of S-NO bond leads to NO release followed by a corresponding biological response. RSNOs found in plasma equilibrate between low molecular weight thiols (GSH and Cysteine, which like to react with membrane thiols) and high molecular weight protein thiols (ex. Albumin) by undergoing transnitrosation. GSNO-containing molecule reacts with other thiols, donating the nitroso functional group to another thiol in a mechanism referred to as transnitrosation, a second order reaction. **Transnitrosation** from an *S*-nitrosothiol which cannot be transported to cells to one that can might facilitate cellular uptake of the nitroso functional group - it is possible that many of the biological effects of GSNO are modulated in this way, making it an important detail from therapeutical point of view. Such modification leads to altered enzyme or receptor activity and there are not many examples where transnitrosation of protein is protective. Responsible for **transnitrosation** reactions (second order reactions) are disulfide forming/isomerizing **enzymes** such as thioredoxin (Trx) and protein disulfide isomerase (PDI) and they are essential for cardio and vascular protection since their role is to reduce intracellular reactive oxygen species.

S-nitrosothiols can be easily divided in two **subgroups**. First would be physiological, represented by for example *S*-nitrosoglutathion and *S*-nitrosoalbumin, which are known for being a physiological stock of NO and can be found circulating in plasma. Seen that albumin is the most abundant plasmatic protein it doesn't come as a surprise that it is expected to act as NO reservoir, transporting it inside the vascular beds (1). CysNO is known to be able to cross the cell membrane through amino-acid transporters and provide an intracellular source of NO, but its formation is likely to result in cellular ion imbalance, via inhibition of plasma membrane channels or enzyme inhibition (6). The second group is the exogenous group, containing all chemically modified versions of physiological *S*-nitrosothiols (*S*-nitroso-*N*-acetylcysteine, *S*-nitroso-*N*-acetyl-penicillamine among others). In tissues and cells, *S*-nitrosothiols have been detected under basal conditions at low but still measurable levels, and typically their concentration increases under pathological conditions (e.g., ischemia, iNOS induction) (7). As already mentioned above, the majority of physiological *S*-nitrosothiols are in equilibrium with reduced GSH through spontaneous nonenzymatic transnitrosation reactions, which result in important second messenger molecule GSNO that transduces NO bioactivity (1).

Thionitrites hold a lot of **advantages** over other classes of NO donors: minimum **toxicity**, less demanding **metabolic** requirements, they do not induce **tolerance** on long term, unique **tissue-selective** capacity to deliver NO (selective for arteries over veins, affinity to injured over intact arteries and to lipophilic areas). Additionally, we could describe them as **antiplatelet** agents, inhibiting aggregation and capable of **direct transfer of NO** without releasing any free NO. This makes them **less reactive** – less susceptible to the oxidative stress due to the covalent **S-NO bond**, which must be nonetheless properly chemically stabilized and provide steric protection against thiol disulfide oxidoreductases. At physiological pH and temperature *S*-nitrosothiols are stable, but the stress factors affecting their decomposition in solution include variable **environmental parameters** such as light, pH, temperature and contaminating transition metal ions (ex. Cu^{2+}) or other reducing agents (sulfur containing compounds, e.g. H_2S) and also hydrolysis (6). The fact that many parameters can vary makes their half-lives clearly condition-dependent and it becomes easier to accept all the variable *S*-nitrosothiols half-lives published.

Many of the mentioned *S*-nitrosothiol compounds have been put to a vast number of tests as potential NO donors and most commonly employed substances in the publications are GSNO, AlbNO and SNAP. Generally, RSNOs have been **proved of being able to** decrease infarct size,

promote faster healing and improve coronary endothelial function (1). Their actions are known also outside the cardiovascular system: able to destroy malignant tissues, heal skin and wound infections, with better efficacies and less side effects. **GSNO** has been showed to have a potential of minimizing liver deterioration, to have neuroprotective properties (regulation of antioxidant and apoptotic enzymes) and might play a role in postponing progression in neurodegenerative disorders. It can also be found in the treatment of glaucoma and was used in wound healing by increasing collagen deposition. Naturally occurring ***S*-nitrosoalbumine** represents lipophilic type of *S*-nitrosothiols, hypothesized to be absorbed in the lipophilic areas of the subendothelium from where it slowly releases the NO over a prolonged period. It presents a good way of targeting NO to areas of endothelial damage, therefore minimizing side effects of non-specific delivery.

In order to deliver NO under a controlled pathway and so overcome drawbacks of commonly used therapeutics, an **appropriate delivery system** has to be established. Some differences were found in the denitrosation rate of different RSNO suggesting that their structure plays a key role in **protecting** NO from degradation (liberation mostly in form of oxidation or radical attack) and its targeted delivery (1). Main key points that must be kept in mind are the following: the surrounding groups should delay the interaction with catalytic proteins (Trx and PDI), and **stability** regarding the molecular skeleton carrying the nitroso moiety should be improved in order to increase the duration of delivery in the blood. The biological effect is strongly related to the **concentration**, therefore increased grafting or good steric protection of the reactive NO part, and thus augmenting the possibility for **sustained release,** are indispensable. The last parameter should target increased molecule lipophilicity seen that sustained vasodilatation was hypothesized being correlated to the retention of **lipophilic** derivatives in sub-endothelial layers of arteries that are known to be lipid rich (1). Such compounds seem to have **affinity** towards injured vessels (8), but particle size is a major limitation since the range of 100 to 200 nm dimension was shown to be able to penetrate and accumulate in the inner lipid layers of arteries (1). Intense investigations and new methods are focusing on **grafting** (vasorelaxant effect is in tight relationship with the number grafted nitroso moieties), "**sandwiching**" (between layers of polymers for controlled release) or **coupling** *S*-nitrosothiols with polymers that would not release NO until a **trigger** provided (e.g. light irradiation or reduction by cellular reductant

(glutathione)), with a view to overcome actual NO-donor's disadvantages and come up with efficient and innovative (nano)formulations.

1.4 Glutathione (GSH) and *S*-nitrosoglutathione (GSNO)

Glutathione, human cell protector, occurs in almost all living organisms in high concentrations and it is our essential water-phase antioxidant and cofactor for antioxidant enzymes with great reducing power. It is a **tripeptide** forming a structure of gama-L-glutamyl-L-cysteinilglicine (γ-Glu-Cys-Gly), from which **cysteine residue** contributes a major part to his reactivity with the –SH group, giving the molecule its electron-donating character. It's highly probable that in mammalian cells amino acid supply from the exteriority of the cells provides a check point in the synthesis of this important nonenzymatic antioxidant, of which concentration in cells attains millimolar levels (9). It is a sulfhydryl (-SH) highly potent antitoxin that exists in two forms: electron rich **reduced form** (GSH) and its **oxidized** form, known as glutathione disulfide (GSSG), whose levels should not exceed 10 % of total cell glutathione. Their ratio may be a sensitive indicator of oxidative stress and therefore **GSH depletion** has been documented in many degenerative conditions (liver and lung diseases, HIV, Hepatitis C), making the cell vulnerable to oxidant attack, and results in cell death - apoptosis (10).

GSH plays multiple **regulatory roles** at the cell levels and is essential both to the functionality and to the structural integrity of the cells, tissues and organ systems. The glutathione status of the cell might be the most accurate indicator of its health and also plays a central role in the functioning of immune cells. Some of cellular functions, linked to the reducing power of glutathione, are protein synthesis, enzyme catalysis, transmembrane transport, receptor action, intermediary metabolism and cell maturation. It is controlled homeostatically, both inside the cell and outside, which means that it is continually self-adjusting. The highest concentrations of glutathione can be found in liver, where it is required by enzymes to convert fat-soluble substances into water-soluble GSH conjugates, facilitating their excretion (10).

S-nitrosoglutathione (GSNO) is its *S*-nitrosated derivative and a good nitrosation agent. It represents an intracellular NO reservoir and one of NO vehicles for its transport throughout the cell, thus expanding its biological activity. Chemically, GSNO can be synthesized in efficient, fast and high yield reaction between GSH and nitrous acid (HNO_2), resulting in an immediate

pink color. The mechanism by which S-nitrosoglutathione is formed from NO in the human body physiologically is still the subject of much enquiry. The process of *S*-nitrosylation enables GSNO to be generated from nitric oxide and reduced glutathione, but the reaction is known to be indirect (Figure 2).

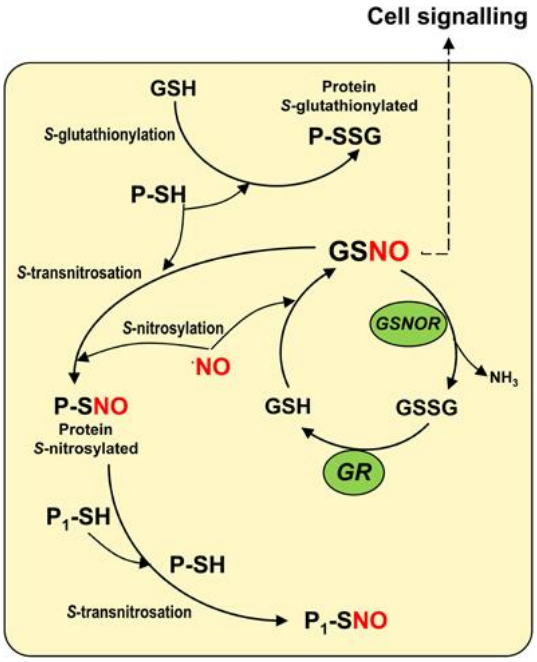


Figure 2. *S*-nitroglutathione (GSNO) metabolism in cells. GSNO reductase decomposes it to GSSG, that is regenerated to reduced GHS by glutathione reductase. GSNO can nitrosate intracellular thiols on proteins, which can cause further transnitrosation reactions. $P_{(1)}$-SH – protein with sulfhydryl group, P-SNO – *S*-nitrosylated protein, GR – glutathion reductase (11).

The direct reaction of GSH with NO does not produce GSNO; it generates glutathione disulfide and nitroxyl anion (NO^-). Only the reaction between NO and molecular oxygen can form GSNO but it is a third order reaction, depending on the c^2(NO). Because of low intracellular oxygen concentrations (tissue level) and also low NO concentrations, it is likely that the *in vivo* NO oxidation by oxygen is a slow and insignificant process (GSNO is readily formed in this way with a yield of 10%, with the reminder forming GSSG) (7, 10, 12). It has been proposed recently that under "low-oxygen conditions" and in low NO environment of cells and tissues, GSNO formation requires binding of GSH to cytochrome C or hemoglobin (metalloproteins), followed by the addition of NO. In any case, in a biological system the nitrosation is not a major fate of NO, and only a small amount of produced NO is converted into an *S*-nitrosothiol. It has been

suggested that mostly hydrophobic environments such as lipid membranes represent important sites for formation of nitrosating species, in particularly because both oxygen and NO prefer to distribute into membranes (7).

The rate of **NO formation** from GSNO is dictated by media conditions - GSNO itself is not directly taken into cells but it is hypothetically decomposed in the extracellular space, from where NO then diffuses across the cell membrane to S-nitrosate target proteins (this mechanism may not be relevant in all cell types). It was observed that there must be **cysteine** present in cell culture media for the cellular metabolism of GSNO (7). The mentioned mechanism of *S*-nitrosothiol uptake is summarized in the picture below (Figure 3).

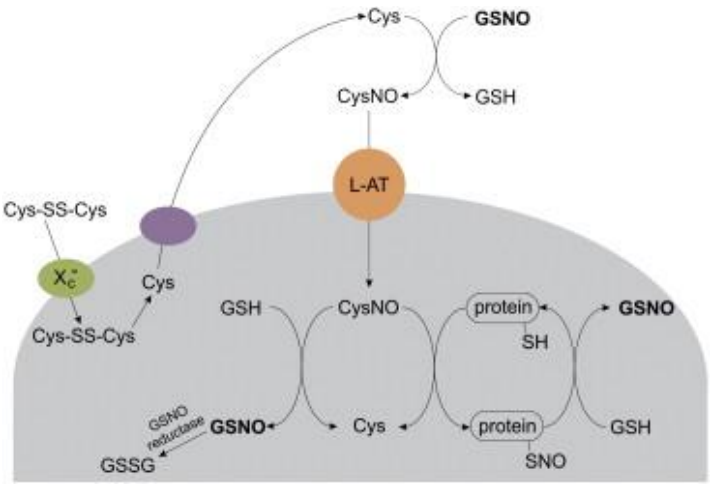


Figure 3. Cellular GSNO uptake. First step is the reduction of Cystine (Cys-SS-Cys) in the cell (transported through X_c^-), followed by its export to extracellular space, where it is transnitrosated by GSNO forming *S*-nitrosocysteine (CysNO). The latter is taken up by cells through an amino acid transporter system L (L-AT), thus transferring the NO group across the plasma membrane. In the cell GSNO will be remade or it will elicit cellular responses. X_c^- - cystine transporter, Cys – Cystine, GSSG – glutathione disulfide (7).

Protein *S*-nitrosation, covalent binding of nitric oxide through transnitrosation to specific cysteine residues in proteins, is known to occur in various (patho)physiological contexts. Its product is defined **as *S*-nitroso proteome**. This modification has an impact on a large number of **signaling processes and cellular events**. Process is finely regulated *in vivo* and the level of nitrosylation is known to change in response to different stimuli - it increases in biological

contexts where NO production is high (e.g. inflammation) and it is directly implicated in several disease states (neurodegeneration, heart failure) (13).

There is no spontaneous **hemolysis** of GSNO to form NO, and in addition, NO formation requires external factors – that is why GSNO should never be referred to as an "NO donor molecule" (7). In the cellular degradation context it is more likely that **biological mechanisms** predominate through **enzymatic reduction** - either the direct reaction of NO/nitrosating agent with GSH happens or the process goes indirectly via formation of protein based/low molecular weight *S*-nitrosothiols followed by transnitrosation to GSH. **Metal-dependent *S*-nitrosation** can happen with regards to transition metal ions and protein metal centers: they are capable of performing a one-electron oxidation of thiol residues, but that is why they are tightly bind in cells to prohibit this kind of chemistry.

Possible **degradation** processes of GSNO are also **non-enzymatic pathways** or reactions with **biological molecules** such as copper ions, iron, ascorbate or thiols, and **enzymes** (tend to be slow, sequestered in cells at very low levels). Compared to the other *S*-nitrosothiols GSNO is a big exception when it comes to stability, significantly less susceptible to **catalytic decomposition** by copper ions. GSSG is presumed to act as a weak copper (II) chelator, preventing catalytic decomposition (14).

Most known enzymes responsible for GSNO degradation are **GSNO reductase** (alcohol dehydrogenase 5, found in brain and lungs – levels of GSNO are lower in the airways of asthmatic patients), **carbonyl reductase,** thioredoxin system (major player in the reduction of low-molecular weight and protein *S*-nitrosothiols) and **γ-glutamyl transpeptidase**, providing novel targets for therapy in some pathological conditions (15).

Until now, there have been more than 20 clinical trials investigating the therapeutic efficacy of GSNO, most based on cardiovascular diseases. GSNO has a major advantage to other NO donors: its effect can be systemic or it can be applied locally without causing systemic disturbances. The best-characterized effect of GSNO in human is his action on platelets (acting primarily as an NO donor, decreasing coagulation and thrombosis). GSNO has been shown to decrease **embolism** after operations and grafting, its beneficial effects extend to **cardiac left ventricular** function, systemic **vasodilatation** and **preeclampsia**. Administered **topically** as a GSNO gel is shown to increase clitoral blood flow (suggested as a **treatment for female sexual**

dysfunction), as an **anti-fungal** agent it improves **onychomycosis** (fungal infection of the nail) and can be used as a therapeutic agent in **cystic fibrosis**.

What is particularly noteworthy with GSNO is, as mentioned above, its correlation with disease pathology in humans – elevated *S*-nitrosothiol levels in fluids with respect to their controls in certain pathologies will likely motivate further clinical trials in this area (7).

1.5 Polymers as carriers

In order to avoid previously described blind alleys and effectively deliver NO, NO-donors can be incorporated in various delivery systems. Direct linking to polymers can be performed or association/incorporation within the polymers resulting in macro, micro or nano sized endproducts or layered structures with retained release. Polymers frequently used are biopolymers that offer wide biomedical applications (eg. Polyurethane coatings for medical devices), can provide prolonged NO release directly at the targeted site and can be easily broken down in the body without toxic by- or endproducts. One of their multiple advantages is also their loading capacity – they can accept substantial amounts of NO with increased stability.

1.5.1 Methocel™

One of the two used polymers in our research is Methocel™, which is characterized by varying ratios of hydroxypropyl and methyl substitution. Methylcellulose (MC) is a cellulose derivative – it consists of numerous anhydroglucose rings that form a carbohydrate skeleton. Each MC glucose molecule exposes three or less hydroxyl groups that can be substituted. Therefore, degree of substitution (DS) gives us the average number of substituted hydroxyl groups (maximum 3.0, but usually from 1.3-2.6). Most known derivatives of methylcellulose are hydroxypropyl methylcellulose (HPMC, with hydroxypropyl substituents) and hydroxyethyl methyl cellulose (HEMC), made to improve some of MCs characteristics, such as water retention etc. Methyl and hydroxypropyl groups are attached to the carbohydrate skeleton via an ether linkage.

Methylcellulose can be described as a hydrophilic white powder able to dissolve in cold water, forming a clear viscous solution or gel, which can swell to 40-times its dry volume. It is not toxic and not digestible, it does not contain protein contaminants that could cause allergies and it has a

nonionic structure that makes it compatible with a wide range of drugs. The distribution of the methyl substituents along the cellulose chains has a powerful influence on its viscosity and solubility in water. Methylcellulose and hydroxypropyl methylcellulose are widely applied in the fields of textile, paper, food (acceptable food additive), pharmaceutical, building and painting industries. Pharmaceutical industry is their large consumer, using them in tablets, granules, solutions, emulsions, to fight against constipation, because of good surface activity in lotions and ointments, and not the least their film-forming properties permit their use in dressing for wounds and burns. Both can be used as coating, granulating and disintegrating agents in tablets, as thickeners, in cell culture or even as buffer additives. Their main properties (mild taste, nonionic, surfactant) give them an excellent potential as carriers for drugs. They can also be used as suspending agents for polymerization (e.g. vinyl chloride monomer polymerization) (16, 17). The chemical structure is presented below (Figure 4).

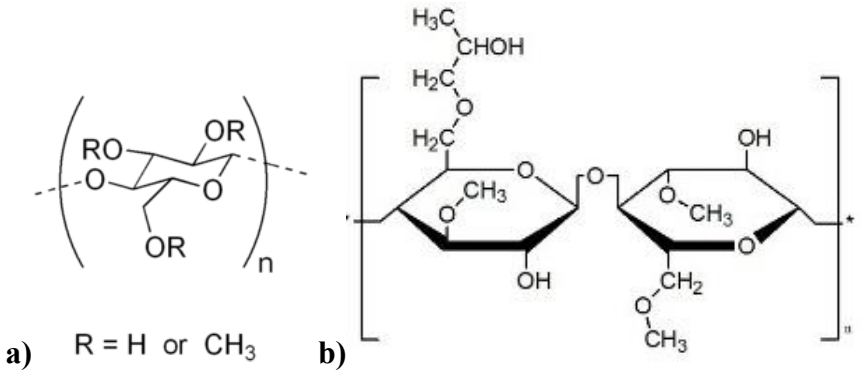


Figure 4. Chemical formula of a) Methylcellulose and b) Hydroxypropyl Methylcellulose (18). Degree of substitution can vary.

The polymer we used in the laboratory is Methocel™ E50 (further on Methocel), a cellulose ether. It is shown that Methocel™ E50 produces one of the smallest particles (19).

1.5.2 Alginate

Alginates are non-toxic polymers following a basic pattern in the form of $(C_6H_7NaO_6)_n$. Widely used in food industry, they received much attention last decades for use in pharmaceutical preparations, particularly as a vehicle for prolonged drug release and potentially useful for oral delivery of micro- and nanoparticles. Anionic (negatively charged), biodegradable and biocompatible polymer represents the perfect substance for preparation of hydrogels and for

encapsulating other delivery systems, such as microspheres and liposomes (20). Alginates are unbranched polysaccharides, composed of 1→4 linked α-L-guluronic acid (G) and β-D-mannuronic acid (M) residues arranged in homopolymeric and heteropolymeric blocks, forming a complex copolysaccharide backbone (Figure 5). Upon hydration a matrix is formed, which produces a gelatinous layer that can act as a drug diffusion barrier. We can find alginate as an important component of brown algae in the intracellular matrix, from where it is commercially obtained, but is also present as an exopolysaccharide of bacteria (e.g. Pseudomonas aeruginosa). The number and sequence of the mannuronate and glucuronate residues vary in naturally occurring alginate. Per structural unit, average molecular weight is about 222 g/mol and the typical average of the macromolecule is about 10,000 - 600,000 g/mol.

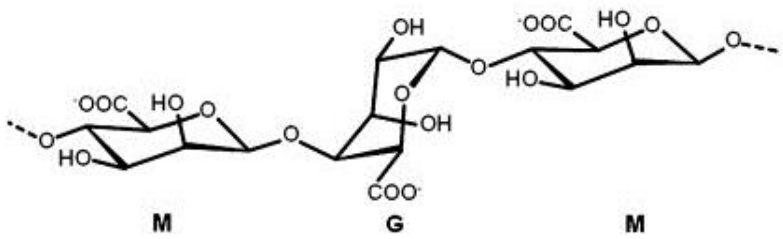


Figure 5. Alginate G and M blocks (21).

Its thickening, stabilizing and gel forming properties make it a good excipient in drug dosage formation. It has an ability to form two types of gel dependent on pH: an ionotropic (in the presence of divalent cations) and an acid gel (in low pH). Hydration of alghinic acid at low pH leads to the formation of a high-viscosity acid gel – so the incorporated acid-sensitive drugs (proteins, nucleic acids) stay safely protected from the gastric juice. The solubility of alginates in water depends on three parameters: pH of the solvent, ionic strength of the medium and presence of ions in the solvent. pH should be above a certain value where the carboxylic groups are deprotonated (two hydroxyl groups also enhance water solubility at pH > 5). In acidic conditions, polysaccharide undergoes a hydrolytic cleavage. Ionic strength has an impact on chain extension and viscosity, and the presence of divalent ions triggers a gel formation (strong bonds between G blocks, weaker between G-M blocks), making alginate ionically crosslinked. In a form of gel, alginate is not soluble; therefore, the release of the drug is dependent on both *dissolution* of the gel and *diffusion* of the drug into the surrounding fluid. Most gel formations in pharmaceutical development base on the use of calcium, forming insoluble Ca^{2+} crosslinked gel.

Nowadays, a new trend has been taking place called controlled derivatization, in order to create "value-added" alginates by tailoring alginate properties. New generation alginates can thus be chemically modified in order to *enhance* its existing properties (e.g. improvement of gel strength and stability, pH sensitivity and release retardant properties by additional crosslinking) or as to *introduce* new, not existing characteristics (e.g. anticoagulant properties, lower critical solution temperature). Alginate's bioadhesive properties make it also advantageous for the site specific delivery to mucosal tissues, protecting the upper gastrointestinal tract from irritations. As we can see, its field of use is expanding and growing, especially when joint with chitosan as mucoadhesive excipient forming various pharmaceutical drug delivery systems.

2. AIM OF OUR RESEARCH / THE OVERALL AIM OF THE STUDY

The aim of this study will be to define, evaluate and improve the synthesis of novel *S*-nitrosothiols as potentially applicable formulations for oral delivery. .Most commonly orally used NO-donors, organic nitrates, are known for the development of tolerance, their toxic metabolites and bad absorption. Previously it was reported that alginate coated chitosan core shell nanoparticles demonstrate promising potential for the delivery through oral route (22), that nitric oxide releasing *S*-nitrosothiol-modified xerogels reduce the platelet and bacterial adhesion and the foreign body response (23) and additionally the efficacy and high absorption rate of thiomers in the non-invasive peptide delivery has been demonstrated by numerous in vivo studies. It has been proven that large alginate polymer fractions (> 48 kD) remain in the circulation and do not accumulate in any of the tissues and are transferred to the bloodstream through the lymphatic system (Flessner et al, 1985) (24). Some work has also already been done in the hosting department concerning conjugates of chitosan and glutathione providing some interesting conclusions. Consequently, our research will be concentrated on the synthesis of thiolated conjugates of Methocel-GSH and alginate-GSH, on the following nitrosation process and on the characterization of the resulting product. We will form covalent bonds between chosen polymers and glutathione with a mixture of appropriate activating agents (EDAC, NHS). Initial studies will be conducted with the help of Ellman's and Griess/Saville reactions, UV/VIS spectrophotometer and Nano/Mastersizer apparatus: with dialysis we will purify our product and further on determinate the success of grafting with Ellman's reaction. Afterwards we will nitrosate the chosen conjugates (alginate-GSH) adding $NaNO_2$ and analyze the reaction yield with Gries/Saville method, followed by UV spectrophotometry. Prepared GSNO and SNOA conjugates will be assessed for their size with Zetasizer and Mastersizer. In addition to the latter measuring molecular weight on the Mastersizer apparatus will be conducted. We will also examine if modification of some parameters can reduce decomposition of our input components.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Table I: List of chemicals

- Obtained from Sigma-Aldrich:

Methocel™ E 50 Premium
Reduced L-Glutathione
1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC)
N-Hydroxysuccinimide (NHS)
Sulfanilamide
N-(1-naphthyl) ethylenediamine (NED)
Ellman's reagent DTNB (5,5'-dithiobis(2-nitro benzoic acid)

- Obtained from Rhône–Poulenc:

Di-Sodium hydrogen phosphate (Na_2HPO_4)
Sodium dihydrogen phosphate (NaH_2PO_4)
Ethylenediamine tetra acetic acid (EDTA)
Mercury (II) chloride ($HgCl_2$)

3.1.2 Solvents, reagents and solutions prepared

Table II: List of solvents, reagents and solution

Double distilled water (ddH_2O)
Ultra purified water
Hydrochloric acid (HCl)
Acetic acid
Sodium Hydroxide (NaOH), 50%

0.1 M phosphate buffer, 5 mM EDTA pH 7.5
Hydrochloric acid solution 0.10 M EDTA 2 mM
Solution of DTNB
Solutions of reduced glutathione of varying concentrations
Acetate buffer pH 2.5
Solutions of *S*-nitrosoglutathione (GSNO) of varying concentrations
Krebs bicarbonate Ringer (KBR) CO_2/HCO_3^- electrolyte solution
Phosphate buffer 10^{-2} M pH 7.4
Solution of sulfanilamide (Griess)
Solution of sulfanilamide and mercuric salt (Saville)
Solution of *N*-(1-naphthyl)ethylenediamine (NED)
Sodium nitrite solution (10^{-2} M)

3.1.3 Laboratory apparatus, equipment, programs and machines

Table III: List of laboratory apparatus, equipment, programs and machines

Spectrophotometer: Cary 50 UV-Vis Spectrophotometer, Laurier research instrumentation
Centrifuge Eppendorf 5415D
Eppendorf Tubes (1.5 mL) Safe-seal microtubes
Filtration system Millipore®
Parafilm© M
pH-Meter 766 Calimatec
pipettes
Tips, pipette (10 µL, 100 µL and 1000 µL)
UV-Cuvettes micro (15 mm)
Water Bath DC3 Memmert
Lyophilisator USIFROID, SMH15 (172x82x80 cm), 370 Kg, 15 dm^2, -60 +60 °C
Zetasizer Nano Series, Malvern instruments, Malvern, UK
Mastersizer, Malvern instruments, Malvern, UK
GraphPad Prism Software version 5 - GraphPad software, San Diego, CA, USA

3.2 Methods

3.2. Linking Glutathione (GSH) to polymers - Thiolation

3.2.1 Synthesis of Methocel™-glutathione conjugates by using EDAC/NHS

The linkage of thiol groups to methylcellulose was achieved by presuming a formation of ester bond between carboxylic acid moieties (-COOH) of GSH and hydroxyl (-OH) groups of our cellulose polymer using modified protocol, taken from cited articles (25, 26) and briefly described below.

100 mg of Methocel™ were weighted and dissolved in 9.2 mL of water (ultra-pure, 18 mΩ cm) and 0.8 mL of 1 M HCl. The pH was slowly adjusted to pH 6 under strong stirring with 1 M NaOH solution. Afterwards, 500 mg of GSH was dissolved in 1 mL of ultrapure water and added to the Methocel™ solution. Thereafter 460 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC, in final concentration of 200 mM) in 0,5 mL of ultrapure water and 280 mg of *N*-hydroxysuccinimide (NHS, in final concentration of 200 mM) in 0,5 mL of ultrapure water were added to the reaction medium. The pH was adjusted to pH 6 with 1 M/5 M NaOH and the solution acquired a pale yellow color. The reaction was kept protected from the light for 15-18 hours under stirring (200 rpm).

The product was then dialyzed in tubing (MWCO/molecular weight cutoff 3.5 kDa) to eliminate the unbound reagents (GSH, NHS, EDAC) in the following sequence and with below solution concentrations (altogether 8 hours):

a. 5 mM HCl 2 h
b. 5 mM HCl + 1% NaCl 2 h
c. 5 mM HCl + 1% NaCl 1 h
d. 1 mM HCl 2 h
e. 1 mM HCl 1 h

Finally, the obtained sample was lyophilized by drying frozen aqueous solution (-56°C, pressure 1.3 bar) and stored at 4°C (refrigerator) until further use.

Controls were prepared in the same way omitting EDAC and NHS during the coupling reaction.

3.2.2 Synthesis of Alginate-glutathione conjugates by using EDAC

The covalent linkage of thiol groups to alginate was achieved by peptide bond formation between amino group ($-NH_2$) of glutathione and carboxylic acid moieties (-COOH) of alginate using modified protocol, taken from cited article (27) and briefly described below.

100 mg of alginate were weighted and dissolved in 10 mL of water (ultra-pure, 18 mΩ cm) and left 30 minutes for hydration to take place. 95.85 mg of EDAC (final concentration of 50 mM) was added to the polymer solution in order to activate the carboxyl groups of the polymer, and left for incubation for 45 minutes. The incubation with EDAC was followed by addition of 50 mg of reduced glutathione (resulting in a weight ratio of polymer: glutathione = 2:1). pH was adjusted to 4 and reaction was allowed to proceed for 2 hours at room temperature under continuous stirring with the help of a magnetic needle. After the time limit, pH was adjusted to 6 and reaction proceeded for an additional hour.

At the end of the reaction time, the aqueous gel-like solution was dialyzed to isolate the alginate-glutathione conjugate. Solution was transferred in a dialysis membrane (MWCO 3.5 kDa) and dialyzed as followed (altogether 6 hours):

a. 1 mM HCl 2 h
b. 1 mM HCl + 1% NaCl 2 h
c. 1 mM HCl + 1% NaCl 1 h
d. 1 mM HCl 1 h

Finally, the obtained sample was lyophilized by drying frozen aqueous solution (-56°C, pressure 1.3 bar) and stored at 4°C (refrigerator) until further use.

Controls to polymers were prepared in the same way, omitting EDAC.

Brief synthesis is outlined on the picture below [Figure 6], proceeding from raw ingredients through the unstable intermediate to the desired conjugate alginate-GSH (and as a byproduct a nontoxic urea derivative), that is easily eliminated throughout normal biological mechanisms of excretion.

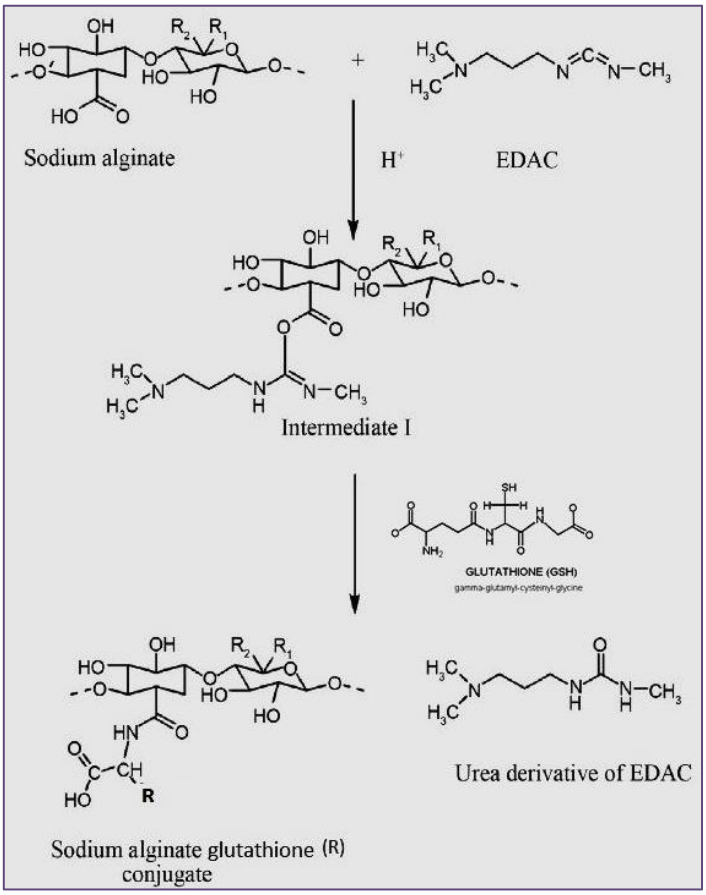


Figure 6. Envisaged synthesis of alginate-GSH conjugates (picture modified, 28).

3.3. Determination of thiol groups

3.3.1 Ellman's reaction

Ellman's reagent (5,5′-dithio-bis(2-nitrobenzoic acid) or DTNB) is a chemical used to quantitate the number (and hence the concentration) of thiol groups in a sample by absorption measurements. The thiol group in its reduced form (only accessible thiols) is oxidized by the acid DTNB, forming a mixed disulfide and liberating the chromophore (yellow colored product): 5-mercapto-2-nitrobenzoic acid (ε = 13 600 M-1.cm-1, λ(absorption maximum) = 412 nm) that can be seen below (Figure 7).

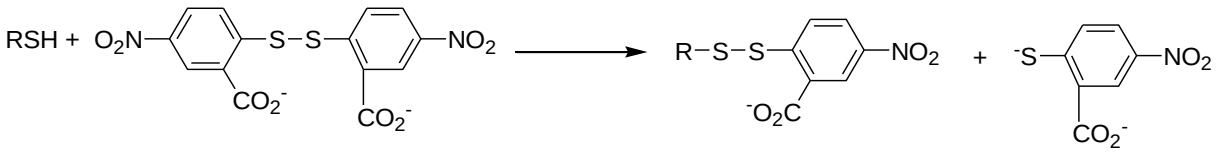


Figure 7. DTNB undergoing thiol-disulfide interchange reaction in the presence of a free thiol (29).

The amount of thiol groups is determined spectrophotometrically by measuring the optical absorbance at 412 nm.

3.3.2. Standard Curve for Ellman's reaction

We have prepared several standard curves, following the protocol described below.

1. Reagents used

A. 0.1 M phosphate buffer, 5 mM EDTA pH 7.5

Weigh 26.80 g of Na_2HPO_4, $7H_2O$ (M_r = 268.07) and 372 mg of disodium EDTA dihydrate (M_r = 372.24) in 1 L of ultrapure water, adjust the pH, 7.50 ± 0.05 using HCl 2 M.

B. Hydrochloric acid solution 0.10 M EDTA 2 mM

Dilute the solution to the nearest hundredth of concentrated hydrochloric acid (37%, d = 1.19) in ultra-pure water and add 0.75 g/L of disodium EDTA dihydrate. Membrane filter of cellulose acetate type 0.2 microns. Discard the reagent if a precipitate appears.

C. Solution of DTNB (must be freshly prepared)

Dissolve 10 mg of DTNB (M_r = 396.3) in 25 mL of phosphate buffer/EDTA pH 7.5.

D. Solution of reduced glutathione 1 mg.mL-1 (3.25 mM)

Dissolve 50.0 mg of GSH (M_r = 307.23; Reference Sigma G-6529) in 50.0 mL of 0.10 M hydrochloric acid. Aliquot 1.5 mL fractions into tubes type eppendorf and freeze at -80 ^{o}C.

E. Solutions of GSH (keep in melting ice for up to 6 hours)

- **SF1 to 100 µg mL^{-1} (325 µM)**

1/10th Dilute the stock solution of GSH in 0.1 M phosphate buffer pH 7.5

- **SF2 to 10 µg mL^{-1} (32.5 µM)**

Dilute 1/10th SF 1 in 0.1 M phosphate buffer pH 7.5

2. Calibration range

The calibration range for the determination of GSH has been prepared in tubes Eppendorf type according to the following table.

Table IV: Calibration tubes for Ellman's reaction standard curve

No of Tubes	**1**	**2**	**3**	**4**	**5**	**6**
SF 2 (µL)	0	100	200	500	750	1000
Phosphate buffer 0.1 M, pH 7.5	1000	900	800	500	250	0
GSH Concentration (µM)	0	3.25	6.5	16.3	24.4	32.5
GSH Concentration (µg mL^{-1})	0	1.0	2.0	5.0	7.5	10

Add 200 µL of DTNB solution to each tube of the test or calibration range.

After 10 min of contact in the dark at room temperature, the absorbance is measured at 412 nm.

The slope of the calibration line should be of the order of 13 600 M^{-1} cm^{-1} (± 10%).

3.3.3 Estimation of bound thiol content in the polymer-GSH conjugates by Ellman's method

In our sample, the amount of thiol groups immobilized on polymer-GSH conjugates was first determined directly from the solution spectrophotometrically using Ellman's reagent (DTNB). To determine oxidized thiol moieties available, disulfide bonds were reduced with $NaBH_4$ and the total thiol groups linked to polymer backbone was determined by Ellman's reagent. GSH was used as a standard reference to make a calibration curve.

As a standard procedure, 5 mg of lyophilized conjugate was dissolved in 3.5 mL of ultrapure water and the polymer was allowed to hydrate for 30 minutes. Thereafter, 6.5 mL of TRIS-HCL 0.05M pH 7.0 was added, making *Solution 1*.

3.3.3.1. Determination of reduced available thiol groups linked to Methocel™ backbone

For determination of available reduced linked thiol groups, 100 µL of *Solution 1* were taken and diluted with 900 µL of [phosphate buffer 0.1 M, EDTA 5 mM, pH 7.5]. Thereafter, 200 µL of Ellman's reagent (DTNB) freshly prepared were added and the solution was kept in the dark for 15 minutes. Absorbance was measured at a wavelength of 412 nm using UV/Vis spectrophotometer. The amount of free thiol groups were calculated from the standard plot, prepared by treating increasing concentrations of reduced glutathione solutions as described above.

3.3.3.2. Determination of total thiol groups linked to Methocel™ backbone

For the determination of all thiol groups in our conjugates, 1 mL of the *Solution 1* was put in a test tube and 1 mL of $NaBH_4$ 4% (w/v) in NaOH 0.05 M was added. The prepared mixture was kept in an agitating bath at 37°C for 1 hour. To destroy the unreacted $NaBH_4$, 200 µL of HCl 5M were added (the reaction was highly exothermic). After 15 minutes, 1 mL of [phosphate buffer 0.1 M, EDTA 5 mM, pH 7.5] was added, followed by a withdrawal of 100 µL, transfer of the aliquot in a test tube and addition of 900 µL of [phosphate buffer 0.1 M, EDTA 5 mM, pH 7.5]. To this solution, 200 µL of DTNB freshly prepared were added and the solution was kept in the dark for 15 minutes before analyzing it by UV/Vis at 412 nm in a plastic cuvettes.

3.3.3.3 Determination of reduced available thiol groups linked to alginate backbone

The same procedure as described in 3.3.3.1 has been applied.

3.3.3.4 Determination of total thiol groups linked to alginate backbone

The same procedure as described in 3.3.3.2 has been applied.

3.4 Synthesis of GSNO and nitrosation of alginate-GSH conjugates (synthesis of SNOA)

Only alginate-GSH conjugates were prepared due to time limited research.

S-nitrosoglutathione synthetically prepared can be obtained as a purified product or without further purification. The latter has been used in our research, described below and taken from the cited article (30).

3.4.1. Synthesis of GSNO (Batch synthesis – brief protocol)

1. Principe:

S-Nitrosoglutathione was synthetized in a solution in acidic medium and stabilized by shifting pH to 7.2.

All the reagents used in this protocol should be of high purity (> 99 %) and deoxygenated using ultrasound bath for the oxygen to be expelled and radical reactions prevented. Ultrapure deionized water (> 18.2 mΩ cm) was used to prepare all solutions.

2. Synthesis:

Briefly, 690 mg of $NaNO_2$ was weighted, dissolved in 50 mL of ultrapure deoxygenated water. Separately, 62 mg of GSH was weighted, put in 10 mL volumetric flask and dissolved in ca. 7 mL of 0.5 M HCl, followed by consecutive addition of first 1 mL of $NaNO_2$ solution (prepared before) and secondly 2 mL of 0.5 M HCl. The reaction was left to proceed at 4°C in the dark, the plateau obtained after 1 h.

Next, 500 µL of NaOH 40% (w/V) was putt into the volumetric flask (the reaction was stopped by the neutralization of the medium), it's content then carefully transferred to a 20 mL volumetric flask, which was filled to final volume with 0,5 M phosphate buffer pH 7.4. The solution was stored at 4°C (refrigerator).

The pH of the final solution was approx. 7.2 and obtained GSNO concentration is ca. 10^{-2} M. The formation and concentration of *S*-nitrosothiol was determined by UV-VIS spectrophotometry at 334 nm (ε = 922 $M^{-1}cm^{-1}$) with a dilution of 1:10 (v/v) with phosphate buffer 10^{-2}M pH 7.4.

3.4.2. Synthesis of *S*-nitrosoalginate (SNOA) from thiolated alginate

All solutions were sonificated for 5 minutes in ultrasonic bath before use.

We decided to make a previous reduction of disulfide bonds between GSH and thiol groups linked to alginate because of low reduced thiol content. Therefore, 50 mg of lyophilized product (thiolated alginate) was dissolved in 4.375 mL of ultrapure water and 8.125 mL of TRIS-HCl 0.05 M pH 6.8. After 30 minutes of hydration, 2.5 mL of $NaBH_4$ 4% (w/v) dissolved in NaOH 0.05 M was added and the solution was incubated at 37°C for 1 hour. Thereafter, 2 mL of 5 M HCl were added, resulting in the reaction of effervescence due to neutralization of $NaBH_4$. After agitation of the sample until the effervescence was no longer seen, the solution was cooled with the addition of 12.5 mL of cooled HCl 0.5 M. The obtained solution was sonificated in ultrasonic bath for 10 seconds, followed by the addition of equimolar amount (calculated from the total thiol groups content) of $NaNO_2$ in 1 mL of ultrapure water. Solution was allowed to react at 4°C for 2 hours. It was then neutralized with NaOH 40% (w/v) (we used 50 % solution in water NaOH in order to minimize the dilution) and then dialyzed in tubing, using a membrane with a cutoff of 3.5 kDa, for 6 hours.

3.5. Determination of nitrosation efficiency by Griess-Saville method and UV spectrophotometry

The selection of our analytical technique will base on the presence of GSH and other *S*-nitrosothiols, always keeping in mind the fact that artifactual formation of GSNO from GSH can take place under those circumstances (presence of nitrite and acidic conditions). Thus, when manipulating with both nitrite and GSH, acidification of the sample should be avoided (it should undergo pre-treatment with ammonium sulfamate or sulfanilamide to remove nitrite). Taking into account the biological mechanisms mentioned metal chelators need to be present, samples protected from light and kept on ice to limit the enzymatic degradation (7).

3.5.1. Griess/Saville protocol – Quantification of nitrites and *S*-nitrosothiols by Griess and Saville/Griess methods

Nitrite and GSNO concentrations were measured using Griess and Griess-Saville methods, taken from cited protocol (31). The final products of the NO oxidation are nonvolatile and stable breakdown products nitrites (NO_2^-) and nitrates (NO_3^-) and thus indicate its presence indirectly (they represent an index of NO production). Their quantification can be made using a spectrophotometric essay based on the Griess and Saville reagents. In Griess reaction, free NO_2^-

reacts with sulfanilamide under acidic conditions and the product, diazonium ion, is linked with NED (*N*-(1-naphthyl)ethylenediamine). This results in the formation of so called chromophoric azo product, which strongly absorbs at 545 nm, and the result obtained gives us the quantity of free nitrates (not linked to the polymer). In the Saville reaction, the NO^+ gets displaced from the thiol by mercuric chloride (mercuric ion), that always reacts with accessible thiols. For the quantification of total nitrates (free and those linked to thiol groups) mercury salt must be used to break the S-NO bond, freeing the nitrates that subsequently react with sulfanilamide (Saville). The reaction is presented on the below picture (Figure 8).

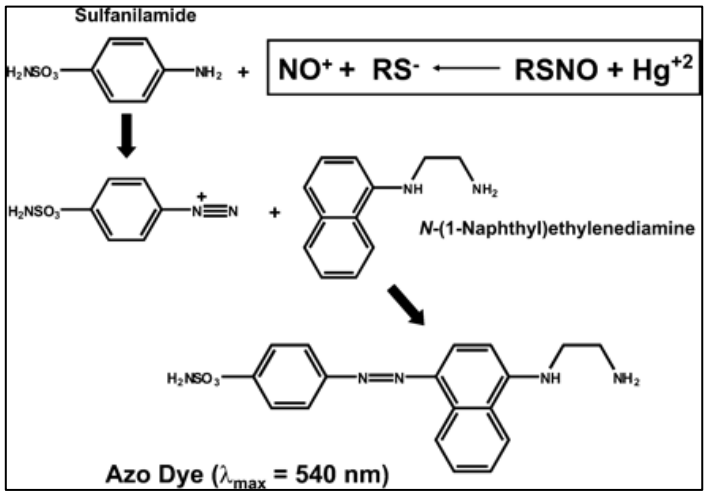


Figure 8. Saville reaction. Nitrosonium ion (NO^+) is displaced by mercury salts. The resulting diazonium salt couples with naphthylethylenediamine (NED) forming azo complex (32).

Sodium nitrate and GSNO were employed for standard curve solutions and fair linear correlations were obtained in the range of 10^{-6} – 10^{-5} M.

3.5.2. Standard Curve for Griess/Saville

1) Principe of the method

Griess method corresponds to a diazotation reaction which occurs in two steps: nitrite ions form a diazonium salt with sulfanilamide, which is fastly linked to *N*-(1-naphthyl)ethylenediamine to give a colored compound with an azoic structure, absorbing at 545 nm.

Saville method corresponds to *S*-nitrosothiols (RSNO) reaction with mercuric ions that produce NO^+: $RSNO + Hg^{2+} \rightarrow RS^{\cdot} + Hg^+ + NO^+$

It is possible to link this reaction with the colorimetric method of Griess.

2) Reagents:

All reagents are stored protected from light at 4°C during 1 month. Water is highly purified (> 18.2 mΩ cm).

A. **Acetate buffer pH 2.5**

Mix 50 mL of pure acetic acid with 400 mL of water, adjust the pH to 2.5 ± 0.05 with sodium hydroxide solution 12 M. Adjust the volume to 500 mL with water.

B. **Phosphate buffer 10 mM pH 7.4**

Dissolve 135 mg of NaH_2PO_4 and 4 mg of disodium EDTA in 100 mL of water; adjust the pH with sodium hydroxide solution 12 M to pH 7.40 ± 0.05

C. **Solution of sulfanilamide (Griess)**

Weight 0.6 g of sulfanilamide in a 100 mL volumetric flask, add 40 mL od hydrochloric acid 1 M and adjust the volume to 100 mL with water.

D. **Solution of sulfanilamide and mercuric salt (Saville)**

Weight 0.6 g of sulfanilamide and 0.2 g of $HgCl_2$, add 40 mL of hydrochloric acid 1 M in a 100 mL volumetric flask and adjust the volume to 100 mL with water. Concentration of mercuric salt in the final volume is 7.4 mM.

E. **Solution of *N*-(1-naphthyl)ethylenediamine (NED)**

Weight 0.6 g of NED in a 100 mL volumetric flask, add 40 mL of hydrochloric acid 1 M and adjust to the volume of 100 mL with water.

3) Standards:

a) **Sodium nitrite solution (10^{-2} M)**

Weight 34,5 mg of $NaNO_2$ in a 50 mL volumetric flask, adjust to the volume with water and dissolve it by a smooth agitation. Store at 4°C for 12 hours maximally.

b) ***S*-nitrosoglutathione (GSNO) solution (4 x 10^{-3}) – stock solution**

Weight 15.5 mg of GSNO chlorhydrate in a 10 mL volumetric flask and adjust to the volume with phosphate buffer 10^{-2} M pH 7.4

Conservation: 12 h at 4°C

Concentration control: dilute the stock solution at 1/10 in phosphate buffer 10^{-2} pH 7.4 and read the absorbance at 334 nm (Control sample: phosphate buffer 10^{-2} M pH 7.4; quartz cuvette).

Molar absorbance: ε = 992 $M^{-1}cm^{-1}$; the exact molar concentration is deduced from C = A/(ε x l) and using this value for the realization of calibration curve, this control will be done before preparing the calibration curve.

4) Quantification:

A. Nitrite quantification

Working solution (WS) 10^{-4} M: dilute the stock solution 10^{-2} M in 1/100 in water (mix with smooth agitation).

Preparation of calibration curve in 1.5 mL Eppendorf tubes:

Table V: Calibration for Griess reaction's standard curve

Tube	**0**	**1**	**2**	**3**	**4**	**5**	**Reference**
WS (10^{-4} M) (µL)	0	10	20	50	70	100	0
Sulfanilamide solution (µL)	200	200	200	200	200	200	200
Acetate buffer pH 2.5 (µL)	1000	990	980	950	930	900	1000

Leave for 3 min protected from the light.

Add 50 µL of NED solution and leave for 5 min protected from light (stability of the colored solution is 48 hours).

Read the absorbance at 540 nm (plastic cuvettes); the calibration curve equation is:

A = a [NO_2^-] (M) + b (R^2 > 0.99, a = 37700 ± 2300 and b = 0.0045 ± 0.0014 (n=5))

B. *S*-nitrosothiols quantification

Working solution (WS) 10^{-4} M: dilute the stock solution 4×10^{-3} M in 1/40 in phosphate buffer 10^{-2} M pH 7.4 (mix by smooth agitation). The dilution has to be modified as a function of GSNO concentration using the absorbance measured at 334 nm.

Preparation of the calibration curve in 1.5 mL Eppendorf tubes:

Table VI: Calibration for Saville reaction's standard curve

Tube	**0**	**1**	**2**	**3**	**4**	**5**	**Reference**
WS (10^{-4} M) (µL)	0	10	20	50	70	100	0
Sulfanilamide + mercuric salt solution (µL)	200	200	200	200	200	200	200
Acetate buffer pH 2.5 (µL)	1000	990	980	950	930	900	1000

Leave for 3 min protected from the light.

Add 50 µL of NED solution and leave for 5 min protected from light (stability of the colored solution is 48 hours).

Read the absorbance at 540 nm (plastic cuvettes); the calibration curve equation is:

$A = a\,[GSNO]\,(M) + b$ ($R^2 > 0.99$, $a = 40200 \pm 4500$ and $b = 0.0066 \pm 0.0030$ (n=5))

3.5.3. Quantification by Griess-Saville

After synthesis of SNOA, 100 µL of sample were taken and diluted with 900 µL acetic acid buffer pH 2.5 to a final volume of 1000 µL. After, 200 µL of sulfanilamide (quantification of free nitrates) or sulfanilamide solution with mercury chloride ($HgCl_2$, quantification of total nitrates) were added, keeping the solution in the dark for three minutes. After, 50 µL of NED was added to each sample and the samples were kept in the dark for 5 minutes before analysis with the UV/Vis spectrophotometer at 540 nm.

Blanks were produced in the same way omitting the SNOA nitrosated polymer, mixing acetic acid pH 2.5 with sulfanilamide/sulfanilamide + $HgCl_2$ with final addition of NED.

3.6. Characterization (of the obtained conjugates)

3.6.1. Particle size

In order to detect size of the synthesized particles, size measurements were made with apparatus Zetasizer Nano Series Version 6.12 (Malvern instruments) by a process called Dynamic light scattering (DLS). It bases on the speed of Brownian motion and relates it to the size of examined particles (Stokes-Einstein equation). When a particle is illuminated by a source of light, the latter is scattered by the object in all directions and a special detectors catches it. Basing on this theory, in the apparatus that we used particles are first illuminated with a laser and subsequently fluctuations in the intensity of scattered light are measured.

For best measures, count rate should be ideally between 400 and 800 and laser obscuration near 5%. The scattering angle is usually of 173°. We took 20 mg of our lyophilized coupled GSH-alginate polymer (not nitrosated) and dissolved it in 4 mL of water. The solution was consecutively diluted during measurement in order to improve our results. The samples were measured at 25°C in polystyrene cuvettes with imaginary refractive indices 1.590 used. Limit of detection for this method is about 6 μm.

3.6.2. Molecular weight

We tried to determine the molecular weight of our conjugates with the same apparatus as cited above. Molecular weight measurements use the process Static light scattering (SLS) to characterize the provided sample. The process is the same as described above, but instead of measuring the time dependent fluctuations, time-averaged intensity of scattered light is considered. Proceeding from this fact, molecular weight (MWt) and the 2nd virial coefficient (A_2) are calculated. A_2 determines the interaction strength between the particles: when $A2 > 0$, particles express *high affinity* for the solvent, and when it is < 0, it is a good indication of particle *aggregation*.

Molecular weight was detected by assessing different concentration of the measured sample and applying the Rayleigh equation (it describes the intensity of light scattered from a particle in the solution). First, the scattering intensity of the analyte is measured – a common standard used is Toluene. As zero concentration measurement we used our solvent (ultra-pure water) and samples

of varying concentrations for all further measurements – in our case 4 solutions of different concentration were prepared.

4. RESULTS and DISCUSSION

For an effective oral delivery there are many obstacles to surmount: acid degradation in stomach, feeble permeability across the gastrointestinal mucosa and presystemic metabolism, which is why we have to carefully choose among wide variety of chemical substances. Micro/nanoparticles, liposomes, mucoadhesive systems were all developed in order to cope with combative human environment. Thiolated polymers, also known as thiomers, have the ability to adhere to the mucosal membrane forming covalent interactions, much stronger than hydrogen bonding or ionic interactions. The liaisons are made between cysteine residues of the mucine and thiol groups of polymers, consequently thiolation of a polymer largely expands the basin of potential polymers that otherwise, even if characterized as excellent and appropriate, may not have been considered. Thiolation is found to be an effective and simple way of improving polymer characteristics (mucoadhesion, enzyme inhibitory potential, retarding drug release, enhancing permeation), making it a potentially effective vehicle for drug delivery (28). Alginate's mucoadhesive properties and biodegradability along with low toxicity, retardant properties and capability to easily link it with other substances could make an interesting combination of necessities for improved drug release/new potential NO donors (33). For all of the reasons stated, in this work we describe carbodiimide mediated coupling of a hydrophilic polysaccharide alginate and omnipresent antioxidant glutathione along with other characterizations made. In this work we strived to provide an insight of potentially efficient oral delivery vehicle of nitric monoxide with sufficient loading capacity that is biocompatible, stable and well absorbed in the intestine or would remain there for prolonged periods of time in order to release the NO donor slowly, allowing it to penetrate in the systemic circulation.

4.1 Methocel™-GSH, alginate-GSH and their characterization

Methocel-GSH conjugates were synthesized at room temperature in changing pH environment. The expected formation of Methocel-GSH conjugates was envisaged to pass through a form of instable intermediate, forming an ester liaison between COOH on the GSH molecule and OH on the HPMC chain. This kind of linkage would be in this milieu theoretically highly possible. Another option, although much less plausible, could present itself in the cyclic cellulose ring – once opened, highly reactive hydroxide groups would be revealed. But this type of reaction

rarely takes place in similar environments, especially when EDAC and NHS are used. These coupling reagents activate carboxyl group (-COOH) making the polymer monomers more reactive in a form of activated ester intermediate. Mildly acidic conditions (pH around 6) provide a suitable environment regarding the stability of this type of bonds. As byproduct formation of thioesters is predicted: the thiol group of one glutathione reacts with activated carboxyl group of another GSH. Being rather sensitive to hydrolysis it is certainly quite possible that they are washed out in the dialysis process. Seen that hydroxypropyl methylcellulose possesses at least one hydroxyl group per ring and methylcellulose three, tight hydrogen bonding is known to take place between cellulose chains and there presented OH groups. This given, HPMC has the characteristics of a hydrophilic polymer, exerting excellent mechanical properties and good medium for particle distribution. GSH ester prodrugs, in which the carboxyl group of the glycine residue is esterified, are thought to be more lipophilic than GSH and have been shown to rapidly raise intracellular GSH levels.

The obtained lyophilized Methocel-glutathione conjugate had the appearance of cotton-like odorless white fibrous structure that remained stable in dry form. It was very hygroscopic and quickly dispersed when put in water, where it easily formed a gel-like transparent solution.

Our second conjugate, alginate-GSH, was synthesized by covalent amide bond formation between negatively charged carboxylic acid groups of sodium alginate and primary amino groups of glutathione. The bridging amide groups along with sulfhydryl unit are commonly encountered in the body and are known for rendering polymers better tolerated.

After lyophilisation the appearance of alginate-GSH conjugate was nearly the same as the one with GSH coupled with Methocel. The lyophilisate had fibrous, cotton-like structure, color of cream and no significant odor. It was easily dispersed in water, was hygroscopic and gave gel-like, slightly orange colored solutions.

Both conjugated products were compared in terms of the amount of immobilized thiol groups on the polymer, calculated from correlations in the below standard curve (Figure 9).

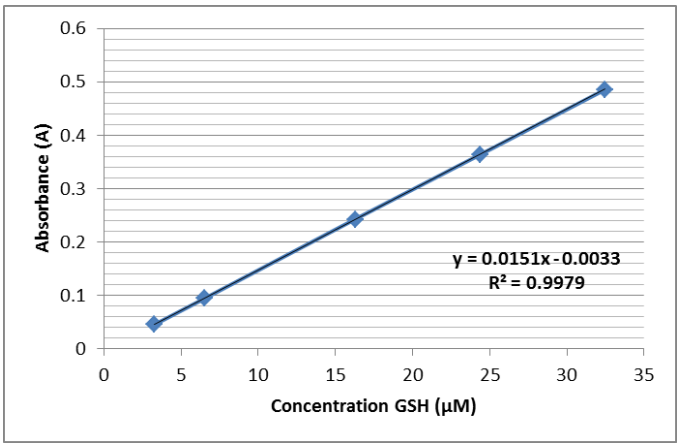


Figure 9. GSH Standard curve, absorbance in linear dependence to concentration of GSH.

The results are presented in the column layout (Figure 10). For Methocel-GSH we can remark that quite a number of reduced thiol groups can be found grafted on the conjugate (third bar).

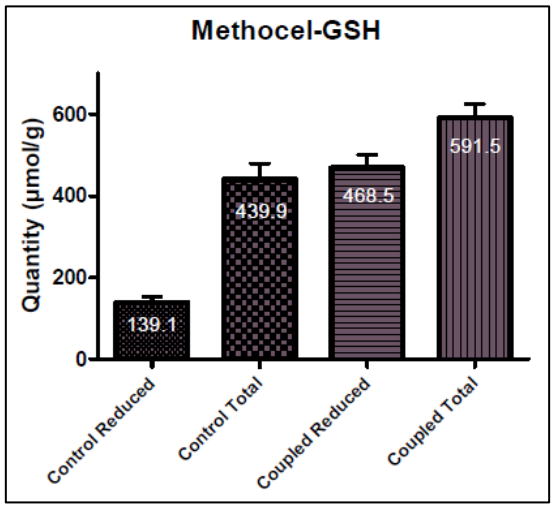


Figure 10. Amount of thiol groups regarding Control and Coupled Methocel samples.
Controls were made without coupling reagents, reduced meaning that only free thiol groups have been measured and total meaning double S-S bonds were broken. The Y axis indicates the quantity of –SH groups per gram of polymer.

What surprises us are the results for the **Control** sample. As it can be observed, the values stay high especially in the **Control** group for total, where we found 439.9 µmol/g of Thiols measured in the sample compared to 591.5 µmol/g found in **Coupled total** group. We would suppose that in **Control** in the absence of any coupling reagent there would be no thiol groups left after the

dialysis process. The only reasonable explanation for this result, seen that HPMC does not possess any thiol group, would be that the molecules of glutathione got embedded in the polymeric matrix. It is commonly documented that hydroxypropyl methylcellulose forms a three dimensional polymeric network, where its chains are intertwined with bridging structures. This makes it easy for the molecules to get enlaced by the polysaccharide chains and form alleged bonds of different strengths with them or among themselves. This theory would provide a satisfying answer to the query why the results for **Control** sample presenting us the free thiols value are of such great extent: after the reduction with nucleophilic sodium borohydrate all the conjugated double bonds get reduced, revealing the free thiol groups that can be linked with DTNB. Since the glutathione molecules are known to have a great tendency to associate, it is not of a surprise that based on the theory above the result after breaking double bonds is so elevated. Concerning the synthesis, according to some publications Methocel should have been stirred for 45 minutes, at which time the balance of the water should be added (19). All in all, we should carefully examine the strength of our dialysis process. If the purification is not long enough or the conditions are not appropriate, it would seem quite obvious that molecules of glutathione are left embedded in the polymeric system. Our dialysis process should also be carried out at non-ambient temperature, such as 10°C or less, knowing that higher temperatures encourage the oxidation of glutathione (27). It should be long enough so at the end of the procedure there would only be the primary amino groups coupled with our polymer. We should question ourselves also about the adequacy of our coupling agent: if some other agent could provide us with bigger yield regarding coupling efficiency.

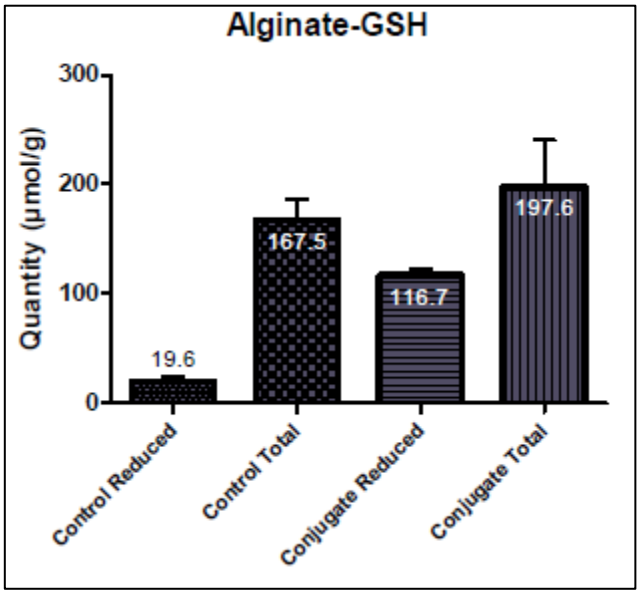


Figure 11: Amount of thiol groups present in Control and Coupled alginate samples. Controls were made without coupling reagents, reduced meaning that only free thiol groups have been measured and total meaning double S-S bonds were broken. The Y axis indicates the quantity of –SH groups per gram of polymer.

In alginate we observe a similar feature. The **Control total** group results are very high, and the overall result for **Conjugate total** group is only 197.6 µmol of thiol groups per gram of polymer. There are quite some variables that could be changed. First of all, it could be possible that there was an inadequate activation of carboxyl groups of sodium alginate, what resulted in low thiolation due to non-activated carboxyl groups. One of possibilities could be also that the coupling reaction is not effective at GSH and EDAC concentrations this low and it should be modified. The reaction should be carried out by adding NHS to the reaction mixture in the coupling reaction, as a possibility to augment the linking yield and overall efficiency.

Jindal and al. (28) described a similar process of coupling Sodium alginate with Cysteine, where alginate was left hydrated overnight (in our research the process lasted only 30 min). For 200 mg of alginate they used EDAC solution of 200 mM concentration, what represents a 2 fold increase in EDAC concentration given that we used 50 mM for 100 mg of alginate. Also time and pH affect the activation. It would be therefore wise to repeat the experiments, changing the parameters and adapting them to the results obtained. Coupling should be repeated and done at lower temperature (at 4 - 10°C) to obtain sufficient GSH bound and reaction time should be

increased, with the goal in mind to achieve a progressive increase in the coupling efficiency of the reaction with time.

Secondly, it is known that the oxidation of thiol to disulfide is inevitable in the synthesis (28), because of the reagents used and the reactive components. This diminishes the overall yield of our reaction. The same researchers found out that the extent of oxidation is increased when pH exceeds 5 (steady increase with increase in reaction pH), or on exposure to light and higher temperature during reaction. We could omit the effects of light exposure and heat in the future experiments, but DTNB, Ellman's reagent, and EDAC require neutral or alkaline pH for good functioning, hence we could only change the coupling reagent. The choice of our reaction pH reflects a compromise between the pH needed for the amine groups to be available and the optimum pH for the carbodiimide activation.

As an important fact we should again highlight the so called intrapolymer chain crosslinking throughout disulfide bond formation. Considering the number of thiol groups available in the environment and contemplating their tendency to more stable and low energy conformations, this is thought to be the fate of a great deal of GSH molecules. As George and Abraham (34) predicted, although in slightly divergent experiment, it could be suggested that crosslinking appears mainly on the outer surface, capturing and thus closing the GSH molecules from the environment and releasing them after the appropriate trigger has been provided (pH for example, that would diminish or amplify the former polyelectrolyte charge). This intra and inter molecular bonds could be the main reason for the high quantity of thiol groups found in our **Control** samples.

One of additional reactions we should carry out would be the evaluation of unbound glutathione with 2,4,6-Trinitrobenzenesulfonic acid (TNBS), a reagent used to detect primary amino groups (10). In this case we would find out if any glutathione got captured in the polymeric network and thus be able to determine the real reaction yield .

4.2 SNOA characterization

S-nitrosoalginate was prepared with nitrosation of formerly prepared alginate-GSH conjugates. We had to know the average quantity of thiol moieties grafted on the alginate backbone so that the calculations for the quantity of the nitrosation agent were exact. Because the amount of thiol

groups before reduction was insignificant, a reduction with sodium borohydrate was formerly carried out. We used at least equimolar concentration of sodium nitrite, thus approximately 200 µmol/g with respect to that of 197.6 µmol/g of available free thiol groups. In some publications multi-fold quantity was used, which was also a part of our essays, but showed no significant differences.

In order to be able to determine the successfulness of our nitrosation afterwards, standard curves were plotted [Figure 12].

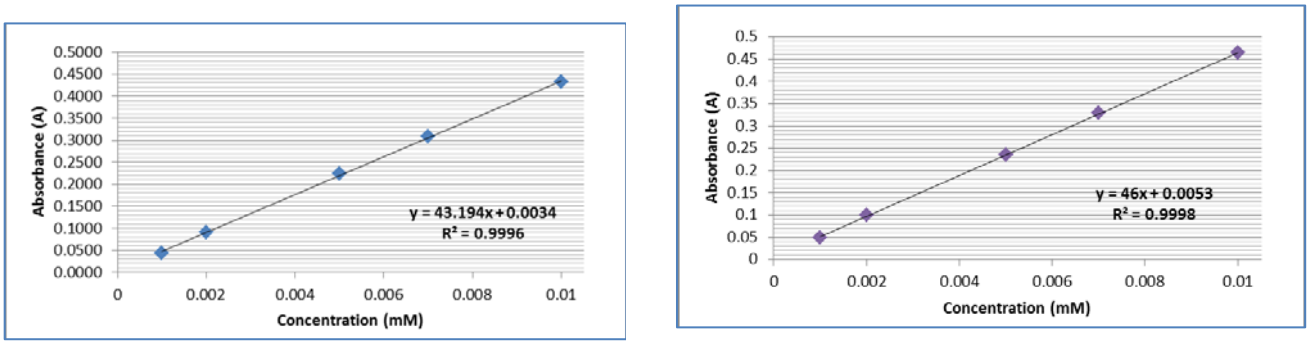


Figure 12. Griess (left) and Saville (right) Standard curve plots.

In this case our control was *S*-nitrosoglutathione. We had quite some problems with its synthesis in view of the fact that it is very sensitive to multiple parameters. Temperature, oxygen concentration, pH, metal ions and light are some variables that had to be controlled and adapted in order for the synthesis to be successful. GSNOs half-life in aqueous solutions is only about 5.5 hours, yet some polymers are able to decrease the degradation rate of GSNO with the formation of hydrogen bridges (based on interactions of the hydroxyl groups). This can also be increased to some extent with an increase of the solution pH to about 8 – 9.

On the graphs we presented time dependent concentration of nitroso group (Figure 13).

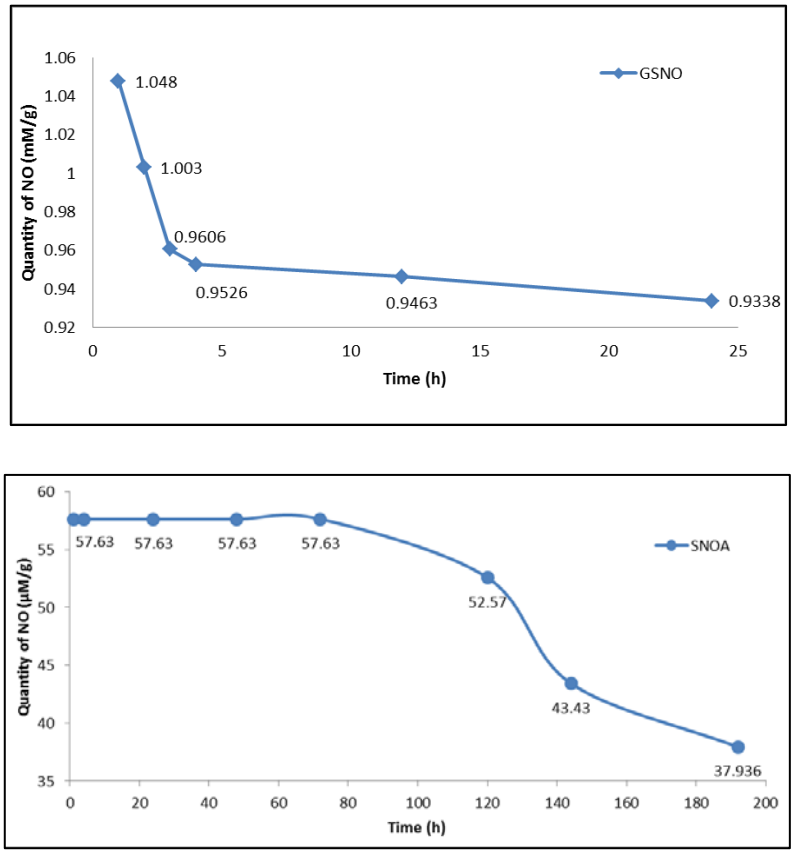


Figure 13. GSNO (above) and SNOA concentration with time
(25°C, normal pressure).

GSNO decay seems to follow the usual omnipresent negative exponential slope. Our product was quite unstable and the concentration began to diminish quickly after the synthesis. Being protected from light, external oxygen and ambient temperature it helped improve its stability and the reaction yield. All reagents had to be put in ultrasonic bath to remove small suspended gas-bubbles and reduce the level of dissolved oxygen below the natural level.

GSNO grafted on alginate polymer seemed to be more stable than GSNO alone. As already mentioned, some polymers can prolong GSNO stability by the formation of hydrogen bridges, which would be plausible in our case since there are oxygen and hydrogen groups present. SNOA was stable for about three days, reaching about the same value in each of consecutive measurements, but after three to four days the concentration started diminishing.

We can see that the overall reaction yield is approximately 25%, regarding initial amount of grafted thiol groups. Parameters should be thoroughly examined in order to provide more profitable result. The main problem would present the fast oxidation of thiol groups and the

sensitivity of NO to the presence of oxygen, therefore the synthesis must be carried out quickly in cold and dark environment. Therefore, it would be best to nitrosate in ice bath and protected from light (35). All the ingredients should be pre-chilled before use and thoroughly deoxygenated. Special care should be given to the recipients in which the final product is stored (should be properly cleaned and pre-chilled before use) and care should be taken that the sampling is fast in order to prevent external oxygen from entering into the solution.

4.3 Interferences

In order to examine if any of the reagents have an impact on the oxidation of glutathione, experiments with EDAC and $NaBH_4$ were carried out. We prepared glutathione solutions (16.3 µM) and treated them with two different concentrations of EDAC and $NaBH_4$ (10 and 100 µM). We have chosen to vary certain parameters: the effects were observed in cold and heated environment at different time intervals. The results are presented in the table below (Table VII).

Table VII: Results of EDAC (above) and $NaBH_4$ impact on oxidation of glutathione at different time intervals.

t(min)	c (GSH) (µM)	c (EDAC) (µM)	c(-SH) (µM)	c(-SH) (µM)
	Input conc.		4-6°C	37°C
5 min	16.3	0	**13.7**	**14.6**
	16.3	10	**10.3**	**9.8**
	16.3	100	**9.9**	**9.4**
30 min	16.3	0	**13.9**	**14.3**
	16.3	10	**9.1**	**9.9**
	16.3	100	**9.0**	**6.6**
60 min	16.3	0	**14.0**	**14.0**
	16.3	10	**9.1**	**8.7**
	16.3	100	**9.0**	**7.2**

t(min)	c (GSH) (µM)	c (NaBH4) (µM)	c(-SH) (µM)	c(-SH) (µM)
	Input conc.		4-6°C	37°C
5 min	16.3	0	**12.3**	**12.2**
	16.3	10	**11.0**	**11.4**
	16.3	100	**12.0**	**11.6**
30 min	16.3	0	**12.1**	**12.4**
	16.3	10	**12.1**	**11.7**
	16.3	100	**13.2**	**11.1**
60 min	16.3	0	**12.6**	**12.9**
	16.3	10	**12.3**	**13.4**
	16.3	100	**12.4**	**12.5**

It can be seen out of diminishment in glutathione concentration when reviewing the upper tables that, whereas $NaBH_4$ didn't really show the impact, EDAC is clearly the main oxidant in the synthesis of our conjugates (the results vary a bit because the concentrations were too small to be able to perform really precise measures). The concentrations of glutathione do not seem to diminish with time, but rather with the growing concentration of the reagent. But we can notice a sizable difference when it comes to exposal of the reagents to higher temperatures. High temperatures seem to somehow accelerate the formation of double bonds. Therefore heating should be sidestepped and the EDAC concentration carefully calculated in forthcoming researches so that unnecessary oxidation would be avoided. Also the synthesis should be according to the requirements of the molecules used. We should think about trying a different coupling reagent, such as succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) as a linker or some other water-soluble imide. GSH is quickly oxidized, that is why at least the dialysis, if not also the synthesis, should be realized in cool environmental conditions (lower than 10°C (20)).

4.4 Kinetics

We questioned ourselves if glutathione is drained out of the dialysis membrane in sufficient amount and to verify this we decided to do a kinetic study of liberation. Considered that different membranes were available among the laboratory inventory, we wanted to identify whether the scope of purification is the same using membranes with pore size 3500 kDa or 12000 – 14000 kDa molecular weight cut off. At the same time we examined if the purification achieves the desired objectives – the plateau phase at the end, which would assure us that the conjugates are free of unbound glutathione. The tables with the results are presented below (Table VIII).

Table VIII: Kinetics of time dependent GSH concentration in two dialysis membranes (A – 3.5 kDa, B – 12-14 kDa). Corrected value is the solution absorbance diminished by blank, inside meaning measured inside the dialysis membrane.

A

3500 kDa/ time (min)	A inside	A corr. inside	c(GSH) (µmol/L)	c(GSH) corr. (µmol/l)	A outside	A corr. outside
1	0.215	0.138	46.2	462	0.084	0.007
2	0.225	0.148	49.54	495.4	0.088	0.011
3	0.22	0.143	47.87	478.7	0.086	0.009
4	0.219	0.142	47.54	475.4	0.091	0.014
5	0.229	0.152	50.21	502.1	0.093	0.016
15	0.217	0.14	46.87	468.7	0.085	0.008
30	0.215	0.138	46.2	462	0.091	0.014
45	0.199	0.122	40.84	408.4	0.086	0.009
60	0.212	0.135	45.19	451.9	0.09	0.013
75	0.196	0.119	39.84	398.4	0.089	0.012
90	0.171	0.094	31.47	314.7	0.091	0.014
105	0.188	0.111	37.16	371.6	0.092	0.015
120	0.169	0.092	30.8	308	0.084	0.007
135	0.17	0.093	31.13	311.3	0.08	0.003
150	0.14	0.063	21.09	210.9	0.083	0.006
165	0.166	0.089	29.79	297.9	0.081	0.004
180	0.159	0.082	27.45	274.5	0.083	0.006
195	0.165	0.088	29.46	294.6	0.086	0.009
210	0.15	0.073	24.44	244.4	0.086	0.009
225	0.146	0.069	23.1	231	0.088	0.011
240	0.146	0.069	23.1	231	0.091	0.014
255	0.145	0.068	22.76	227.6	0.088	0.011
270	0.145	0.068	22.76	227.6	0.085	0.008
285	0.147	0.07	23.43	234.3	0.088	0.011
300	0.142	0.065	21.76	217.6	0.08	0.003
315	0.131	0.054	18.08	180.8	0.089	0.012
330	0.11	0.033	11.05	110.5	0.081	0.004
345	0.104	0.027	9.038	90.38	0.08	0.003
360	0.116	0.039	13.06	130.6	0.083	0.006
Blank	0.077					

B

12000-14000 kDa/ time (min)	A inside	A corr. inside	c(GSH) (µmol/L)	c(GSH) corr. (µmol/l)	A outside	A corr. outside
1	0.209	0.132	44.19	441.9	0.079	0.002
2	0.207	0.13	43.52	435.2	0.082	0.005
3	0.219	0.142	47.54	475.4	0.081	0.004
4	0.211	0.134	44.17	441.7	0.082	0.005
5	0.209	0.132	44.19	441.9	0.086	0.009
15	0.214	0.137	45.86	458.6	0.085	0.008
30	0.184	0.107	35.82	358.2	0.081	0.004
45	0.206	0.129	43.18	431.8	0.084	0.007
60	0.163	0.086	28.79	287.9	0.078	0.001
75	0.165	0.088	29.46	294.6	0.082	0.005
90	0.183	0.106	35.48	354.8	0.083	0.006
105	0.167	0.09	30.13	301.3	0.076	0.005
120	0.172	0.095	31.8	318	0.087	0.01
135	0.143	0.066	22.09	220.9	0.086	0.009
150	0.154	0.077	25.78	257.8	0.085	0.008
165	0.134	0.057	19.08	190.8	0.089	0.012
180	0.139	0.062	20.75	207.5	0.087	0.01
195	0.141	0.064	21.42	214.2	0.088	0.011
210	0.147	0.07	23.43	234.3	0.088	0.011
225	0.155	0.078	26.11	261.1	0.089	0.012
240	0.14	0.063	21.09	210.9	0.093	0.016
255	0.141	0.064	21.42	214.2	0.087	0.01
270	0.14	0.063	21.09	210.9	0.088	0.011
285	0.135	0.058	19.42	194.2	0.088	0.011
300	0.112	0.035	11.72	117.2	0.081	0.004
315	0.108	0.031	10.38	103.8	0.078	0.001
330	0.108	0.031	10.38	103.8	0.084	0.007
345	0.097	0.02	6.695	66.95	0.082	0.005
360	0.096	0.019	6.38	63.8	0.073	0.004
Blank	0.077					

After presenting the above results in a form of graph the below curves were formed (Figure 14).

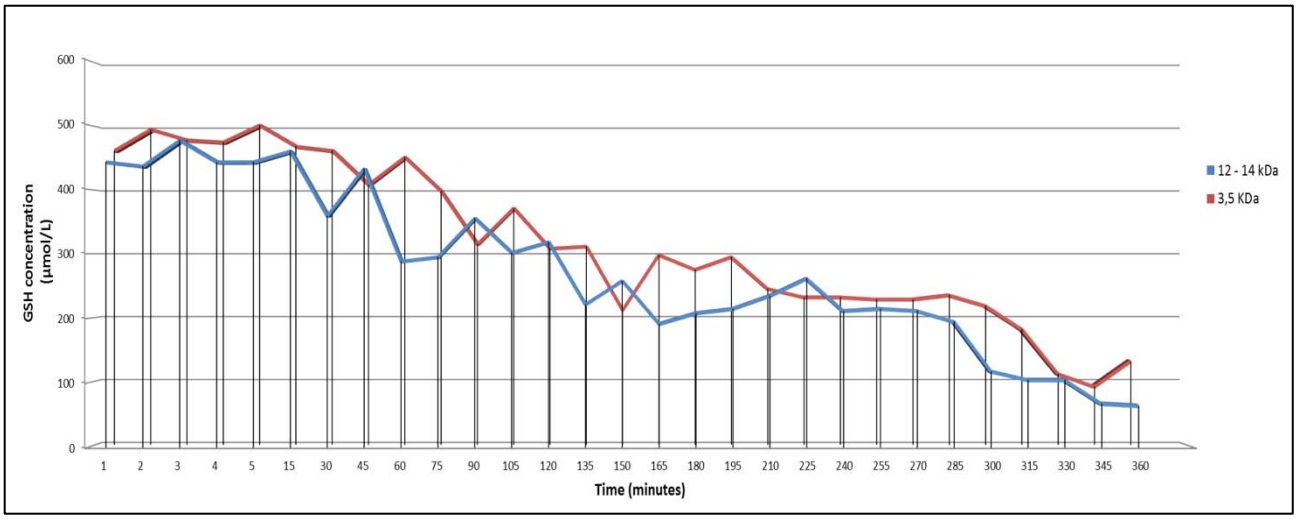


Figure 14. Kinetics of time dependent GSH concentration in the two dialysis membranes

The results between curves vary because of the multiple parameters affecting the measurements. It can be clearly seen from the curves that the MWCO of membrane pores does not affect the changing GSH concentration in the membrane. But what is even more important is that the plateau phase has not been reached. This observation confirms the initial hypothesis stated above suggesting that some molecules could have stayed embedded in the polymeric network in case of a too sparing dialysis. The dialysis process should last at least 24 hours at room temperature or 10°C (both temperatures should be examined), also filtering of the resulting solution should be considered in order to remove the aggregated particles (36). According to Fonseca and al., for best results alginate should be purified before any chemical modification, with dialysis against deionized water for 3 days, followed by stirring with activated charcoal, then filtered, lyophilized and stored (37). Jindal and al. went even further in their experiments of coupling sodium alginate with cysteine. They initialized the dialysis with 1 mM HCl (pH 4) lasting two days, when they replaced the dialyzing fluid with fresh medium, containing also 1% of NaCl. This was repeated twice, for 24 hours each. Finally, the purification process was carried out against 1 mM HCl for 2 more days. At all times of polymer preparation and purification the experiments were carried out in the dark as much as possible so as to minimize disulfide bond formation (28). Therefore, in this experiment the purification was elongated over 6 days, providing a solid ground to assert that all of the free glutathione was washed out. It is right to question if the long lasting process

that Jindal and al. used is not excessive. The ester linkage is more easily broken than the amide bond so the ester drugs are less stable in a solution and cannot be stored for as long as amides. Therefore, we should carefully examine if the 6-days purification would not destroy our samples and hydrolyze the newly formed bonds, especially in the case of Methocel-GSH because of ester bonding.

4.5 Particle size

As a therapeutically applicable formulation it is essential to know what happens once the molecules reach the intestine. One option is that GSNO coupled with alginate monomer reaches the circulation after penetrating the mucus barrier. This would represent quite a challenge seen the negative charge of alginate and the cluster formation. A depolymerization of alginate should be considered, although that could result in lower stability of the conjugates and their deposition in the non-specific zones could appear. The other possibility is that GSNO molecule is released in a form of single GSNO when the polymer is covalently attached to the mucus, where it promotes penetration of the pharmaceutical ingredient through and between cells. Therefore, GSNO is absorbed and coupled with transporters in the circulation. A part of uptake-friendly intestine is known to exist, called Peyer's patches. It is less protected by mucus and the most exposed to the internal milieu, representing minimal mucus barrier and the most ideal place for the uptake of foreign molecules (20). This could serve as a major getaway for the adsorption of negatively charged small-sized protein conjugates (smaller than 1 μm show best absorption rate). That is why particle size measurements clearly show whether the formulation is on the right track when the absorption is in the question. Our results are presented hereinafter.

4.5.1 Measures of Lyophilisate Control and Coupled samples

The solutions for particle size measurements were prepared after lyophilisation. After dissolving 20 mg of our lyophilized product in 4 mL of water the solution was consecutively diluted according to the needs of the process, primarily for the purpose of reaching the appropriate count rate. The samples were measured at 25°C in polystyrene cuvettes, using imaginary refractive indices of 1.590. Measurements of Control sample had shown that data quality was too poor for

distribution analysis. Furthermore, along with the sample the cumulant analysis was too polydisperse, so the results were not gathered. Because in range figure was low we assumed that large sedimenting particles are present (which was confirmed with apparatus stating sedimentation process taking place), along with the fact that Z-average was larger than upper size analysis limit (10109.6 > 10000 nm).

Measurements of Coupled sample also gave us very polydisperse results: in percentage most representative sizes were distributed on the interval between 1591 and 2674 nm. Due to high Z-average values we decided to study the particle size also on Mastersizer, particle sizing instrument that measures particles in the size range from 0.02 to 2000 µm. In the measurements for both batches, Control and Coupled samples, we noticed that the size measures decrease with time (Figure 15).

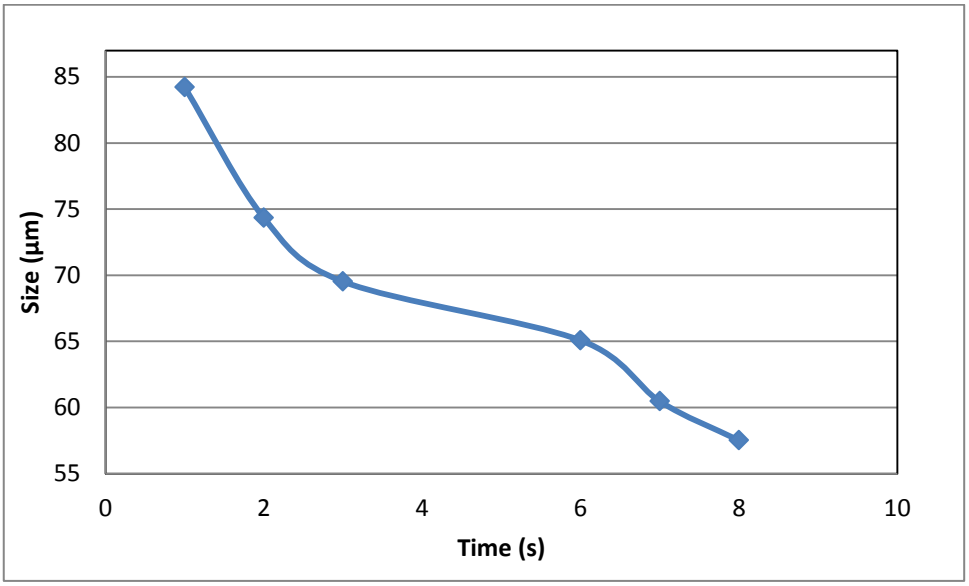


Figure 15. Consecutive size measurements show size diminishment.

The decreasing results could have different background. First of all, molecules or conjugates could form complexes that would result in particle agglomeration. That would be particularly true in the Control sample, seen that alginate molecules are known to have a strong tendency to self-assemble. There is also the natural tendency of systems known, continuously attempting to

minimize the free energy and form clusters. Secondly, seen that our polymer has adhesive and hydrophilic properties, there is a possibility that with time more and more bigger particles got fixed to the walls and to the stirrer placed in the middle of the measuring cell, which made overall result smaller. Somehow problematic could also be the liquid in which we dispersed our sample (water) due to its polar properties – it could increase the affinity of polymer molecules and provoke grouping or, quite simply, degradation of our conjugates. We also verified the zeta potential, which was measured negative because of overall negatively charged alginate.

Seen that lyophilisation has been frequently proven to change particle size, its physical properties (deformation) and as such cause particle agglomeration and clumping, we decided to measure our liquid samples that did not undergo lyophilisation process.

4.5.2 Measures of Liquid Control and Coupled samples

When measuring freshly prepared liquid samples on Zetasizer (measures were made once dialysis process was finished or not later than the following day), we noticed that particles were distributed in two or more size groups. In the Control sample there were more or less two representative size groups to be found, the particles measuring from 46.9 to 137.1 nanometers and a group of particles sized from 629.8 to 814.2 nanometers. For Coupled, broad size distribution was present with a lot of small sized groups, where the group sized from 583.3 to 954.4 nanometers was the most representative in percentage.

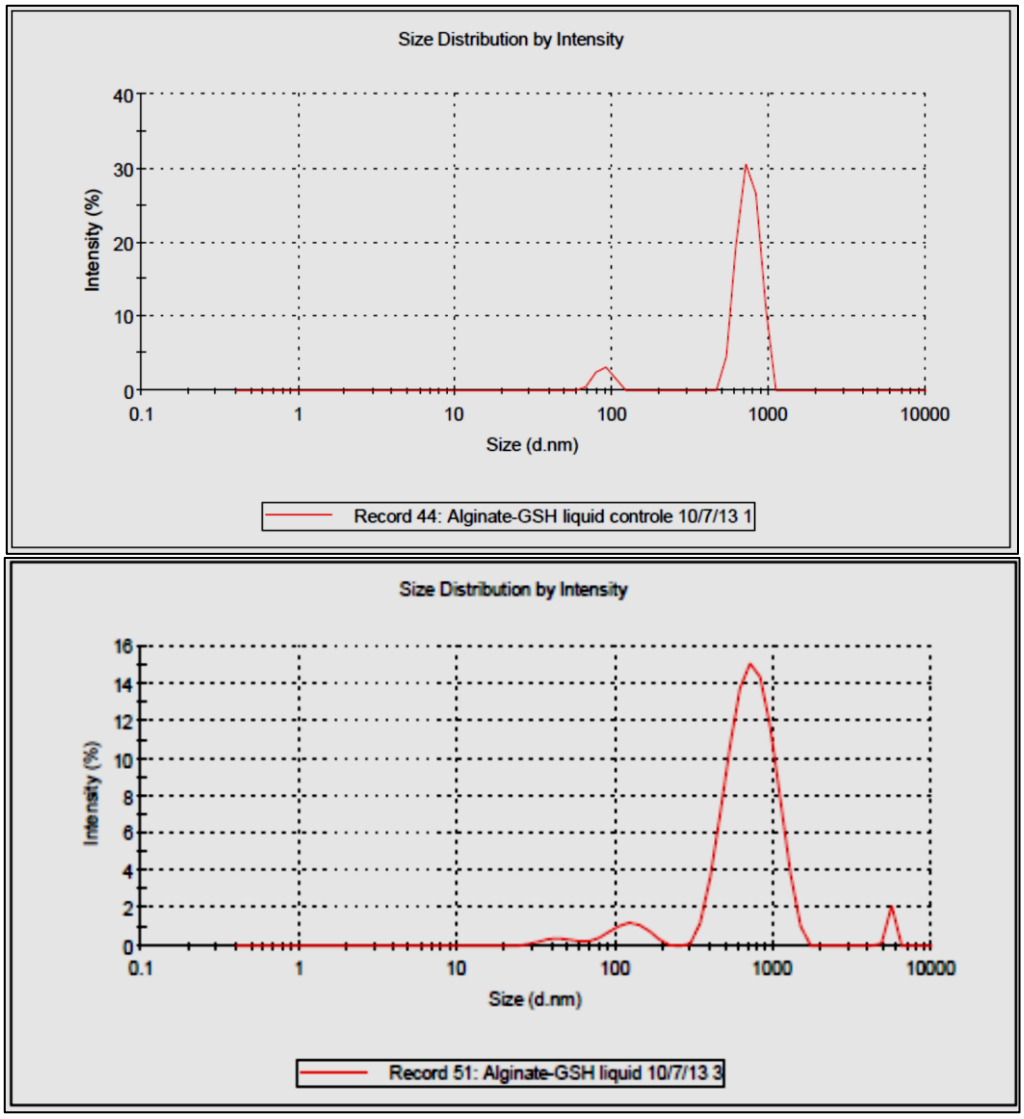
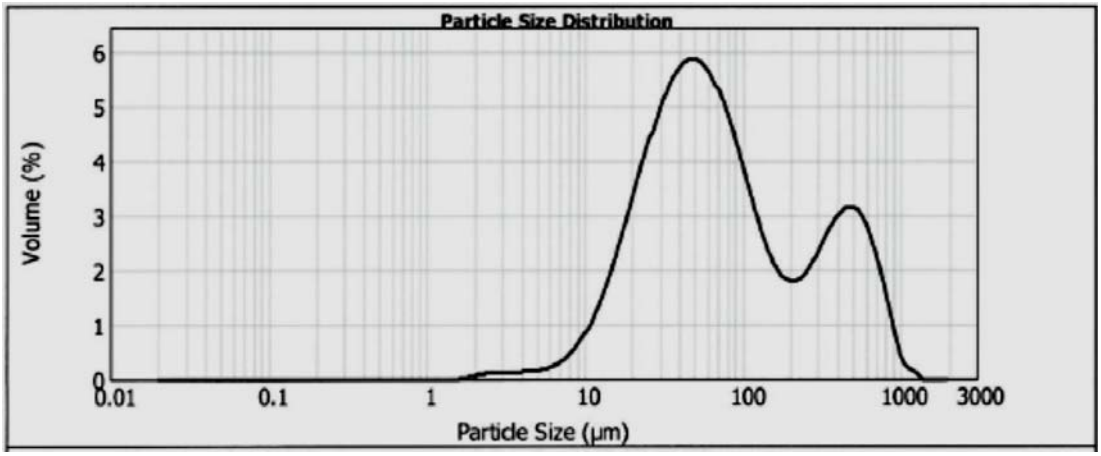


Figure 16. Size distribution by Intensity for Liquid Control and Coupled on Zetasizer

After measurements made on the Mastersizer we noticed that results from consecutive measurements were more stable compared to precedent measures of lyophilized product – decrease rate diminished and average size interval was shown to be 59.461 - 68.86 µm (64.160±4.7) µm for Control and 32.547 – 38.456 µm (35.50±2.956) µm for Coupled. We noticed a significant difference between lyophilized and liquid Coupled samples: the liquid Coupled sample results were approximately 2 times smaller than lyophilized ones. This could be caused by physiological modifications in lyophilisation known as lyophilisation-induced aggregation that is a common phenomenon in protein-based samples. In addition to that, average

particle size of Coupled sample was approximately 2 times smaller than Control. It can be therefore pointed out that GSH potentially diminishes the polymer's affinity to self-assembly by changing the overall zeta potential or providing steric hindrance. One might also deduce that the conjugates were characterized by more covalent cross-links, preventing excessive swelling. After measuring the zeta potential in control and conjugates the upper hypothesis was confirmed with values -48.9 for Control and -35.8 for Coupled sample.

However, despite the fact that the samples were freshly prepared, the parameters required to obtain good results were still not fully reached: our sample remained to have a very broad size distribution (graphs on the next page) – it was too polydisperse for distribution and cumulant analysis and thus very likely not appropriate for DLS measurements. The instrument reported the presence of large or sedimenting particles (aggregates), sample fluorescence and absorbance. The graphs from Metasizer analysis are presented below (Figure 17), giving us a generalized overview of the particle population.



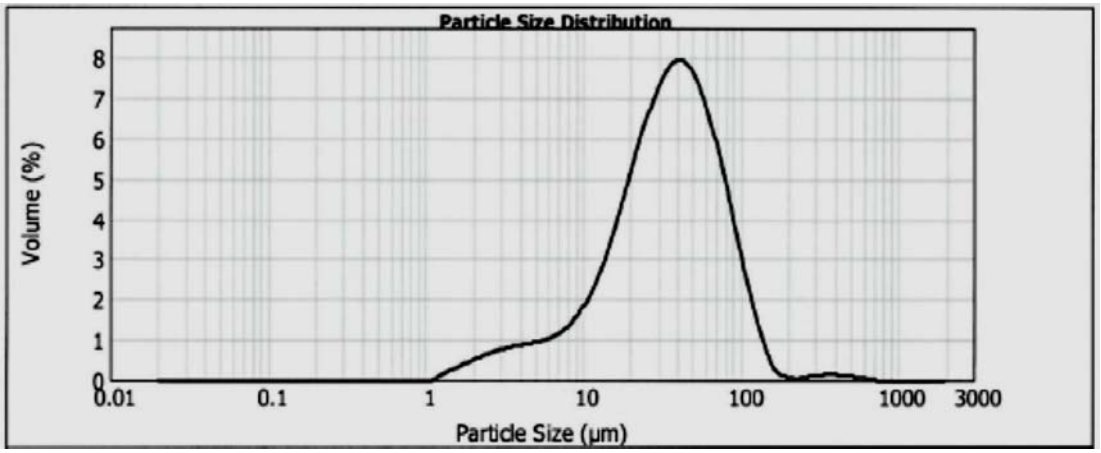
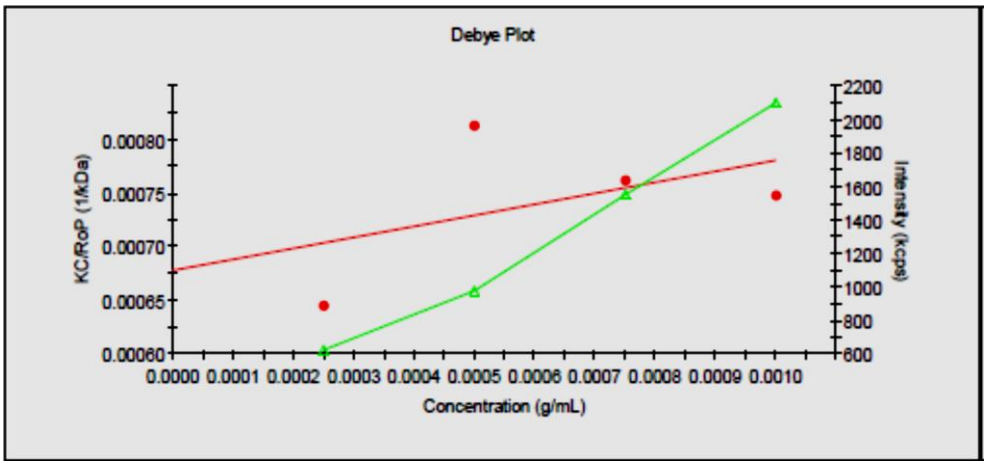


Figure 17. Size distribution by Volume for Liquid Control (above) and Coupled measured on Mastersizer

4.5.3 Measures of Molecular weight

When measuring the molecular weight we encountered a similar problem as stated below, that is why we could not take into consideration the given results. After preparing four solutions of different concentrations (0.25, 0.5, 0.75 and 1 mg/mL) and measuring their values, the results were not linear and consequently the molecular weight measurements had an unsure value. The reason can be found in high polydispersity index of our sample, when measuring small and big objects gives a false result on average molecular weight that cannot be taken into account. Count rate and size were both increasing – again we may conclude that the sample was aggregating, which could also be due to the solvent used for the measures (purified water). It can be seen from the pictures below (Figure 18) that the correlation coefficient was very low.



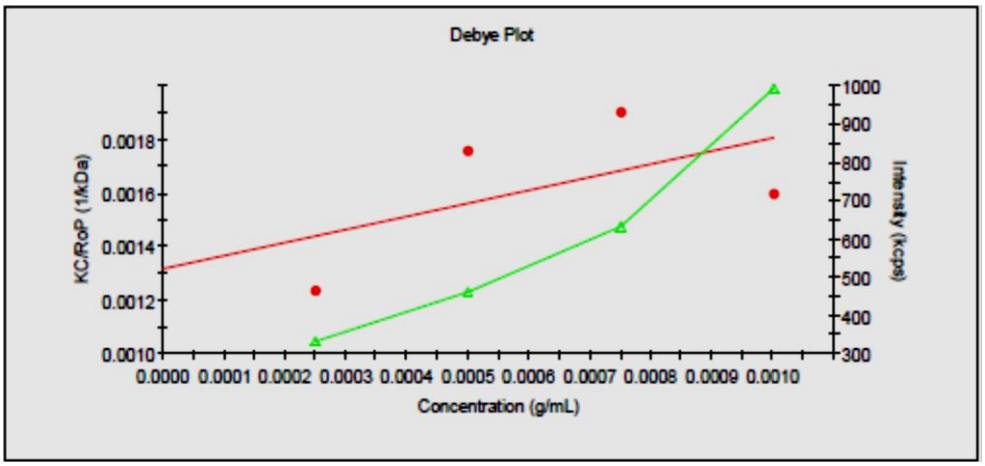


Figure 18: Molecular weight measurements of Liquid Control (above) and Coupled. The scattered light was measured at a single angle (90°) at multiple sample concentrations. Green line represents the intensity of scattered light. The intercept of the Debye plot is used to determine the molecular weight.

In the beginning in low concentrations the affinity between molecules seemed to be poorly developed and the particles seem soluble. With the growing concentration it seems like there would be a limit as a "critical micelle concentration" after which particle agglomeration takes place, forming less-soluble clusters of particles. Polymer concentration has a great impact on the phase behavior of alginate molecules. At low concentrations of the polymer its chains remain localized, but as the concentration increases attractive interactions arise between the polymer chains, creating aggregates and assemblages. This kind of interactions can produce percolation-like behavior that results in the formation of lamellar, bicontinuous or liquid crystalline structures (38). In order to verify the solubility or hypothesize any other structure formation, we should prepare solutions with the following concentrations: 0.375 and 0.625 mg/mL. This would give us an answer about solubility changes and a good average of the molecular weight of our product. Also diminishing the particles polydispersity should be inevitable for correct measures.

For information purposes the molecular weight for Control was measured 757±161 kDa and for Coupled 1470±178 kDa. For pure alginate the average molecular weight is about 222 g/mol per structural unit and the typical average of the macromolecule is about 10,000 - 600,000 g/mol.

5. Conclusion

As it has been highlighted throughout this work, developing a NO delivery system suitable for successful biomedical application remains a very interesting though at the same time challenging field being under thorough investigation throughout the last years. Drugs joint with different scaffolds such as biopolymers in nano/micro sizes or encapsulated in nanostructures often offer sustained release, improved solubility and protection of the active substance from aggressive environment. But due to their instability RSNOs need a vehicle to be used in medical applications and require a formulation that would offer protection in the physiological invasive media. George and Abraham (34) state in their publication that alginate exhibits various properties that make it an ideal candidate for intestinal delivery, the latter being supported by the fact that in the stomach conditions alginate shrinks forming an insoluble "alghinic acid skin" – tight viscous gel that resists dispersion and swelling at low pH values. The charges and functional groups in the molecule get reoriented and form intra- and inter-molecular hydrogen bonds between water molecules, glutathione and the oligosaccharides of Sodium alginate. When pH rises, carboxyl groups become ionized (COO^-), causing intermolecular electrostatic repulsion of polymeric chains, resulting in high "porosity" and thus high diffusion rate of dispersed molecules (38, 39). Polymer chain mobility, caused by the presence of GSH molecules that interrupt the polymer matrix continuum, is proved to liberate polymer chains to move freely (water penetration is facilitated) and could result in entanglement with mucine molecules. While unmodified alginate can only form non-covalent bonds with mucosal surface, via hydrogen bonds, van der Waals forces and ionic interactions (28), in this study we have seen that polymer conjugates can be readily coupled with glutathione (augmenting the possibility to link with the mucus forming covalent bonds) and thereafter nitrosated, forming potential NO carriers that thus still have a lot of space for improvement after our initial research. The biomedical potential of our conjugate is despite constrains clearly noticeable: Alginate is a biodegradable non-invasive and non-allergenic material, able to conjugate with other molecules, appropriate for oral delivery, resistant to acid environment and able to form strong bonds. Being a mucoadhesive polymer it swells and can thus fill the crevices of the mucous membrane, contributing to the effective surface area in contact with the intestinal mucosa and yielding a high local concentration of the drug, diffusing through the polymeric network. In general the low-molecular

weight entities which are able to diffuse through the membrane are absorbed by the transcellular pathway mostly in the area called Peyer's patches. The drug can thus penetrate its way to the arteries, having affinity to injured over intact arteries and to lipophilic areas, acting as antiplatelet agent and being able to decrease infarct size, promote faster healing and improve coronary endothelial function.

In the study we demonstrated that the liaisons between alginate – glutathione and Methocel – glutathione can be formed. Our furtherly examined conjugates of alginate-GSH hold a great potential for oral route delivery, since alginate closes up when exposed to highly acid environment. Polymer is biocompatible and stable, providing a relatively slow release. The –SH loading is sufficient, we may therefore conclude that in the intestine the liaisons can be made between cysteine residues of the mucine and thiol groups of polymers that are not nitrosated. We should use NMR spectroscopy to analyze the subtle substitution patterns on the polymer backbone, in order to see in which percent the groups get substituted. With respect to SNOA, the conjugate seemed to be more stable than GSNO, confirming the fact that some polymers can prolong GSNO stability, and it was proven to have NO molecules grafted. The morphology should be examined using SEM (Scanning electron microscope) or TEM (Transmission electron microscopy). Overall reaction yield was of 25% and in the future experiments we should attempt to increase its profitability. For best results the synthesis must be quicker, performed in cold and dark environment. The dialysis process should be longer and carried out at non-ambient temperature to efficiently purify our samples and filtering of the resulting solution should take place before proceeding with the experiment. To the reagents used special attention should be addressed and their suitability considered, varying different parameters such as effect of concentration, temperature and their contribution to the oxidation of input ingredients (GSH, NO). Different linker could be chosen to augment the percentage of linked glutathione. We assume the formation of aggregates with time and lyophilisation and should therefore find a proper process to depolymerize the polymer and thus try to equalize the size distribution of our particles that were defined as polydisperse (steric hindrance was found to diminish the zeta potential comparing Control to Conjugate results, so additional experiments should be carried out). Broad size distribution probably gave us a false result on average molecular weight with low correlation coefficient, thus these measurements should be repeated.

In order to boost the extent of thiolation, Jindal and al. (28) demonstrated that the rate of hydrolysis of intermediate and the scope of disulfide bond formation are pH dependent, the highest rate being measured when the reaction pH during first 2 hours did not go beyond 4. Therefore this was included in the synthesis of alginate conjugates. But there are quite some important details that we brought together after the thorough literature examination, which were not considered, and could improve the yield and quality of our synthesis; such as hydrating alginate overnight, preparing fresh EDAC each time and especially as already stressed a couple of times longer dialysis time – we had noticed in many publications that the dialysis was carried out in a far greater time frame, lasting for 6 days in total. The reaction should be carried out in dark in order to minimize disulfide bond formation. Also we could ask ourselves a question about the effect of ambient temperature taking into account the temperature sensibility of glutathione. All in all, the physicochemical properties of the conjugates need further improvement.

In the literature it was identified as reliable to synthesize nanoparticles or beads in combination with Chitosan, because of easier preparation and great compatibility of physical properties. Encapsulation in alginate/chitosan nanoparticles has been shown to increase the stability of GSNO and diminish its decomposition at physiological temperature (35). We could try to direct our further researches in the way of symbiosis with appropriate molecules in order to improve our formulation and form less polydisperse particles. The most positive quality of recent NO donors is that the drugs can be tailor-made so that one may suit the disease target: their lipophilicity can be changed, they also hold a great deal of promise in specific medical applications such as coatings for catheters and stents, which will likely diminish a number of complications regarding restenosis or thrombosis over a significant period of time. We are expecting many innovative and exciting applications in the future of alginate use in pharmaceutical technology, at the same time attempting to deliver the correct quantity of NO to the right place for the proper length of time.

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