



Miniaturized devices for bioanalysis : case of nitric oxide stored as S-nitrosothiols in biological fluids

Abdul Ghani Ismail

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Miniaturized devices for bioanalysis: case of nitric oxide stored as S-nitrosothiols in biological fluids

Par Abdul Ghani ISMAIL

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Dirigée par Fethi BEDIOUI et Anne VARENNE

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Preface

***NB:** This thesis manuscript entiteled "Miniaturized devices for bioanalysis: case of nitric oxide stored as S-nitrosothiols in biological fluids" is written in English language except a french summary at the beginning. It is composed of four chapters in addition to a general introduction and a conclusion.*

This work resulted in five publications:

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- 2. Ismail, A.; D'Orlyé, F.; Griveau, S.; Bedioui, F.; Da Silva, J. A. F.; Varenne, A.; Anal. Bioanal. Chem. 2015, 407, 6221-6226.*
- 3. Ismail, A.; D'Orlyé, F.; Griveau, S.; Varenne, A.; Bedioui, F.; Electroanal., 2015, 27, 2857-2863*
- 4. Baldim, V.; Ismail, A.; Taladriz-Blanco, P.; Griveau, S.; de Oliveira, M.; Bedioui, F.; anal. Chem., 2016, 88 (6), 3115-3120*
- 5. Ismail A.; de Oliveira Araújo M.; L. S. Chagas C.; Griveau S.; d'Orlyé F.; Varenne A.; Bedioui F.; K. T. Coltro W.; Analyst, 2016, 141, 6314-6320*

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LIST OF ABBREVIATIONS

μPAD	Microfluidic Paper-based Analytical Device
AE1	Anion exchange protein 1
AlbSNO	S-nitrosoalbumin
AuNP	Gold nanoparticle
Aβ	Amyloid β
BBB	Blood brain barrier
BGE	Back ground electrolyte
BH ₄	Tetrahydrobiopterine
BSA	Bovine serum albumine
BW	Buffer waste reservoir
C ⁴ D	Capacitively coupled contactless conductivity detection
CE	Capillary electrophoresis
CGMP	Cyclic guanosine monophosphate
COC	Cyclic-olefin copolymer
CVD	Cardiovascular diseases
CySNO	S-nitrosocysteine
CySSyc	Cysteine glutathione disulfide
DAF	4,5-diaminofluorescein
DAF-FM	4-amino-5-methylamino-2',7'-difluorofluorescein
DAN	2,3 diaminonaphthalene
DARPA	Defense advanced research projects agency
DDAB	Dihexadecyl dimethyl ammonium bromide
DEA- NONOate	Diethylammonium (Z)-1-(N,N-diethylamino)diazene-1-ium-1,2-diolate

LIST OF ABBREVIATIONS

DG	Drying gas
DIGE	Difference gel electrophoresis
DLS	Dynamic light scattering
DMF	Dimethylformamide
DNIC	Dinitrosyl iron complexes
DTNB	5,5-dithio-bis-(2-nitrobenzoic acid)
DTPA	Diethylenetriaminepentaacetic acid
Dyneon THV	Polymer of tetrafluoroethylene, hexafluoropropylene and vinylidene fluoride
EC	Electrochromatography
ECD	Electrochemical detection
EDRF	Endothelial derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial NO synthase
EOF	Electro osmotic flow
ESI	Electro spray ionization
GC	Gas chromatography
GE	Gel electrophoresis
GGT	Γ -glutamyl transferase
GSH	Reduced glutathione
GSNO	S-nitrosoglutathione
GSNOH	Aminoxyl radical
GSO ₂ H	Glutathione sulfinic acid
GSO ₃ H	Glutathione sulfonic acid
GSOH	Glutathione sulfenic acid
GSSG	Oxidized glutathione
GTN	Glyceryl trinitrate

LIST OF ABBREVIATIONS

GTP	Guanosine-5'-triphosphate
HbFe(II)	Deoxygenated hemoglobin
HbFe(II)O ₂	Oxygenated hemoglobin
HbS	Sickel-cell hemoglobin
HbSNO	S-nitrosohemoglobin
HcySNO	S-nitrosohomocysteine
HETP	Height equivalent to a theoretical plate
HMWSNO	High molecular weight S-nitrosothiols
HONO	Nitrous acid
HPDP	N-[6-biotinamido hexyl]-3'-(2'pyridyldithio)propionamide
HPLC	High performance liquid chromatography
Hsp	Heat shock protein
HT-29	Human colon adenocarcinoma cells
IAA	Iodoacetic acid
IEF	Isoelectric focusing
iNOS	Inducible NO synthase
IR	Infra-red
ITP	Isotachophoresis
LIF	Laser induced fluorescence
LMWSNO	Low molecular weight S-nitrosothiols
LOD	Limit of detection
MALDI	Matrix-assisted laser desorption/ionization
MCE	Microchip capillary electrophoresis
MEEKC	Microemulsion electrokinetic chromatography
MEKC	Micellar electrokinetic chromatography
MMTS	Methyl methanethiosulfonate
MQ	Menadione

LIST OF ABBREVIATIONS

MS	Mass spectrometry
N ₂ O ₃	Dinitrogen trioxide
NACysNO	S-nitroso-N-acetyl-L-cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NEM	N-ethylmaleimide
NG	Nebulising gas
nNOS	Neuronal NO synthase
NO	Nitric oxide
NO ⁺	Nitrosonium ions
NO ₂	Nitrogen dioxide
NONOates	Diazeniumdiolates
NOS	Nitric oxide synthase
O ₃	Ozone
OPA	O-phthaldehyde
o-PD	O-phenylenediamine
PC	Polycarbonate
PDMS	Polydimethylsiloxane
pI	Isoelectric point
PLA	Poly(lactic acid)
PMMA	Poly(methyl methacrylate)
PPIs	Proton pump inhibitors
PR ₃	Phosphines
PS	Polystyrene
PSNO	Protein nitrosothiols
PTFE	Polytetrafluoroethylene
PTM	Post-translational modification
PU	Polyurethane

LIST OF ABBREVIATIONS

RAC	Resin assisted capture
RBCs	Red blood cells
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RS*	Thiyl radical
RSH	Thiol
RS-N=PR ₃	S-substituted aza-ylides
RSNO	S-nitrosothiols
RSSR	Thiol disulfide
SDS m-CGE	Sodium dodecyl sulphate micro-capillary gel electrophoresis
sGC	Guanylyl cylase
SIM	Single ion monitoring
SNAP	S-nitroso-N-Acetyl-D,L-penicillamine
SW	Sample waste reservoir
T _g	Glass transition temperature
TNB	2-nitro-5-thiobenzoic acid
UME	Ultramicroelectrodes
UV	Ultra-violet

GLOSSAIRE

AuNPs	Nanoparticules d'or
BPM	Bas poids moléculaires
CG	Chromatographie en phase gazeuse
CHES	Acide N-Cyclohexyl-2-aminoethanesulfonique
CLHP	Chromatographie liquide à haute performance
DEA NONOate	Diethylammonium (Z)-1-(N,N-diethylamino)diazene-1,2-diolate
EC	Electrophorèse capillaire
EOF	Flux électro-osmotique
GS*	Radical thiyl
HPM	Haut poids moléculaires
PMMA	Poly (méthyl méthacrylate)
RSNO	S-nitrosothiols
SM	Spectrométrie de masse

DICTIONARY

17- β estradiol	The most potent naturally occurring ovarian and placental estrogen in mammals; it prepares the uterus for implantation of the fertilized ovum and promotes the maturation of and maintenance of the female accessory reproductive organs and secondary sex characters.
Acetylcholine	A substance that is released at the junction between neurons and skeletal muscle fibers, at the nerve endings of the parasympathetic nervous system, and across synapses in the central nervous system, where it acts as a neurotransmitter
Achalasia	A condition in which the muscles of the lower part of the oesophagus fail to relax, preventing food from passing into the stomach.
Alveolar collapse	A condition where the alveoli are deflated down to little or no volume resulting in reduced or absent gas exchange
Amyotrophic lateral sclerosis	A nervous system (neurological) disease that causes muscle weakness and impacts physical function.
Anesthetic	A substance that induces insensitivity to pain.
Angina pectoris	Chest pain due to an inadequate supply of oxygen to the heart muscle. The pain is typically severe and crushing, and it is characterized by a feeling of pressure and suffocation just behind the breastbone. Angina can accompany or be a precursor of a heart attack.
Angiogenesis	The growth of blood vessels from the existing vasculature. It occurs throughout life in both health and disease, beginning in utero and continuing on through old age.
Anti-oxidant	A substance that inhibits oxidation or reactions promoted by oxygen, peroxides, or free radicals
Arthritis	Inflammation of one or more of your joints. The main symptoms of arthritis are joint pain and stiffness, which typically worsen with age. The most common types of arthritis are osteoarthritis and rheumatoid arthritis.
Autacoids	Biological factors which act like local hormones, have a brief duration, and act near the site of synthesis

Autocrine	Form of cell signaling in which a cell secretes a hormone or chemical messenger (called the autocrine agent) that binds to autocrine receptors on that same cell, leading to changes in the cell.
Behavioral activity	The observable response a person makes to any situation
Bradykinin	A kinin that is formed locally in injured tissue, acts in vasodilation of small arterioles, is considered to play a part in inflammatory processes, and is composed of a chain of nine amino acid residues
Calmoduline (CaM)	Calcium-modulated protein is a multifunctional intermediate calcium-binding messenger protein expressed in all eukaryotic cells. It is an intracellular target of the secondary messenger Ca^{2+} , and the binding of Ca^{2+} is required for the activation of Calmodulin. Once bound to Ca^{2+} , Calmodulin acts as part of a calcium signal transduction pathway by modifying its interactions with various target proteins such as kinases or phosphatases
Catalase	An enzyme found in most living cells that catalyzes the decomposition of hydrogen peroxide to water and oxygen. They are formed from 4 peptidic chains each one composed of more than 500 amino acids. They contain iron atoms inside heme which constitute the active site of protein.
Catecholamine	Any of various amines (as epinephrine, norepinephrine, and dopamine) that contain a dihydroxy benzene ring, that are derived from tyrosine, and that function as hormones or neurotransmitters or both.
Cell signaling	Part of a complex system of communication that governs basic cellular activities and coordinates cell actions
Chagas disease	Also known as American trypanosomiasis, is a potentially life-threatening illness caused by the protozoan parasite <i>Trypanosoma cruzi</i>
Constitutive enzyme	Produced constitutively by the cell under all physiological conditions. Therefore, they are not controlled by induction or repression

Cystic fibrosis	An inherited disorder that causes severe damage to the lungs and digestive system. It affects the cells that produce mucus, sweat and digestive juices. These secreted fluids are normally thin and slippery. But in people with cystic fibrosis, a defective gene causes the secretions to become thick and sticky. Instead of acting as a lubricant, the secretions plug up tubes, ducts and passageways, especially in the lungs and pancreas.
Cytochrome oxidase	One of a superfamily of proteins which act as the terminal enzymes of respiratory chains. The two main classes are cytochrome c oxidases, and quinol oxidases. The common features are: There are two catalytic subunits, I and II. Subunit I contains two heme centers.
Cytochrome P450	A group of enzymes involved in drug metabolism and found in high levels in the liver. These enzymes change many drugs, including anticancer drugs, into less toxic forms that are easier for the body to excrete
Cytokines	Any of a number of substances, such as interferon, interleukin, and growth factors, which are secreted by certain cells of the immune system and have an effect on other cells.
Dinitrosyl iron complexes (DNICs)	They have been recognized as storage and transport agents of nitric oxide capable of selectively modifying crucial biological targets via its distinct redox forms (NO^+ , $\text{NO}^\bullet$ and NO^-) to initiate the signaling transduction pathways associated with versatile physiological and pathological responses
Endothelial cells	Cells that form the lining of the blood vessels
Gastrointestinal reflux	Digestive disorder that affects the lower esophageal sphincter (LES), the ring of muscle between the esophagus and stomach
Gastroparesis	Disorder affecting people with both type 1 and type 2 diabetes in which the stomach takes too long to empty its contents (delayed gastric emptying). The vagus nerve controls the movement of food through the digestive tract
Hemoglobin	Red protein responsible for transporting oxygen in the blood of vertebrates. Its molecule comprises four subunits, each containing an iron atom bound to a haem group

Hirschsprung's (HIRSH-sproongz) disease	Condition that affects the large intestine (colon) and causes problems with passing stool. Hirschsprung's disease is present when a baby is born (congenital) and results from missing nerve cells in the muscles of part or all of the baby's colon
Histamine	A compound which is released by cells in response to injury and in allergic and inflammatory reactions, causing contraction of smooth muscle and dilation of capillaries
Hormone	The chemical messengers of the endocrine system and are transported by blood to distal target cells
Huntington's disease	Progressive brain disorder that causes uncontrolled movements, emotional problems, and loss of thinking ability (cognition)
Hyperaemia	The process by which the body adjusts blood flow to meet the metabolic needs of its different tissues in health and disease
Hypoxia	Deficiency in the amount of oxygen reaching the tissues
Inducible enzyme	An enzyme that is expressed only under conditions in which it is clear of adaptive value, as opposed to a constitutive enzyme which is produced all the time
Inflammatory bowel disease (IBD)	Involves chronic inflammation of all or part of your digestive tract. IBD primarily includes ulcerative colitis and Crohn's disease. Both usually involve severe diarrhea, pain, fatigue and weight loss. IBD can be debilitating and sometimes leads to life-threatening complications
Ischemic heart disease	Disease characterized by reduced blood supply to the heart
Joint	The point where two or more bones meet. There are three main types of joints; Fibrous (immoveable), Cartilaginous (partially moveable) and the Synovial (freely moveable) joint
Lipid peroxidation	The oxidative degradation of lipids. It is the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism

Macrophages	Important cells of the immune system that are formed in response to an infection or accumulating damaged or dead cells. Macrophages are large, specialized cells that recognize, engulf and destroy target cells
Metalloprotein	A protein molecule bound to a metal ion
Methemoglobin	Stable oxidized form of haemoglobin which is unable to release oxygen to the tissues, produced in some inherited abnormalities and by oxidizing drugs
Multiple sclerosis or MS	A long-lasting disease that can affect your brain, spinal cord, and the optic nerves in your eyes. It can cause problems with vision, balance, muscle control, and other basic body functions. MS happens when your immune system attacks a fatty material called myelin, which wraps around your nerve fibers to protect them. Without this outer shell, your nerves become damaged. Scar tissue may form
Myoglobin	A red protein containing haem, which carries and stores oxygen in muscle cells. It is structurally similar to a subunit of haemoglobin.
Neurodegenerative disease	An umbrella term for a range of conditions which primarily affect the neurons in the human brain. any neurodegenerative diseases including amyotrophic lateral sclerosis, Parkinson's, Alzheimer's, and Huntington's occur as a result of neurodegenerative processes.
Neurotransmitter	The chemical messengers found in the nervous system that specifically do the transmission across the synaptic cleft and act on a postsynaptic membrane
Neutrophil	Type of white blood cell, a granulocyte that is filled with microscopic granules, little sacs containing enzymes that digest microorganisms
Nitrile hydratase	Mononuclear iron or non-corrinoid cobalt enzymes that catalyse the hydration of diverse nitriles to their corresponding amides.
NMDA receptor	A type of glutamate receptor that participates in excitatory neurotransmission and also binds N-methyl-D-aspartate; may be particularly involved in the cell damage observed in individuals with Huntington diseases
Nociceptive	Relating to or denoting pain arising from the stimulation of nerve cells (often as distinct from that arising from damage or disease in the nerves themselves)

Paracrine	A form of cell-cell communication in which a cell produces a signal to induce changes in nearby cells, altering the behavior or differentiation of those cells.
Penile erectile	The state of the penis when it is filled with blood and becomes rigid. The penis contains two chambers called the corpora cavernosa, which run the length of the organ, are filled with spongy tissue, and are surrounded by a membrane called the tunica albuginea.
Phosphorylation	The addition of a phosphoryl group (PO_3^{2-}) to a molecule. Phosphorylation and its counterpart dephosphorylation, turn many protein enzymes on and off, thereby altering their function and activity. Protein phosphorylation is one type of post-translational modification.
Pneumonia	Swelling (inflammation) of the tissue in one or both of your lungs. It's usually caused by an infection.
Portal hypertension	An increase in the blood pressure within a system of veins called the portal venous system. Veins coming from the stomach, intestine, spleen, and pancreas merge into the portal vein, which then branches into smaller vessels and travels through the liver.
Post-translational modification	Refers to the covalent and generally enzymatic modification of proteins during or after protein biosynthesis. Proteins are synthesized by ribosomes translating mRNA into polypeptide chains, which may then undergo PTM to form the mature protein product. PTMs are important components in cell signaling.
Pre-eclampsia	A condition in pregnancy characterized by high blood pressure, sometimes with fluid retention and proteinuria.
Pulmonary hypertension	A type of high blood pressure that affects the arteries in your lungs and the right side of your heart.
Pyloric stenosis	Uncommon condition in infants that blocks food from entering the small intestine.
Sepsis	Potentially life-threatening complication of an infection. Sepsis occurs when chemicals released into the bloodstream to fight the infection trigger inflammatory responses throughout the body. This inflammation can trigger a cascade of changes that can damage multiple organ systems, causing them to fail.

Septic shock	Serious medical condition that occurs when sepsis, which is organ injury or damage in response to infection, leads to dangerously low blood pressure and abnormalities in cellular metabolism.
Serotonin	An organic compound formed from tryptophan and found in animal and human tissue, especially the brain, blood serum, and gastric mucous membranes, and active in vasoconstriction, stimulation of the smooth muscles, transmission of impulses between nerve cells, and regulation of cyclic body processes
Stroke	Is a "brain attack". It can happen to anyone at any time. It occurs when blood flow to an area of brain is cut off. When this happens, brain cells are deprived of oxygen and begin to die. When brain cells die during a stroke, abilities controlled by that area of the brain such as memory and muscle control are lost.
Substance P	A neuropeptide that consists of 11 amino acid residues, that is widely distributed in the brain, spinal cord, and peripheral nervous system, and that acts across nerve synapses to produce prolonged postsynaptic excitation. It involved in regulation of the pain threshold.
Synaptic plasticity	The biological process by which specific patterns of synaptic activity result in changes in synaptic strength and are thought to contribute to learning and memory. Both pre-synaptic and post-synaptic mechanisms can contribute to the expression of synaptic plasticity.
Template bleeding time	A bedside test to determine the presence of abnormal delays in blood clotting, in which a small cut is made in the skin, and the time it takes for bleeding to stop is measured.
Tuberculosis (TB)	A potentially serious infectious disease that mainly affects lungs. The bacteria that cause tuberculosis are spread from one person to another through tiny droplets released into the air via coughs and sneezes.

Vasopressin

A hormone secreted by cells of the hypothalamic nuclei and stored in the posterior pituitary for release as necessary; it stimulates contraction of the muscular tissues of the capillaries and arterioles, raising the blood pressure, and increases peristalsis, exerts some influence on the uterus, and influences resorption of water by the kidney tubules, resulting in concentration of urine. Its rate of secretion is regulated chiefly by the osmolarity of the plasma. Also prepared synthetically or obtained from the posterior pituitary of domestic animals; used as an antidiuretic. Called also antidiuretic hormone.

RÉSUMÉ DES TRAVAUX DE THÈSE

I. Introduction

Le monoxyde d'azote (ou l'oxyde nitrique), NO, est l'une des plus petites molécules les plus intéressantes en biologie. Il joue plusieurs rôles importants se rattachant au système cardiovasculaire, immunitaire, neuronal, respiratoire, et digestif. NO est un radical libre et de ce fait il intervient dans plusieurs réactions dans le milieu biologique et en particulier avec les métaux, les thiols et les autres radicaux libres. Il est stocké naturellement au niveau des peptides et des protéines via sa réactivité avec les groupements thiols en se liant par exemple à la cystéine pour former les S-nitrosothiols (RSNOs). Les RSNOs peuvent être distingués en deux groupes : haut ou bas poids moléculaires (HPM et BPM, respectivement) comme la S-nitrosoalbumine (AlbSNO) et la S-nitrosohemoglobine (HbSNO), d'une part, et le S-nitrosoglutathion (GSNO) et la S-nitrosocystéine (CySNO), d'autre part. Il a été remarqué que dans plusieurs situations pathologiques, les concentrations des RSNOs HPM et BPM varient [1]. De ce fait, il est intéressant de pouvoir disposer de méthodes analytiques robustes, sensibles, sélectives et rapides pour les quantifier. L'ordre de grandeur des RSNOs en milieu biologique va des nano au micromolaire [2,3]. Ces concentrations en RSNOs représentent un challenge important pour leur détermination par les méthodes analytiques. Les méthodes existantes de quantification des RSNOs sont basées soit sur la détection directe des RSNOs sans traitement chimique préalable, laissant intacte la liaison RS-NO (spectrométrie de masse, détection par fluorescence, RMN, ou MS des produits de réaction sélective entre RSNOs et phosphines), soit sur leur détection indirecte, impliquant une coupure de la liaison RS-NO (électrochimie, chimiluminescence, dosage par échange de biotine (biotine-switch assay), colorimétrie, fluorescence). La rupture de la liaison RS-NO peut être photo-assistée sous l'action de la lumière, chimiquement catalysée par l'action d'ions métalliques (Hg^{2+} , Cu^{+}) ou thermiquement activée. Les méthodes indirectes sont les plus sensibles mais elles possèdent des limitations au niveau de leur sélectivité [4,5].

En tout état de cause, l'amélioration des méthodologies analytiques existantes pour les RSNOs passe par un couplage de méthodes séparatives comme la chromatographie liquide à haute performance (CLHP), la chromatographie en phase gazeuse (CG), et l'électrophorèse capillaire (EC) avec des méthodes spécifiques et sensibles. Ce couplage peut tirer profit de la miniaturisation de plus en plus élaborée des méthodes de séparation. L'intégration des différentes étapes chimiques et analytiques dans un microsysteme permet en effet un gain de temps d'analyse et une diminution de volume d'échantillon mis en jeu. L'optimisation de l'étape de décomposition, dans le cas de la détection indirecte, représente un challenge. Toutes ces étapes doivent être compatibles pour la détection des RSNOs à des faibles concentrations ($\approx$ nM) [2,3].

Le projet à long terme de l'équipe est de développer un microsysteme analytique intégrant toutes les étapes d'analyse sur un même micro-dispositif, à savoir les étapes d'injection, de séparation, de décomposition et de détection des RSNOs de différents poids moléculaires, (**Figure 1**). L'objectif de la thèse est d'effectuer cette réalisation en étudiant chaque étape séparément afin de les maîtriser individuellement, pour enfin les assembler et les intégrer dans un microsysteme. La séparation électrocinétique qui est la plus adéquate pour l'intégration dans un microsysteme va être développée et la détection sera faite électrochimiquement, ce qui relève les défis du couplage entre une détection électrochimique et une séparation électrocinétique.

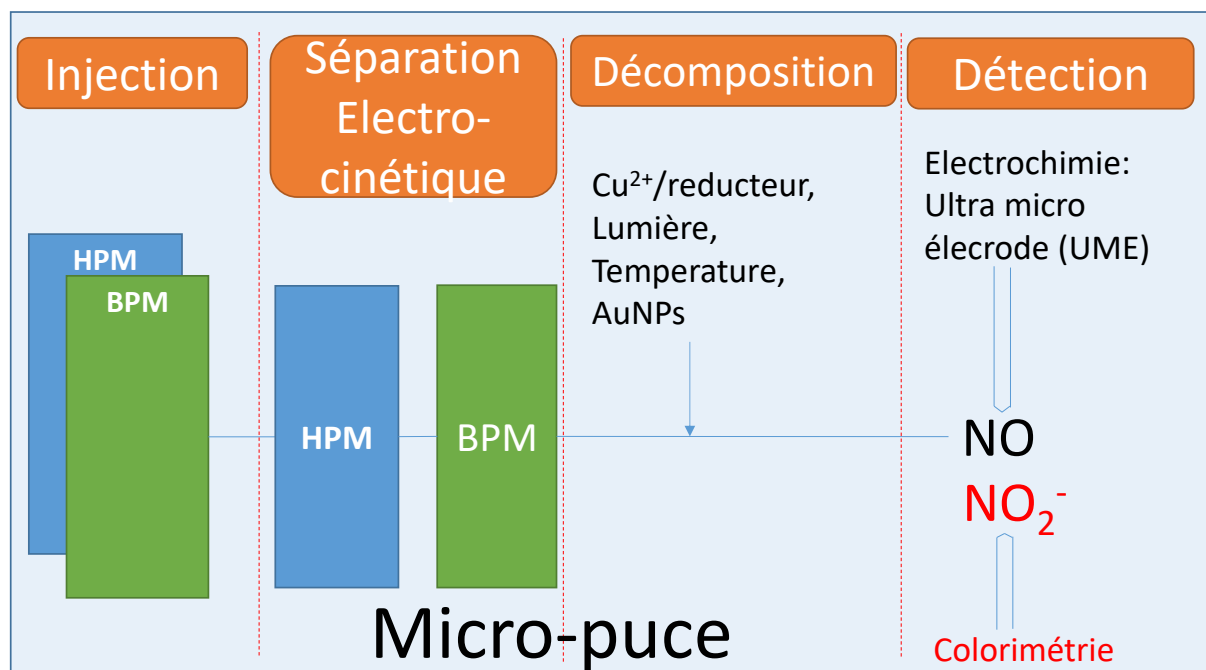


Figure 1 : Schéma du microsysteme envisagé pour l'analyse des RSNOs dans les fluides biologiques comprenant quatre étapes : injection, séparation, décomposition et détection.

Après une présentation concise de l'état de l'art sur le rôle biologique de NO et des RSNOs ainsi que des méthodes de séparation, décomposition, détection utilisées habituellement pour leur quantification, nous présenterons les différentes approches étudiées dans ce travail pour la miniaturisation des systèmes d'analyse et la caractérisation des échantillons de GSNO. La première étude a été menée par électrophorèse capillaire couplée à la spectrométrie de masse et à la détection conductimétrique sans contact. Par la suite, plusieurs méthodes de décomposition des RSNOs seront étudiées telles que celles mettant en œuvre l'ion Cu^+ , la lumière et les nanoparticules d'or (AuNPs). Enfin nous présenterons les résultats obtenus dans le cas de la miniaturisation en développant dans un premier temps un système miniaturisé en papier pour l'analyse de la décomposition des différents RSNOs et dans un deuxième temps l'intégration d'une séparation électrocinétique et d'une détection électrochimique dans un canal microfluidique pour la détection de GSNO.

II. Etat de l'art

L'état de l'art est présenté dans le premier chapitre. Il est constitué de trois parties, traitant de la biologie de NO et des RSNOs (effet biologique, formation, réactivité, transport, décomposition), des différentes méthodes de quantification des RSNOs (directes et indirectes), de la miniaturisation des systèmes analytiques et de ses applications en microfluidique dans le cas de NO et RSNOs. Nous reprenons ici les principaux éléments permettant d'introduire nos travaux.

A. Biologie de NO et des RSNOs

Il a fallu près de 150 ans après la synthèse de la nitroglycérine contenant NO en 1847, alors utilisée comme explosif et comme traitement contre l'angine de poitrine, pour découvrir le mécanisme cellulaire par lequel NO intervient. NO est le deuxième messager cellulaire responsable de la vasodilatation induite par la nitroglycérine. Outre l'intervention de NO dans le système cardiovasculaire (vasodilatation, inhibition de l'agrégation des plaquettes, et adhésion des leucocytes) son rôle est de plus en plus démontré dans le traitement du système digestif (contre les pathogènes et la motilité intestinale), de l'inflammation, du cancer, du

système nerveux central ainsi que des maladies neurodégénératives (Parkinson, Alzheimer, Huntington...) et du diabète [6-10].

NO est un radical qui peut réagir avec plusieurs molécules présentes dans le milieu biologique. Ses cibles principales sont : i) les métaux et les métallo-enzymes, spécialement celles qui contiennent du fer et du cuivre, pour lesquelles il peut modifier leur activité (par activation ou inhibition), ii) les autres radicaux et le dioxygène et ses dérivés réduits, donnant lieu soit à une espèce plus nuisible soit à une neutralisation, et iii) les thiols, conduisant à la formation des RSNOs [11,12].

La fixation de NO aux protéines et peptides pour former les RSNOs offre la possibilité de le stocker dans les liquides biologiques pour l'amener à réagir dans un endroit éloigné de son site de production. En effet, les RSNOs sont plus stables que NO. NO peut alors être échangé entre différents RSNOs par une réaction nommée S-transnitrosation basée sur une attaque nucléophile d'un thiol (R_2S sur le R_1SNO qui conduit à la formation de R_2SNO et R_1S). Seule la CySNO peut pénétrer dans les cellules où elle peut échanger NO avec les autres thiols déjà présents et former alors des RSNOs intracellulaires. En règle générale, les RSNOs agissent plutôt par transnitrosation avec la cystéine de la protéine fonctionnelle que par libération de NO et diffusion de ce NO libéré vers son site d'action [13].

Plusieurs mécanismes de formation des RSNOs en milieu biologique sont proposés [14] : i) auto-oxydation de NO en N_2O_3 suivie d'addition de thiolate, ii) oxydation du thiol suivie par l'addition de NO et iii) nitrosylation directe par simple addition de NO et de RSH. Le mécanisme par lequel les RSNOs sont formés dépend de la concentration du thiol et de NO (**Figure 2, pour le cas du glutathion**). Il existe aussi d'autres méthodes de nitrosation comme celles mettant en jeu les métaux de transition présents dans les enzymes (céruloplasmine, peroxydase, cytochrome c oxydase, et autres hémoprotéines) [12], la décomposition des complexes de di-nitrosyle de fer par des ligands thiolates [15] et la S-nitrosation par l'anion nitrite dans l'estomac ou les poumons (après dismutation du nitrite en milieu acide) [16]. La dernière méthode qui est surtout connue pour la formation des RSNOs HPM est la transnitrosation à partir des RSNOs BPM [12].

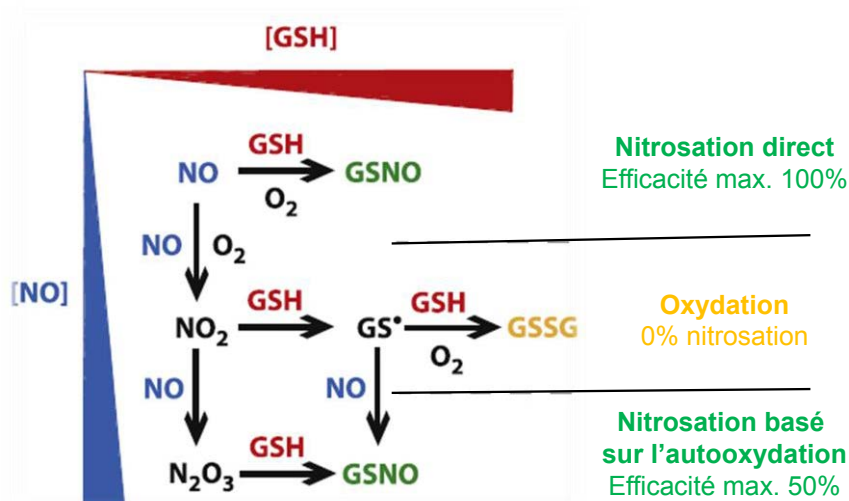


Figure 2 : Mécanisme de formation de GSNO selon la concentration en glutathion (GSH) et en NO. GSSG : glutathion oxydée. Adapté de [14]

La concentration en RSNOs *in vivo* varie selon les situations pathologiques entre une dizaine de nanomolaire et moins de dix micromolaires [1-3,17]. Elle peut augmenter dans le cas de l'arthrite, du diabète, de la sclérose en plaque, de la pré-éclampsie, de la pneumonie... En revanche elle diminue dans le cas d'autres maladies comme la fibrose kystique, l'asthme, l'hypoxie néonatale...[1] De ce fait, la détermination des concentrations des RSNOs dans les milieux biologiques constitue une clef de diagnostic pour de nombreux cas cliniques.

De plus, plusieurs types des RSNOs ont été synthétisés et utilisés au moins dans des modèles animaux pour le traitement de diverses pathologies (cardiovasculaire comme dans les accidents vasculaires cérébraux et l'angioplastie coronaire [18-20], les plaies ischémiques [21], le traitement de l'onychomycose [22]). Plusieurs formulations ont été proposées : intraveineuse, sous cutanée, topique, orale, vaginale...[23] et le suivi de leur stabilité dans le temps et au cours de leur stockage constitue aussi une clef analytique importante.

B. Décomposition et quantification des RSNOs

Dans les milieux biologiques il existe plusieurs enzymes qui sont capables de décomposer les RSNOs (glutathion peroxydase, thioredoxine, protéine disulfure isomérase, carbonyle réductase...). Il est également connu que les RSNOs sont sensibles à la décomposition par les ions métalliques (Cu^+ , Hg^{2+}), l'ascorbate, la chaleur, la lumière, et l'or [24,25].

La quantification des RSNOs peut être obtenue par des méthodes directes ou indirectes (**Figure 3**). La méthode est dite directe lorsque la liaison RS-NO est préservée, et indirecte si cette liaison est coupée [5].

La spectrométrie de masse (SM) et la spectroscopie Ultra-Violet (UV) ont été utilisées pour la détection des RSNOs souvent en couplage en ligne ou hors ligne avec l'HPLC [26]. La SM offre l'avantage d'être sélective et sensible, mais nécessite un traitement de l'échantillon pouvant induire une perte de NO pendant le traitement de l'échantillon; elle est aussi techniquement encombrante mais elle a l'avantage d'être sélective. L'UV est un mode de détection peu sensible à cause du faible coefficient d'extinction moléculaire des RSNOs ($\epsilon=0,9 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ à 340 nm), ce qui limite ses applications biologiques.

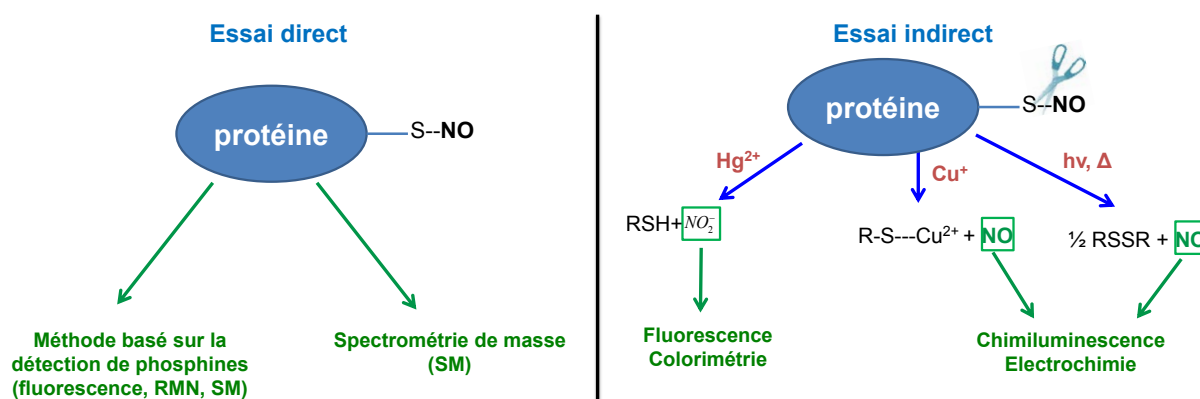


Figure 3 : Méthodes directes et indirectes pour la détection des RSNOs.

Les méthodes indirectes sont basées sur la décomposition des RSNOs en NO ou nitrite. La colorimétrie est la méthode de référence pour détecter les RSNOs. Saville [27] a découvert cette méthode basée sur la détection de la coloration rose à 540 nm d'un composé diazoïque qui résulte de la décomposition des RSNOs par Hg^{2+} en présence du réactif de Griess (sulfanilamide avec N-naphtyl éthylène diamine en milieu acide). Cette méthode est robuste mais nécessite l'utilisation de sels de mercure. Elle n'est cependant pas assez sensible pour détecter la plupart des concentrations physiologiques des RSNOs et est donc considérée comme une méthode semi quantitative pour les RSNOs. De plus, cette méthode indirecte n'est pas sélective.

Les sondes de fluorescence qui ont été développées pour la détection du NO dans les cellules peuvent aussi être utilisées pour la détection des RSNOs après leur décomposition par le mercure (II). Cette méthode est très sensible mais une réactivité croisée avec d'autres molécules présentes en milieu biologique peut entraîner des contre-performances [2].

La chimiluminescence est une des méthodes qui consistent à détecter NO [2]. Elle est basée sur la détection de photons émis par NO₂ à l'état excité après réaction de NO avec de l'ozone en phase gaz. Cette méthode est très sensible mais sa réalisation *in vitro* et ne permet pas de suivi en temps réel. Dans le cas des RSNOs, elle nécessite leur décomposition en NO ce qui peut engendrer des interférences avec d'autres molécules présentes dans le milieu comme les nitrites, les nitrates, et les N-nitrosamines. De plus, cette méthode requiert des appareillages encombrants.

Le *biotine switch assay* [28] est une méthode basée sur le marquage des thiols issus de la décomposition des RSNOs par la biotine suivie par une précipitation. Les protéines biotinylées sont visualisées directement à l'aide d'avidine-HRP (horseradish peroxidase) après SDS-PAGE et « western blot »; alternativement, les protéines biotinylées sont précipitées à l'avidine ou la streptavidine monomérique, immobilisées et identifiées par Western blot pour les protéines d'intérêt ou la spectrométrie de masse pour toutes les autres. Cette méthode nécessite le blocage de tous les autres thiols « libres » (non présents sous la forme de RSNO). Elle souffre des limitations de l'efficacité et de la spécificité de ce blocage. Le *biotine switch assay* ne nécessite pas d'équipements spéciaux et est capable d'identifier les sites de nitrosation des protéines.

L'électrochimie est une méthode de détection très intéressante [29] car elle permet le suivi de NO, en temps réel, qui peut être réalisé *in vivo* ou *in vitro*. Elle est spécifique et sensible. Les électrodes peuvent être modifiées pour être plus sélectives et cela de différentes manières comme l'immobilisation de membranes sélectives pour NO ou d'un catalyseur pour diminuer le potentiel de détection du NO en dessous de celui des autres molécules interférentes, telles que le nitrite.

Les différentes méthodes de détection des RSNOs sont résumées dans le **Tableau 1**, leurs performances et la nature des échantillons biologiques analysés sont également précisées.

Tableau 1 : Comparaison de différentes méthodes de détection de NO. Adapté de [3,26,30]

	Méthode analytique	Espèce détectée	LOD	Caractère analytique principal	RSNO analysé	Echantillon biologique	Gamme de concentration
Méthodes directes	Réactions avec phosphines et MS	Adduit sélectif	~0,01 μM	Spécificité	GSNO	Lysat cellulaire	0,7-3,9 μM
	HPLC-MS	GSNO	2,5 nM	Spécificité	GSNO	Plasma	Non détecté <LOD
	CE-UV	GSNO	10 μM	Spécificité	GSNO	Plasma épiné avec CySNO 0.2 mM	Endogène <LOD
Méthodes indirectes	Spectrophotométrie (Saville assay)	Colorant azoïque	500 nM	Sensibilité faible	RSNOs BPM	Plasma	RSNO 5,9 μM
	Chimiluminescence	NO_2	pM	Sensibilité forte	RSNOs BPM, AlbsNO	Sang artériel	0,05-2,5 μM
						Plasma	0,5 nM-7 μM
						Sérum	35-930 nM
	Fluorimétrie	Marqueur Fluorescent	nM	Sélectivité faible	RSNOs BPM, AlbsNO	Sang artériel	0,25-0,56 μM
	GC-MS	NO_2^- dérivé	200 nM	Sélectivité	AlbsNO	Plasma	0,2-9 μM
	Système CLHP-Saville	Colorant azoïque	0,1 μM	Sensibilité	RSNOs	Plasma	0,18 $\pm$ 0.15 μM
	CLHP avec détection fluorimétrique	Fluorescente	20 nM	Sélectivité	RSNOs BPM	Plasma	0,09-0,3 μM
	Sonde ampérométrique de NO	NO	10 nM	Sélectivité	RSNOs BPM	Plasma	0-3 μM
						Sérum	$\approx$ 500 nM

C. Miniaturisation et microfluidique

Les systèmes microfluidiques ont été introduits dans les années 1930 avec la chromatographie sur papier [31]. Les travaux les concernant ont explosé dans les années 1990 suite à la découverte de l'électrophorèse capillaire et du programme militaire des Etats-Unis pour la microfluidique. La miniaturisation offre de nombreux avantages : i) moins de consommables et d'échantillon, ii) rapidité, iii) intégration de plusieurs fonctionnalités, iv) mise en parallèle d'opérations d'analyses multiples, v) réduction des dimensions d'équipements, vi) réduction de consommation de puissance électrique et vii) portabilité [32].

Plusieurs matériaux peuvent être utilisés en microfluidique. Ces matériaux ont des propriétés différentes et sont dans la plupart des cas à base de silicone, verre, polymères thermoplastiques ou élastomères et papier. Le choix du matériau se fait selon le profil de microsystème souhaité. Le **Tableau 2** récapitule les principales caractéristiques recherchées.

Tableau 2 : Comparaison des différents matériaux utilisés pour les dispositifs microfluidiques, adaptée de [33]

Propriété	Matériaux			
	Verre	Silicon	PDMS	Papiers
Aspérité de surface	Très bas	Très bas	Très bas	Modéré
Flexibilité	Non	Non	Oui	Oui
Structure	Solide	Solide	Solide, perméable au gaz	Fibreux
Rapport surface / volume	Faible	Faible	Faible	Haut
Flux de fluide	Forcé	Forcé	Forcé	Action capillaire
Sensibilité à l'humidité	Non	Non	Non	Oui
Biocompatibilité	Oui	Oui	Oui	Oui
Usage unique	Non	Non	Non	Oui
Biodégradabilité	Non	Non	Dans une certaine mesure	Oui
Haut débit de fabrication	Oui	Oui	Non	Oui
Homogénéité du matériau	Oui	Oui	Oui	Non
Prix	Modéré	Haut	Modéré	Faible
Investissement initial	Modéré	Haut	Modéré	Faible

Dans le cadre de l'utilisation des systèmes microfluidiques en analyse, une étape de séparation est souvent nécessaire et elle peut se faire en utilisant au sein des dispositifs microfluidiques de la chromatographie ou de l'électrophorèse. Cette dernière est la plus pratique et la plus utilisée à cause du contrôle du flux de manière électrocinétique et non hydrodynamique et de l'absence de remplissage des microcanaux. Il existe plusieurs modes de séparations électrocinétiques : l'électrophorèse de zones, l'électrochromatographie, la chromatographie électrocinétique micellaire, l'électrophorèse sur gel, l'isoélectrofocalisation, et l'isotachophorèse. Aussi, plusieurs modes d'injection existent pour l'échantillon : hydrodynamique (application d'une pression) ou électrocinétique (application d'un champ électrique). Les injections électrocinétiques permettent de mieux contrôler les flux de fluides dans les microcanaux et sont les plus utilisées. On distingue couramment plusieurs types d'injection électrocinétique qualifiés de « floating », « pinched », et « gated ». Les caractéristiques de chacun de ces types d'injections sont résumées **Figure 4** [34]. L'injection « floating » a l'inconvénient de diffusion de l'échantillon au moment de l'injection (entre S et SW), ceci est bien montré par l'absence

de « plug » carré idéal et l'apparition d'une zone blanche vers les réservoirs B et BW (**Figure 4A**). La **Figure 4B** montre l'injection « pinched » entre S et SW. On peut remarquer que la zone d'injection n'est pas homogène, ce qui donne une distribution asymétrique. La **Figure 4C** montre l'injection « gated » où le plug d'injection est déterminé par le temps d'injection et les potentiels utilisés.

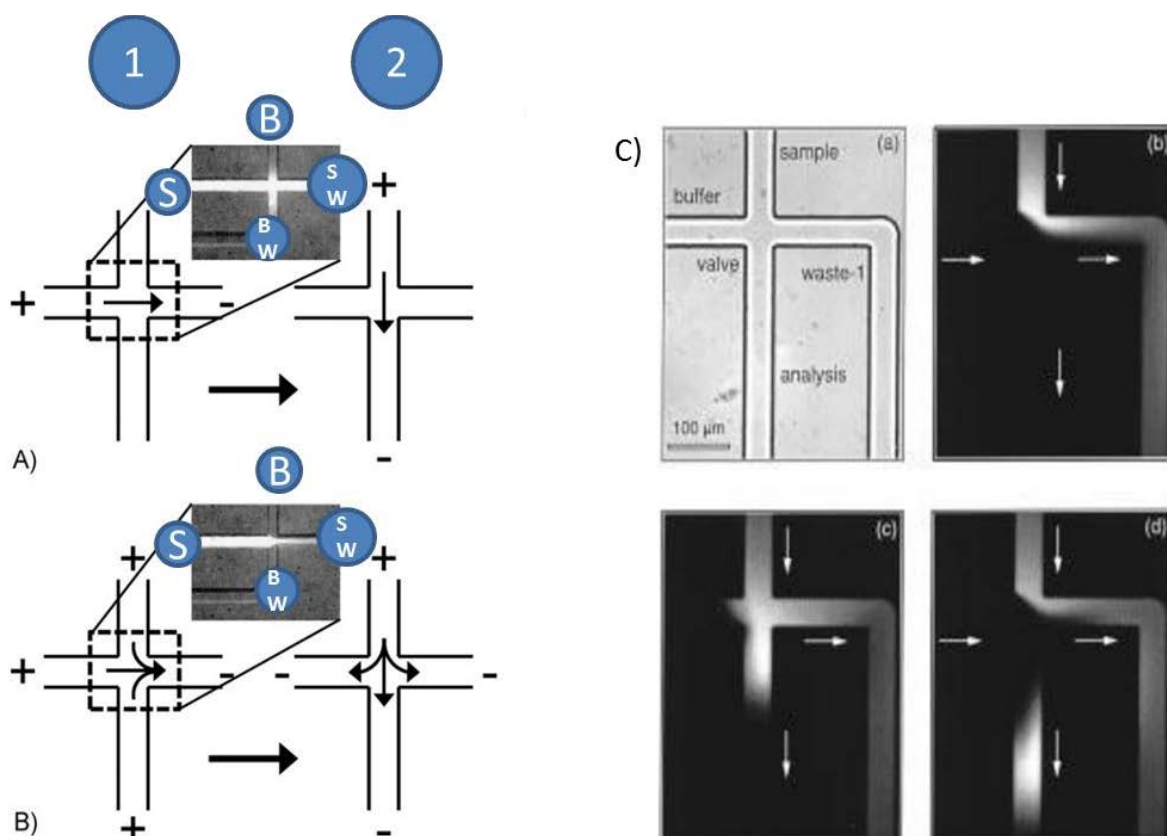


Figure 4 : Comparaison des injections dites «floating» (A), «pinched» (B), et «gated» (C). En (C) l'image en clair représente le positionnement des valves (a), les images en fluorescence de l'injection « gated » dans les étapes successives de chargement (b), d'injection (c), et de séparation (d). La couleur blanche matérialise l'échantillon fluorescent contrastant avec l'électrolyte support incolore. Adapté de [35,36]

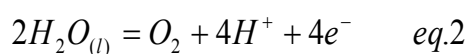
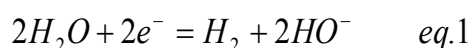
Plusieurs techniques de détection ont été considérées et développées, intégrées ou couplées aux microsystèmes comme les détecteurs optiques (fluorescence, absorbance, spectroscopie raman, chimiluminescence, électrochimiluminescence...), la spectrométrie de masse et l'électrochimie (ampérométrie et conductimétrie). La détection par fluorescence est la plus répandue grâce à sa sensibilité élevée, mais elle nécessite dans certains cas le marquage des molécules. La spectrométrie de masse est une technique universelle qui peut être couplée à une séparation électrocinétique ou chromatographique en sortie de microcanal. Elle permet d'obtenir des informations qualitatives et quantitatives, mais elle rend difficile la portabilité du système car elle est encombrante. L'électrochimie représente une méthode très intéressante car elle s'intègre totalement dans les microsystèmes. Dans ce cas, l'ampérométrie est la méthode

la plus utilisée parmi les techniques électrochimiques. Le positionnement de l'électrode par rapport au canal peut être à l'origine de certaines complications car le courant généré pendant l'étape de séparation est de l'ordre du μA tandis que celui mesuré pour la détection est de l'ordre du pA au nA , et ce dernier peut être fortement perturbé. Pour éviter cela, trois positionnements de l'électrode de détection peuvent être imaginés (**Figure 5**) [37].

- le positionnement en sortie de canal (end channel) (**Figure 5.1**) qui permet de s'affranchir de l'influence du champ électrique engendré par l'électrophorèse mais conduit à l'élargissement des pics de l'électropherogramme.

- le positionnement dans le canal (in-channel) nécessite l'utilisation d'un potentiostat dédié à la détection qui soit électriquement isolé pour permettre de mesurer le courant faradique de la détection sans mesurer de composante liée à la séparation électrocinétique. Dans ce cas, le découplage n'est pas nécessaire (**Figure 5.2**).

- le positionnement hors canal (off channel) où l'électrode est située dans le canal mais un « découpleur » est utilisé afin de dévier le champ électrique lié à l'électrophorèse avant l'arrivée de l'échantillon à l'électrode de travail. Le champ électrique est appliqué dans le canal de séparation jusqu'au découpleur uniquement. Ceci implique une petite longueur de canal dans laquelle ne règne aucun champ électrique. Cette opération conduit à la formation de gaz par électrolyse de l'eau au niveau du découplage (du dihydrogène par polarisation positive et du dioxygène par polarisation négative (**eq. 1 et 2**)), qui doit être absorbé efficacement par le découpleur (**Figure 5.3**) sinon la formation de bulles d'air dans le canal nuira à la séparation.



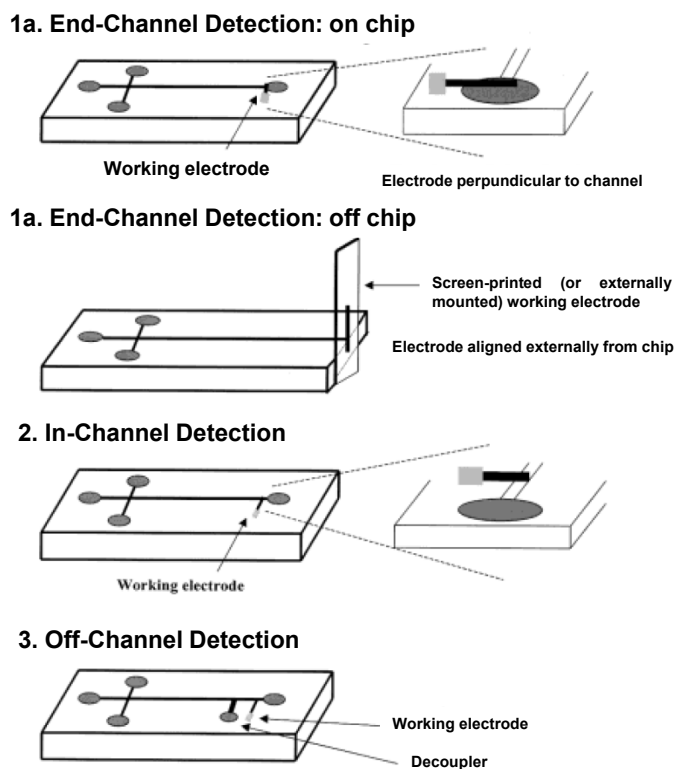


Figure 5 : Positionnement des électrodes en détection ampérométrique. Adapté de [37]

Dans le cas de la détection de NO et des RSNOs dans les microsystèmes, Gunasekara et al. [38,39] ont développé plusieurs approches pour la détection de NO en utilisant l'électrophorèse couplée à l'ampérométrie. Gunasekara et al. [38,39] ont séparé des marqueurs de stress nitrosatif dont le NO et nitrite font partie mais ils n'ont pas séparé les RSNOs. Hunter et al. [40] ont proposé un dispositif mettant en jeu la décomposition des RSNOs par la lumière et la détection de NO par ampérométrie sans séparation préalable. Dans ce dernier cas, la sensibilité dans PBS a été de 22,6, 25,5 and 5 pA / μM et les LOD de 60, 60 and 280 nM pour GSNO, CySNO and AlbSNO, respectivement. Cependant le temps nécessaire à la décomposition est relativement long, ce qui n'est pas compatible avec une séparation électrophorétique. Wang et al. [41] ont développé une approche mettant en œuvre une détection par fluorescence induite par laser après l'étape de séparation par électrophorèse sur gel pour détecter des protéines nitrosylées et marquées par une sonde fluorescente. Malgré l'inconvénient du marquage, la limite de détection est de 1,3 pM qui est excellent par rapport aux concentrations attendues pour les RSNOs (micro et nanomolaire) et la méthode a été appliquée seulement aux protéines S-nitrosylées, et non aux RSNOs BPM. D'autres méthodes électrophorétiques avec une détection par fluorescence ont également été proposées comme celles de Wang et al. La séparation par électrophorèse sur micro puce bidimensionnelle des protéine nitrosylées a été effectuée en

moins de deux minutes à partir de prélèvement de cobayes atteints de cancer et de la maladie d'Alzheimer, mais aucune séparation des RSNOs HPM et BPM n'a été affectuée [42].

III. Résultats

A. Analyse de la décomposition dans le temps de GSNO par électrophorèse capillaire : cinétique et identification des produits de décomposition

Dans ce chapitre, nous avons utilisé la technique d'électrophorèse capillaire couplée à l'UV et à la spectrométrie de masse (EC-SM) pour la caractérisation des produits de décomposition de GSNO. L'objectif de cette étude entre dans le cadre de l'évaluation de la stabilité des composés RSNOs en général au cours de leur stockage et de leur utilisation dans diverses applications. Un tampon de force ionique 20 mM de carbonate / bicarbonate de pH 8,5 a été utilisé. Chaque pic (courant ionique total en fonction du temps) peut être analysé par spectrométrie de masse (% d'abondance en fonction de m/z) pour identifier le poids moléculaire des espèces présentes. Pendant l'analyse d'un échantillon solide âgé de six mois de GSNO, trois pics ont été observés par UV, le dernier pic étant partiellement dédoublé lors de la détection par SM. En effet, la détection SM se situe à 80 cm de l'entrée du capillaire, tandis que la détection UV n'est qu'à 20 cm de celle-ci. Trois produits de décomposition (GSSG, GSO₂H, et GSO₃H) en plus de GSNO ont ainsi été identifiés par (EC-SM) (**Figure 6**). L'attribution de ces trois produits est basée sur leur poids moléculaire et sur les adduits résultants (**Tableau 3**). Les structures de ces produits sont indiquées dans la **Figure 7-I**.

L'analyse d'un échantillon de GSNO fraîchement synthétisé et d'un échantillon âgé de 6 mois (stocké à l'état solide à -20°C) a montré une diminution des concentrations de GSNO et de GSO₂H par 3 fois et de 20 %, respectivement. En revanche, les concentrations de GSSG et GSO₃H ont augmenté de 20 % et 600 %, respectivement. Ces résultats montrent qu'il est possible d'identifier une voie d'oxydation de GSNO à l'état solide (**Figure 7**). Ceci a un impact biologique important car ces produits peuvent par exemple inhiber les glutathion transférase-like, famille d'enzymes qui détoxifie les composés xénobiotiques. Cette méthodologie peut être

appliquée aux liquides physiologiques afin d'identifier de possibles interférents, en cas d'effet toxique de GSNO dans des pathologies ou en cas d'administration en quantité importante.

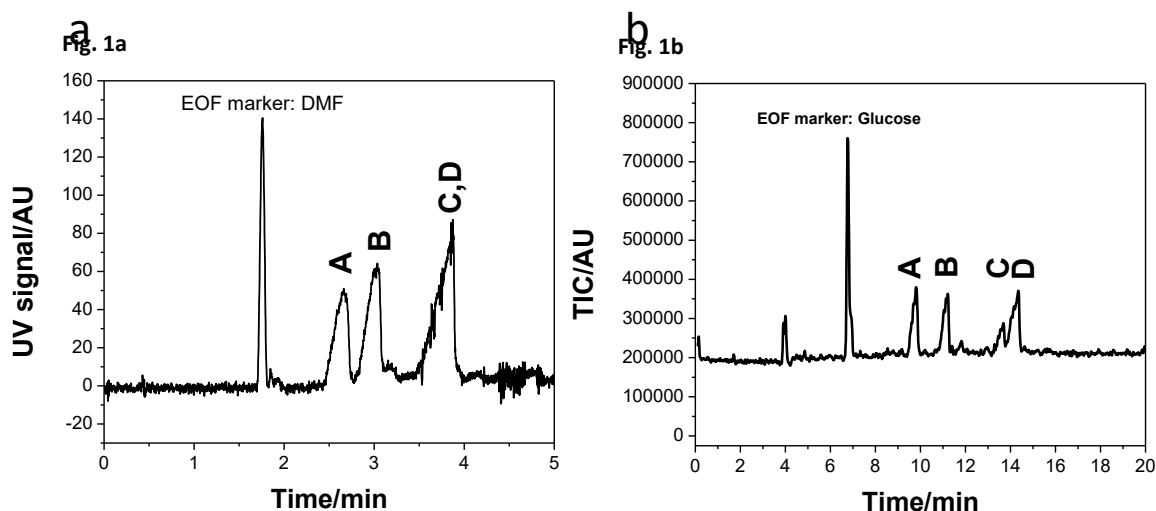


Figure 6 : Les électrophérogrammes UV et SM correspondant à l'analyse de GSNO à 4,61 mM dans l'électrolyte support après stockage à l'état solide pendant 6 mois. Electrolyte support : tampon carbonate d'ammonium (force ionique= 20 mM, pH 8,5). Injection : marqueur neutre (30 mbar, 2s), électrolyte support (50 mbar, 3s), échantillon (50 mbar, 3s), électrolyte support (50 mbar, 2s). Capillaire : diamètre interne 75 μ m, longueur totale 80 cm, longueur jusqu'à la fenêtre de détection UV 22 cm (Fig 6a) et au spectromètre de masse (SM) 80 cm (Fig 6b). Tension de séparation 20 kV. SM en mode d'ionisation positive. Attribution des pics : A : GSNO, B : GSSG, C : GSO₂H, et D : GSO₃H. Marqueur neutre (DMF 0.02 %, Glucose 5mM).

Tableau 3 : Spectre de masse et abondance (%) relative des ions fragments en courant ionique total pour les pics identifiés. X représente A, B, C, ou D selon la colonne d'intérêt dans le tableau.

Ion Assignment	A: GSNO		B: GSSG		C: GSO ₂ H		D: GSO ₃ H	
	m/z	% ab.	m/z	% ab.	m/z	% ab.	m/z	% ab.
[X+H] ⁺	337,1	100	613,1	100	340,1	100	356,2	100
[X+Na] ⁺	359,2	16	635,2	27	362,2	41	378,1	24
[2X+H] ⁺	673,1	16	-	-	679,1	6	711,1	13
[2X+Na] ⁺	695,2	10	-	-	701,0	7	733,0	7
[X+2Na-H] ⁺	-	-	657,1	4	384,1	12	400,1	5
[X+3Na-2H] ⁺	-	-	-	-	406,0	6	-	-
[(X+H)-NO] ⁺	307,1	8	-	-	-	-	-	-
[X+2H] ²⁺	-	-	307,1	61	-	-	-	-
[X+Na+H] ²⁺	-	-	318,2	6	-	-	-	-
[(X-O)+H] ⁺	-	-	-	-	324,2	8	-	-
[(X-O)+Na] ⁺	-	-	-	-	346,3	11	-	-

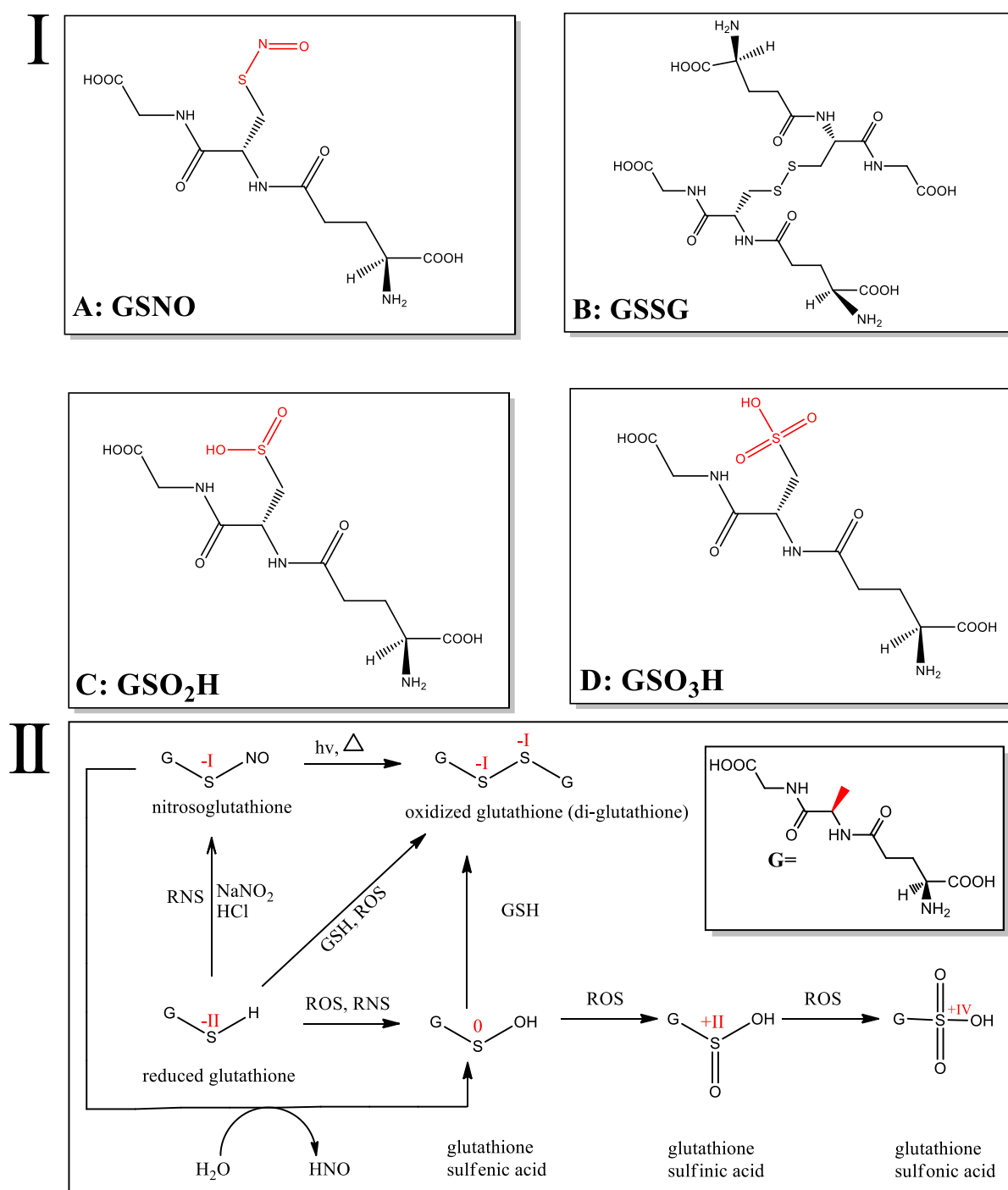


Figure 7 : I) Structure des produits identifiés A, B, C, D. II) voie de décomposition de GSNO. Les numéros rouges au dessus de chaque atome de soufre représentent leur état d'oxydation.

L'étude de la décomposition d'une solution de GSNO sous l'effet de la lumière et de la température a été effectuée par électrophorèse capillaire couplée à une détection conductimétrique sans contact (EC-C⁴D). Cette méthode permet de détecter toute molécule ayant une mobilité différente de celle de l'électrolyte support. Elle permet de plus un choix de tampons plus important qu'avec la EC-SM, car différents électrolytes, tels ceux à base de sodium, phosphate, borate, ne sont pas compatibles avec la détection SM. Dans cette étude,

nous avons utilisé un tampon CHES (acide N-Cyclohexyl-2-aminoethanesulfonique) comme électrolyte support. Le GSNO et ses produits de décomposition précédemment identifiés par EC-SM, ont été détectés par EC-C⁴D (**Figure 8**). Il est important de noter que la séparation par EC-C⁴D donne une meilleure résolution que par EC-SM et EC-UV

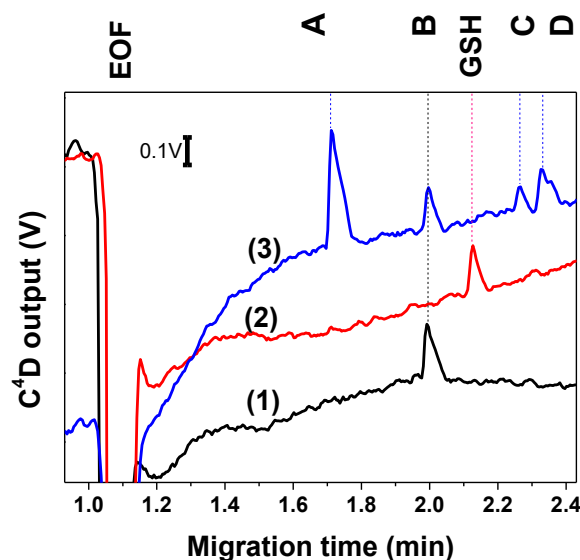


Figure 8 : Electropherogramme correspondant à l'analyse de solutions étalon de GSH et GSSG et d'un échantillon de GSNO âgé de 4 mois. Tous les échantillons ont été préparés dans CHES (C =20 mM, pH 9. Injection hydrodynamique : 3s, 11kPa ; électrolyte support : 20 mM CHES (pH 10) ; tension : 27kV, capillaire : 47 cm (37 cm effectif), diamètre interne 75 μ m. Détection : 600 kHz, 1,9 V_{pp}. (1) GSSG à 157 μ M, (2) GSH à 356 μ M, (3) GSNO échantillon à 1 mM (pureté 66 % déterminée par détection colorimétrique à 336 nm en utilisant $\epsilon=920 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

Après décomposition par la lumière et par chauffage, la composition de la solution a été analysée par EC-C⁴D en utilisant des produits de références. Nous avons montré que la cinétique de la décomposition par la lumière est du premier ordre. La constante de vitesse calculée est $(4,9 \pm 0,3 \times 10^{-1} \cdot \text{s}^{-1})$ soit dix milles fois plus grande que celle mesurée par Sexton et al. [43] $((3,40 \pm 0,17) \times 10^{-3} \text{ s}^{-1})$ (**Figure 9**). Ceci peut être lié à la variation de la constante de décomposition selon l'intensité de lumière utilisée et selon le positionnement par rapport à l'échantillon.

L'analyse des produits de décomposition thermique (à 80°C) a montré que la réaction est d'ordre 0 avec une constant de vitesse $k_{\text{obs}, 80^\circ\text{C}} = 4,34 \pm 0,14 \times 10^{-6} \text{ mol} \cdot \text{L}^{-1} \cdot \text{S}^{-1}$ (**Figure 10**). Ces résultats peuvent être expliqués par la différence dans le mécanisme de décomposition entre la lumière et la chaleur. Les mécanismes restent un sujet de discussion : de Oliveira *et al.* [44] a suggéré un mécanisme radicalaire pour la décomposition par chaleur qui implique le radical thiyl (GS^*), tandis que Singh et al. [45] ont reporté que GS^* n'est pas impliqué par la décomposition par la chaleur au contraire de la décomposition par la lumière.

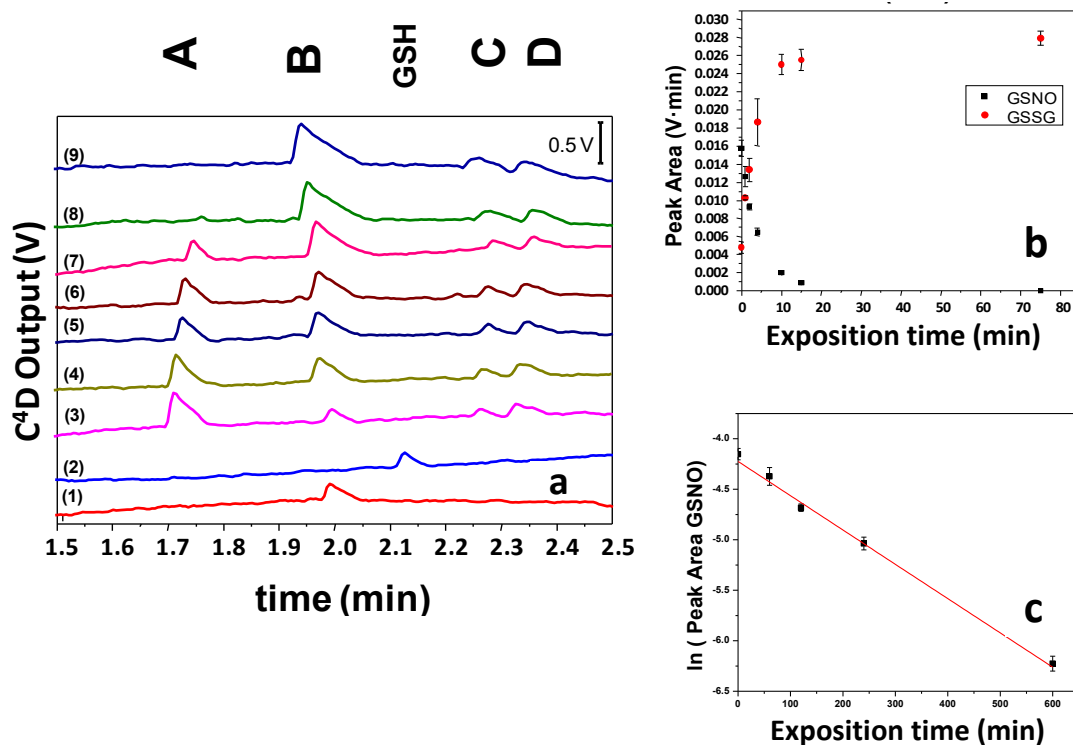


Figure 9 : Etude de la cinétique de décomposition de GSNO induite par lumière. a) Electrophoregramme correspondant à l'analyse de 1) GSSG à 102 μM , 2) GSH à 77 μM et (3-9) GSNO à 1 mM (pureté 66 %) déterminé par détection colorimétrique à 336 nm en utilisant $\varepsilon = 920 \text{ M}^{-1} \cdot \text{cm}^{-1}$) après un temps de décomposition de 3) 0 min, 4) 1 min, 5) 2 min, 6) 4 min, 7) 10 min, 8) 15 min, et 9) 75 min. b) Représentation de l'aire des pics de GSNO et GSSG en fonction du temps d'exposition. Tous les échantillons ont été préparés dans CHES ($C=20 \text{ mM}$, pH 9). c) Représentation du logarithme népérienne de l'aire des pics de GSNO en fonction du temps d'exposition. Injection hydrodynamique : 3s, 11kPa ; électrolyte support : CHES ($C=20 \text{ mM}$, pH 10) ; tension de séparation : 27kV, capillaire : 47 cm (37 cm effectif), diamètre interne 75 μm . Détection : 600 kHz, 1,9 V_{pp}.

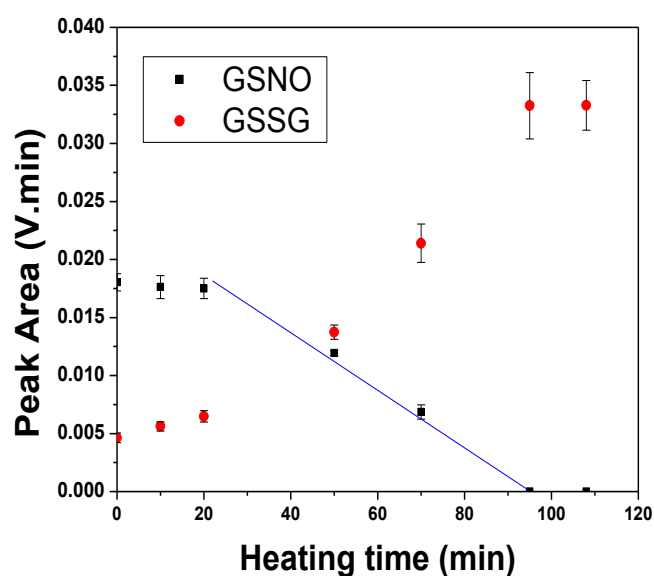


Figure 10 : Etude de la décomposition thermique d'échantillon de GSNO (pureté 66 %). Représentation de l'aire des pics électrophorétique de GSNO et GSSG en fonction du temps de chauffage. Conditions opératoires : voir Figure 9

Une autre application de notre méthode EC-C⁴D est l'étude de la transnitrosation. La transnitrosation est une réaction très connue en biologie. Les protéines fonctionnelles captent le NO des RSNOs BPM ce qui modifie leur action. Le corps humain peut être symbolisé comme un bassin qui contient différents RSNOs et protéines nitrosylées en équilibre [12,46]. L'analyse des produits de la réaction de transnitrosation entre le GSNO et la cystéine a été réalisée. Les résultats sont en accord avec la littérature qui indique qu'une molécule de NO est échangée entre GSNO et la cystéine pour donner CySNO (**Figure 11**) et GSH [12]. La **Figure 11** montre les différents pics visualisés. On observe que lorsque la concentration en Cys augmente (de (3) à (7)) les concentrations en CySNO et GSH augmentent et celle en GSNO diminue selon **eq. 3**



En conclusion, les deux méthodes EC-UV-SM et EC-C⁴D développées sont complémentaires. Le couplage EC-UV-SM a permis d'identifier les composés de décomposition du GSNO à l'état solide et proposer un chemin de décomposition. Le couplage EC-C⁴D a permis leur analyse rapidement (<2,5 min) et une étude de la cinétique des réactions de décomposition et de transnitrosation.

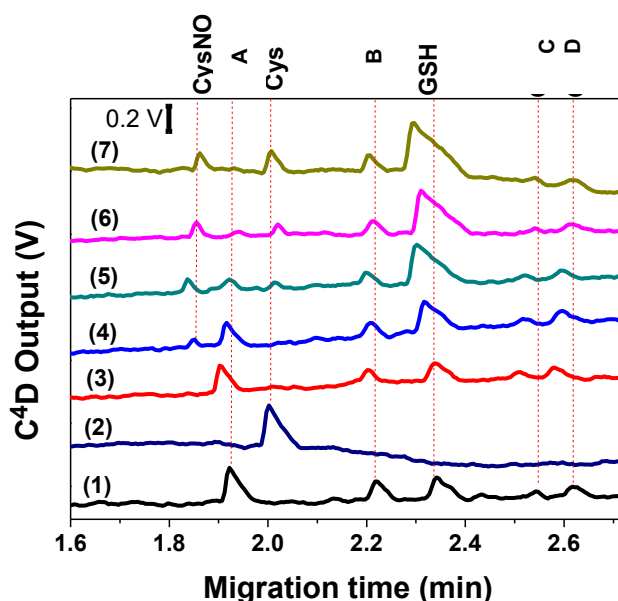


Figure 11 : Etude de la transnitrosation entre le GSNO et la Cystéine. Electrophérogrammes correspondant à l'analyse des solutions contenant 1) GSNO à 331 μ M + GSH à 90 μ M, 2) cystéine à 495 μ M, (3-7) GSNO à 331 μ M + cystéine à des concentrations variables (76 μ M, 152 μ M, 305 μ M, 381 μ M et 495 μ M, de 3 à 7 respectivement). Conditions opératoires : voir.Figure 9

B. Analyse de la décomposition des RSNOs par le Cu^+ en milieu réducteur ou en présence de nanoparticules d'or

L'analyse de la décomposition de GSNO par la lumière et par la chaleur décrite ci-dessus montre que le temps de décomposition est trop long pour que ces deux procédures puissent être appliquées dans un microsysteme (15 min et 1h30min, respectivement). De ce fait, d'autres méthodes de décomposition ont été développées et évaluées. Ces méthodes sont basées sur la décomposition de GSNO par les métaux, et en particulier par Cu^+ en utilisant un sel de cuivre (II) et un réducteur chimique tel que le glutathion, ou des nanoparticules d'or. La détection du produit de décomposition qui est NO est alors réalisée soit par colorimétrie soit par ampèrométrie.

1) Décomposition des RSNOs par Cu^+ en milieu réducteur

Il a été rapporté dans la littérature que la décomposition de GSNO par Cu^+ , produit après réduction de CuSO_4 par GSH, donne NO et GSSG [24,47,48]. Cette procédure nécessite l'utilisation de l'EDTA comme agent chélatant afin de minimiser l'effet indésirable de traces de métaux présents dans les solutions d'analyse et qui conduisent à la décomposition spontanée des RSNOs. Les rapports CuSO_4 / GSNO et GSH / GSNO ont été mentionnés comme étant des facteurs importants pour le contrôle de la réaction de décomposition [49]. Dans cette étude, nous avons réexaminé l'effet de chacun de ces paramètres en utilisant la méthode colorimétrique dite de Griess, pour laquelle le réactif de Griess (sulfanilamide et N-(1-naphthyl)ethylenediamine en milieu acide) réagit avec le nitrite pour produire une coloration rose absorbant à $\lambda = 540 \text{ nm}$ [50].

Dans un premier protocole, la concentration de GSNO a été fixée à environ $40 \mu\text{M}$ tandis que celle de CuSO_4 varie de 0 à $1200 \mu\text{M}$ en présence de GSH résiduel ($0,25 \mu\text{M}$, $20 \mu\text{M}$, $50 \mu\text{M}$, et $100 \mu\text{M}$) (**Figure 12**). La **Figure 12** montre l'absorbance normalisée par rapport au maximum d'absorbance de chaque courbe en fonction de la concentration de CuSO_4 . Pour les concentrations inférieures à $440 \mu\text{M}$ en CuSO_4 , la décomposition de GSNO reste négligeable ($\approx 2\%$) dans le cas de très faibles concentrations en GSH ($0,25 \mu\text{M}$). Ceci peut s'expliquer par la complexation de Cu^{2+} par l'EDTA ($450 \mu\text{M}$ initialement rajouté). Pour des concentrations

plus élevées en CuSO_4 , la décomposition reste constante. Pour une concentration de GSH de $20\ \mu\text{M}$, l'évolution de l'absorbance normalisée en fonction de la concentration de CuSO_4 est la même que celle obtenu dans le cas de traces de GSNO mais la valeur absolue de l'absorbance est plus élevée, c'est-à-dire la conversion en nitrite est plus efficace en présence de cette concentration de GSH (d'où l'étude de l'importance de GSH dans le paragraphe suivant). Pour des concentrations de GSH supérieures à $20\ \mu\text{M}$, la décomposition a lieu à des faibles concentrations de CuSO_4 , puis le taux de la décomposition diminue pour des concentrations supérieures à $600\ \mu\text{M}$ en CuSO_4 (**Figure 12**). Ces résultats montrent que la décomposition la plus performante pour une concentration de GSNO d'environ $40\ \mu\text{M}$ a lieu pour une concentration en CuSO_4 de $600\ \mu\text{M}$, quelle que soit celle de GSH étudiée. Alors cette concentration de Cu^{2+} (ainsi qu'une concentration plus élevée ($1\ \text{mM}$)) va être utilisée pour tester un deuxième protocole.

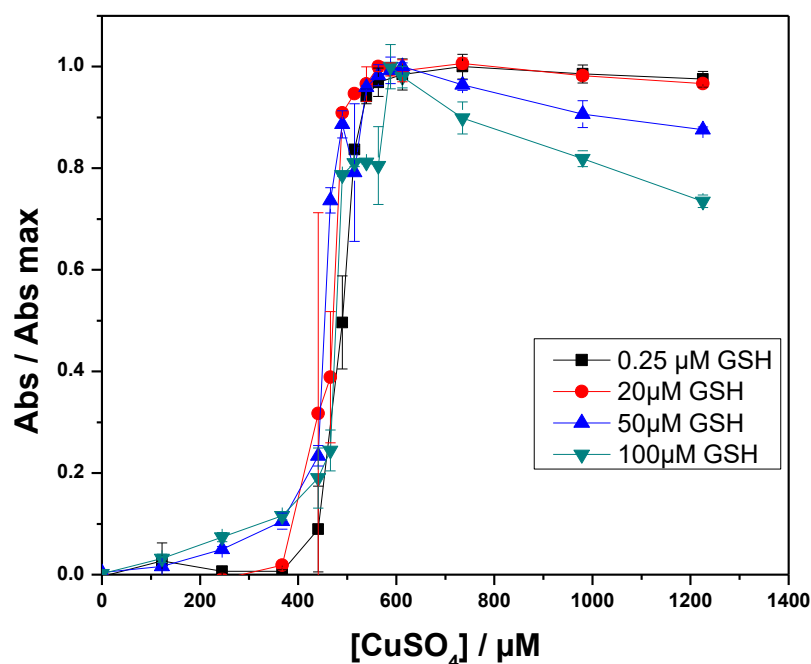


Figure 12 : Représentation de l'effet de l'augmentation de la concentration de GSH sur la décomposition normalisée (chaque courbe a été normalisée par rapport à son max d'absorbance) de GSNO ($39\ \mu\text{M}$) par Cu^+ (concentration variable de CuSO_4 entre 0 et $1200\ \mu\text{M}$) dans PBS $0.1\ \text{M}$ (pH 7,4) + EDTA ($450\ \mu\text{M}$). (n=3)

Ce second protocole a été développé pour évaluer la décomposition de GSNO à différentes concentrations ($\approx 20\ \mu\text{M}$, $\approx 50\ \mu\text{M}$, et $\approx 80\ \mu\text{M}$) par une solution CuSO_4 à $600\ \mu\text{M}$ et $1000\ \mu\text{M}$ en présence de GSH (teneur comprise entre de 0 à $200\ \mu\text{M}$). Les résultats décrits **Figure 13** montrent qu'une concentration de $20\ \mu\text{M}$ en GSH conduit à un maximum de décomposition de

GSNO, quelle que soit sa concentration, et que les concentrations élevées de GSH ($> 100 \mu\text{M}$) ainsi que les faibles concentrations ($< 10 \mu\text{M}$) conduisent à de faibles taux de décomposition toujours en utilisant des concentrations de CuSO_4 supérieures à $450 \mu\text{M}$ (**Figure 13**). Ceci peut s'expliquer par le fait que lorsque la concentration en GSH est très faible la réduction de Cu^{2+} en Cu^+ est trop faible pour décomposer tous les GSNO, et lorsque la concentration en GSH est élevée il y a complexation de Cu^+ par GSH pour former un complexe inactif Cu^+ / GSH . L'effet de la concentration en GSH augmente quand la concentration en GSNO est faible. Il faut donc optimiser le rapport GSH / GSNO et $\text{Cu}^{2+} / \text{GSH}$. Ces résultats prouvent l'importance de prendre en considération le niveau de GSH présent en solution pendant la décomposition par le cuivre. Il existe en effet un risque de sous-estimer le niveau des RSNOs des échantillons biologiques lorsque la teneur en GSH dans le milieu extracellulaire est d'au moins $5 \mu\text{M}$ et de l'ordre du millimolaire dans le milieu intracellulaire. En prenant ces critères en considération, les meilleures conditions pour la décomposition de GSNO par la méthode $\text{Cu}^{2+} / \text{GSH}$ sont : une concentration en GSH de $20 \mu\text{M}$ et une concentration en CuSO_4 $1000 \mu\text{M}$.

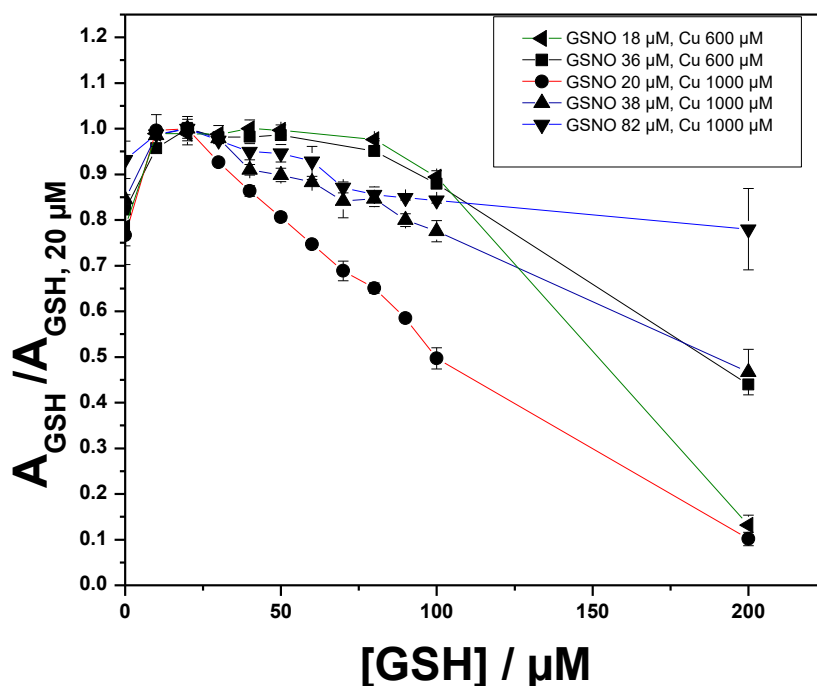


Figure 13 : Représentation graphique de l'effet de la concentration en GSH sur la décomposition normalisée (chaque courbe a été normalisée par rapport à son maximum d'absorbance) de GSNO (20, 38, et $82 \mu\text{M}$) par CuSO_4 à $600 \mu\text{M}$ ou $1000 \mu\text{M}$ dans 0.1 M de PBS (pH 7,4) contenant de l'EDTA à $450 \mu\text{M}$.

Cependant, la détection colorimétrique ne permet pas un suivi en ligne et en temps réel. Ainsi nous nous sommes intéressés à la détection électrochimique qui offre la possibilité d'un suivi

en ligne et temps réel et une limite de détection très basse, de l'ordre du nanomolaire [29]. Dans ce but, nous avons élaboré une ultra microélectrode sélective vis à vis de NO en déposant à la surface une membrane de poly(eugénol) et poly(phénol) suivant des procédures bien établies au sein du laboratoire [51]. La calibration de cette électrode pour la détection de NO issue de Diethylammonium (Z)-1-(N,N-diethylamino)diazene-1,2-diolate (DEA NONOate) est faite de la manière suivante : un ampérogramme de libération de plusieurs concentrations de DEA NONOate dans un tampon PBS ($C=0,1$ M, pH 7,4) a été effectué. Le maximum de libération par DEA NONOate correspond à une concentration de NO calculé théoriquement selon les modèles cinétiques [52]. La valeur de courant de ce maximum est corrélée à la concentration calculée de NO (**Figure 14**). Ceci a permis de constater que :

- la décomposition par le cuivre (maximum atteint après 10-20 s) est beaucoup plus rapide que celle par la lumière et par la chaleur, et elle semble donc prometteuse pour une décomposition en microsystème.
- deux zones de linéarité de la courbe dose-réponse (courant mesuré en fonction de la concentration en GSNO). La partie linéaire observée pour les faibles concentrations en GSNO possède la même pente que celle obtenue pour la courbe de calibration de NO issue de la décomposition totale et quantitative de DEA-NO. Ceci signifie que la décomposition de GSNO dans ces conditions est totale en NO. La pente de la partie linéaire correspondant aux concentrations plus élevées en GSNO est plus faible. Ceci peut s'expliquer par la variation des rapports CuSO_4 / GSNO et GSH / GSNO au cours du calibrage. Cette variation peut en effet affecter l'efficacité de la décomposition comme montré par l'étude spectrophotométrique précédent. Cependant, l'augmentation de la concentration en CuSO_4 (3 mM vs 1 mM) a également conduit à une diminution de la pente observée (1,4 vs 1,8 pA / μM). Ceci peut être expliqué par une complexation de NO par le cuivre.

Afin d'améliorer la sensibilité de la détection de NO, nous avons remplacé la membrane de polyeugénol-polyphénol par une membrane à base de polyeugénol uniquement. Ceci a conduit à une augmentation de la sensibilité d'un facteur 3 (4,9 vs 1,4 pA / μM). De la même façon, la limite de détection a également été abaissée de 0,6 μM (membrane polyeugénol-polyphénol) à 0,1 μM (polyeugénol).

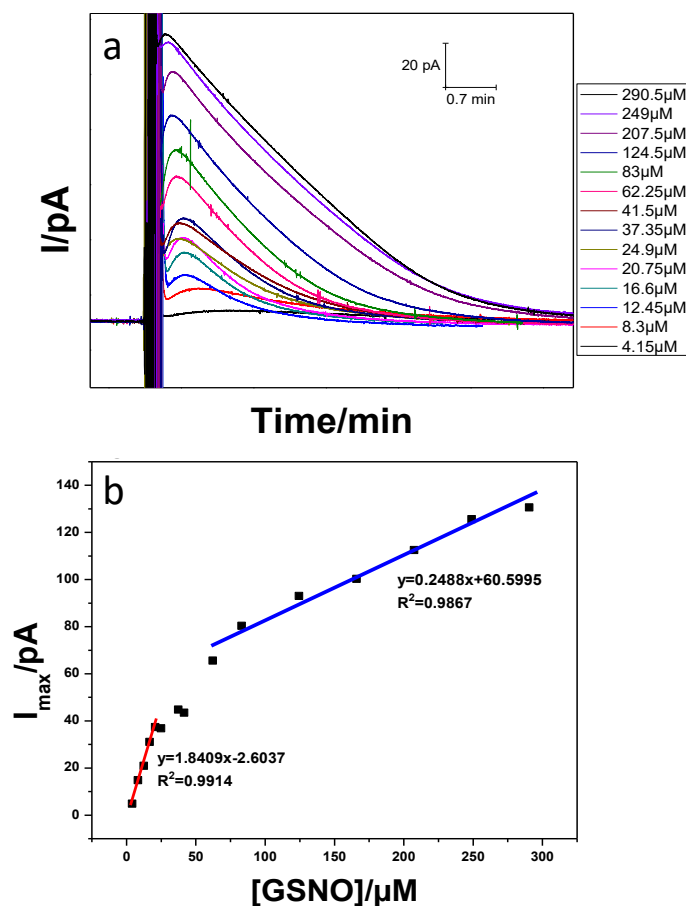


Figure 14 : a) Ampérogrammes mesurés par une ultra microélectrode sélective de NO à 0,8 V vs Ag / AgCl . CuSO₄ (1000 μM) est ajouté à des concentrations différentes de GSNO (de 4 μM à 290 μM) + PBS (C=0,1M ; pH 7,4) + EDTA (450 μM) + GSH (20 μM). ; b) courbe de calibration obtenue à partir des données de (a).

2) Décomposition des RSNOs en présence de nanoparticules d'or

Le principe de décomposition des RSNOs par les nanoparticules d'or (AuNPs) est basé sur le fait qu'en présence d'AuNPs, les RSNOs sont décomposés et libèrent NO qui est par la suite détecté à une ultra microélectrode sélective. L'idée d'utiliser les AuNPs, l'optimisation du protocole de fabrication des AuNPs, la preuve de concept de la décomposition telle que décrite ci-dessus et les premières courbes de calibration ont été faites par V. Baldim. Il a travaillé sur le même sujet dans le cadre de sa thèse de doctorat de l'Université de Campinas (Brésil) au cours de son séjour au laboratoire à Paris dans le cadre du projet CAPES-COFECUB coordonné par le laboratoire. Dans le cadre de mes propres travaux de thèse, j'ai évalué la répétabilité des mesures des courbes de calibration et de la détection des RSNOs en milieu biologique (plasma reconstitué) après optimisation du protocole de blocage des thiols. Ces thiols ont été à l'origine

de difficultés de détection des RSNOs dans le plasma lors des premiers tests de détection de NO car ils induisent la passivation des AuNPs suite à leur adsorption.

La concentration des solutions de AuNPs fabriquées et utilisées est entre 7 et 11 nM et leur diamètre hydrodynamique moyen de 16 nm. Le nombre de molécules contenant des fonctions thiols qui peuvent se lier à une nanoparticule d'or a été déterminé : 1500 pour GSH et 18 pour l'albumin. La méthode utilisée pour cette détermination consiste à d'ajouter une concentration de GSNO qui sature les nanoparticules déjà traitées par des concentrations croissantes de GSH ou AlbSH. Le courant obtenu sera proportionnel à la surface d'AuNP qui n'est pas occupée par les thiols. Celui-ci nous permet de déterminer la concentration en thiols nécessaire pour complètement occuper la nanoparticule (quand on obtient plus de courant en ajoutant GSNO). En connaissant les dimensions de la surface et la concentration en nanoparticules, on peut calculer le nombre de thiols par nanoparticule. La courbe de calibrage de la détection électrochimique de NO issue de la décomposition de GSNO par les AuNPs est linéaire avec une pente un peu plus élevée que celle obtenue lors de la décomposition par le cuivre (6,64 vs 4,9 pA / μ M). Le temps nécessaire pour atteindre le maximum du signal ampérométrique est plus court (5s vs 10-20 s) (**Figure 15**)

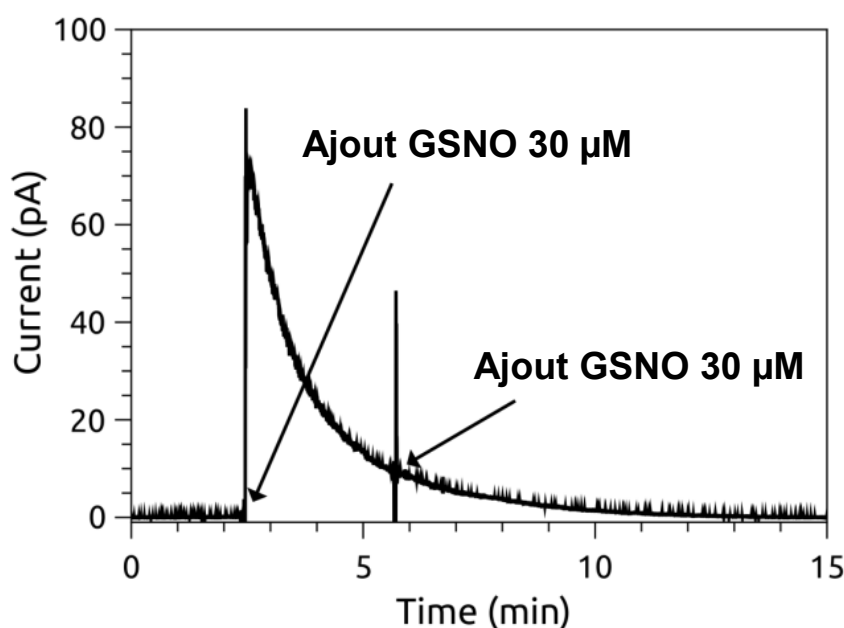


Figure 15 : Courant observé après l'addition de GSNO jusqu'à condition finale de 30 μ M sur des AuNPs (dispersion de 9 μ M)). Une deuxième addition de GSNO ne donne aucun courant additionnel.

En résumé, la méthode de décomposition par le cuivre (I), même si elle est un peu plus lente et moins sensible que celle mettant en jeu les AuNPs, offre l'avantage d'être utilisable dans des systèmes miniaturisés car la décomposition en présence d'AuNPs nécessite un grand volume d'AuNPs (2 mL) ce qui n'est pas compatible avec un système microfluidique.

C. Miniaturisation

Etant donné les avantages majeurs qu'offre la miniaturisation (en termes de réduction des volumes de l'échantillon et des réactifs, d'analyse rapide, d'intégration de plusieurs fonctionnalités, et de réduction des coûts de fabrication et de consommation d'énergie) nous avons orienté nos travaux vers l'implémentation des procédures de décomposition, séparation et détection dans des micro-dispositifs. Cette section comprend deux parties. La première partie correspond à la mise en œuvre de la miniaturisation pour la décomposition des RSNOs et la détection colorimétrique en utilisant le principe du « *point of care device* » et un dispositif papier. La deuxième partie présente une approche préliminaire visant à intégrer les étapes de séparation, décomposition et détection des RSNOs dans un microsystème verre / polymère.

1) Détection colorimétrique dans un dispositif microfluidique d'analyse à base de papier

L'idée d'utiliser l'approche d'un dispositif microfluidique d'analyse à base de papier, l'optimisation du protocole de fabrication et la preuve de concept de la décomposition ont été réalisées lors de mon séjour à l'Université de Goiânia (Brésil ; Pr W Coltro) dans le cadre du projet CAPES-COFECUB coordonné par le laboratoire et avec Maryllia (Brésil, étudiante de pharmacie) qui a travaillé sur le même sujet dans le cadre de son Master. Dans le cadre de mes propres travaux de thèse, j'ai évalué la miniaturisation de détection des RSNOs par la méthode de Saville et par la lumière, normalement effectué par spectrophotométrie.

Les dispositifs microfluidiques d'analyse à base de papier ont été réalisés en forme d'étoile en utilisant l'impression de cire sur papier. Ce dispositif a été choisi pour réaliser la décomposition simultanée des RSNOs dans différentes zones en utilisant dans chacune d'entre elles un protocole de décomposition différent : Hg^{2+} , lumière visible, UV ou infrarouge. Ainsi, des solutions étalons de GSNO, CySNO, et AlbsNO ont été utilisées. Le réactif de Griess a été utilisé dans chacune de ces zones afin de révéler la couleur indicatrice du composé diazoté, lui-même indicateur du nitrite issu de la décomposition. Un scanner a été utilisé pour capturer la photo couleur du dispositif, par la suite analysée à l'aide du logiciel Corel photo-paint qui permet d'avoir les intensités d'une couleur spécifique (rose par exemple) (**Figure 16**). L'intensité de la couleur est proportionnelle à la quantité de nitrite formé et par conséquent des RSNOs initial.

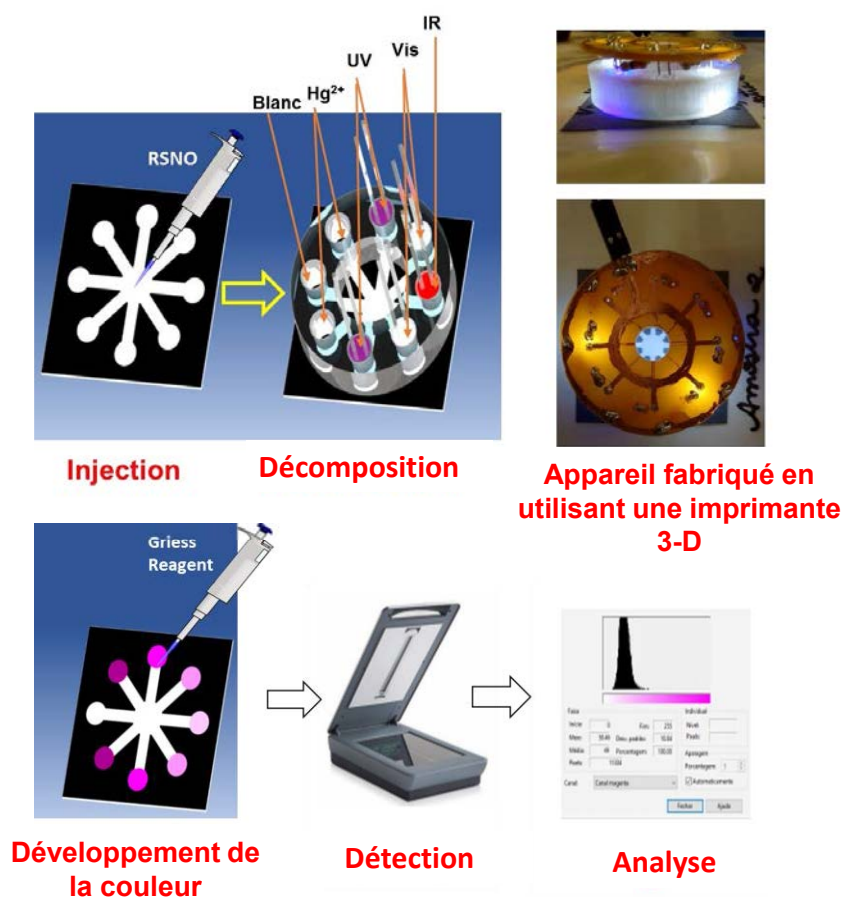


Figure 16 : Illustration des différentes étapes de détection des RSNOs sur dispositifs microfluidiques d'analyse à base de papier : injection des RSNOs au milieu, décomposition (lumière et sel de mercure), addition du réactif de Griess, scan et analyse par le logiciel Corel photo-paint. Des courbes de calibration peuvent alors être établies pour déterminer les concentrations dans des échantillons inconnus.

Dans un premier temps, l'anion nitrite a été utilisé comme référence, afin de vérifier la linéarité des courbes d'étalonnage en utilisant les microsystèmes. Elle a donné une courbe linéaire de pente 0,36 A.U / μM avec un R^2 supérieur à 0,99. Dans le cas de GSNO, des courbes d'étalonnage linéaires ont été obtenues pour sa décomposition par Hg^{2+} , lumière UV, Visible, et infra-rouge. La méthode la plus sensible est celle qui consistait à décomposer GSNO par le Hg^{2+} (pente la plus élevée). La décomposition par Hg^{2+} a été démontrée comme quasi-totale, elle est quantitative car la valeur de la pente est très proche de celle obtenue dans les mêmes conditions pour l'anion nitrite. La décomposition par UV après 20 min d'illumination conduit à une décomposition partielle, celle induite par irradiation dans le domaine du visible est encore moins efficace que par UV et finalement l'illumination infra-rouge ne conduit à aucune décomposition (**Figure 17a**).

Concernant le CySNO, la décomposition par illumination par lampe UV pendant 5 minutes est totale et identique à celle induite par Hg^{2+} . La décomposition par illumination dans le domaine du visible est moins importante que les deux procédures précédentes, mais elle reste meilleure

que la décomposition de GSNO par la lumière IR. L'infra-rouge ne conduit à aucune décomposition (**Figure 17b**). Concernant l'AlbSNO, la décomposition est totale uniquement avec Hg^{2+} (**Figure 17c**).

En conclusion, un microsystème a été mis au point et a permis d'étudier de façon simple la décomposition de plusieurs RSNOs sous l'effet de différents paramètres. La décomposition par Hg^{2+} est quantitative et la plus rapide pour tous les RSNOs. Les RSNOs BPM, GSNO et CysNO, sont plus sensibles vis à vis de la décomposition par lumière que les RSNOs HPM tels que AlbSNO.

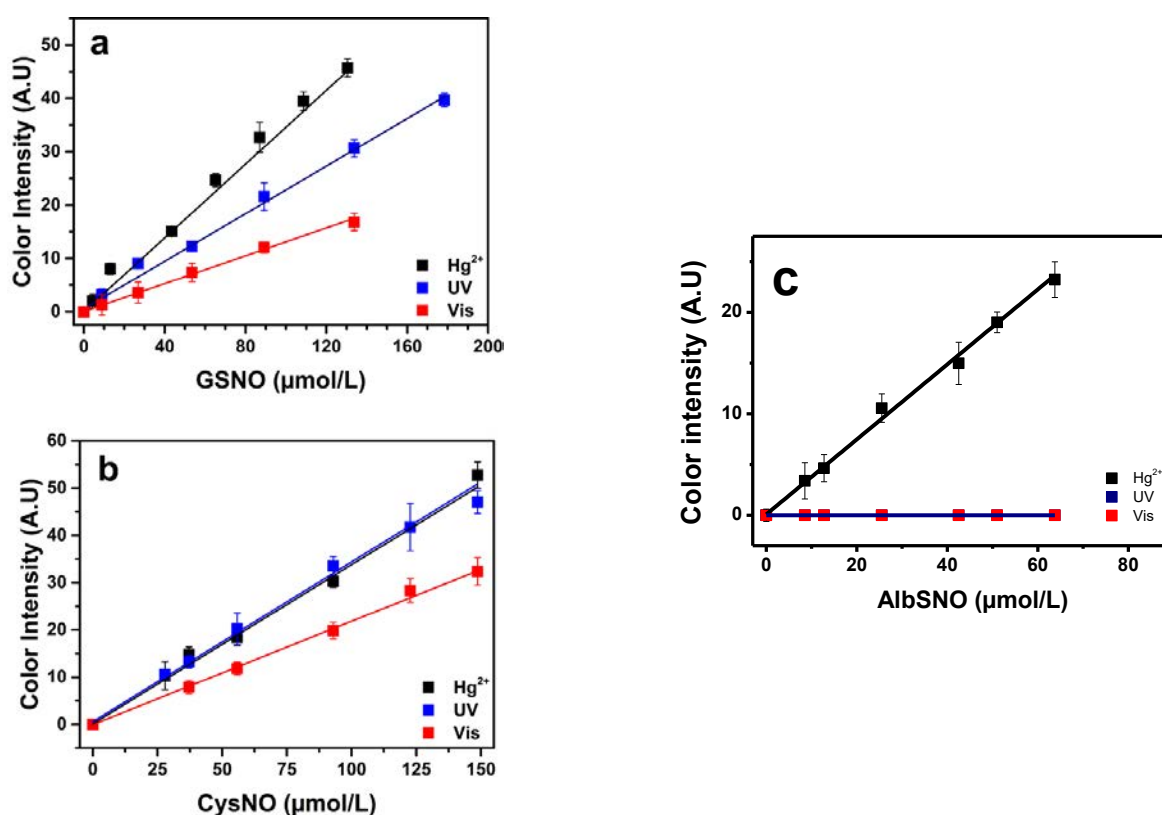


Figure 17 : Courbes dose-réponse caractéristiques de la décomposition de a) GSNO, b) CysNO et c) AlbSNO mesurée en dispositifs microfluidiques d'analyse à base de papier après décomposition par Hg^{2+} , lumière UV et visible.

La possibilité de détection des RSNOs dans des échantillons de plasma a aussi été évaluée. Le plasma a une couleur légèrement jaune à cause des protéines et spécialement de l'albumine ce qui engendre une difficulté de mesure basée sur l'approche colorimétrique développée ici. De ce fait, il est nécessaire de procéder soit à une ultrafiltration, soit à une étape permettant d'éliminer les protéines de HPM. Cette procédure conduit aussi à l'élimination des HPM-RSNOs. Ceci nous a conduits à développer une méthodologie qui permette de quantifier les

différents composants du plasma (**Figure 18**). Cette méthodologie peut être résumée comme suit :

- Décomposition des RSNOs avant filtration du plasma : dans ce cas l'ensemble des RSNOs HPM et BPM seront quantifiés, en plus du nitrite déjà présent ;
- Décomposition des RSNOs après filtration (ou déprotéinisation) du plasma : dans ce cas seuls les RSNOs BPM seront quantifiés, en plus du nitrite déjà présent ;
- Absence de décomposition : dans ce cas seul le nitrite initialement présent dans le plasma est quantifié.

Les concentrations de chaque composant (nitrite, RSNOs HPM et BPM) peuvent ainsi être obtenues par simple soustraction des données obtenues suivant ces trois protocoles (

Tableau 4). Les résultats montrent que seul les nitrites et RSNOs peuvent être détectés à cause de leur concentration plus élevée que la limite de détection. La concentration en nitrite se situe entre 37 et 58 μM et en AlbsNO entre 5 et 16 μM . Cela est en accord avec des rapports précédents par Stamler et al. [53] et autres ([2] et références incluses). Dans certaines maladies, la concentration en RSNOs BPM augmente au delà de 3 μM , alors ils pourront être détectés.

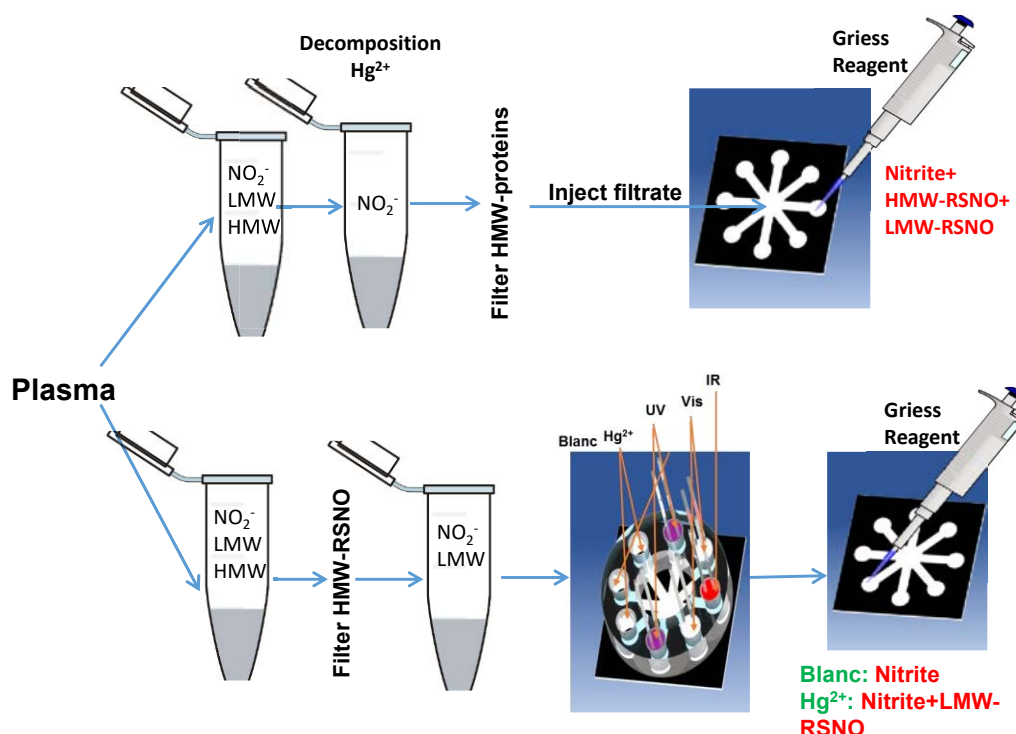


Figure 18 : Schéma de la méthode mise en oeuvre pour détecter les RSNOs dans le plasma.

Tableau 4 : Concentrations en nitrite et RSNOs HPM et BPM dans les échantillons de plasma mesurées en dispositif microfluidique d'analyse à base de papier

Sample	Nitrite (μM)	RSNOs HPM (μM)	RSNOs BPM (μM)
1	49,9 $\pm$ 2,3	14,0 $\pm$ 2,9	< LOD
2	49,5 $\pm$ 3,8	4,5 $\pm$ 2,4	< LOD
3	37,5 $\pm$ 3,3	5,7 $\pm$ 2,7	< LOD
4	58,5 $\pm$ 3,4	16,2 $\pm$ 3,6	< LOD

2) Détection électrochimie des RSNOs en microsystème après séparation par électrophorèse de zone.

Comme la méthode de détection (électrochimique, colorimétrique...) ne permet pas de discriminer les différents RSNOs, il faut intégrer dans un microsystème une étape de séparation des RSNOs avant leur décomposition et leur détection. Les microsystèmes en verre et / ou polymère sont mieux adaptés pour la séparation électrophoretique que ceux en papiers (en terme de contrôle de l'injection de l'échantillon, qualité de séparation et robustesse [54,55]). Nous avons donc entrepris une première étude d'intégration de l'étape de séparation avec un microsystème fait maison en poly (méthyl méthacrylate) (PMMA) avec une ultra micro électrode de platine (diamètre 25 μm) pour l'étape de détection. Ayant rencontré des problèmes de sensibilité et de répétabilité, les études ont ensuite été effectuées sur un système commercial Verre / Su-8 (polymère photorésistant chargé négativement) en intégrant des électrodes en platine. Le principal inconvénient du système verre / Su-8 étant sa non-transparence en fluorescence, un microsystème en copolymère de cyclo oléfine (COC) a enfin été utilisé pour optimiser l'injection.

La micro-fabrication du premier microsystème a été réalisée au laboratoire du Pr. Alberto Fracassi (Université de Campinas, Brésil) au cours de mon séjour dans le cadre du projet COFECUB-CAPES coordonné par l'UTCBS. Le microsystème fabriqué est à base de poly (méthyl méthacrylate) (PMMA) et la gravure a été réalisée par laser CO₂. Le collage des différents composants a été réalisé par voie thermique au four (25 min à 120 °C). L'électrode de travail (capteur) est un disque de Pt de 25 μm de diamètre placé à la sortie du canal pour obtenir une configuration « end-channel » (**Figure 19**). Les dimensions des canaux ont été déterminées par profilométrie de 100 μm largeur, 80 μm profondeur et 25 mm longueur.

La détection du paracétamol (marqueur d'électroosmose détectable par ampérométrie) a été réalisée après injection en mode « floating » et séparation électrocinétique (**Figure 20**).

L'interférence électrique entre le dispositif délivrant le champ électrique nécessaire à l'injection et la séparation, et le potentiostat de mesure du courant électrochimique au niveau du capteur a conduit à la très forte diminution de la sensibilité du signal (en imposant la terre du dispositif délivrant le champ électrique dans le réservoir de détection, on note une grande chute du courant détecté). La détection de pic du paracétamol a permis d'estimer la mobilité électroosmotique à $2,5 \times 10^{-5} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$.

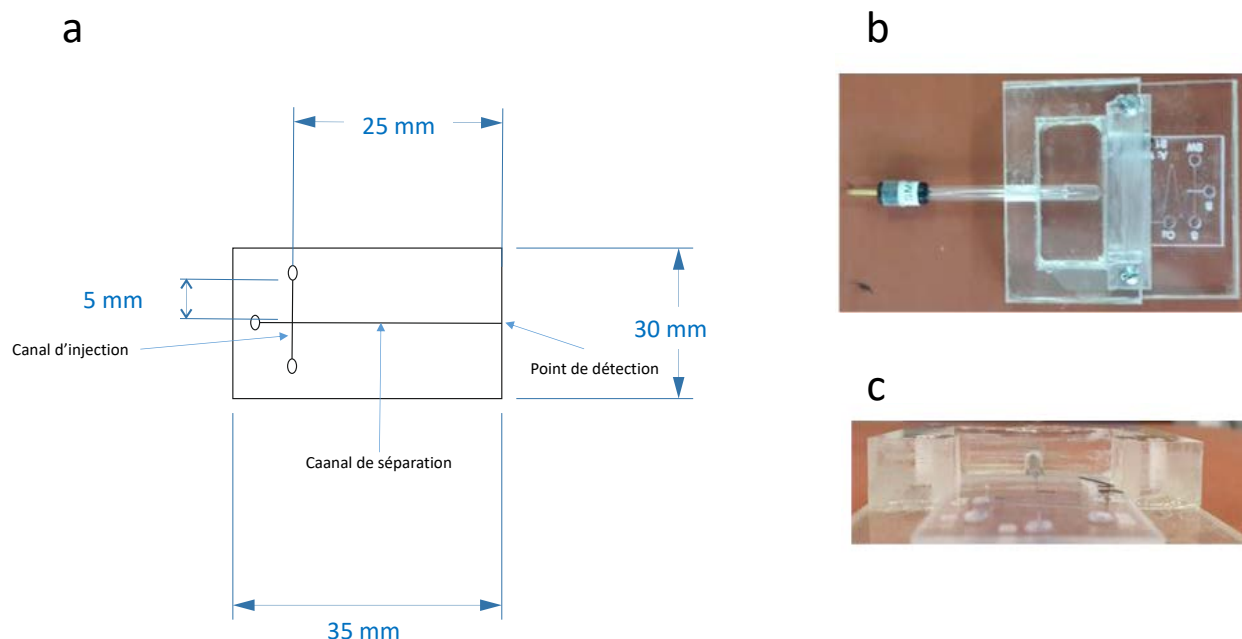


Figure 19 : Visualisation au dessus (a) ou de côté (b) de la configuration du microsystème

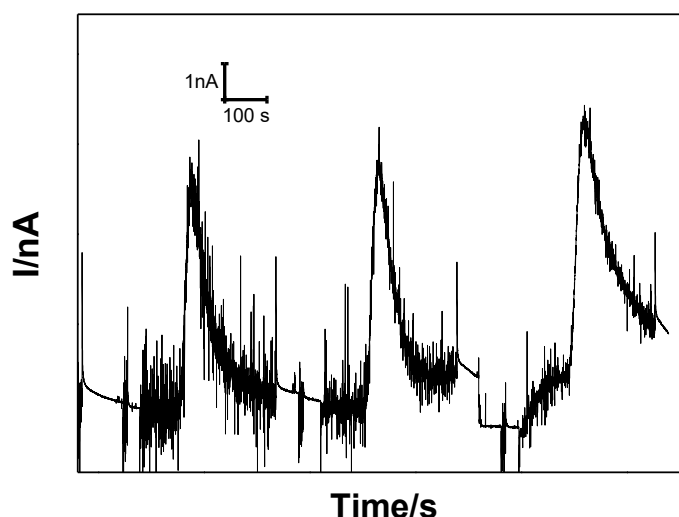


Figure 20: Profils électrophorétiques correspondant à l'injection séquentielle (10s à 1kV) du paracétamol (5,2 mM) dans un tampon PBS ($C=20\text{mM}$, pH 7,4) dans le canal d'injection, puis séparation sous 1 kV dans le canal de séparation de microsystème en PMMA (3 injections) et une détection amperométrique $E=0.8 \text{ V vs Ag / AgCl}$.

Afin d'améliorer les conditions de l'analyse, il a été envisagé i) d'utiliser un potentiostat isolé pour la détection, qui élimine l'interférence électrique avec le potentiostat haute tension utilisé pour l'injection et la séparation, ii) d'utiliser le mode d'injection dit « gated injection », et iii) de changer la nature et la configuration du microsysteme en utilisant un microsysteme en verre / Su-8 avec des électrodes intégrées et la dimension des canaux plus petite. Les résultats effectués dans ces directions ont montré que :

- L'utilisation d'un potentiostat blindé sans fil nous permet d'éviter l'interaction entre la tension de séparation et la tension de détection. Ce potentiostat nous permet aussi de travailler en mode « in-channel » en cas de nécessité. Ces conditions peuvent détruire un potentiostat conventionnel [56].
- Comme mentionné précédemment, l'injection en mode « gated » limite les phénomènes de diffusion pendant l'injection et de fuite de la solution échantillon pendant l'étape de séparation électrophorétique. Ces deux phénomènes ont pour effet un élargissement des profils électrophorétiques. L'optimisation des conditions d'injection a été réalisée à l'aide d'une molécule fluorescente (coumarine 334) permettant l'observation de son transport dans le microsysteme par microscopie de fluorescence (**Figure 21**). La formation de bulles de gaz a été observée lorsque la tension appliquée pour la séparation est élevée. La formation de ces bulles d'air dépend du courant de la séparation qui à son tour dépend de la force ionique de l'électrolyte support et des dimensions des canaux. En utilisant une puce en PMMA (100 μm largeur, 80 μm profondeur et 25 mm longueur.) et un tampon PBS (10 mM, pH =7,4), les consignes de tensions maximales appliquées sont $V_1=250\text{ V}$ et $V_2=300\text{ V}$ (V_1 et V_2 sont les tensions aux réservoirs de l'échantillon et de l'électrolyte respectivement). Un autre système en COC a été utilisé pour étudier l'influence de la dimension du canal. Pour ce système (50 μm largeur, 50 μm profondeur et 87 mm longueur), les tensions maximales V_1 et V_2 sont de 500 et 400 V, respectivement. La **Figure 21** montre l'injection « gated » qui a été faite avec le microsysteme en COC.
- L'approche qui consistait à réduire la taille de la section du canal grâce à l'utilisation d'électrodes intégrées a été réalisée en utilisant une micropuce commerciale de Micrux[®] qui a deux avantages par rapport au système précédent, micro-fabriquée au laboratoire : i) les canaux sont de plus petites dimensions (20 x 50 μm vs 80 x 100 μm pour le PMMA), et ii) l'intégration des électrodes est faite d'une manière plus optimisée avec des dimensions précises. Le système est en verre-Su8 ce qui permet de générer des

écoulements électro-osmotiques plus importants. De plus, ce dispositif est peu perméable aux gaz.

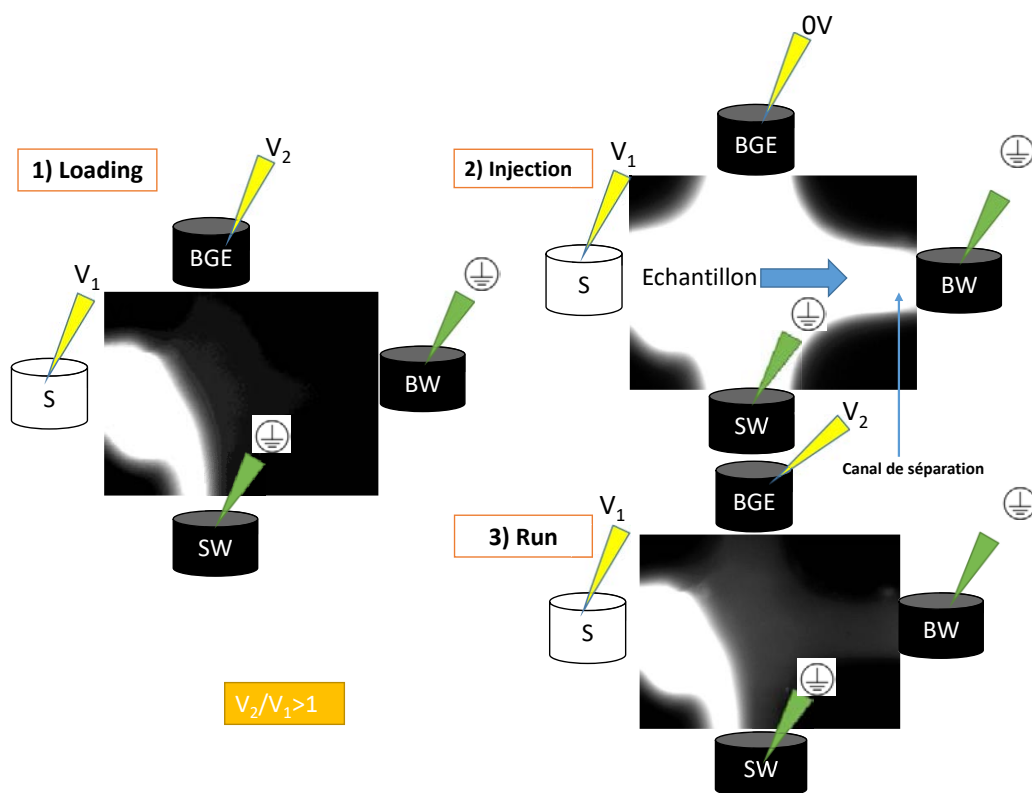


Figure 21 : Illustration du mode d'injection «Gated » en microscopie de fluorescence. Conditions opératoires : puce en COC (section du canal 50 μm x 50 μm , longueur : 87 mm) ; échantillon coumarine 334 ($c = 5\text{mg} / \text{L}$ dans BGE / EtOH 99 / 01 (v/v)) imagé en blanc ; électrolyte support PBS (C 20 mM, pH= 7,4) imagé en noir. A) étape de chargement nécessaire pour que le volume d'échantillon circulant entre le réservoir d'échantillon (S) et le réservoir d'électrolyte support (SW) soit de nature homogène. B) Etape d'injection où l'échantillon remplit le volume de la croix d'injection. C) Début de l'étape de séparation où le volume d'échantillon remplissant la croix d'injection est entraîné dans le canal de séparation. S : réservoir d'échantillon, SW : réservoir poubelle de l'échantillon, BGE : réservoir de l'électrolyte, DW : réservoir poubelle de l'électrolyte.

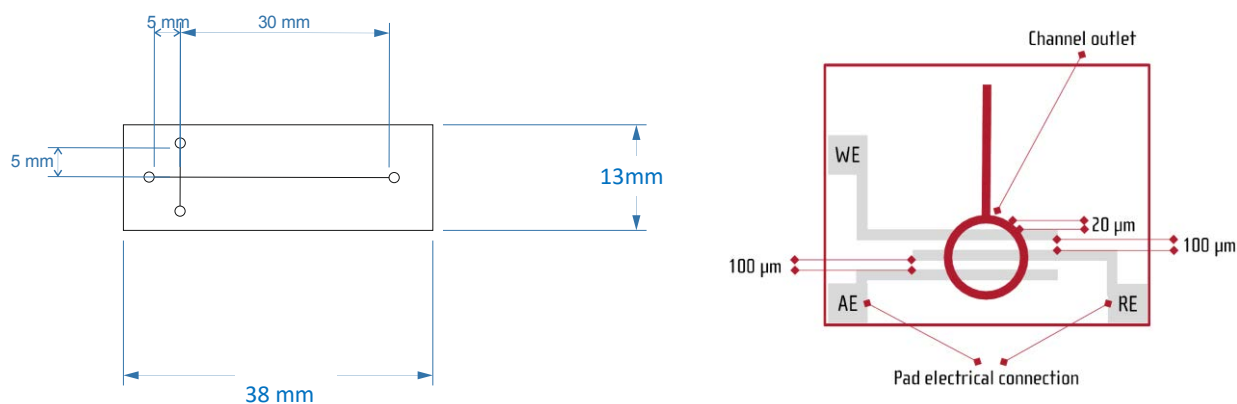


Figure 22 : Schéma et dimensions des puces Micrux® commerciales intégrant un jeu de trois électrodes de Pt dans le puits de sortie du canal de séparation. WE : électrode de travail, AE : contre électrode, RE : électrode de référence.

La mobilité électroosmotique a été déterminée par l'utilisation du paracétamol qui est neutre à pH 5,6. L'injection était de type « gated » ($V_1=1000V$, $V_2= 1200V$) pendant 0,5s. La mobilité électro osmotique calculée dans ce dispositif est dix fois plus faible que celle obtenue avec l'appareil conventionnel (capillaire de silice) à pH 8,5 ($9,5$ vs $75 \times 10^{-5} \text{ cm}^2 \cdot V^{-1} \cdot s^{-1}$). Cette variation peut être due à la différence de pH (5,6 vs 8,5), la nature du tampon (arginine / acide acétique à 20 mM vs carbonate / bicarbonate à la même concentration), et la nature des matériaux des deux microsystèmes. De plus la sensibilité est meilleure : les résultats montrent que pour une concentration de paracétamol de 1 mM, le rapport signal sur bruit (S / N) est supérieur à 100, tandis qu'il était de 4 dans le système en PMMA pour une concentration en paracétamol de 5 mM. La détection du nitrite se fait en mode contre-électroosmotique à une mobilité de $-27,8 \times 10^{-5} \text{ cm}^2 \cdot V^{-1} \cdot s^{-1}$ à pH 5,8.

La stratégie suivie pour détecter le GSNO avec ce système est la suivante : Hg^{2+} est introduit dans le réservoir en sortie de canal, là où le détecteur est placé de telle sorte que la décomposition instantanée de GSNO par Hg^{2+} se produise au niveau du capteur. En appliquant l'injection de GSNO (1200 V, 1000 V) dans un tampon carbonate pH 8,5 (comme celui employé en EC-SM), aucun signal ampérométrique n'a été observé. Ceci peut s'expliquer par le fait que la mobilité électrophorétique de GSNO est plus élevée que la mobilité électroosmotique en valeur absolue. La même difficulté apparaît lorsque la valeur du pH est diminuée. Ainsi, une inversion du champ électrique a été effectuée. Lorsque le Hg^{2+} est introduit comme précédemment (au niveau du détecteur), il migre dans ces nouvelles conditions expérimentales vers le puits d'entrée du microsystème. Ainsi, la zone de Hg^{2+} rencontre celle de GSNO au sein du canal de séparation, induisant la décomposition du GSNO. Dans les conditions étudiées, il apparaît alors au détecteur un composé à une mobilité identique de celle du nitrite.

Lorsque le Hg^{2+} est introduit dans le réservoir de l'échantillon, le même pic est identifié qui a la même mobilité et sensibilité que celui du nitrite. Ces résultats semblent indiquer que la mobilité de GSNO est identique à celle de nitrite dans les conditions expérimentales étudiées et que la décomposition est instantanée (**Figure 23**).

On peut uniquement conclure que l'on peut faire une analyse quantitative en deux temps :

- analyse de l'échantillon sans Hg^{2+} : on quantifie uniquement le nitrite
- deuxième analyse en ajoutant Hg^{2+} : on quantifie le GSNO décomposé et nitrite, donc par soustraction on peut quantifier le GSNO

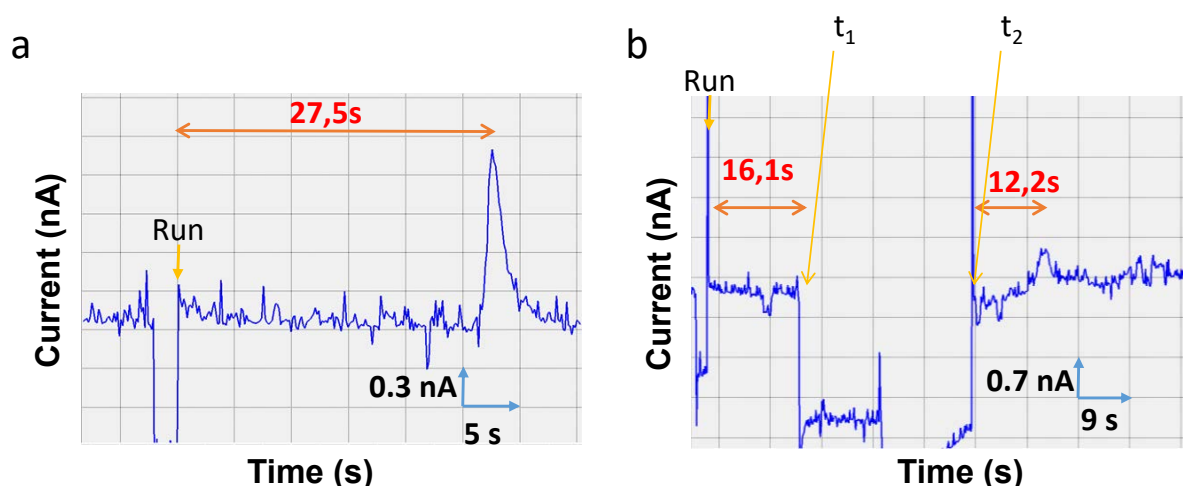


Figure 23 : Electropherogramme de l'injection de GSNO (3 mM) en « gated injection » (-1000, -800 V) pour 1s. a) le Hg^{2+} est déjà présent dans le puit de sortie. b) en t_1 : arrêt du champ électrique, injection de Hg^{2+} et stabilisation de l'électrode face à la nouvelle solution, 16.1 s après le début de la séparation du GSNO, t_2 application à nouveau du champ électrique. On voit la même allure avec nitrite.

L'objectif initial étant de pouvoir quantifier chaque RSNO, il faudrait pouvoir faire une séparation effective de ces RSNOs.

Un nouveau protocole a alors été mis en oeuvre, en décalant dans le temps l'introduction de Hg^{2+} dans le puits de détection, à un temps pour lequel le GSNO a presque atteint le détecteur. Cependant le pic identifié sort toujours au même temps que celui du nitrite. Une des limitations principales de ce nouveau protocole est qu'il faut attendre dizaine de second après l'introduction du Hg^{2+} pour que l'électrode se stabilise face à la nouvelle solution, ce qui peut induire des modifications importantes sur la qualité de la séparation.

Ainsi, notre objectif étant de développer un microsystème intégré qui permette une séparation efficace des différents RSNOs avant leur décomposition (afin de discriminer ces différents RSNOs), l'étape de décomposition doit être située le plus en aval du microcanal. Comme un temps de stabilisation est nécessaire pour le mercure avant de l'utiliser pour la décomposition, nous proposons le design d'un dispositif dans lequel l'introduction de Hg^{2+} s'effectuerait à partir d'un autre canal perpendiculaire au canal principal. Ainsi la décomposition serait effectuée juste avant le détecteur (**Figure 24**)

Ainsi, nous avons montré la faisabilité du couplage de la séparation électrocinétique avec la détection électrochimique. Une optimisation de l'intégration de l'étape de décomposition est alors nécessaire, et un autre défi à relever sera la sensibilité de détection des RSNOs. La limite de détection atteinte durant cette étude est de l'ordre de la dizaine de micromolaire pour le nitrite et du millimolaire pour les RSNOs tandis que les concentrations en RSNOs sont de l'ordre du micromolaire et en dessous.

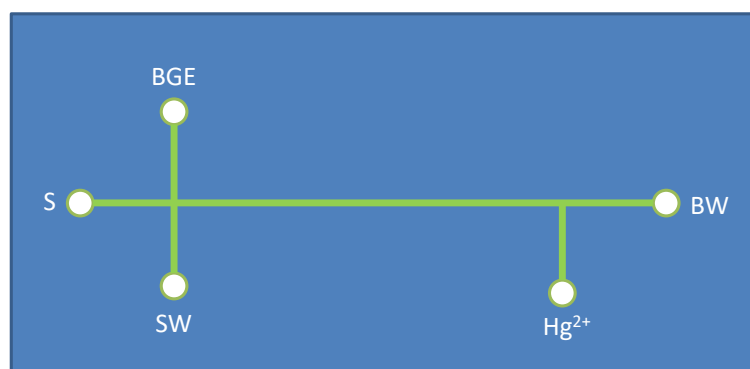


Figure 24 : Schéma du microsystème propose ou un réservoir du Hg²⁺ est ajouté. Séparation va procéder entre les réservoirs S, SW, BGE, and BW. Après, le potentiel va être appliquer entre S, SW, BGE, and Hg²⁺ alors les RSNOs se décompose en nitrite et on détecte les nitrites qui viens des RSNOs diffèrent.

IV. Conclusion et perspectives

Cette thèse a eu pour objectif d'utiliser la microfluidique pour détecter le NO dans les milieux biologiques, stocké sous forme des RSNOs. Plusieurs méthodologies nécessaires pour cette détection ont été développées. Une méthode EC-SM pour identifier la composition et les produits de décomposition de GSNO a été développée. Ces résultats nous ont permis de proposer des voies de décomposition. La séparation de GSNO et de son produit de décomposition, de la Cysteine et de CySNO a été optimisée avec une méthode EC-C⁴D conventionnelle. Différentes techniques de décomposition (Cu²⁺ / GSH, nanoparticules d'or, lumière, température...) des RSNOs ont été étudiées afin de choisir la plus rapide et la plus adaptée à une mise en œuvre en système microfluidique. Lors de ce travail d'optimisation, différentes méthodes de détection ont été utilisées comme la spectrophotométrie, l'électrochimie, et la chimiluminescence. La miniaturisation de la détection des RSNOs a ensuite été menée en microsystème papier avec une détection colorimétrique dans le but de produire un « point of care device ». Enfin, la détection de GSNO a été faite en un système microfluidique intégrant une réaction de décomposition par Hg²⁺.

Les perspectives de ce travail concernent entre autres (1) l'optimisation du protocole d'injection et de l'étape de séparation des RSNOs, (2) la mise au point de l'intégration de la méthode Cu²⁺ / GSH avec des électrodes à membrane sélective dans le microsystème, (3) de l'étude d'un protocole de concentration d'AuNPs pour décomposer les RSNOs dans des volumes faibles comme ceux employés en microfluidique, (4) une étape en ligne de préconcentration des

RSNOs en microchip en utilisant par exemple l'isotachophorèse ou des effets de différence de champs électriques pour améliorer la sensibilité du diagnostic. Ce travail sur la sensibilité pourrait aussi éventuellement venir d'améliorations des potentiostats afin de détecter des concentrations plus faibles sans être affecté par le champ électrique dû à la séparation.

GENERAL INTRODUCTION

Nitric oxide (NO) is considered as the first gaseous signal transduction molecule [11,57]. It is a diatomic free radical that has extremely a small free trajectory (100-200 μm) and short half-life (<1 s) in biological fluids. The addition of NO to functional proteins (or S-nitrosation reaction) is as important as phosphorylation in its consequences on cellular activities [58] and this necessitates the transport of NO to the targets in biological fluids. In order to be transported and stored in biological fluids, NO binds to the sulfhydryl groups of peptides and proteins forming S-nitrosothiols (RSNOs; thionitrites). RSNOs have been shown to occur endogenously in various biological systems (respiratory, cardiovascular, neurological, digestive...). RSNOs play important roles in several physiological functions (vasodilatation and relaxation [59-61], antiplatelet aggregation [20,59,62-66], antimicrobial [67], regulation and signaling of protein function etc.) and physiopathological events (neurodegenerative diseases such as Parkinson and Alzheimer) [68,69], apoptosis [70], cancer, asthma, chronic obstructive pulmonary disease [71], preeclampsia [72] and diabetes[17]).

RSNOs can be of biological or artificial origin. They can be formed in many pathways for example through (i) radical recombination between NO and a thiyl radical ($RS^{\bullet}$), (ii) transition metal catalyzed pathway, (iii) transnitrosation reaction from low molecular weight RSNOs (LMW-RSNOs, such as S-nitrosogluthathione and S-nitrosocysteine) to high molecular weight RSNOs (HMW-RSNOs, such as nitrosoalbumin and nitrosohemoglobin) [47]. HMW-RSNOs, perform their biological activity by transferring NO to LMW-SNO that can penetrate cells and act on functional proteins [73].

RSNOs can be classified based on many criteria such as natural abundance and molecular weight. They are either present *in vivo* (S-nitrosohemoglobin, S-nitrosoalbumin, S-nitrosogluthathione, S-nitrosocysteine) or can be synthesized (S-nitroso-N-acetylpenicillamine, S-nitrosocaptopril, S-nitroso-N-acetyl-L-cysteine) as candidates of pharmacological molecules, acting as NO donors. In all cases, they are considered as stocks of NO and then act on functional proteins either by releasing NO [74] or mostly by transnitrosation reactions [75].

RSNOs exist in biological media at concentrations that vary between tenth of nanomolar to less than ten micromolar ([2,3] and references therein). The variation of RSNO concentration has been shown to occur in many diseases [1]. There is no gold standard method to determine the biological concentrations of RSNOs. Even presently the same sample can give different results

using different detection methods. This raises the challenge to develop a robust, sensitive, selective and rapid method to detect RSNOs. Several analytical methods have been developed over the years to detect RSNOs. They can be direct if the RS-NO remains intact or otherwise indirect. Indirect methods are more sensitive but can suffer from several drawbacks such as: i) length and complexity of the procedure increasing the likelihood of artefacts, ii) selectivity problems (especially from nitrite usually present in biological fluids) and iii) employment of toxic Hg^{2+} in millimolar concentration in many of these assays, which impose safety issues for the operator and the environment [76].

Indirect methods are based on a two-step protocol: decomposition of the RS-NO bond followed by detection of the decomposition products (NO, nitrite or thionyl moiety) using electrochemical, spectrophotometric, or fluorescent methods, gas chromatography coupled to mass spectrometry, biotin switch methods or chemiluminescence assays [2,50,77-80]. According to the application purposes, one of these methods can be selected. For example, chemiluminescence is a very selective and sensitive real time detection method but is only applicable for *in vitro* studies [81]. The colorimetric method (Saville method) is widely employed as it is very reproducible, but it cannot be used for real time analysis and presents low sensitivity and selectivity [2]. Mass spectrometry (MS) requires preliminary sample treatment and can underestimate RSNO amounts due to possible signal suppression or sample decomposition [80]. Electrochemical methods represent direct, real time, and label-free detection techniques that can be used for *in vivo* applications ([3] and references cited herein). All the indirect detection methods benefit from the fact that RSNOs can be decomposed through different pathways such as metal cation catalysis [24], ascorbic acid assisted reduction [47], heat [24,44], infrared, ultraviolet [82,83] or visible light assisted decompositions [43,60,82,84]. However, they mainly lead to partial and non-reproducible decomposition that can be detrimental for accurate detection. Furthermore the decomposition process can be multiple: homolytic cleavage giving rise to the formation of unstable $\text{NO}^{\bullet}$ and $\text{RS}^{\bullet}$ that can lead to nitrite and other end-products, or heterolytic cleavage leading to RS^- and NO^+ which rapidly forms nitrite.

Direct methods include MS [80,85] and detection of phosphines after their selective reaction with RSNO by MS, NMR, or fluorescence. The former can induce loss in NO during sample handling and during MS detection. The latter, even though highly selective, can induce a reaction with nitroxyl (HNO). Also it has the limitation of the stability of the formed aza-ylide compound, which varies depending on the RSNO being determined. Other direct characterization methods of RSNOs utilize a separation method coupled to one of these

detection methods, such as high performance liquid chromatography (HPLC) with UV detection[26,45,86,87], HPLC with electrochemical detection [88] capillary zone electrophoresis (CE) with UV detection [89-92], capillary gel electrophoresis with laser induced fluorescence [93].

The improvement of the existing detection techniques by coupling with a separation method such as high performance liquid chromatography (HPLC), gas chromatography (GC) or capillary electrophoresis (CE) is helpful in selectivity enhancement, therefore in determining different RSNOs compositions. Conventional techniques necessitate most of the time several separation steps (sample pretreatment, separation, decomposition and detection). One way to improve these methods for RSNO quantitation would be to integrate them into miniaturized devices to form a lab on a chip device. This miniaturization should integrate the separation, decomposition and detection within the same microsystem and would offer many advantages over conventional analytical devices including: i) low sample and reagent consumption, ii) faster analysis time, iii) low power consumption, iv) low cost, v) reduced risk of contamination and vi) global affordability and portability[94,95]. The optimization of the decomposition step, in the case of indirect methods, represent an additional challenge to the ones of coupling the separation with the detection.

The long time project of the team is to integrate on a micro device all analysis steps including injection, decomposition, separation and detection for RSNOs of different molecular weights in a miniaturized device (**Figure 25**).

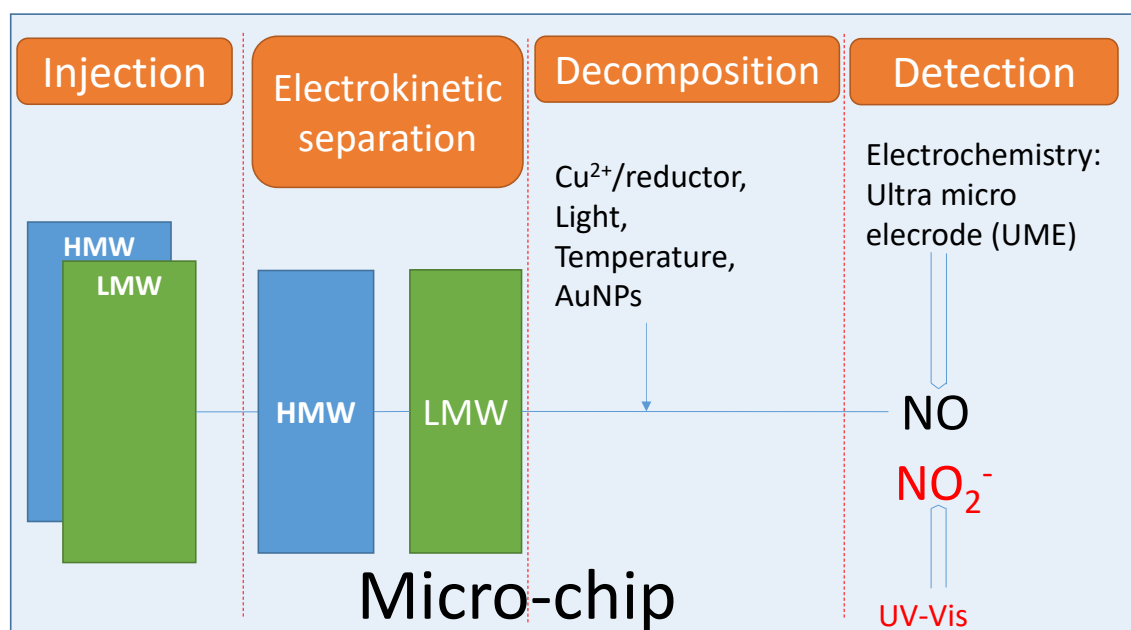


Figure 25: Schematic representation of the objectives of the PhD project

The objective of this PhD work was develop and optimize the methodologies for each step and then to assemble and integrate them in a microfluidic device. As we aim at developing a simple and efficient miniaturized diagnostic device, the separation will be performed electrokinetically and the detection electrochemically. As for electrokinetic separation, CE was chosen over liquid chromatography because of its higher efficacy, ease of application of electrokinetic injection in comparison to pressure injection and ease of fabrication of microchip because no filling with microparticles is required. Detection was set to be electrochemical because of its simplicity (no need to derivatization) and portability in comparison to other detection methods, which need bulky instrumental detection (fluorescence and MS). Work was done principally to determine the best decomposition pathway among the possible ones (light, metallic ions, heat and gold nanoparticles).

In the first chapter, the current knowledge about biosynthesis and biological activities of NO and RSNOs is presented. Then, the synthesis, reactivity, decomposition and analysis methods for RSNO are developed. Finally we provide a short bibliography on miniaturization: the materials and techniques used and what is achieved for RSNO detection in microfluidic devices.

CHAPTER I: STATE OF ART ON NITRIC OXIDE AND S- NITROSOTHIOLS

I. Chemo and Bio-Properties of Nitric Oxide and Nitrosothiols

A. Nitric Oxide (NO)

1) History of NO discovery

Nitroglycerine, a nitric oxide based drug, was firstly synthesized in 1847 by *Ascanio Sobrero* (**Figure 26**). At that time it was not known as a drug but as an explosive. Sobrero has tasted this product and found it was sweet, pungent and aromatic. He published his results in *comptendu* and described “great precaution should be used, for a very minute quantity put upon the tongue produces a violent headache for several hours” [96]. After two years, Constantin Hering pursued nitroglycerine as a homeopathic remedy for headaches. In 1863, Alfred Nobel has made his famous Nobel patent detonator or blasting cap for detonating nitroglycerin. The Nobel patent detonator used a strong shock rather than heat combustion to ignite the explosives. Nobel recognized that nitroglycerine, which is in a liquid state, is volatile so he tried to mix it with silica and he turned the liquid into a malleable paste called “dynamite” [97].

In 1867, Lauder Brunton, the father of modern pharmacology, used amyl nitrite (another NO based drug) to relieve angina pectoris, and noted a pharmacological resistance to repeated doses. Then in 1876, William Murrell first used nitroglycerine for treating angina after it was well known for its antihypertensive properties. The ironic fact is that in 1890, Nobel’s physician recommended him to take nitroglycerine in order to treat his intense angina pectoris. At that time Nobel refused to take it. In 1896 he wrote “isn’t it the irony of fate that I have been

prescribed NG1 [nitroglycerine] to be taken internally! They call it Trinitrin, so as not to scare the chemist and the public”.

The mysterious cellular mechanism of vasodilatation by nitroglycerine was not discovered until 150 years after its first synthesis (**Figure 27**). The story started with Ferid Murad (Associate Professor in the Departments of Medicine and Pharmacology at the University of Virginia) in 1970 where he studied the relation between NO and cyclic guanosine monophosphate (cGMP) [98]. In 1977, he discovered that nitroglycerine releases NO. He hypothesized that this NO acts on soluble guanylyl cyclase thus increasing cGMP and causing muscular relaxation. Indeed, he saw an increase in cGMP when delivering NO gas to soluble and particulate preparations from various tissues. The effect was dose-dependent and was observed with all tissue preparations examined. This idea was original in the manner that a gaseous molecule can control muscle contraction and he suggested that hormones, neurotransmitters and other endogenous factors can act on cGMP via NO released by endogenous precursor. He thought also that NO could be a second messenger inside the cells, but at that time he did not have the experimental evidence and tools to prove it [99]. The idea of a reactive radical that can activate an enzyme and be a second messenger was not accepted by many physiologists. Robert Furchgott and John Zawadzki recognized the importance of endothelial cells for the action of acetylcholine (a potent vasodilator). Without endothelial cells, acetylcholine was not able to dilate the blood vessels *in-vitro*. They proposed that muscle relaxation due to bradykinin, histamine, ATP and ADP was due to the same unstable relaxing substance, which they named endothelial derived relaxing factor (EDRF). They thought that EDRF was prostacycline or other prostanoids. In 1981, they suggested that nitroglycerine and amyl nitrite act by transforming NO to a thiol thus forming RSNOs, which are unstable and react with guanylyl cyclase and activate it to produce cGMP [100]. In 1987, Ignaro et al. stated “EDRF from artery and vein is either NO or a chemically related radical species” after a series of experiments that showed that both arteries and veins are excited and inhibited by similar reagents with similar mechanisms [101,102]. This was one of the factors that led to the explosion of scientific work on this domain, NO was now formally classified as a biologically signaling molecule similar to phosphorylation and other signaling pathways. Just two months before Ignarro’s discovery Moncada et al. [103] showed that EDRF and NO are the same molecule using similar techniques to those used by Ignaro et al (**Figure 27**).

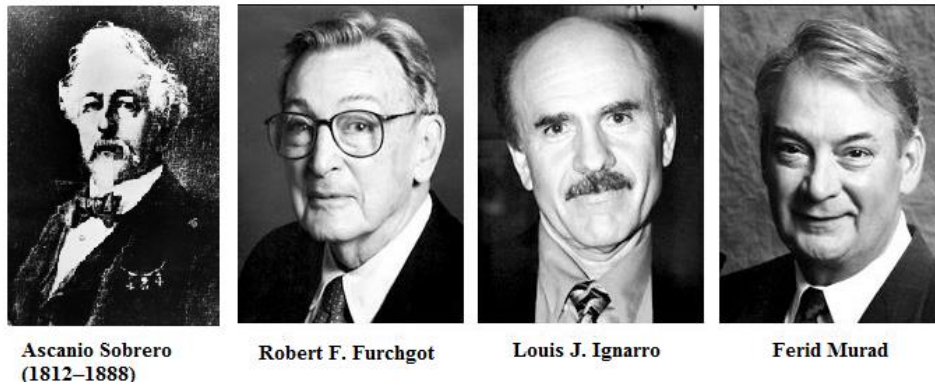


Figure 26: Ascanio Sobrero (the discoverer of nitroglycerine) and the three Noble prize laureate in 1998

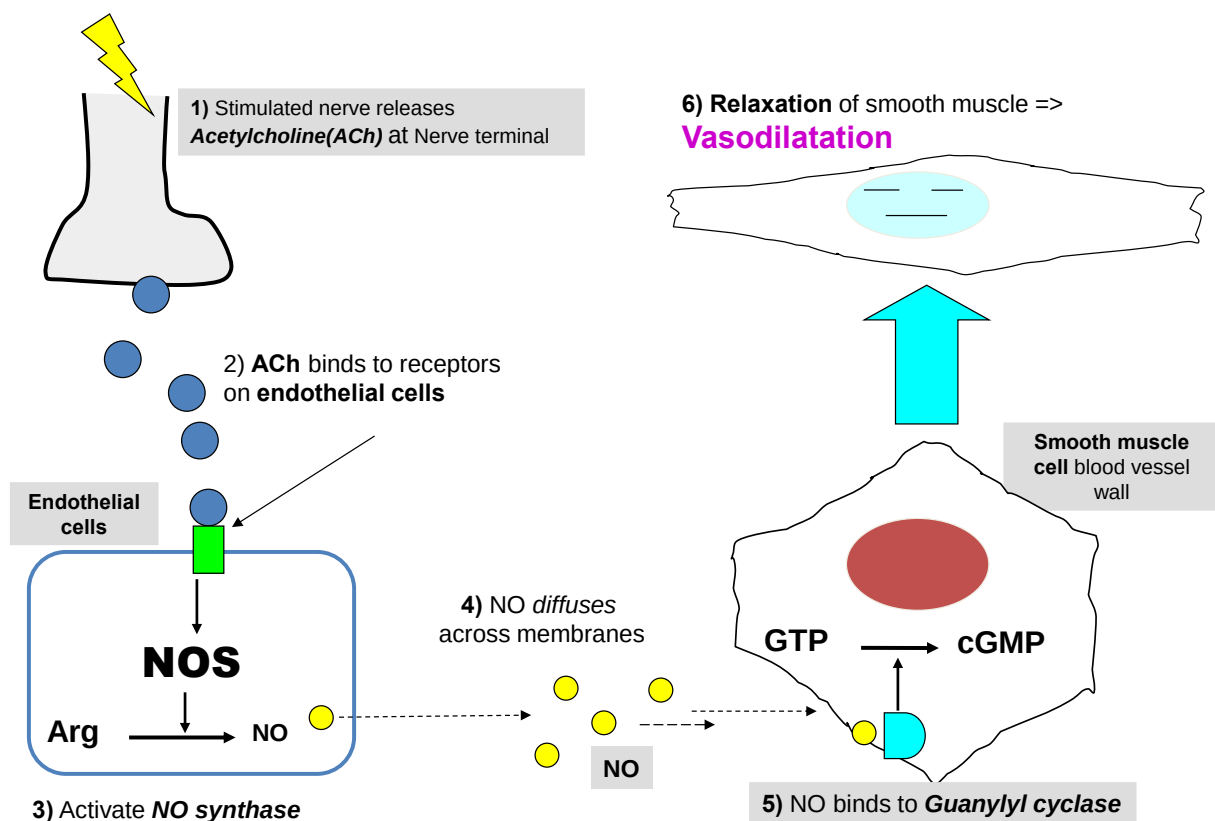


Figure 27 schematic presentation of the sequence leading to vasorelaxation where a stimulation of nerve lead to the production of acetylcholine that bind to its receptors on endothelial cells and leads to the production of NO by nitric oxide synthase (NOS) enzyme. This NO diffuses through the membrane of muscular cells and binds to guanylyl cyclase thus producing cGMP starting from GTP. This cGMP provokes vasorelaxation.

Despite this important discovery of Moncada that was made before Ignarro et al., in 1998 the Noble prize in physiology or medicine was given to Ignarro, Murad and Furchgott (Figure 26). This happened after 150 years after the first use of NO based molecules (nitroglycerine) as explosives. Nowadays there are more than 20000 papers dealing with this subject.

2) NO and NO derivatives characteristics

NO is one of the smallest gaseous molecules. It is produced industrially as chemical intermediate, naturally by electric discharges [e.g. lightening], and released as an air pollutant from: i) car engines [104], ii) fossil fuels power engines [105], iii) and high hydrogen content (HHC) syngas non-premixed jet flames [106]... It is also produced biologically in human body and has many actions that are referred to its chemical properties.

NO has one unpaired electron so it is a free radical and is paramagnetic. It is neither a very reactive free radical nor deleterious when compared to other radicals such as hydroxyl ($\text{HO}^\bullet$) and superoxide radicals (O_2^-). Its paramagnetic properties permit it to react only with paramagnetic species. It is uncharged so it has weak nucleophilic character and it can function as an autocrine or paracrine signaling molecule. The average free pathway covered from its site of production until reacting with other cellular constituents is 100-200 μm [11].

Nitrogen atoms in NO (**Figure 28**) can have different oxidation states, as summarized in **Table 1**. As an example, the intracellular targets and bioactivity in pulmonary system of these different possible molecules are also presented in **Table 1**.



Nitric oxide

Figure 28: structural formula of NO molecule

3) Biological synthesis of NO

NO is synthesized in biological systems either enzymatically or non-enzymatically. Enzymatic synthesis originates from L-arginine by a family of enzymes including nitric oxide synthase (NOS), or by enzymatic reduction of nitrite and nitrate.

i. Enzymatic Synthesis of NO by Nitric Oxide Synthase

NOS enzymes are responsible for most of enzymatic synthesis of NO. They are members of the cytochrome P450 family. They contain two main domains: reductase and oxygenase. These domains necessitate many cofactors such as tetrahydrobiopterine (BH_4), FAD and FMN that help in the transfer of electrons from NADPH into the heme complex at the catalytic side of the enzyme (**Figure 29**) [107]. It produces NO by transforming L-arginine into L-citruline.

Table 1: Different products that can be formed from NO with their principle targets and pulmonary bioactivity. Adapted from [13,108]

Nitrogen oxidation state	Name	Symbol	Intracellular target	Bioactivity	Comments
-3	Ammonia	NH ₃		Diverse structural functions in pulmonary biology (proteins, buffers)	Metabolic waste product
-1	Hydroxylamine	NH ₂ OH		Intermediate in nitrogen oxide reduction	Product of nitroxyl reaction
0	Nitrogen	N ₂		Prevention of alveolar collapse	
+1	Nitroxyl	HNO or NO ⁻	Transition metals nucleophiles e.g. thiols	Smooth muscles relaxant	Angeli's salt product; act as electrophile
+1	Nitrous oxide	N ₂ O		Anesthetic , denitrification intermediate	Laughing gaz
+2	Nitric oxide	NO	sGC, CcOx, NHI, Thyl radicals	Vascular smooth muscle relaxant, involved in immune cytotoxicity and antimicrobial activity	Uncharged free radical, product of NO synthetase
+3	Nitrite	NO ₂ ⁻	Heme	Oxidation end product, denitrification substrate	Can be reduced to NO in presence of Iron
+3	Nitrosonium	NO ⁺	Thiolate anion	NO group equivalent, bioactive as RSNO and Fe-NO, versatile cell signaling moiety	Formally oxidized NO, does not exist at neutral pH
+3	Peroxynitrite	ONOO ⁻	Thiols, transition metals	Cytotoxic NO product with antimicrobial activity	Formed upon reaction of NO with superoxide, nitrating agent
+3	Dinitrogen trioxide	N ₂ O ₃	Thiols, amines	Nitrosating species, nitrosonium donor	Nitrosation agent; formed from reaction of NO with O ₂ and from protonation and further reaction of nitrite
+4	Nitrogen dioxide	NO ₂	Thiols, phenolics (tyrosine)	Involved in lipid peroxidation, toxic free radical	Free radical, strong oxidizing agent, nitrating agent
+4	Dinitrogen tetroxide	N ₂ O ₄		Nitrosating species	
+5	Nitrate	NO ₃ ⁻		Denitrification substrate, non-reactive oxidation end product	

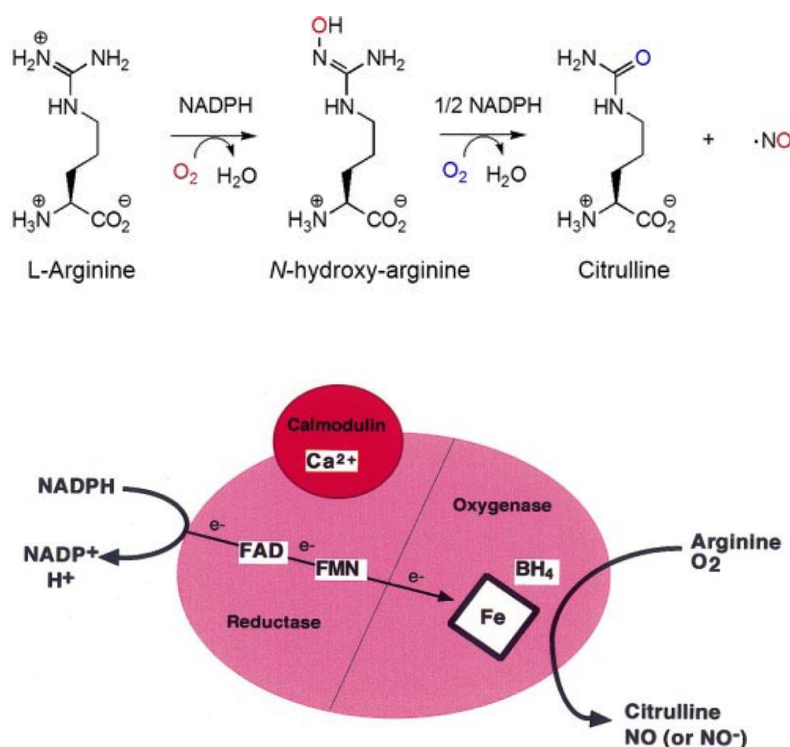


Figure 29: Reaction mechanism of transformation L-arginine to L-citrulline (upside). Electrons (e⁻) are donated by NADPH to the reductase domain of the enzyme and proceed via FAD and FMN redox carriers to the oxygenase domain. There they interact with the heme iron and BH₄ at the active site to catalyze the reaction of O₂ with L-arginine, generating citrulline and NO as products. Electron flow through the reductase domain requires the presence of bound Ca²⁺/CaM (downside). FAD: Flavin adenine dinucleotide, FMN: Flavin mononucleotide, BH₄: Tetrahydrobiopterin, CaM: calmoduline, Adapted from [109,110].

There are three types of NOS. The first NOS to be discovered, purified and cloned was located in the brain and was thus called neuronal NO synthase (nNOS) or NOS 1. The second one to be cloned was isolated from macrophages and named NOS2 or inducible NO synthase (iNOS) because it is already induced in many tissues by pro-inflammatory cytokines. The third NOS to be cloned is found in the endothelium and is therefore named endothelial NO synthase (eNOS) or NOS 3 [9]. It is obvious that the naming of the three NOS came from the tissues where they were first discovered not from the tissue where they are exclusively present, because a specific NOS can be present in many other types of tissues and cells [7]. NOS 1 and 3 are constitutive while NOS 2 is inducible. NOS enzymes can be divided into calcium dependent such as nNOS and eNOS or calcium independent such as iNOS. All NOS enzymes bind to calmodulin (calcium modulated protein). Calmodulin only binds to calcium dependent enzymes when calcium concentration is high (half-maximal activity between 200 and 400 nM), while in calcium independent enzymes, calmodulin binds to calcium at extremely low concentrations (below 40 nM) due to a different amino acid structure of the calmodulin-binding site [111]. When calmodulin binds the enzyme, it triggers electron flow between the reductase and the

oxygenase domains of NOS, which can be viewed equivalent to NO synthesis (**Figure 29**). NOS enzymes also differ in the NO amount produced. iNOS, induced by inflammation, produces the largest amount (in nM vs in pM). The main differences between NOS enzymes are presented in the **Table 2** below.

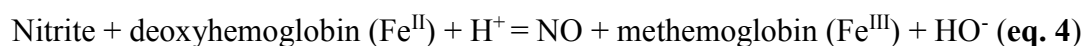
Table 2: Properties of NOS1, 2 and 3. [7,105,107,112]

	NOS 1	NOS 2	NOS 3
Other name	Neuronal NOS	Inducible NOS	Endothelial NOS
Dependence on calcium	Elevated calcium (70-100nM)	Normal cell calcium level (30-70 nM)	Elevated calcium (70-100nM)
Trigger	Sheer stress, acetylcholine, histamine, bradykinin, substance P, glutamate on NMDA receptor in CNS and 5 hydroxytryptamine (serotonin), 17 β estradiol	Cytokines, bacterial toxins	Sheer stress, acetylcholine, catecholamines, vasopressin, histamine, bradykinin, substance P, glutamate on NMDA receptor in CNS and 5 hydroxytryptamine (serotonin), 17 β estradiol, autacoids
NO output	Picomolar	Nanomolar	Picomolar
Localisation	Non-adrenergic non-cholinergic nerves, cardiac and skeletal muscles, smooth muscles, epithelium, neutrophils, cells of macula densa in kidney and adventitial layer of penile arteries	Macrophages, neurons, epithelium, other cells after induction	Endothelium of blood vessels, post junctional smooth muscles and Neurons
Phenotype of knockout	Gastric dilation Stasis	Increased susceptibility to infections and tumors Altered inflammatory response	Impaired vasodilation
Physiological role	Regulation of neuronal excitability and firing, long term potentiation or depression of synaptic plasticity, memory and learning process, motility in gastro intestinal track	Host defense, inflammation	Vasodilation, hyperaemic response, anti-adhesive (platelets, leucocytes), mucosal integrity, electrolyte and water absorption
Relation to disease	Decrease in pyloric stenosis, achalasia, hirschprung's disease, penile erectile	Hypotension, cardiodepression and vascular hyporeactivity in septic shock, increase in inflammatory bowel disease, gastroenteritis, sepsis	Decrease in portal hypertension

ii. Enzymatic synthesis of NO by reduction of nitrite and nitrate [105]

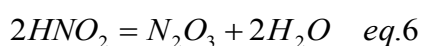
The end-products of oxidation of NO are nitrite and nitrate. They act also as a biological reservoir for synthesis of NO when needed [112-114]. Ischemic heart has shown to synthesize NO starting from nitrite [114]. Under many other conditions, especially ischemic ones, different enzymes (hemoglobin, myoglobin, xanthine oxido-reductase, mitochondrial cytochrome oxidase, aldehyde dehydrogenase 2, cytochrome P450 reductase and cytochrome P450) can catalyze the reduction of nitrite and nitrate to produce NO [105]. For example, in ischemia,

when hemoglobin is deoxygenated, its conformation changes from R (in oxygenated state) to T (in deoxygenated state). Under these conditions and at pH 6.4, it shows its maximum catalytic activity in reduction of nitrite, which results in vasodilatation due to NO production [115] (**eq. 4**). Thus, hemoglobin works in regulatory mechanism to counterbalance the hypoxia and deliver more O₂ to tissue by inducing vasodilation. Indeed de-oxyhemoglobin is more than 30 times stronger reductant than de-oxyhemoglobin.



iii. Non-enzymatic NO synthesis

Non enzymatic NO-synthesis mainly takes place in the stomach where the acidity is very high (pH 1.5-3.5). The production of NO protects the animal from pathogenic organisms. Under acidic conditions, nitrite forms nitrous acid (pKa 3.2, **eq. 5**) and then dinitrogen trioxide (**eq. 6**) undergoes homolytic cleavage to give NO and NO₂ radicals (**eq. 7**).



In Summary, NO can be produced *in vivo* starting from L-arginine by NOS enzymes or it can be produced by enzymatic and non-enzymatic pathways starting from nitrite. Three types of NOS were discovered that vary in properties and in amount of produced NO.

4) Biological effects of NO

NO has many biological activities such as vascular relaxation, cytotoxicity of phagocytic cells and cell to cell communication in central nervous system, detailed in the following sections [116].

i. Role in cardiovascular system

NO has a very important role in the vascular endothelium. It regulates vascular tone (playing the role of anti-ischemia / anti-hypertension), platelet aggregation (anti-thrombosis), leukocyte adhesion and endothelial junctional permeability (anti-atherosclerosis) [6]. **Figure 30** summarizes the effects of NO on cardiovascular function.

NO prevents ischemia and hypertension by two main pathways: promoting the production of cGMP, and increasing angiogenesis. NO plays the role of vasodilator by increasing the

concentration of cyclic guanosine monophosphate (cGMP). After its production, NO fixes on the iron-heme of soluble guanylyl cyclase (sGC), changing its conformation and permitting it to transform guanosine-5'-triphosphate (GTP) to cGMP. The increase of cGMP concentration prevents the entry of calcium thus leading to vasorelaxation (**Figure 27**). This vasorelaxation is important in maintenance and enhancement of coronary perfusion and peripheral blood flow, as well as in decreasing systemic vascular resistance, and decreasing hypertension in systemic and pulmonary vascular beds ([6,117] and references therein).

NO exerts its anti-atherosclerosis effect by: decreasing oxidative stress (scavenging superoxide radicals, inhibiting some enzymes involved in oxidative stress such as NADPH oxidase, inhibiting lipid peroxidation and inhibiting gene expression of oxidizing enzymes) and inhibiting atherogenesis signaling processes that leads to leucocyte adhesion [6]. It has also antithrombotic properties, by inhibition of platelet adhesion and aggregation [117]. This is due to the upregulation of cGMP by NO, increasing the phosphorylation of proteins that regulate the process of platelet adhesion [6]. Finally, NO is involved in cardiovascular diseases (CVD) that have been correlated to impairment of endothelial function [118]. Indeed, this impairment is due to a decrease in NO availability, which has several possible causes: i) abnormalities in sheer stress (which is a stimulant of NOS activity), ii) increase of concentration of asymmetrical dimethyl arginine, an eNOS inhibitor, iii) inactivation of NO by reactive oxygen species (ROS) and iv) increased amounts of vasoconstrictors such as angiotensin-II, endothelin 1 and norepinephrine.

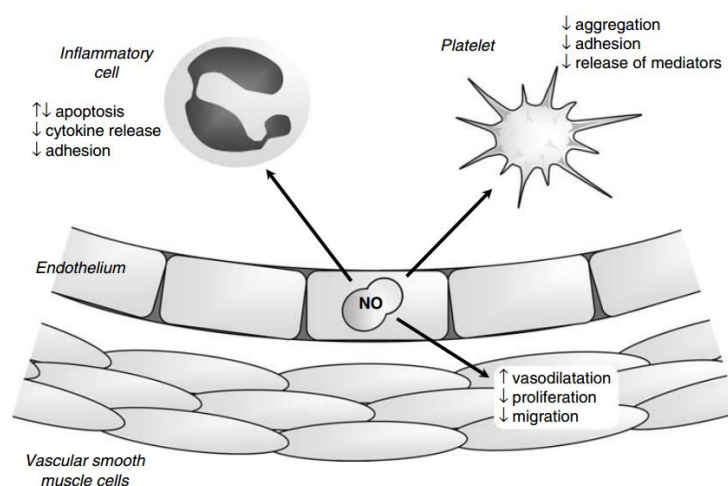


Figure 30: Effects of NO in cardiovascular system. Adapted from [119]

ii. Role in digestive system

NO plays an important role in digestive system: i) it protects against pathogens (see non enzymatic NO production paragraph), ii) it is also considered as non-adrenergic non-cholinergic inhibitory neurotransmitter in the gut, iii) it increases gastric accommodation and emptying, iv) it inhibits small bowel motility in animals v) it is involved in postprandial hyperaemia and in hyperaemia after gastrointestinal reflux and vi) it has a role in the relaxation of ileo-colonic junction [7].

A decrease of NO concentration due to impairment of NOS activity can lead to digestive problems [7,120,121] such as esophageal achalasia [122], hypertrophic pyloric stenosis, internal and sphincter achalasia, hypertrophic pyloric stenosis, diabetic gastro-paresis, colonic dysfunction, Chagas disease and Hirschsprung's disease [7]. Under physiological concentrations, NO promotes absorption of water from the gut, while under pathological conditions (high NO concentration) it promotes diarrhea associated with inflammation of gut.

iii. Role in inflammation

NO has anti-inflammatory role when it is present at normal physiological conditions while it has a proinflammatory mediator role in pathological conditions when it is overproduced [123]. NO has indirect role in several inflammatory diseases such as chronic arthritis, inflammatory bowel disease and organ damage and inflammation triggered by chemicals and drugs. Inflammation occurs usually after activation of iNOS, which produces a large amount of NO. Its damaging action is based on the formation of peroxynitrite, which triggers oxidative damage of DNA [123-125].

iv. Role in cancer

NO can initiate or protect from cancer depending on its quantity, the type of iNOS expressing cells (tumor, inflammation, stromal), the cellular sensitivity to its toxic activity and the status of the p53 tumor suppressor gene in the tumor cells [9]...

NO overproduction by iNOS affects several signaling pathways in cancer [126]. First, NO stimulates tumor angiogenesis i) by stimulating angiogenic and lymphogenic factor expression, ii) by inhibiting the expression of endogenous anti-angiogenic factors such as thrombospondin-1 and iii) by stimulating blood vessel maturation via the recruitment of perivascular cells. Secondly, it enhances migration and invasion of tumor cells. Finally it induces tumor damage, which leads to DNA mutation and clonal transformation [9].

v. Role in central nervous system and neurodegenerative disorders

NO is essential to the central nervous system (CNS). It is important for vasodilation of the vascular bed thus allowing blood to reach the cells in CNS (NO produced by eNOS). It also plays the role of neurotransmitter and is involved in synaptic plasticity, modulation of neuroendocrine function, memory and behavior. Under various pathological conditions (ischemia, neurodegenerative disorders), the amount of NO produced increases by activation of iNOS. High amounts of NO cause deleterious effects, which can aggravate a situation. Oxidative stress occurring in many neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's, multiple sclerosis (MS) and amyotrophic lateral sclerosis leading to damage of neurons, which are non-renewable cells [9].

In spinal cord, NO and cGMP have emerged as a pro-nociceptive molecules. They contribute basically to sensitization during both inflammatory and neuropathic pain. However, they are not involved in basal pain perception, which serves as an essential early warning. This means that blocking this pathway can be beneficial in the treatment of inflammatory and neuropathic pain [127].

The decrease in base levels of NO synthesis in the endothelium of microvasculature in brain is a common feature of aging and cerebrovascular diseases. This decrease leads to impaired vasodilation which leads to a decrease in regional blood flow and production of oxidative stress. These are common features in Alzheimer's disease, for example. The deregulation of NO synthesis leads also to blood brain barrier (BBB) and permeability dysfunction, oxidative stress, chronic regional hypo-perfusion and an increase in amyloid β ($A\beta$) production. In inflammation and chronic hypo-perfusion these effects could be increased, thus accelerating the neurodegenerative process and the vicious cycle continues [6]...

vi. Role in diabetes

Hyperglycemia favors the induction of iNOS expression thus increasing NO concentration. Also it is characterized by an increased production of superoxide. This makes the ideal situation for the transformation of NO to peroxynitrite with all its deleterious effects, especially on β cells in the pancreas (that secrete insulin) and on endothelium of cardiovascular system. Most diabetics suffer mostly from microvasculature problems that lead to diabetic complications: nephropathy, neuropathy and retinopathy. The production of peroxynitrite is believed to be on the basis of these micro-vascular problems. In other words NO has a role in the pathogenesis of diabetes and its complications [9].

vii. Role in immunity

NO is produced by phagocytes (cells that ingest harmful foreign bodies) as antimicrobial agent. Large amounts of NO (200-1000 nM) produced by iNOS react with superoxide (produced also by phagocytes) in order to form peroxynitrite and other RNSs that kill the pathogens by interacting with its enzymes, plasma membranes and DNA [10,128]

In summary, the role of NO in different parts of our body depends on its level as illustrated in **Figure 31**:

- 1) At physiological concentrations (low levels of NO), NO principally binds to heme proteins such as soluble guanylate cyclase (sGC) on which it exerts its beneficial activity, also it exerts its antioxidant properties.
- 2) Intermediate levels of NO stimulate wound healing processes. NO targets are principally non-heme iron and thiols thus promoting tissue repair by increasing anti apoptotic and progrowth responses, as well as increase immunosuppressive and angiogenic factors.
- 3) High levels of NO, produced by inducing iNOS, exert anti-pathogenenic and antitumor effects. This level of NO is anti-proliferative and causes cell cycle delay. Prolonged NO exposure can cause apoptosis and increases in RNS levels and damage proteins, which result in nitrosative stress [8]. (**Figure 31**)

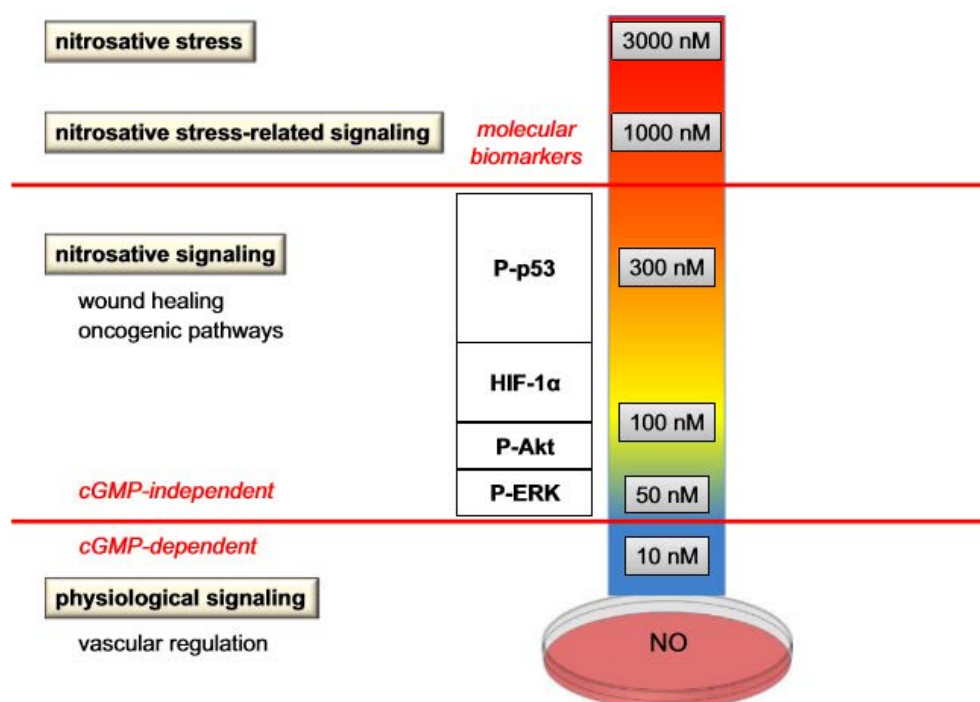


Figure 31: NO biological actions correlated with its concentration and molecular mechanisms. Adapted from [8]

5) Targets of NO in biological system

There are three main targets of NO in biological systems: 1) metals / metalloproteins, 2) Low molecular weight cellular targets (especially free radicals) and 3) thiols / thiol proteins [129].

i. Reactions of NO with metals / metalloproteins

The basis of NO binding to metals is simple addition. NO is an amphoteric ligand, this means it can bind as NO^+ (isoelectronic with CO thus having linear bond) or as NO^- (isoelectronic with O_2 thus having bent bond) [130]. The nature of the bond between NO and the metal and the reactivity and the kinetics of the nitrosyl complex, depend on the energy in the d-orbital of the metal. This energy is determined by the nature and oxidation state of the metal, the conformation and the ligands binding to the enzyme containing the metal ion, as well as the coordination number of the metal [129,131]. For example, NO has higher affinity for the ferrous heme (II) than ferric heme (III) [11,132].

The most famous target is the ferrous-heme in the soluble guanylate cyclase (sGC). When the ligand's binding site in the ferrous heme is occupied by NO, a conformational change in the sGC is induced. This conformational change is propagated into the catalytic side of the enzyme thus triggering the transformation of GTP to cGMP, which will lead further to muscle relaxation (Figure 32).

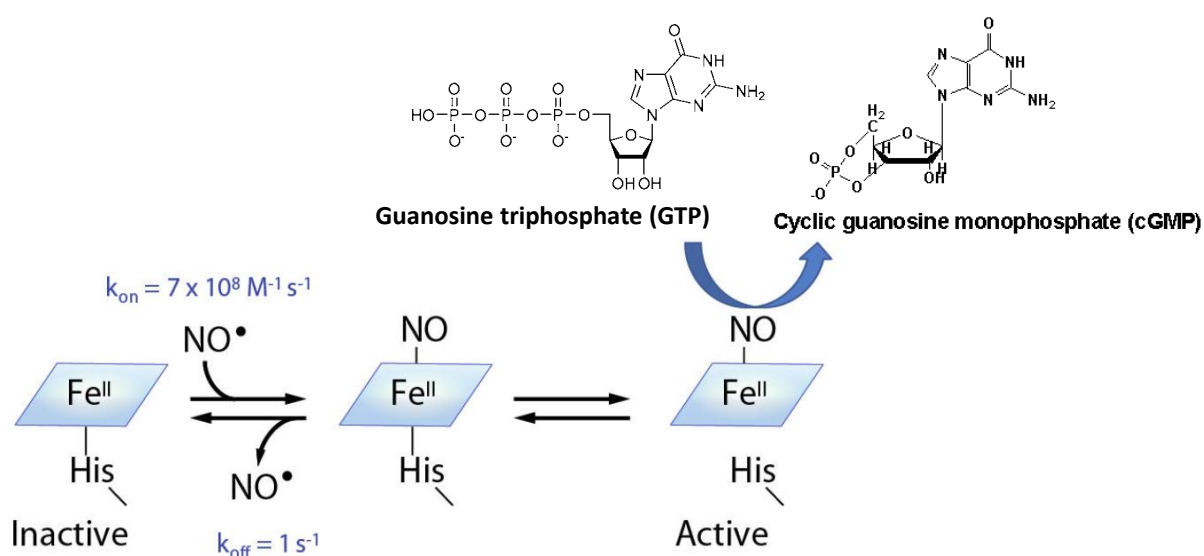


Figure 32: Classical single site model of sGC activation by $\text{NO}^\bullet$. This view is currently being substituted by more complex models to account for the properties of the pentacoordinated-NO complex, which is strongly affected by the availability of ATP, the GTP substrate, or excess $\text{NO}^\bullet$ [11].

NO can inhibit the activity of many metalloenzymes such as cytochrome P450 [133], cytochrome oxidase (responsible for mitochondrial oxygen consumption), nitrile hydratase and catalase (responsible for H_2O_2 breakdown) ([132,134] and references therein). Another famous activity of NO on metalloproteins is related to the heme (in hemoglobin) where it can bind the oxygenated (HbFe(II)O_2) and the deoxygenated form (HbFe(II)). NO binds the heme HbFe(II) with a higher affinity than O_2 . The binding of NO to Fe (II) of the heme is favored in deoxygenated state (T) rather than the oxygenated state (R) (**Figure 33**). Conversely, the binding of NO to Cys β 93 of Hb is favored in the oxygenated state (R) rather than the deoxygenated state (T) [135]. In lungs when Hemoglobin is oxygenated, it undergoes allosterical modification and transforms from T form (deoxygenated state) into R form (oxygenated state). This transformation promotes the transfer of NO^+ from the heme to the thiol on the hemoglobin thus forming RSNO particularly Cys β 93 of Hb. When blood goes into the tissue, hemoglobin deoxygenates or undergoes heme-oxidation, this makes the HbSNO unstable and promotes the transfer of NO^+ from hemoglobin into a receptor cysteine in the cytosolic N-terminus of the membrane-spanning anion exchange protein 1 (AE1), this transfer is called transnitrosation (**Figure 34**). Then this NO^+ is exported from the red blood cell by one of two possible processes: either direct cell-cell transfer or the formation of S-nitrosoglutathione or S-nitrosocysteine. This process is important in providing a rapid response of blood supply depending on metabolic needs [1] (**Figure 33**)

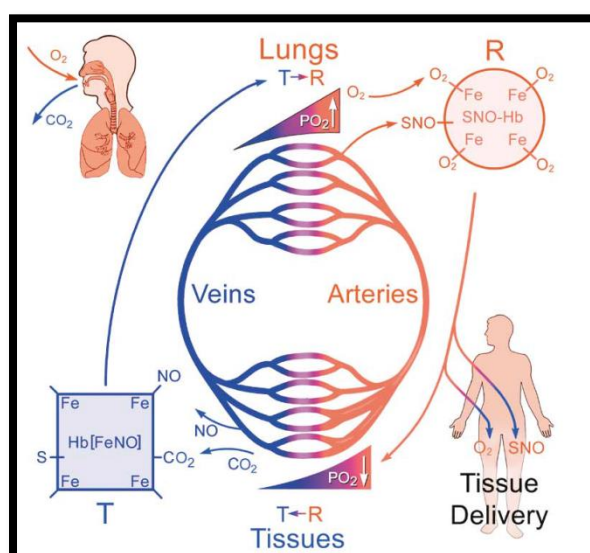


Figure 33: Variation of nitrosylation between SNO:FeNO based on the oxygenation level of Hb [135]

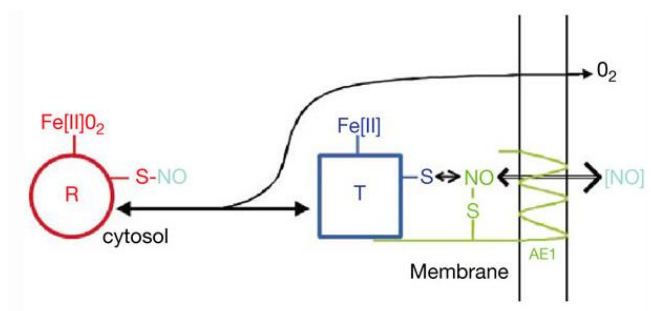
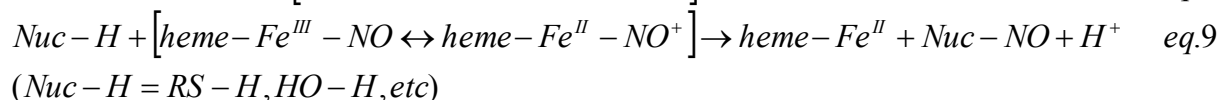
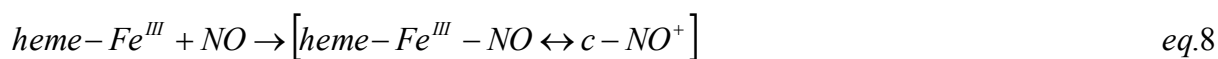


Figure 34: AE 1 transport system. Left part is inside RBC and right side in plasma. Adapted from [136]
 NO can also bind to methemoglobin (ferric (III) hemoglobin). When binding Fe(III), NO is partially positive, this means it has the form Fe(II)-NO⁺ which is linear. Hb in this case undergoes reductive nitrosylation during which it is reduced into HbFe(II) while NO is oxidized into nitrite by the attack of nucleophilic species such as water or into RSNO by the attack of a thiol (eq. 8-9) [129,130].



NO can also bind to ferric and ferrous myoglobin in the same manner as hemoglobin. It can also bind to non-heme iron (e.g. iron-sulfur proteins and ferritin) forming dinitrosyl iron complexes (DNIC)[Fe(NO)₂(X)₂]⁻ [137] that may act as reservoirs of NO bioactivity [1] **(Figure 35)**

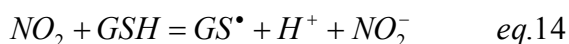
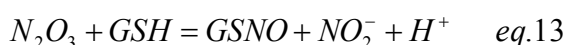
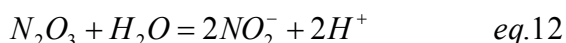


Figure 35: Generic structures of dinitrosyl iron complexes (DNIC). DNIC are formed primarily from the chelatable iron pool (CIP) [11].

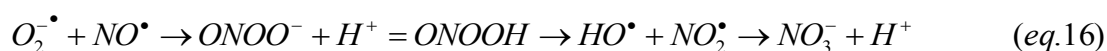
NO can bind also to metals other than the iron such as manganese and cobalt [131]. These metals exist in metalloproteins.

ii. *Reactions of NO with low molecular weight chemicals*

Oxygen gas is paramagnetic, it has two unpaired electrons in the ground state making it vulnerable to reaction with NO; this reaction is called auto-oxidation of NO. This reaction is somewhat unusual because it is second order in NO and third order overall ($-d[NO] / dt = 4k_{aq}[NO]^2[O_2]$ with $k_{aq} = 2 \times 10^6 \text{ M}^{-2} \cdot \text{s}^{-1}$ in aqueous solution at 25°C) [138], thus in the presence of high amounts of NO the half-life of NO is very short [139]. The end product of the reaction is nitrite, but the reaction has many steps during which there is formation of unstable compounds such as nitrogen dioxide NO_2 and dinitrogen trioxide N_2O_3 [139,24]. (eq. 10-11). NO_2 and N_2O_3 are on the basis of the formation of RSNOs in biological system (nitrosation). They can nitrosate thiols more effectively than NO that is relatively inert (eq. 12-15). In biological medium this reaction of N_2O_3 with thiol (eq. 13) is most likely to occur in the hydrophobic regions of the cellular membrane where it is 300 times faster than the surrounding hydrophilic medium and accounts for more than 90% of NO oxidation [140].



NO can react spontaneously and rapidly with the superoxide radical to form peroxynitrite [141]. This reaction competes with superoxide dismutase in eliminating superoxide [142]. The peroxynitrite formed can decompose to hydroxyl radical and nitrogen dioxide radical NO_2 [143]. Hydroxyl radical can react again with NO_2 to yield nitric acid but this reaction is less likely to occur because the rate is much slower than other reactions of hydroxyl radicals (eq. 16).



NO can also react with hydroxyl radical resulting in nitrous acid (HONO) that undergoes further transformation to nitrite [144]. Beside this, it can react with carbon centered radicals ($R^\bullet$) to give the corresponding C-nitroso compound ($R-NO$) [145]. Moreover NO can react with lipid peroxidation products (Lipid radical and lipid peroxy radical) thus terminating the process of peroxidation. This process of protection has been seen in macrophages [146] and the

endothelium [147] to protect from the formation of plaques in atherosclerosis. NO has shown to be more protective of lipid peroxidation than α -tocopherol (vitamin E) [148].

Figure 36 summarizes the reaction that NO can make in its cationic, neutral and negative forms.

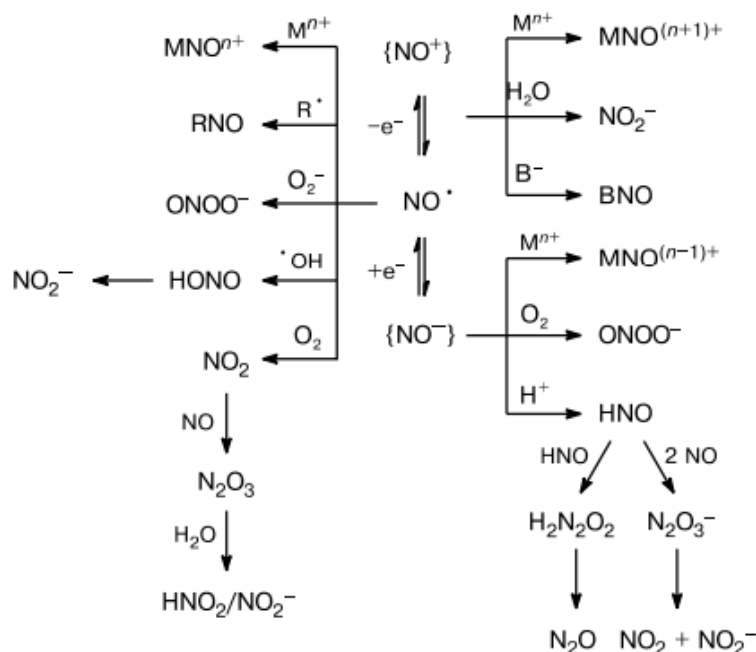


Figure 36: Possible reactions of NO^+ , NO and NO^- . Adapted from [144]

iii. Reaction with thiols:

The transfer of NO^+ to the thiol of an amino acid (usually cysteine) in a protein is named S-nitrosation; while if the addition was of NO it is called nitrosylation [145]. S-nitrosylation plays an important role in signal transduction pathways [149-151]. There are many pathways that can lead to the formation of RSNOs, most of them occur after the formation of a nitrosating molecule derived from NO interactions with O_2 , oxygen peroxide or metal centers. This will be discussed in detail in section 1). Here we are interested in the direct interaction of NO with thiols. Despite the fact that many researchers do not think there is direct reaction between thiol and NO, Gow et al [152] have proposed the formation of GSNO from GSH and NO in the presence of an electron acceptor which can be O_2 (eq. 17-19). Kolesnik et al [14] proved that this reaction is the major pathway for formation of GSNO at submicromolar concentrations of NO. At first GSH reacts with the free NO to form aminoxyl radical (GSNOH) (eq. 17), then an electron acceptor such as NAD^+ or O_2 can transform it to GSNO (eq. 18-19). This is a first order reaction in contrast to other S-nitrosation reactions [12].



B.NO-donors drugs

NO gas is very difficult to handle because it can transform into noxious NO₂ in presence of small amounts of O₂ (thus a complete exclusion is demanded). Nevertheless it is used in some particular cases for treatment of pulmonary hypertension in adults [153] and in neonates [154] where it is delivered via inhalation. In order to benefit from NO effects, numerous NO-donor drugs have been synthesized. These NO-donors should stabilize the radical NO until it is needed to exert its effect. These NO-donor drugs are designed to release NO in biological system by various mechanisms and they can be divided into several categories depending on their structural formula: organic nitrates that are already in use for different cardiovascular treatments, iron-nitrosyl complexes, sydnonimines, c-nitroso compounds, furoxans and secondary amine / NO complex ions. We will describe in the following section each family and its use [107].

1) Organic nitrates (RONO₂s)

Organic nitrates have been used for years (since 1879) and they are the most used drugs in the treatment of cardiovascular diseases such as angina pectoris [155]. The most famous clinically used drug is nitroglycerine or glyceryl trinitrate (GTN) (see section 1)), there is also isosorbide mono- and di-nitrate, triethanolamine trinitrate biphosphate and pentaerythrityl tetranitrate [156] (**Figure 37**). They can decompose to release NO by enzymatic or non-enzymatic mechanisms. They decompose in alkaline pH but are stable in slightly acidic medium, which is a major difference between them and NONOates [156]. Their principle of action is the vasodilation of the coronary arteries of the heart thus relieving angina pectoris [107]. In addition they inhibit platelet aggregation and improve the left ventricular function in patients with congestive heart failure [157]. They can be divided either into short-acting or long-acting nitrate preparations. Short acting nitrates (sublingual or spray nitroglycerine) have fast onset of action (1-5 min), and are thus suitable for immediate relief of effort or rest angina. GTN and isosorbide dinitrate exist in both long acting and short acting formulations. Long acting nitrates

(eg, isosorbide dinitrate, mononitrates, transdermal nitroglycerin patches and nitroglycerin ointment) have onset of action 20-60 min and are used for prevention of recurrent angina [158]. GTN is mostly used to relieve the pain of acute angina while isosorbide mononitrate is slow releasing and is used for chronic angina. Isosorbide dinitrate was developed in order to treat heart failure in African Americans (specific to a racial group) [159]. GTN ointment is used for anal fissures [160]. The main problem with long acting nitrates preparations in therapeutics of prophylaxis of angina is the tolerance [161]. The patient should have a nitrate-free period (which is usual at night) in order for the drug to be effective next day [155].

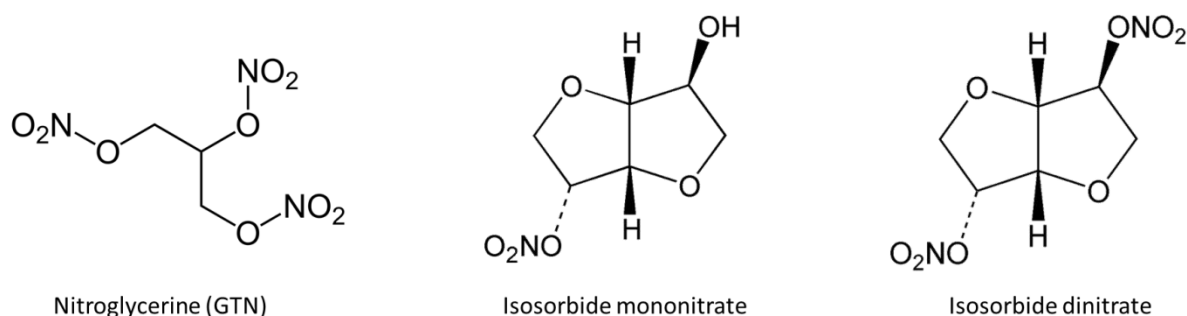


Figure 37: structure of organic nitrates.

2) NONOates (Diazeniumdiolates):

The NONOates represent another family of NO donors of general formula presented in **Figure 38**. They store NO and release it in a pH dependent manner without need for light or redox activation [107], in contrast to organic nitrates they decompose at acidic pH [156]. Their release is not controlled by enzymes or catalyzed by thiols or biological tissue unless specifically designed to [119]. They have many biological actions such as vasodilation, inhibition of platelet aggregation and inhibition blood coagulation ([119] and references therein). Spermine-NONOate has been shown to have beneficial effects in animal models of erectile dysfunction [162]. DETA NONOate produced an antidepressant-like effect and significantly increased hippocampal neurogenesis in animal models [163]. Isopropylamine NONOate (HNO donor drug) was compared to GTN in terms of vaso-relaxation and anti-aggregatory effect *in vitro*. More specifically in isolated carotid arteries and washed platelets [164]. DEA NONOate has shown also comparable effects to isopropylamine NONOate *in vivo* vasorelaxant activity [165]

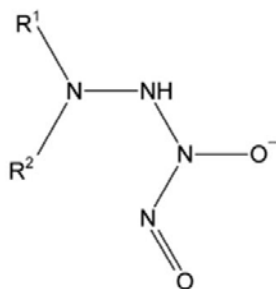


Figure 38: General condensed structural formula of NONOate

3) C-nitroso compounds:

The c-nitroso compounds represent a small family of NO-donor drugs that are not widely used. The nitroso group binds to the carbon of an alkyl or arene to form nitrosoalkanes and nitrosoaranes, respectively. They decompose to liberate NO upon exposure to light [107]. They have been shown to react with hemoglobin, myoglobin and heme-model complexes [166].

4) Iron nitrosyl complexes:

Sodium nitroprusside (**Figure 39**) is used as a medicine for hypertensive crisis because of its potent vasodilator properties. It exerts its action after being reduced by thiol or other reducing agents in the body. It is an NO^+ donor (nitrosating electrophilic species) that can interact with ketones, amines and thiols. It can also decompose using light to release NO [107]. Its major drawback is its need to be administered intravenously, its decomposition once it is in solution by photolysis and its potency which makes dose titration difficult [167].

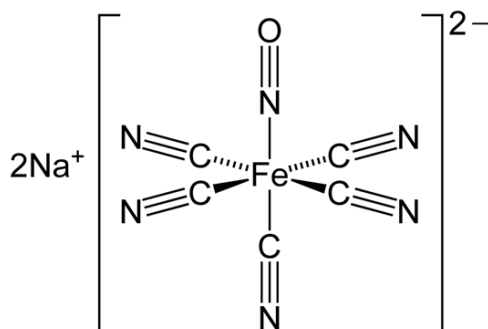


Figure 39: Structure of sodium nitroprussid

5) Furoxans

Furoxans represent an important class of NO donors that is capable of producing high levels of NO *in vitro* and inhibit growth of tumor *in vivo* ([168] and references therein) (**Figure 40**). The thiol-mediated mechanism of generation of NO from furoxans involves the production of nitroxyl (NO^-) followed by the oxidation of nitroxyl to NO [169]. They represent NO-hybrid drug compounds in which two drugs can be associated. They were associated with dihydropyridine (a class of calcium antagonists), $\alpha 1$ and $\beta 1$ antagonists (for vasodilatory cardiovascular effects), histamine H2 receptor antagonist, Proton pump inhibitors (PPIs) and metronidazole (protection against gastric ulcers)...[119]

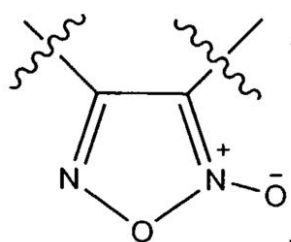


Figure 40: Structure of furoxans

6) S-nitrosothiols

The S-nitrosothiols represent biological reservoirs of NO. Their physico-chemical properties and their biological effects are treated in following section **I.C** in details

7) Other NO-hybrid donors

SNO-captopril, NO-NSAID and many other similar drug combination have been used either to add beneficial effect to the existing drug (additional vasodilation and antiplatelet activities) or to counter balance one of its side effects (NO prevents aspirin related ulcers) ([119] and references therein).

In conclusion the best NO donor in medical practice is the one that can generate NO at the right time and the right bodily location. An ideal polymeric-based NO donor could offer considerable promise for biomedical applications in which local delivery of NO is desired [156]. NO donors that are already used clinically (GTN, Nitroprusside, Isosorbides...) have three major drawbacks: i) they are only considered to have a weak antiplatelet effect at therapeutic concentrations [170], ii) the tolerance caused by long term or high dose use of nitrates [157] and iii) it is uncertain if they can prevent post-operative spasm and thrombosis in the saphenous vein and internal mammary artery grafts, and how long the effect of established NO donor drugs persists [171].

C.S-nitrosothiols

S-nitrosothiols (RSNOs) are the most stable forms of bioactive NO in vivo [172]. There are several pathways for the formation of RSNOs, some of which occurs at physiological and physiopathological conditions. Others are produced at laboratory scale for preparing standards of RSNOs. RSNOs can be divided into low molecular weight (LMW) and high molecular weight (HMW) RSNOs (**Table 3**). Although there is no defined border in terms of molecular mass, it is common to use the term “low molecular weight” for peptides and aminoacid S-nitrosothiols (such as S-nitrosogluthathione (GSNO) and S-nitrosocysteine (CySNO)) and “high molecular weight” for s-nitrosylated proteins (such as S-nitrosoalbumin (AlbSNO) and S-nitrosohemoglobin (HbSNO)). S-nitrosation is very important in proteins in which the thiol is important for activity such as creatine kinase [173], glyceraldehyde-3-phosphate dehydrogenase [174] and glutathione reductase [175].

1) Formation of RSNOs in-vivo

NO can diffuse freely through the plasma membrane due to its chemical properties (uncharged, small gaseous molecule). Once inside the cell it can activate several enzymes or can form nitrosating reagents by the reaction with O₂ or via other catalyzed mechanisms.

S-nitrosylation is classified as a post-translational modification (PTM) of proteins for cellular signaling analogous to phosphorylation, palmitolation, glutathionylation, acetylation and glycosylation [176]. Computational analysis suggests that 70% of proteins can undergo S-nitrosation [177], but only 3000 proteins have been identified experimentally [178]. The exact

mechanism of RSNOs formation is ambiguous, several mechanisms have been suggested to explain the formation of RSNOs in human body such as: i) auto-oxidation of NO followed by addition to thiolate, ii) oxidative nitrosylation (oxidation of thiol of cysteine followed by addition of NO), iii) direct nitrosylation, iv) transition metal ion / protein nitrosation (ceruloplasmine, peroxidase, cytochrome-c-oxidase, hemoproteins), v) transnitrosation, vi) decomposition of low molecular weight dinitrosyl iron complexes (DNICs) with thiolate ligand and vii) nitrite mediated S-nitrosation. These mechanisms have been reported for the formation of both LMW- and HMW-RSNOs [179]. The predominance of one pathway over the other depends on the NO concentration [14] and thus the place where nitrosation occurs [8]. In the cellular membrane and specific proteins, hydrophobicity facilitate the accumulation of NO thus increasing its concentration in these areas[140,180]. Other factor that can play a role is the nucleophilicity of the thiol, which depends on its pKa and on its neighbor amino acids that render it more or less susceptible to nucleophilic attacks by nitrosating species [181]. The availability of O₂ and O₂⁻ also plays a role. Moreover the antioxidant level of the cell can affect the pathways and the formation of RSNOs [182] (**Figure 41**). S-nitrosylation regulates protein interactions, which permits to affect cell signaling functions [176]. It can also change protein activity and subcellular location of target proteins [183].

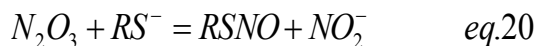
Table 3: classification of different RSNOs

	Natural	Synthetized
Low Molecular Weight (LMW)	-GSNO (S-nitrosoglutathione) -CysNO (S-nitrosocysteine)	-NACysNO (S-nitroso-N-acetyl-L-cysteine) -SNAP (S-nitroso-N-Acetyl-D,L-penicillamine (trtiary structure)) -HcysNO (S-nitrosohomocysteine) -S-nitroso-B,D-thioglucose -S-nitroso-3-mercaptopropanoic acid -S-nitrosomercaptoethylamine -S-nitroso-N-Acetyl-D,L-penicillaminyl glycine methyl ester
High Molecular weight (HMW)	-AlbSNO (S-nitrosoalbumin) (in plasma) -S-nitrosohemoglobin (in erythrocyte)	

i. Auto-oxidation of NO followed by addition to thiolate

As discussed before (section 3)) the auto-oxidation of NO leads to the formation of nitrosating products: nitrogen dioxide (NO₂) and dinitrogen trioxide (N₂O₃) (**eq. 6-7**). The first pathway of

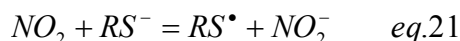
RSNOs formation can be via the reaction of N_2O_3 , which is a potent nitrosating agent, and thiolate ion by transferring (rather than releasing) NO^+ and producing RSNO according to **eq. 20**



The other fate of N_2O_3 is hydrolysis to yield nitrite (**eq. 12**). This pathway cannot be the major pathway under physiological conditions because: i) firstly NO_2 is very reactive radical and it can react with many other molecules other than NO, such as thiols (**eq. 14**). This means that NO concentrations should be high in order to compete with other targets of NO_2 , and consequently N_2O_3 will be formed. Cellular concentrations of thiols are much higher than that of NO. ii) Secondly the reaction between NO, O_2 and RSH gives only 20% RSNOs and leads to formation of disulfides (RSSR). This pathway gives no clear explanation of the formation of this large amount of RSSR [12,182].

ii. Oxidative nitrosylation

In oxidative nitrosylation the thiol is oxidized by NO_2 or by other oxidizing agent ($HO^\bullet$ and $HOO^\bullet$) to give thiyl radical (**eq. 21**). Thiyl radical then reacts with other NO to produce RSNO (**eq. 22**) [182].



The reaction of thiyl radical with NO has several other competing reactions (**eq. 23-26**). The concentration of O_2 plays an important role in favoring the pathway of formation of RSNOs. At low dioxygen concentration, more RSNOs are prone to be formed [182]. Many proteins and enzymes are known to have a cysteine thiyl radical that is necessary to their activity [184], thus adding NO can modify the activity of the enzymes.



iii. *Direct nitrosylation*

This third pathway was already described (iii) and involves the direct addition of NO to the thiol followed by one-electron oxidation by O₂.

Kolesnik et al. [14] have studied the formation of GSNO in vitro and they deduced that its formation by one of the three above mentioned pathways depends on NO and GSH concentrations. At low (micromolar) [NO] and high (millimolar) [GSH] direct nitrosation will outcompete autoxidation, and a yield of 100% GSNO may be expected. Without direct nitrosation GSNO yields are expected to approach 0% under such conditions. When the NO concentration is raised, autoxidation will become more prominent, resulting in a mixture of oxidation and nitrosation with a limiting value of 50% for the GSNO yield at high [NO]. Please note that superoxide is assumed to be scavenged effectively; in the absence of SOD the maximal GSNO yield is expected to be 50% at both high and low [NO] [39].

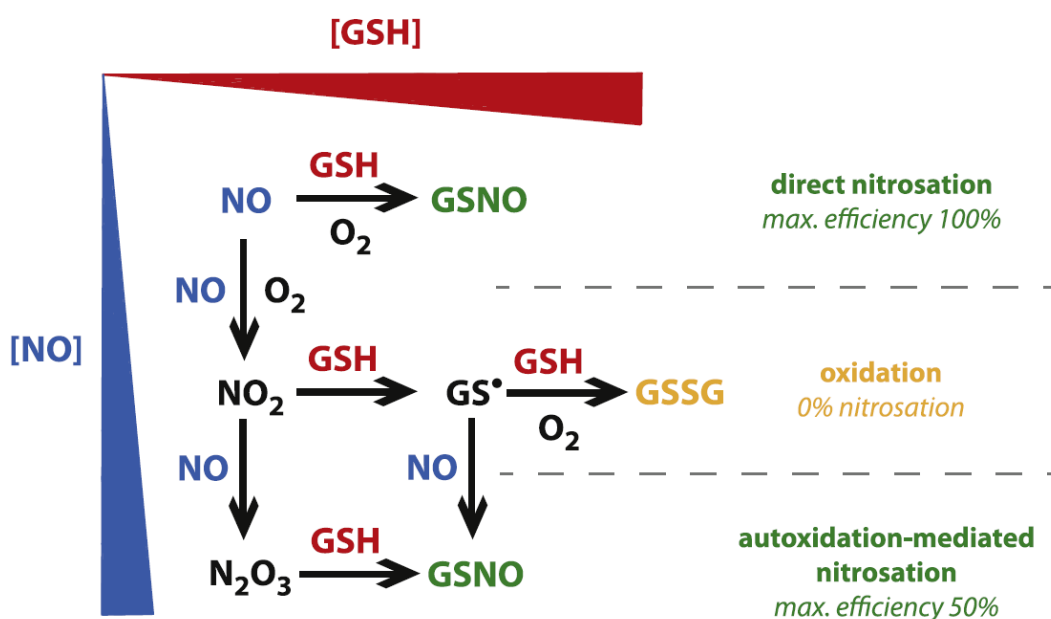


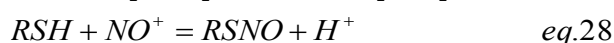
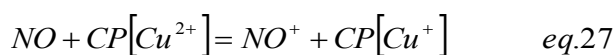
Figure 41: Illustration of the way in which GSH nitrosation and oxidation are expected to depend on the concentrations of NO and GSH. Adapted from [14]

iv. *Transition metal ion / protein nitrosation*

Despite the fact that RSNOs are known to decompose in presence of some metal ions such as copper ions (Cu²⁺), Stabauer et al. [185] reported the formation of AlbSNO and HbSNO starting from Alb and Hb in presence of NO gas and Cu²⁺ but not Fe³⁺. GSNO could not be formed by this method because of dimerization of GSH in the presence of Cu²⁺. The mechanism is similar to oxidative nitrosylation where the metal ion oxidizes the thiol to a thyl radical and then NO

recombines directly with thyl radical to form RSNO [185]. Vanin et al. [186] showed also the catalysis of GSNO and CySNO formation from gaseous NO and the corresponding thiol in presence of ferrous ion (Fe^{2+}). The proposed mechanism was by the formation of nitrosonium ions (NO^+) NO / metal complex and then NO^+ attack the thiol to form RSNO.

Many metalloproteins such as ceruloplasmin, peroxidase, cytochrome c oxidase and hemoproteins were shown to catalyze S-nitrosation. Ceruloplasmin is a metallo-enzyme that contains copper. It exerts its action by making metal / NO complex attracting the electrons from NO to be NO^+ and thus facilitating its transfer to RSH in order to produce RSNO (eq. 27-28). This mechanism occurs at physiological conditions but is responsible only for part of RSNOs formation and cannot be responsible to all RSNOs formation in every part of the body because it is not present inside cells [12]. Ceruloplasmin is important in pathological conditions where there is nitrosative stress and production of large amounts of NO because it helps in NO detoxification and forms a buffer pool of RSNOs [1].



Hemoglobin is a heme-protein, well-known for its nitrosative properties. Its properties of nitroston were described in section (I.A.5.i)

Peroxidases represent potential RSNOs generating enzymes but there is not enough experimental data on them. They act by one of the aforementioned mechanisms (oxidizing thiol then addition of NO, metal / NO complex then transferring NO^+ , or nitrosation by of N_2O_3 after oxidation of nitrite to NO_2 and its combination with NO).

Cytochrome-c was shown to have GSNO synthase activity *in-vitro*. The mechanism of S-nitrosation exerted by this metallo-enzyme is represented in **Figure 42**. The ferric heme center binds to thiol (step a), then this complex is attacked by NO resulting in hydroxyl amino radical complex bound in the vicinity of the heme (step b); This bound radical then reduces ferric heme to ferrous heme and RSNO is formed by electron transfer process (step c). In presence of high NO concentrations, the hydroxyl amino radical complex bound to heme can react with the bound radical to yield GSSG, nitrous oxide and regenerate ferric-cytochrome c (step d) [187].

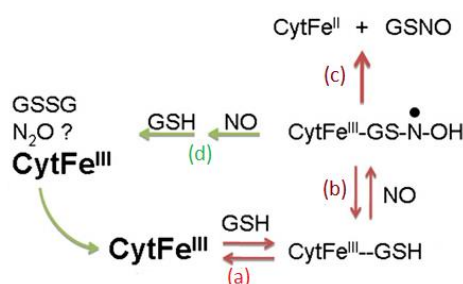


Figure 42: Cytochrome C GSNO synthase activity. Red arrows represent the favored pathway; green arrows represent high NO concentration. Scheme adapted from [187]

v. Transnitrosation

RSNOs do not exert their function only by releasing NO in the vicinity of functional protein, they also act mostly by transnitrosation [13]. Transnitrosation is the transfer of nitroso group from one RSNO to another as shown in **eq. 29**. This reaction involves the attack of the thiolate anion on the nitroso nitrogen. RSNOs can make S-transnitrosation with LMW-RSNOs or the thiol moiety of the cysteine of a functional protein [188]. S-nitrosation is controlled by the i) cellular location of the reaction, ii) the steric hindrance, which is determined by the conformation of the protein involved, iii) and by the pKa of the thiol which controls the nucleophilicity ([13],[12] and references therein). It is a reversible second order reaction [151] and it is not always fast ([12] and references therein). It is responsible for RSNOs activity *in-vivo* [189,190]. Transnitrosation is involved in signal transduction downstream from its site of generation. For example following eNOS activation, it is S-nitrosylated, followed by transnitrosylation of downstream targets such as NOS-interacting protein, tubulin and heat shock protein (Hsp) 90 [176]. These targets interact with one another by NO⁺ transfer.

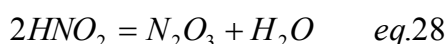


vi. Decomposition of low molecular weight DNICs with thiolate ligand

Di-Nitrosyl Iron Complexes (DNIC's) (**Figure 35**) are suggested to play important role in steady state of SNO in physiological and physiopathological “nitrosative stress” conditions. They are exported from plasma membrane [15]. They can thus contribute to nitrosylation in cell membranes or in plasma. DNIC are composed of clusters of NO with pools of non-heme iron (e.g. Iron-sulfur proteins and ferritin) and they can nitrosate the sulfhydryl groups on proteins by NO⁺ transfer.

vii. *Nitrite mediated S-nitrosation*

Nitrite mediated S-nitrosation occurs under physiological conditions (stomach, asthmatic airways, ischemic tissue (pH5.5)) as well as in laboratory preparations of RSNOs [16]. The principle of this reaction is the protonation in acidic medium of nitrite to give nitrous acid (HNO_2) and subsequently dinitrogen tri-oxide N_2O_3 molecules [191] (eq. 27-28). N_2O_3 is a potent nitrosating reagent that can transfer the NO^+ to the thiol resulting in RSNO (eq. 13)



2) RSNOs trans-membrane trafficking

Although GSNO cannot diffuse through the cellular membrane, it was shown that an increase in GSNO extracellular concentrations leads to an increase in intracellular concentration [192]. This can occur by more complex mechanism through which GSNO or other RSNO make transnitrosation with cysteine in the extracellular environment in order to form CySNO (**Figure 43**). Then CySNO crosses the membrane by the help of a specific transporter system T (L-AT) [192,193]. Once in the cell, CySNO makes transnitrosation with GSH and other thiols to give GSNO and RSNOs, respectively. Subsequently the cascade of the reactions of transnitrosation continues depending on cell type and other factors discussed elsewhere (section I.C.1).v) (**Figure 43**).

Other transport systems have been identified such as erythrocytic anion exchange protein 1 (AE 1) in red blood cells (RBCs) where nitrosohemoglobin (HbSNO) exposes NO to vascular structures through this transport system, which leads to vasodilation [136]. (see section I.A.5).i, **Figure 34**).

Other transport system could be present, but the knowledge about the transport of NO across membrane is still in its infancy [176].

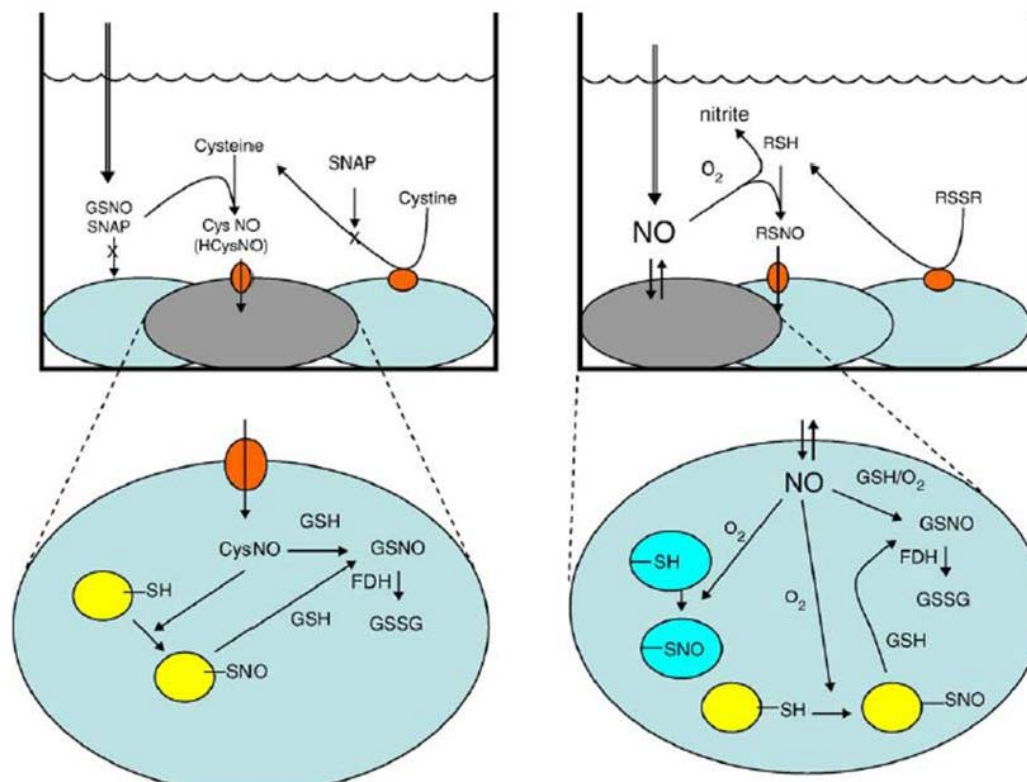


Figure 43: The left graph represents transport and metabolism of RSNOs from extracellular medium to intracellular medium with existing transnitrosation reactions that occurs outside and inside the cell. Only CysNO can transverse the membrane. Inside the cell GSNO is metabolized by many enzymes (formaldehyde dehydrogenase is presented here but it could be others). The right graph represents how NO transverse the membrane and its possible reactions that could increase intracellular RSNOs concentration. SNAP: S-nitroso-Nacetylpenicillamine. It is possible such S-nitrosation occurs at buried sites (blue) as well as exposed sites (yellow) and that the buried RSNOs are poorly reparable by the GSH / FDH system. Adapted from [192]

3) Decomposition of RSNOs

RSNOs are sensitive to decomposition by several pathways. They can decompose enzymatically or by metal ions (Hg^{2+} , Cu^+ , Fe^{2+}), by light (UV, visible, IR), by chemical reduction (ascorbic acid) and by heat. While each of these methods can occur *in-vitro*, only enzymatic, copper and ferrous pathways can occur *in vivo*. Herein the decomposition pathways are described in details [24].

i. Enzymatic denitrosylation

Denitrosylation corresponds to the removal of NO from a protein or peptide. This is important but this aspect of NO-based signaling is not well studied. Denitrosylation i) activates some enzymes such as eNOS, caspase-3 and I κ B kinase- β , ii) inhibits other enzymes, iii) affects protein–protein interactions, as in the case of beta-arrestin, iv) preserves protein or cellular

function in case of nitrosative stress and v) facilitates or inhibit signal transduction ([194] and references therein). Several enzymes control the denitrosylation of RSNOs such as: glutathione peroxidase [195], copper-(I)-dependent enzyme [73], thioredoxine [196-198], protein disulfide isomerase [199,200], GSNO-reductase [201], carbonyl reductase [202], xanthine / xanthine-oxidase [203,204], γ -glutamyl transferase [183] and Cu / Zn superoxide dismutase [205]. Products formed from this denitrosylation reaction vary according to the enzyme; they include alternate RSNOs, oxidized thiol, NO, HNO, peroxynitrite, hydroxylamine and ammonia [176,196,199]. Both cellular location and kinetics are determinant of denitrosylation signaling effects [176].

ii. *Decomposition by metal ions*

Several metallic ions have been shown to decompose RSNOs. Saville [27] was the first to describe RSNOs decomposition using mercuric and silver ions. The mechanism by which mercuric ions decompose GSNO is illustrated in **Figure 44**. The metal ion (here mercury) has a strong affinity to the sulfur, thus it binds to it reversibly forming the complex (I), which is susceptible to nucleophilic attack by water molecules, thus producing nitrous acid on one side and a mercuric-thiol complex on the other side. This reaction laid the foundation for quantification of RSNOs using the so-called “Saville Method”. Swift et al. [206] studied the kinetics of decomposition by mercuric and silver ions. They found that i) it follows first order kinetics with both reactants, ii) there is very little variation in rate constant depending on RSNO structure and iii) mercury (II) nitrate is 1000 times more reactive than mercury (II) chloride over a range of substrates.

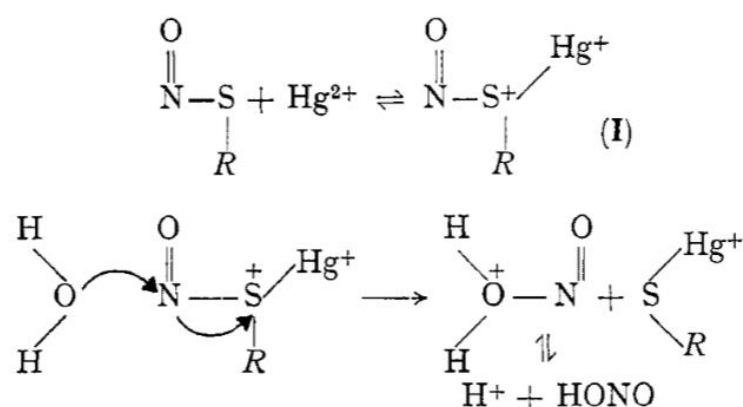


Figure 44: Decomposition mechanisms of RSNOs involving mercuric ions. Adapted from [27]

McAninly and coworkers [207] extensively studied the kinetics of decomposition of RSNOs by different metal ions using spectrophotometry as shown in **Figure 45** for Cu^{2+} . Their results

show that RSNOs decompose in contact with Cu^{2+} and Fe^{2+} more than with Ag^+ , while they do not decompose when in contact with Zn^{2+} , Ca^{2+} , Mg^{2+} , Ni^+ , Co^{2+} , Mn^{2+} , Cr^{3+} or Fe^{3+} . Other researchers have shown that RSNOs can decompose by Cu^{2+} and Fe^{2+} [124,208]. Decomposition involving Cu^{2+} is first order $m=k[\text{Cu}^{2+}][\text{RSNO}]$ [24]. EDTA (Cu^{2+} and Cu^+ chelator) addition has been proven to block this reaction. Neocuproine (Cu^+ selective chelator) also blocks the reaction [124,209-211], while cuprizone (Cu^{2+} selective chelator) does not block the reaction [209]. This shows that the reaction is due to Cu^+ and not to Cu^{2+} [210,211].

Mercuric decomposition was more rapid and gives directly NO^+ while Cu^{2+} gives NO (eq. 32). The pH dependence is very unusual and may be due to equilibria with ligand replacement [212]. For example Cu(II) is usually hexahydrated $[\text{Cu}(\text{OH}_2)_6]^{2+}$ but in basic medium it can form $[\text{Cu}(\text{OH}_2)_5\text{OH}]^+$ and $[\text{Cu}(\text{OH}_2)_4(\text{OH})_2]$. The pH dependence can also affect GSH (pI (GSH / GS^-) = 5.93). Free hydrated Cu^{2+} is not the only source of Cu^+ ; bound Cu^{2+} to amino acids, peptides and proteins can also be a possible source because in biological medium the concentration of free Cu^{2+} is very low.

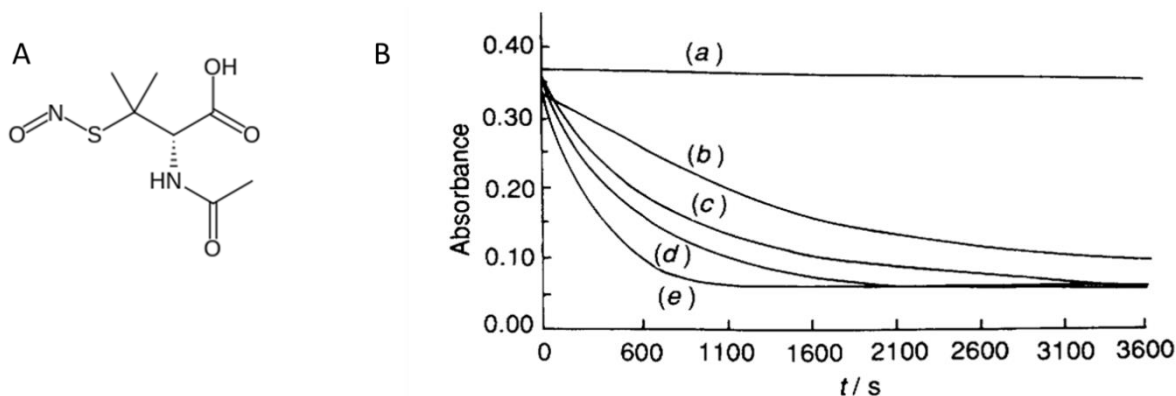


Figure 45: (A) Structure of SNAP. (B) Absorbance time plots for the decomposition of SNAP ($5 \times 10^{-4} \text{ mol.dm}^{-3}$), (a) no added Cu^{2+} , (b) $[\text{Cu}^{2+}] 1 \times 10^{-5}$, (c) $[\text{Cu}^{2+}] 5 \times 10^{-5}$, (d) $[\text{Cu}^{2+}] 1 \times 10^{-4}$ and (e) $[\text{Cu}^{2+}] 5 \times 10^{-4} \text{ mol.dm}^{-3}$. Adapted from [212]

The decomposition of RSNOs by Cu^+ is regulated by three factors: i) the concentration of copper ions, ii) the concentration of thiols and iii) the concentration of RSNOs.

Cu^{2+} ions undergo two principle reactions with thiols:

-in the presence of high thiol concentration the dithiolate complex (**Figure 46**) formation reaction (eq. 33) is more dominant than the reaction of reduction to Cu^+ (eq. 34) [213].

-In the presence of low concentrations of thiol the reduction is more efficient than complexation.

The ratio Cu^{2+} / RSH plays an important role in the decomposition. So the effect of added thiols can lead either to stabilization of the RSNOs or acceleration of the decomposition. In addition, as the concentration of copper increases, the reaction becomes much faster, and induction periods (period for the reaction to start) progressively disappear [214].

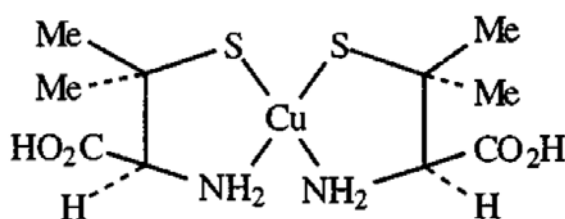
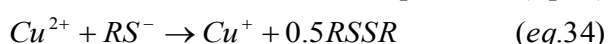
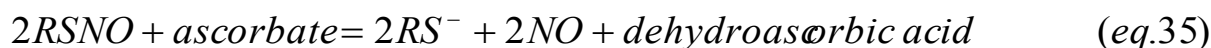


Figure 46: Copper dithiolate complex. Adapted from [24]

The increase of some RSNOs concentration can lead to decrease of their decomposition by copper. This stabilizing effect is due to the complexation of the copper by the oxidized thiols thus playing the role of EDTA. GSSG is well-known to exert this action [215].

iii. Decomposition by ascorbate

RSNOs can be decomposed by ascorbate. The mechanism at physiological pH depends on the concentration of ascorbate. The physiological concentration of ascorbate varies and depends on the food intake. Normal physiological concentration of ascorbic acid is between 34 and 144 μM in plasma and between 100 and 500 μM in brain [216]. It can be higher than 1mM at pharmacological physiological concentrations after vein infusion [217]. At low concentrations ($<10^{-4} M$), ascorbate plays the role of reducing agent of Cu(II) to Cu(I) then Cu(I) decomposes RSNOs as discussed above. At high concentration ($10^{-3} < [ascorbate] < 10^{-1}$) the ascorbate is a nucleophile that attacks the nitrogen of the nitroso group in RSNOs leading to nitrosated ascorbate (O-nitrosoascorbate) and thiol (RSH). O-nitrosoascorbate then decomposes, by free-radical pathway, to give dehydroascorbic acid and NO according to eq. 35 [218,24]

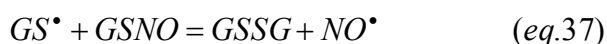


The reaction between ascorbate and RSNOs at physiological pH is very slow and the kinetics depend on the RSNO and on the concentration of ascorbate [28,219]. For example, with 1 mM ascorbate, the half-life is 230 min for GSNO and 3530 min for SNAP [28]. The reaction follows second order kinetics and the rate constants are in the order $GSNO > SNAP > AlbsNO$ [28]. The rate constant of the reaction varies sharply with pH [47]. As the pH increases, the nucleophilic attack of ascorbate becomes faster, this is simply due to the nucleophilicity of ascorbate. Ascorbic acid can be monoanionic or dianionic depending on pH (pKa values 4.25 and 11.75) [47]. The rate constant increases $\sim 10^6$ times as the pH increases from 3.6 to 13.6 (**annex 1**) [220]. At physiological pH, the rate constant is $11.5 \times 10^{-3} \text{ L.mol}^{-1}\text{s}^{-1}$.

iv. *Decomposition by light*

RSNOs absorb in the visible (450-550 nm) and UV (330-340 nm) corresponding to $n-\pi^*$ and $\pi-\pi^*$ transitions, respectively [79,221]. The extinction coefficients are small and the color of RSNOs can vary from green to orange / pink depending on the dihedral angle made by the S-N bond [221]. For example, GSNO is a pink solid at room temperature with extinction coefficients at 340 and 545 nm of 922 and $15.9 \text{ cm}^{-1}.\text{mM}^{-1}$, respectively [222].

RSNOs undergo hemolytic cleavage by ultraviolet and visible light to produce thiyl ($GS^\bullet$) and NO radicals [24] (**eq. 36**). Sexton et al. [43] have described the kinetics of light decomposition as a first order kinetic with $k_{\text{obs}} = (4.9 \pm 0.3) \cdot 10^{-7} \text{ s}^{-1}$. This thiyl radical either reacts with GSNO to produce NO, or it can react with O_2 to give peroxyradical that can produce NO after reacting with GSNO (**eq. 37-39**) [24].

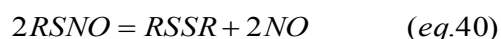


Light (photolysis) is an alternative method used for the decomposition of RSNOs, without the necessity of addition of external reagent to the sample such as copper or ascorbic acid. Usually photolysis is coupled to chemiluminescence and electrochemical detection to detect RSNOs [40,84,223]. A filter that prevents light below 300 nm from hitting the sample is usually used in order to avoid NO formation from nitrate, also the lamp power should not exceed 100 W because heat generated from the lamp can result in spurious signals [85]. The decomposition using ultraviolet light has been shown to overestimate NO concentrations due to generation of

NO from nitrate facilitated by reduced thiols [83]. The sensitivity is proportional to the intensity of the light [40]. HMW-RSNOs such as AlbSNO are more stable to light decomposition than LMW-RSNOs such as GSNO and CySNO. Also, GSNO is more stable than CySNO [40]. Sexton et al. have studied the kinetics of light decomposition and found it of first order with $k_{obs} = (4,9 \pm 0,3) \cdot 10^{-7} \text{ s}^{-1}$ [43]

v. Decomposition by heat

RSNOs are thermo-sensitive. In absence of light and in presence of metal chelators, they continue to decompose. This can be attributed to decomposition by heat. Heat decomposition involves hemolytic cleavage to produce NO and GS* radicals [24,44,224] and thus equations (eq. 36-39) occurs. Conversely, Singh et al. [45] reported that, in the absence of light, the decomposition doesn't involve thiyl radical formation (RS*). The overall equation is presented in eq. 40



4) RSNOs in health and disease

i. RSNOs therapeutic effects

RSNOs play important biological roles in every part of the body. They have similar biological roles as NO but not the same. In the cardiovascular system they have been shown to be potent vasodilators [225] and selective inhibitor of platelet aggregation [226]. In respiratory system S-nitrosylation is involved in every single aspect of lung cell-biology. It has effect on i) gene expression, ii) protein expression, folding and degradation and iii) protein activity and function. It regulates through these effects, cellular replication, ion-channel activity, cell-cell signaling, host defense and apoptosis ([13] and references therein). They can act through an NO-dependent pathway by releasing NO or in NO-independent pathway by S-transnitrosation [2,12,192]. (

Table 4)

GSNO has been examined or as a therapeutic for several conditions. It has been administered as an intravenous infusion [227,228], subcutaneous microparticules [18], aerosol [229], polymer nanocomposite particles for oral delivery [230], topical cream [231], polymeric vaginal films [232] and poly vinyl alcohol cream [233]. GSNO was used for its cardiovascular

beneficial effects (treatment of stroke [18,19],inhibition of platelet activation in coronary angioplasty [20] and antithrombotic effects ([234] and references therein). It improved hemodynamics in preeclampsia [227]. Topical treatment with GSNO of intracellular Leishmania major ulcers in BALB / c mice suppressed lesion growth, reduced the parasite load and induced healing [231]. Topical treatments also have induced healing of ischemic wounds [21]. Indeed it helps in treating onychomycose due to its antifungal properties [22]. GSNO has been shown to be a more potent vasodilator than theophylline [235]. It shows benefits in many respiratory diseases such as asthma and cystic fibrosis [229]. It was also able to prevent experimental cerebral malaria ECM at lower doses with minimal side effects than an NO donor, dipropylene triamine-NONOate (DTPA-NO) [236]. It was also used for female arousal sexual disorders where they enhanced the blood infusion in female rat models [232]. Moreover, GSNO showed therapeutic potential for Th17-mediated autoimmune diseases, where it was efficient in an animal model of multiple sclerosis after an oral administration [237].

Table 4: Nitrogen oxides in respiratory biology adapted from [13]

Respiratory effect	NOS involved	RSNO signaling involved	Human RSNO studies performed	NO radical involved independently of RSNO
Respiratory control	Yes	Yes	No	Opposite effect as that of RSNO
Ventilation-perfusion matching	Yes	Yes	Yes	Presumed
Pulmonary hypertension	Yes	Yes	Yes	Presumed
Human airway smooth muscle tone and asthma	Likely	yes	Yes	Unlikely, although NO radical is a biomarker
Cystic fibrosis	Likely	yes	Yes	Only as a substrate for denitrification
Adult respiratory distress syndrome	likely	likely	No	Opposing effects

Nitrosoalbumin (AlbSNO) has also been tested via intravenous injection for its cardiovascular effects especially protection of ischemic tissue injury ([238] and references therein). It showed more sustainable lowering of blood pressure than nitrates. It delays template bleeding time that is associated with inhibition of platelet aggregation [239]. Local administration of AlbSNO

inhibits intimal proliferation and platelet deposition following balloon injury to the rabbit femoral artery [240]. Intra-peritoneal injection of AlbSNO in model animals has shown promising results for the treatment of acute lung injury in sickle cell disease. It prevents thrombus formation and reduce hypoxia-induced consequences [238].

S-nitrosohemoglobin (HbSNO) has been suggested to be a blood substitute in a cell-free SNO-hemoglobin containing solutions that mimics blood because it has shown antihypertensive properties compared to Hb infusions [241]. In sickle cell anemia, S-nitrosation of Sickle-cell hemoglobin (HbS) has shown to increase O₂ sensitivity and to lessen vasoconstriction and thrombosis, thus countering sickling. Supplementing the patient with S-nitrosated RBC could help in acute phases [242,1].

In order to benefit from these effects of RSNOs, future work should be done to minimize side effects due to non-specificity of RSNOs delivery. This could be done by using specific formulations for targetting to specific organs or limiting its access to central nervous system. The chemical stability can be changed by fabrication of synthetic RSNOs which have secondary or tertiary, rather than a primary carbon structure. Therapies using RSNOs are promising future interests especially as they avoid the tolerance induced by nitrates especially in cardiovascular therapeutics.

ii. RSNOs as diagnosis indicator

The variation of the concentration of different RSNOs in biological fluids can lead and / or be a marker of some diseases. The degree of S-nitrosation can increase or decrease depending on the disease. For example, in systemic sclerosis and in Raynaud's disease patients, the amount of RSNOs in plasma was less than in healthy volunteers [172], while in pre-eclampsia patients the amount of RSNOs was higher in patients than in healthy volunteers [72]. Cerebrospinal fluid of patients with multiple sclerosis have elevated RSNOs compared to their respective controls [243]. Indeed the same effect is present in airway lining fluid in asthmatics treated with budesonide [244]. More examples are given in **Table 5**. It is worth mentioning that RSNOs, RNS and NO do not have always same effect and correlation in disease. As a conclusion the amount of different RSNOs species should be monitored as a key in the diagnostic of several diseases.

Table 5: Elevated and depressed levels of RSNOs in human diseases. Adapted from [1]

	Disease	Finding	Suspected mechanism
Elevated	Arthritis	SNO's in joints	Local NO production
	Bronchopulmonary dysplasia	Lung SNO-proteins	Induction of iNOS
	Diabetes ^(a)	SNO-Hb (RBC)	Defective release
	Inflammatory lung disease	SNO's in exhaled-breath condensate	Unknown
	Multiple sclerosis ^(a)	Anti-SNO-Cys antibodies	Unknown
		SNO4s in cerebrospinal fluid	Induction of iNOS
	Pre-eclampsia	SNO-albumin in plasma	Impaired SNO release
	Sepsis ^(a)	SNOs in RBCs, plasma	Induction of iNOS
	Tuberculosis ^(a)	Plasma SNOs	Macrophage production
	Hypercholesterolemia	Plasma SNOs	Increased synthesis in plasma
	Pneumonia	Airway SNOs	Induction of iNOS
	Stroke	S-nitrosylation of MMP-9	Activation of nNOS, NMDA receptors
Depressed	Abnormal perinatal transition	Umbilical arterial plasma SNOs	Unknown
	Asthma	GSNO in airway lining fluid	Accelerated metabolism
	Cystic fibrosis	GSNO in airway lining fluid	Decreased NO / GSH substrates
	Hypoxemic neonates	Tracheal SNOs	Synthetic defect
	Pulmonary hypertension	SNO-Hb (RBC)	Low O ₂ saturation
	Pre-term birth	SNO-proteins in lung	Decreased NOS expression

^(a): disease for which an animal model produced similar findings.

II. Methods of quantification of RSNOs

The ideal method to quantify RSNOs is a method that i) has a LOD in the nanomolar range in order to detect LMW- RSNOs and in the micromolar range in order to HMW-RSNOs, ii) is selective to NO (no detection of nitrite or other biological molecule), iii) that does not under- or over-estimate RSNOs because of matrix or other effects, iv) can detect online the progression of NO variation and v) can be potentially implemented into the body for in-vivo detection.

A. Sample pretreatment

Sample pretreatment plays a key-role in quantification of RSNOs. Several precautions should be taken in order to prevent RSNOs decomposition and thus loss of NO [245]:

- i) Samples should be handled with N-ethylmaleimide (NEM), or methyl methanethiosulfonate (MMTS) [246], or iodoacetic acid (IAA) [247,248] in order to block the thiol groups (SH) and prevent artificial S-nitrosation that could occur in the presence of nitrite in acidic medium or transnitrosation (between AlbSNO and GSH for example) ([245] and references therein, [246])
- ii) Metal chelators such as ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) should be used in order to prevent decomposition of RSNOs by metal ions normally present in biological fluids such as Cu^{2+} and Fe^{2+} ([245] and references therein)
- iii) Inhibitors of γ -glutamyl transferase (GGT) such as serine / boric acid complex should be used because this enzyme has been shown to decompose GSNO ([245] and references therein)
- iv) In order to avoid light and heat decomposition, samples should be treated in chilled tubes ($2-4^{\circ}\text{C}$) and be handled in the dark ([245] and references therein).

B. Direct vs indirect methods

Several chemical and biochemical methods have been developed since the 1990's for RSNOs quantification [2]. They can be classified as direct and indirect methods. In the direct methods, the RS-NO bond is conserved, while in the indirect methods, byproducts are detected after RS-NO decomposition (**Figure 47**).

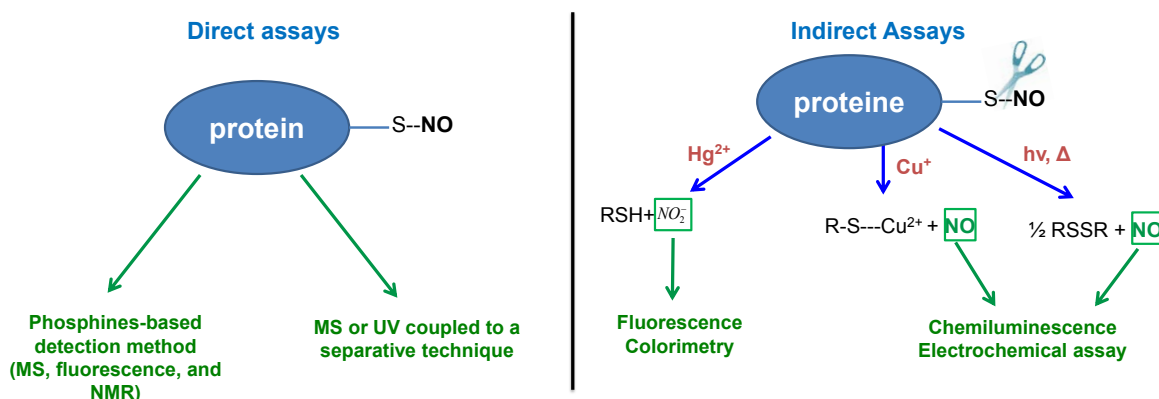


Figure 47: strategies for direct and indirect detection methods

1) Direct Methods

i. Phosphines-based detection method

The method is based on the detection by fluorescence, ^{31}P -NMR or HPLC-MS, of the end product resulting from the reaction between RSNOs and phosphines (PR_3) (**Figure 47**). Several types of the reaction between RSNOs with PR_3 have been reported in the literature [30,249-253]. The basis of these reactions is that RSNOs react with PR_3 to produce reactive S-substituted aza-ylides ($\text{RS-N}=\text{PR}_3$) preserving the original S-N bond linkage of the RSNO that undergoes further reactions to give a stable RSNO-based adducts [5]. Among these reactions, only the Staudinger ligation sequence (**Figure 48**) subsequent chemistry does not damage the S-N bond so it is considered as the direct labeling, other approaches form indirect labeling after destruction of S-N bond linkage [5]. These molecules are highly selective to SNO bond and do not react with thiols. Many limitations face these methods such as cross-reactivity between RSNO and nitroxyl (HNO) and the stability of the aza-ylide depending on the corresponding RSNO [254].

Staudinger Ligation:

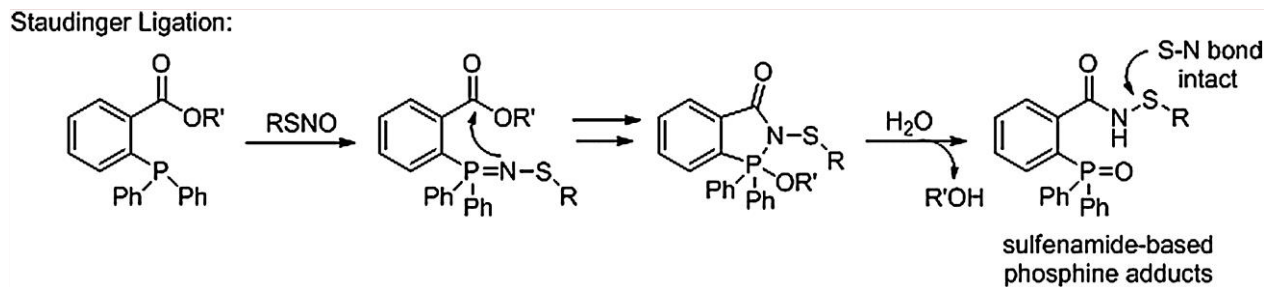


Figure 48: Staudinger ligation principle. Adapted from [5]

2) Indirect methods

Indirect methods involve the decomposition of RSNOs followed by the detection of i) nitrite by spectrophotometry after reaction with the Griess reagent (sulfanilamide with N-(1-Naphthyl)ethylenediamine) in acidic medium), or by fluorimetry after reaction with specific reagents (4,5-Diaminofluorescein (DAF), pentafluorobenzylbromide, or 2,3-diaminonaphthalene (DAN)...), of ii) NO by electrochemistry or chemiluminescence or iii) of the nascent thiol, after the elimination of NO and blocking of other free thiols, by labeling it then quantifying it by MS (Biotin switch assay).

i. Colorimetric (Saville reaction)

The Saville reaction is based on the decomposition of RSNOs by mercuric (II) ions. The resulting NO^+ produced from decomposition by mercury ions react with sulfanilamide to yield a diazonium derivative. This diazonium further reacts with N-(1-Naphthyl)ethylenediamine to produce a pink colored azo-dye that has the maximum absorption at 540 nm (**Figure 49**). The method is highly reproducible, simple, inexpensive and robust. It is considered as a reference of comparison for other methods. Its main limitation is its sensitivity to detect biological RSNOs and its range of linearity is 0.5-100 μM [255]. Therefore, it is generally not a usefull for assay in many biological RSNOs. High toxicity of mercuric salts is another challenge. Without a separation step, the interference of nitrite, which is present in large concentrations can limit the application [85]. Although this method was used to determine RSNOs in biological systems [256,257], it is considered as semi-quantitative technique [85].

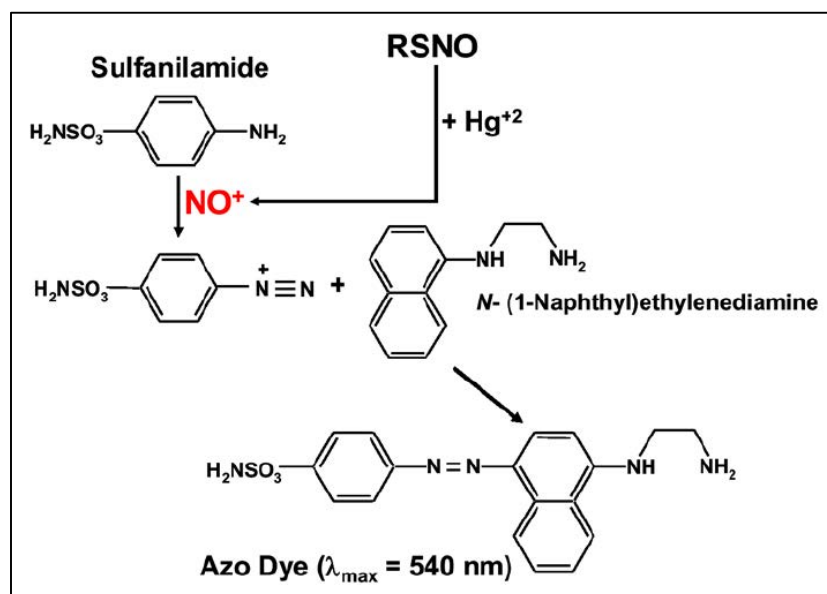


Figure 49: Principle of Saville reaction: Decomposition of RSNOs by Hg^{2+} leads to NO^+ . The reaction between NO^+ and sulfanilamide leads to the formation of a diazonium that reacts with N(1-Naphthyl)ethylenediamine finally forming a colored azo-dye strongly absorbing at 540 nm. After series of reactions with Griess reagent at pH 7.4 a colored Azo-dye that absorbs strongly at 540 nm. Adapted from [50]

ii. Fluorescence detection

Fluorescent probes have been developed for the detection of reactive oxygen and nitrogen species inside cells [258]. Many fluorescent molecules have been used such as 4,5-diaminofluorescein (DAF-2) [259], pentafluorobenzylbromide, or 2,3 diaminonaphthalene (DAN) [260]. The principle is either a reaction between oxidized NO with the non-fluorescent probe, or the reaction between oxidized non-fluorescent molecules with NO, yielding to the

fluorescent form of the molecule. RSNO decomposition by mercury leads to the release of NO^+ that reacts with the fluorescent molecule diaminonaphthalene (DAN) (**Figure 50**). Several differences exist between these fluorescent molecules in terms of pH stability, photo-bleaching, selectivity and sensitivity to NO. For example 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) is superior in these characteristics to the first generation compound DAF-2 [258]. DAF-2 represents a more reliable probe than DAN *in-vitro* because the already used fluorometers for other bioassay are compatible with excitation wavelength of DAF-2 [50]. The main limitations of use of these fluorescent molecules are: i) specificity: they can produce signals by reaction with other molecules (e.g. ascorbate, dehydroascorbate, thiols, urate [261]), ii) variation of intensity based on the redox state and iii) mercuric chloride can cause spectral change to DAF-2 that are overlayed with NO signals [50]. To avoid these problems, negative controls should be carefully done and be coupled with separation technique such as CE-LIF and LC-MS [258].

Determination of RSNOs has been made with mercuric decomposition and DAN, rather than DAF because of interaction between mercuric chloride and DAF. The detection range was between 50-1000 nM [255]. In other study, GSNO was determined by HPLC-DAF detection. Firstly it was separated from other interfering compounds, then a post-column, on-line enzymatic hydrolysis of GSNO by immobilized γ -glutamyl transferase was made followed by fluorescence detection [76]. Other detection methods include derivatization of GSNO with o-phthalaldehyde (OPA) followed by HPLC separation. After derivatization the extinction coefficient is increased 5 fold in UV detection. The LOQ was 100 nM of GSNO in human plasma ultrafiltrate. The limitation of this method is that GSH should be eliminated prior to analysis in samples containing cellular GSH [262].

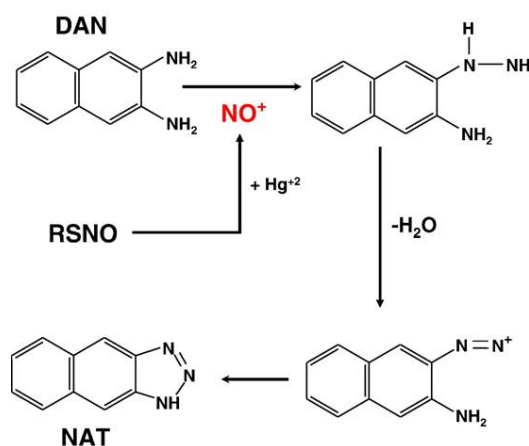
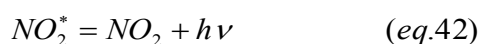
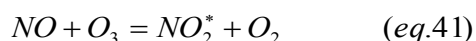


Figure 50: Principle of fluorimetric detection of RSNOs. DAN=diaminonaphthalène, NAT=naphthotriazole. Adapted from [50].

iii. Chemiluminescence

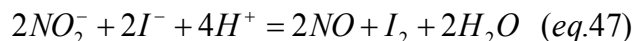
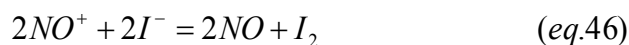
The principle of chemiluminescent detection of NO is summarized in **Figure 51**. The reaction between NO and ozone (O₃) yields to nitrogen dioxide in excited state (NO₂*). The NO₂* decay back to NO₂ produces light (hν) in the red and infrared regions (600-3200nm) (**eq. 41-42**). The intensity of the light emitted is proportional to the amount of NO allowing its quantification [79]. This reaction is highly specific to NO due to its particular properties of reaction with O₃ with respect to other possible interfering molecules and its existence in gaseous state contrary to nitrite and nitrate [263].



The sample is introduced in liquid or gaseous form (exhaled air) into the sample zone (a purge vessel). After that, decomposition in the sample zone to give NO is done. The produced NO is carried by an inert gas (Argon or helium) into the reaction cell where it reacts with ozone.

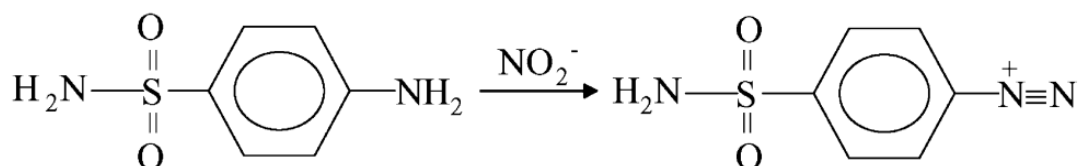
The main methods for RSNOs decomposition are

- i) Light decomposition
- ii) Triiodide I₃⁻: the reaction is based either on the reduction of RSNOs by triiodide according to equations below (**eq. 43-46**) or on the transfer of RSNOs to nitrite by the help of mercury (II) then nitrite is converted to NO as shown in **eq. 47**

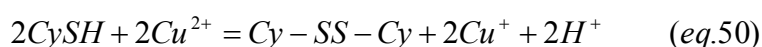
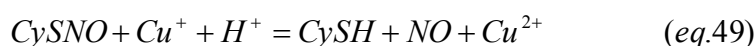


The detection limit is 1 pmol (which can be equivalent to 1 nM depending on the volume injected). This method has the limitation of interference of nitrite (because it is performed in acidic medium) (**eq. 47**) [264], the problem of foaming with proteins and the problem of forming a potent nitrosative compound NOI. The nitrite interference was suggested to be solved by the addition of sulfanilamide thus forming a diazonium cation, but this treatment can distort proteins and form or destroy SNO thus falsifying the results [265]. The foaming problem can be reduced by addition of antifoaming agent and by using glacial acetic acid instead of HCl.

Potassium ferricyanide has been used for the oxidation of Fe(II)-heme and thus prevention of NO capture. Although many researchers believe that this method should be considered only as complementary or should be avoided ([85] and references therein), others have investigated this issue and concluded that this assay is a satisfactory method in terms of specificity and sensitivity for RSNOs analysis in biological fluids ([79] and references therein, [264]). Subsequent methods were studied at neutral pH to avoid nitrite interference problem [79], also copper does not reduce nitrate or nitrite to NO.



- iii) Cu^+ / Cysteine: Cysteine plays two roles: the transnitrosation reaction where it takes NO from other RSNOs, and the role of Cu^{2+} reductor (eq. 48-50)



The pH should be monitored and controlled to avoid acidic conditions thus permitting the detection of nitrite (by formation of RSNOs). A major disadvantage of this method is the low efficiency (~35%) of detecting protein nitrosothiols (PSNO) ([79] and references therein)

- iv) Cu^+ / Cysteine / CO: the addition of CO prevents the scavenging of NO by heme by binding to it. The use of CO is dangerous in case of gas escape, therefore, precautions should be taken. It might also make exothermic reaction with hopcalite traps thus melting it and ignite an ozone scavenging cassette ([85] and references therein).
- v) Cu^{2+} / ascorbate decomposition: ascorbate plays the role of reductor of Cu^{2+} to Cu^+ , Cu^{2+} is recycled after reaction of Cu^+ with RSNOs; Cu^{2+} / ascorbate method decompose HMW-RSNOs and LMW-RSNOs unlike Cu^{2+} / Cys method which detects only LMW-RSNOs [266]; the problem of plasma foaming production at neutral pH was reported [48]; acidification decreases foaming but increases nitrite interference which can be decreased by the addition of sulfanilamide [79].

The chemiluminescence method has succeeded in detection of picomoles of NO in biological samples. It cannot be used for online detection of NO production or in-situ detection. It has many limitations that can be corrected by several ways: i) nitrite contamination which can be corrected by using nitrite-free water and washing well the glassware, ii) precision in peak integration in the software provided with the NO Analyzer can be corrected by using origin software, iii) foaming can be corrected by antifoaming product and iv) variability of sensitivity between machines which can affect reproducibility between laboratories, this effect can be decreased by calibration of each analyzer by standard nitrite solutions[264].

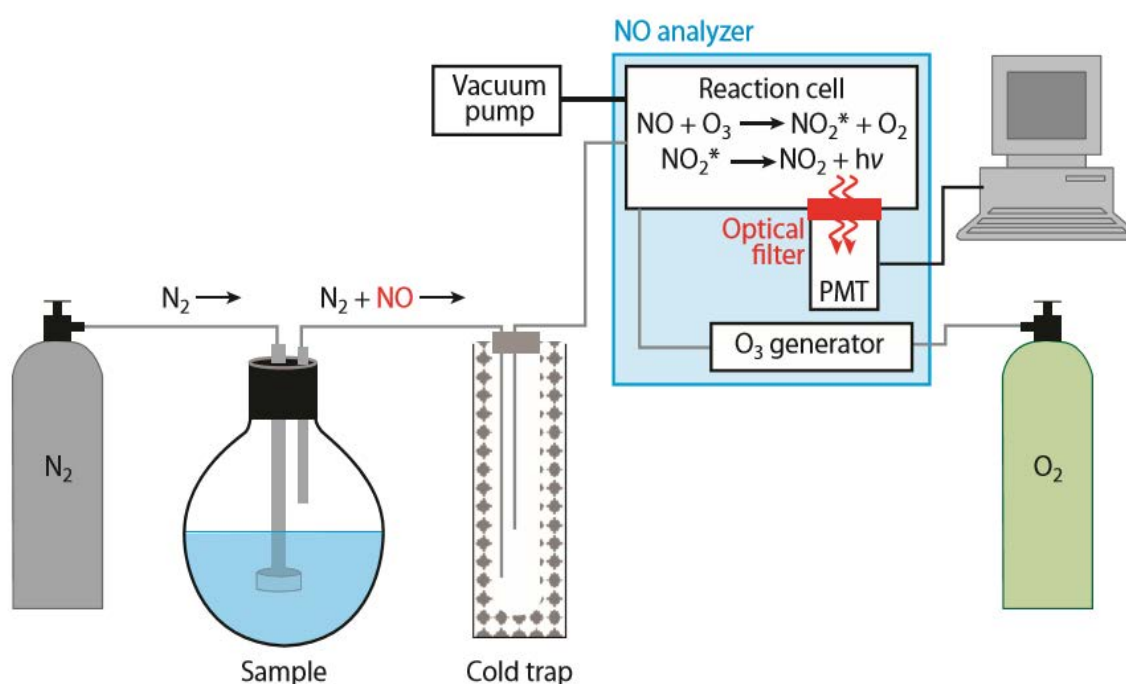


Figure 51: Principle and set-up for NO detection using chemiluminescence. Adapted from [77]

iv. Biotin Switch Assay (BSA) and derived methods

This method was first developed by Jafferay et al. [267] and is based on transforming protein nitrosated cysteines into biotinylated cysteines that could be easily detected using streptavidin or a specific antibody (**Figure 52**). At first HMW-RSNOs are captured by resin assisted capture (SNO-RAC) then reduced thiols are blocked under denaturing conditions (SDS, heat) by an adequate agent such as S-methylmethanethiosulfonate (MMTS), N-ethylmaleimide (NEM), or iodoacetic acid. The second step is the decomposition of the NO bond by ascorbate or other agents such as DTT leading to thiols. The blocking step was necessary to discriminate between the thiols initially present and the nascent thiols resulting from reduction of SNO. The third and last step is the conjugation of nascent thiol to biotin or fluorophore using N-[6-biotinamido

hexyl]-3'-(2'pyridyldithio)propionamide (biotin HPDP) or Dylight 488 maleimide, respectively. The resulting product can be analyzed by i) Western blot or direct MS after selective precipitation using streptavidin or anti-biotin antibodies [268], ii) difference gel electrophoresis (DIGE) using fluorescent labels [268] or iii) one or two dimensional gel electrophoresis with subsequent identification by liquid chromatography-tandem mass spectrometry (LC-MSMS) [5,41,269]. The use of DTT for decomposition instead of ascorbic acid, fluorescent for detection instead of biotin-HPDP and DIGE with fluorescence or CGE with LCMSMS for detection instead of Western blot are modifications of the original BSA method [268].

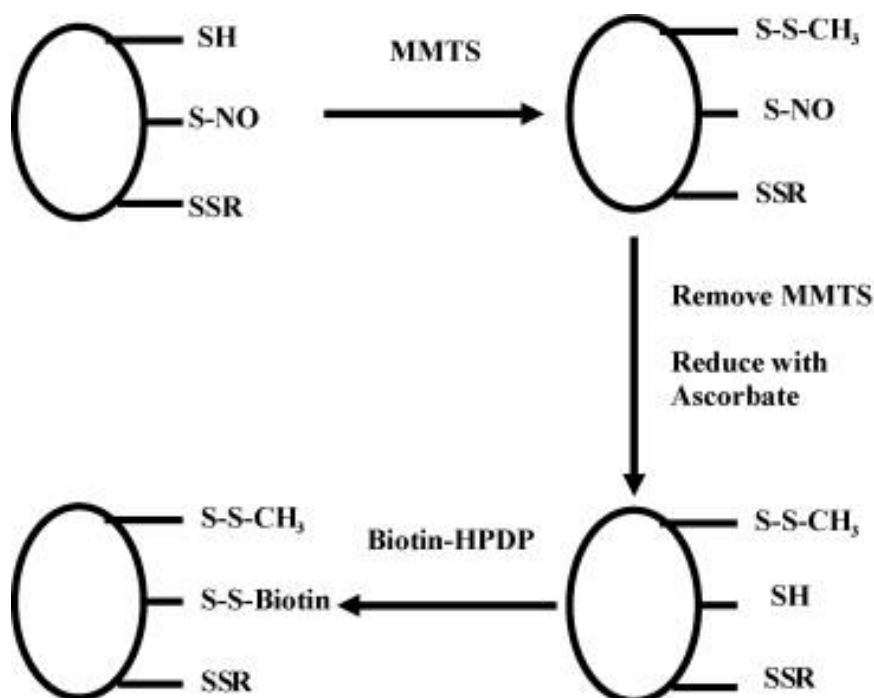


Figure 52: Biotin switch assay. Adapted from [268]

The limitation of this assay is in the efficiency and specificity of the second step (decomposition of PS-NO) which is of wide controversy. Zhang et al. [270] have shown that a high amount of ascorbate and a long incubation time is necessary to obtain complete reduction. Incomplete reduction of PS-NO by ascorbate can lead to an underestimation of HMW-RSNOs concentration. Huang et al. have reported that the use of ascorbate can increase biotinylation and false positive results [271]. There is some evidence that ascorbate decomposition is not specific to RSNOs since it can reduce sulfenic acids (RSOHs) and some disulfides, leading to false identification of HMW-RSNOs sites [272,273]. Zhang et al. have reported that it is

possible to report erroneously HMW-RSNOs at a concentration three times higher than the endogenous one, due to the ascorbate effect [192]. Ascorbate can also act in cooperation with Cu^{2+} to increase the amount of decomposition and specificity of PS-NO [266]. Another limitation is the sensitivity of the method, which depends on many factors: antibody (or streptavidin) dilution, the development conditions, the chemiluminescence substrates and the exposure time to film or camera [268].

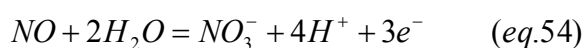
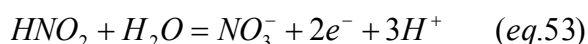
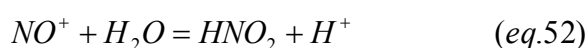
The BSA is more qualitative than quantitative (relatively quantitative). However, contrary to other methods, it identifies the site of nitrosation on proteins. The advantage of this technique is that it does not necessitate special equipment [5].

v. Electrochemistry

Electrochemistry can be implemented for RSNOs detection through the detection of NO released by RSNOs decomposition. Only one study reported on the direct RSNOs detection thanks to their reduction at gold and glassy carbon electrodes in acidic medium at pH 4 [274]. They showed that the main product of reduction is not NO at physiological pH. Very little application of RSNOs electrochemical reduction (between -0.6 and -0.9 V vs Ag / AgCl) is found in literature, probably due to the possible interferences of other molecules and to the low sensitivity of the method. In addition, the detection of the resulting NO necessitate other method such as chemiluminescence and there is possibility to detect molecules other than NO such as nitrite, nitroxyl, nitrogen dioxide and ammonium depending on the pH used [274]. Thus, the most used strategy consist in chemical decomposition of RSNOs into NO or nitrite and then the detection of the resulting molecules by electrochemistry. The advantages of electrochemical detection are the real time monitoring, amenability to miniaturization, the ability to enhanced selectivity, sensitivity, lower LOD and enhanced special resolution [77]. Several groups have worked on the detection of NO itself. Amatore et al. [275-278] worked extensively on detecting NO using UME in cellular proximity. The use of ultramicroelectrodes (UME), electrodes having one dimension less than 25 μm [279], has permitted the positioning of electrodes near the surface of the cells in restricted areas and the detection of elements of oxidative stress such as NO in microvolumes [278].

NO can be oxidized or reduced depending on the potential utilized. It can undergo mild cathodic reduction by gaining one electron at potentials from -0.5 to -1.4 V vs Ag / AgCl ([280] and references therein). The reduction leads to the formation of nitroxyl anion, which is unstable in aqueous medium and leads to the formation of N_2O after a series of chemical reactions. The

biological applications of NO reduction are limited since they necessitate special requirement in such as a very low pH and they have low sensitivity. In addition, O₂ is a major interferent because it is more easily reduced than NO which has led to the abandonment of this method of detection ([29,280] and references therein). Direct electrooxidation of NO is the most used form of electrochemical detection of NO. It oxidizes at potentials more than 0.8 V vs Ag / AgCl. On bare electrode, NO is oxidized by losing one electron to become NO⁺ (**eq. 51**). NO⁺ is not stable and gives nitrite (**eq. 52**). The oxidation potential of nitrite is lower or slightly higher than NO (60-80 mV [280] or 120 mV [281]) depending on the electrode. The formed nitrite can undergo further oxidation to give nitrate by losing two more electrons (**eq. 53**). This means that NO undergoes three electrons oxidation process (overall **eq. 54**).



The high oxidation potential required for NO oxidation leads to selectivity problems because a lot of biological molecules such as nitrite, dopamine, urea, hydrogen peroxide, dopamine, carbon monoxide, norepinephrine and epinephrine [282,283] can be oxidized at such high potentials. The electrode materials usually employed are platinum [284], platinum alloy (90% Pt, 10% Ir) [285], gold [286], glassy carbon [282] and carbon fibers [287]. The electrode material and surface characteristics affect the potential at which NO is oxidized, the selectivity, the sensitivity, the signal stability and the quality of the ensuing analytical measurements [283]. For example, the platinization of the platinum electrode surface gives faster electron transfer and lowers NO reduction potential [77,288].

To better explain the selectivity concept, **Figure 53** shows the normalized oxidative current as a function of the potential applied [275]. In order to detect NO, a potential of 650 mV vs ECSS is needed, But at this potential O₂⁻, H₂O₂ and ONOO⁻ are detected. In other conditions (electrode and electrolyte) nitrite is more easily oxidized than NO thus in while detecting NO, nitrite would also be detected. To overcome the problems of selectivity two main approaches have been developed by the use of membrane selective to NO and / or the use of electrocatalytic membranes that decrease the potential of NO oxidation.

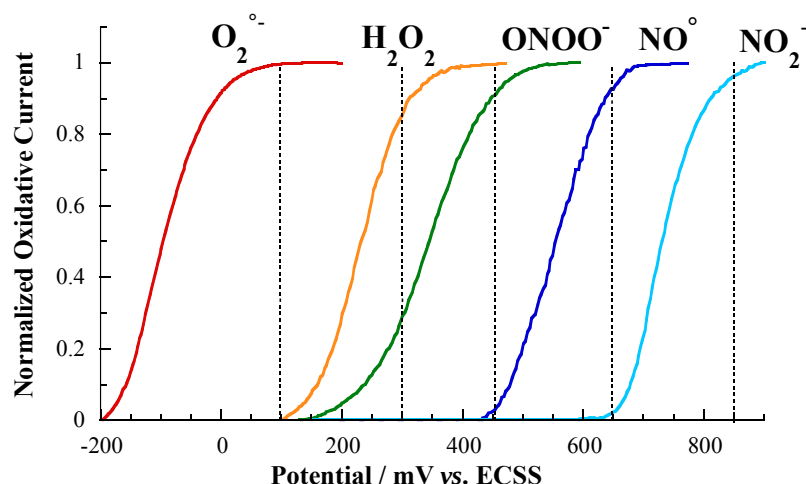


Figure 53: Normalized steady-state voltammograms (20 mV s^{-1}) obtained for the electrochemical oxidations of H_2O_2 , $ONOO^-$, NO° and NO_2^- solutions (each at 1 mM in PBS) at platinized carbon-fiber microelectrodes. Dotted lines define the potentials offering the best sensitivity and selectivity of detection for each. Adapted from [275]

The first “NO sensor” in brain tissue was Shibuki’s electrode [284] (**Figure 54**). He succeeded in detecting NO by modification of the Clark’s electrode that was developed to detect O_2 . The gas permeable membrane used was wax printed and sealed twice with chloroperene rubber. This approach improved the selectivity against nitrite but lacked reproducibility. The linear range was very narrow ($1\text{-}3\mu\text{M}$). The sensitivity varied a lot over time and over sensors ($2.5\text{-}106.3 \text{ pA} / \mu\text{M}$), which made measurements unstable. Further modifications of this electrode by covering with a stainless steel holder have decreased the LOD to 15 nM and the linear range to $0.05\text{-}0.4 \mu\text{M}$ but reproducibility remained an obstacle [280]. The miniaturization of such sensor is rather difficult, because of its complex structure and the necessity for filling solution [283].

The second approach of NO sensor was based on the immobilization of a NO permeable membrane directly on the metal without the use of internal solution (**Figure 54**). This electrode is amenable to miniaturization and uses a multilayer membranes that permits selectivity against possible biological interferences. This NO sensor can thus be used in biological medium [283].

Several kinds of membranes have been deposited on electrodes. Anionic membranes prevent anions such as nitrite and ascorbate, cationic membranes prevent positive molecules like dopamine from reaching the electrode metal surface and being oxidized [283]. These membranes act by electrostatic repulsion improving selectivity against interferences. Ichimori et al. [285] used a Pt / Ir electrode coated with nitrocellulose membrane in order to increase the mechanical robustness of the electrode. Bedioui et al. [286] used microelectrodes of gold for the first time for detection of NO. The linearity was between $10\text{-}100 \mu\text{M}$ and the membrane

was composed of Nafion. Nafion prevents negative ions from detection but is not impermeable to positive and neutral species. Other membranes were evaluated, including polycarbazole, polydimethylsiloxane, polystyrene (PS), fluorinated xerogel, polytetrafluoroethylene (PTFE) and o-phenylenediamine (o-PD) ([77,283] and references therein). Multilayered polymers could be deposited to ensure the impermeability to the largest amount possible of analytes except NO. A compromise should be found between selectivity and sensitivity since thicker membranes lead to lower sensitivity. Although multilayered polymers permit the selectivity against most interferents, CO interference is not avoided. Only a Shibuki's style NO / CO sensor developed by Lee et al. [289] were able to obtain it. This electrode is composed of two disk-shaped platinum working electrodes with Ag / AgCl counter / reference electrode covered with an expanded poly-(tetrafluoroethylene) (Tetra-tex) gas-permeable membrane. The electrode surface is modified differently in such a manner to detect selectively each gas.

Electropolymerisation represents an elegant method for polymer deposition on electrode surface since it permits controlling the thickness of film and covering small irregular spaces [290]. The monomers usually used are eugenol, phenol, aniline, o-PD [216]. NO is hydrophobic so it needs certain degree of hydrophobicity for the membrane to cross it. The less hydrophobic the membrane, the less sensitive the NO sensor. The less thick the membrane, the more sensitive and less selective the NO sensor becomes.

The third approach has the same structure as the second type except that an electrocatalyst (metallo-porphyrins or metallo-phtalocyanines) is used to improve the electron transfer inducing a negative shift in the cyclic voltammogram by 0.15 V and an increase in sensitivity ca 2-3 times [29,291] (**Figure 54**). The electrocatalyst layer is deposited directly on the metal; other layers (size or charge exclusion) can be coated on the electrocatalyst layer to give more selectivity.

A fourth rarely used approach is a composite material made from catalyst and permselective membrane [216].

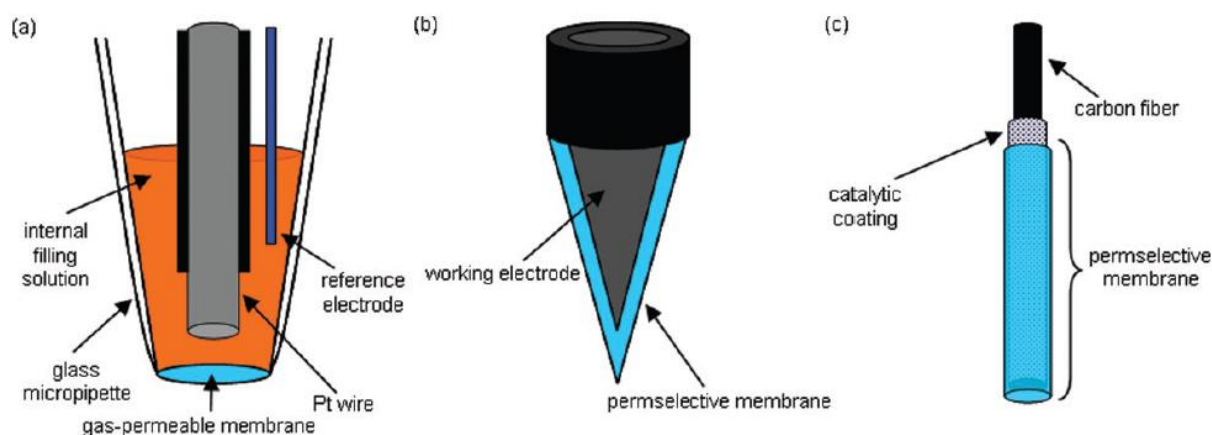


Figure 54: General types of electrochemical NO sensors. Adapted from [283].

The electrode material and the conditions of polymerization affect the properties of an electrochemically prepared polymer modified electrode [281]. The potential necessary for NO detection depends on the nature of the substrate, the electrocatalytic properties of the membrane and on the surface roughness [283]. The electrochemical techniques usually used for NO detection are: simple amperometry, pulsed chronamperometry, differential normal pulse amperometry and differential normal pulse voltamperometry [290].

As stated above, RSNOs decomposition to NO is necessary for their electrochemical detection. The decomposition is accomplished mostly by Cu^+ catalyst. Several methods have been used (**Table 6**) i) Cu^+ can be added directly using CuCl ii) Cu^{2+} can be added with a reducing agent such as thiol or ascorbic acid to give Cu^+ or iii) Cu metal can be oxidized to produce Cu^+ . For example, Meyerhoff et al. [292-295] have used a strategy where the catalyst is immobilized in a polymeric membrane near to the NO sensor. The catalysts used were copper, organoselenium and organotelluride nanoparticles. **Table 6** summarizes the reported RSNOs detection methods using electrochemical approaches, including the RSNOs detected and analytical performances.

Table 6: Different methods of decomposition by copper to detect RSNOs. Adapted from [3]

RSNOs decomposition	RSNOs sensor configuration	E (V / ref)	LOD	RSNO tested	Sensor lifetime	Interfering compounds tested	Biological samples
Catalyst: $\text{Cu}(\text{NO}_3)_2$ + thiol (L-cysteine or glutathione)	ISO-NO WPI	0.8 V	≈ 50 nM	GSNO, SNAP, S-nitrosated bovine serum albumin	n.d.	Nitrosomorpholine (no response up to 1 nM), N-nitroso-N-methylurea (no response up to 1 mM), Isoamyl nitrite (no response up to 0.7 mM), Nitroglycerol (no response up to 4 mM), NO_2^- (no response up to 50 μM)	–
Catalyst: CuCl	ISO-NO Mark II WPI: carbon fiber (=100 nm diameter) / Nafion / WPI membrane	0.865 V	≈ 10 nM	GSNO, SNAP, AlbsNO	n.d.	NO_2^- (no response at 50 μM), AA (no response at 50 μM), L-Arg (no response up to 100 μM), DA (no response at 10 μM), $\text{NH}_3(\text{g})$ (no response at 1 μM), $\text{CO}_2(\text{g})$ (no response at 1 μM), $\text{CO}(\text{g})$ (no response at 1 μM)	–
Catalyst: CuCl	Pt disk (200 μM diameter) / poly-Cu(II)TAPc / Nafion	DPV	≈ 4 nM	GSNO	n.d.	NO_2^- (no response at 10 μM), UA (no response at 100 μM), AA (no response at 100 μM), 5-hydroxyindole-3-acetic acid (no response at 10 μM), 3,4-dihydroxyphenylacetic acid (no response at 10 μM), DA (peak at 0.3 V above 1 μM), Epinephrine (above 1 μM , peak observed at 0.3 V), 5-hydroxytyptamine (above 1 μM , peak observed at 0.3 V)	Whole blood
Catalyst: Cu(II)-ligand complex or Cu(II)phosphate or Cu(0) particles as the copper source + ascorbate	Platinized Pt disk (250 μM diameter) / PTFE / Copper-based catalytic membrane	0.75 V	n.d.	SNAP, SNAC, CysNO, GSNO, AlbsNO	10 days	NO_2^- (no response from 0.1 to 100 μM)	Whole blood
Catalyst: organoselenium	Platinized Pt disk (250 μM diameter) / PTFE / organoselenium catalytic hydrogel	0.75 V	<0.1 μM	SNAC, SNAP, SPA, CysNO, GSNO, AlbsNO	10 days	n.d.	Whole blood
Catalyst: organotelluride	Platinized Pt disk (250 μM diameter) / PTFE / organotelluride based hydrogel	0.75 V	<0.1 μM	SNAP, GSNO, CySNO, AlbsNO	>1 month	n.d.	Whole blood
Catalyst: organoselenium	Platinized Pt disk (250 μM diameter) / PTFE / organoselenium	0.75 V	<20 μM	GSNO, CySNO, AlbsNO	10 days	NO_2^- : sensitivity ratio $\text{S}(\text{NO}_2^-)/\text{S}(\text{NO}) = 10^{-6}$, AA: sensitivity ratio $\text{S}(\text{NO})/\text{S}(\text{AA}) = 10^{-6}$, N-nitroso-1-proline sensitivity ratio: $\text{S}(\text{N-nitrosoproline})/\text{S}(\text{NO}) = 10^{-4}$, $\text{NH}_3/\text{NH}_4^+$	Whole blood
Catalyst: electrochemically oxidized Cu(0) in the presence of ascorbate	Micrometric ring-disc: central disc (50 μM diameter) = Cu(0), ring = NO sensor (gold / poly-eugenol / polyphenol)	0.70 V	n.d.	GSNO	n.d.	NO_2^- (sensitivity ratio: 9×10^{-4}), H_2O_2 (sensitivity ratio: 3.9×10^{-2}), AA (sensitivity ratio: 1.8×10^{-2})	GSNO in serum

3) Separation techniques (HPLC, GC, CE) coupled to direct or indirect methods

Selectivity is an issue in RSNOs detection. In biological samples five main NO sources exist: i) nitrite, ii) nitrate, iii) RSNOs, iv) Fe-nitrosyl (DNIC) and v) N-nitrosamines. The decomposition method cannot differentiate between RSNOs and N-nitrosamines thus it will be a problem for indirect methods. For direct methods such as UV-visible absorption, RSNOs absorb in UV (330-340 nm) with low extinction coefficients ($\epsilon=0.9 \text{ mM}^{-1}.\text{cm}^{-1}$ around 340nm for GSNO) in a wavelength range at which a lot of other biological molecules can absorb and therefore it is not possible to determine each RSNO concentration. A separation step permits one to obtain selectivity even if the detection method is not selective. The low extinction coefficient does not permit detection of RSNOs below 1 μM [26], thus most of the developed techniques are based on the indirect detection of RSNOs by conversion of the SNO moiety into nitrite by HgCl_2 or the reduction to NO and finally the detection of NO itself or the nitrite formed from NO. Several groups have developed high performance liquid chromatography coupled to ultraviolet, mass spectrometric, or electrochemical detection methods to analyze RSNOs [26,45,262,296-298]. For example, the glutathionyl moiety has been detected after reduction of GSNO by β -mercaptoethanol [262]. In other techniques, RSNOs are monitored directly by UV without derivatisation [26].

Tsikas et al. [26] developed a HPLC-MS-MS technique for the quantitation of RSNOs with a LOQ of 2.8 nM. Other HPLC-MS techniques were used but most of them are offline methods. This means that aliquots of the HPLC were collected and then analyzed by MS in order to identify and quantify RSNOs. The essential thing is to be aware while using acidic mobile phase, for the possible formation of RSNOs from nitrite and thiols. A control using spiked thiols should be made in order to assure that no artificial RSNOs are formed [85] (**Table 7**). Tsikas et al. [262] developed a GC-MS method to study the metabolism of GSNO and CySNO in isolated human erythrocytes. They quantified GSNO and CySNO externally added to plasma by using their labeled ^{15}N analogue. Firstly, RSNOs are decomposed by Hg(II) thus providing nitrite. Then a derivatization reaction is done using pentafluorobenzyl bromide. The resulting compounds are analyzed by GC-MS allowing RSNO quantification (**Figure 55**). Later, the same authors developed a method to detect AlBSNO in human plasma. For this, the sample pre-treatment consisted in extraction followed by a decomposition step using either Hg^{2+} or Cu^{2+} / cysteine (see **section I.C.3.ii**). The subsequent derivatisation was the same as for GSNO and

CySNO. The results showed that the two methods of decomposition are the same in effectiveness and specificity [212].

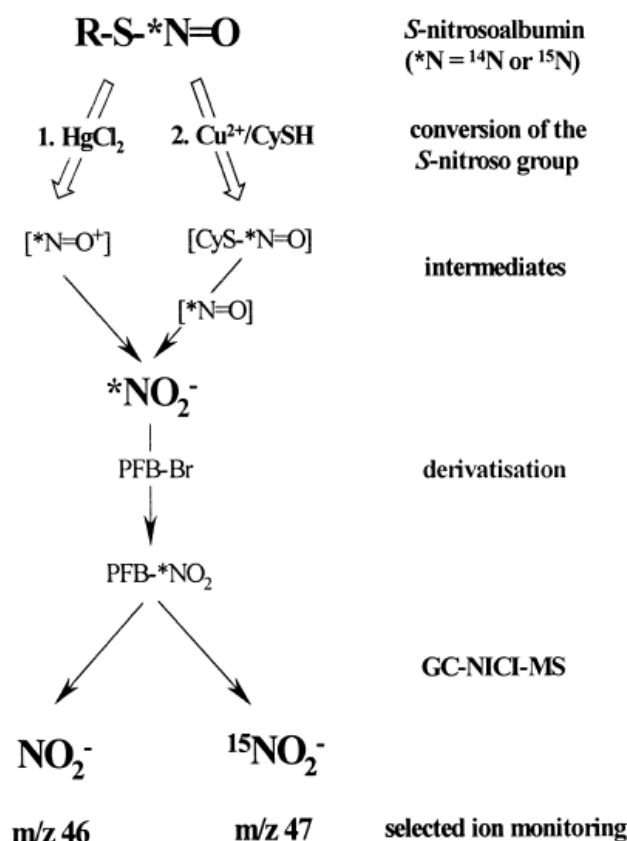


Figure 55: Principle of GC-MS analysis of RSNOs. Adapted from [299]

Methods using capillary zone electrophoresis coupled to UV detection [89,91,92,300] and capillary gel electrophoresis coupled to laser induced fluorescence [41] have also been developed. Stamler et al. [92] separated several RSNOs from their derivatives by CE in acidic pH. Detection was made by absorbance at 200 nm and 340 nm (specific to RSNOs). The migration times were between ~2.5 and ~18 min. Samples were artificial and their concentration was in the 100 μM range. Trushina et al. [300] have also used CE-UV with acidic pH to separate GSNO from GSH and GSSG. The separation time was long (more than 30 min) and the background electrolyte was high ionic strength (0.32 M), which led to a very high current 249 μA for 11 kV. Authors claimed a reproducible method despite this big aberrant electric current from CE standard current ranges. Samples were artificial and the concentrations used were in mM range. Messina et al. [91] developed a method for separation and quantification of GSNO from red cell-extract by CE-UV in acidic pH. The detection limits were 10 μM for GSH and GSNO and 5 μM for GSSG in less than 25 min. Misiti et al. [89] have separated GSNO, GSH

and GSSG in less than 25 min. They separated them from the acid extracts of erythrocytes treated with 0.2 mM CySNO. For all these methods, the preliminary sample treatment should avoid the loss of S-NO bond and the formation of artificial S-NO bond. The main drawback of coupling to UV detection is the LODs which are in low micromolar range [301]. For this, it was advantageous to make an indirect detection for which a lower LOD can be obtained.

In Summary, several techniques have been used in order to quantify RSNOs. Each technique has its own advantages and limitations. Different concentrations of RSNOs have been reported in the literature for real biological samples depending on the technique used and on the pre-analytical steps for sample separation. **Table 8** summarizes these techniques. The advantages of electrochemical detection are: the real time monitoring, the amenability to miniaturization, the ability to enhanced selectivity, sensibility and lower LOD [77]

CHAPTER I : STATE OF ART

Table 7: Different HPLC and GC conditions for detection of RSNOs

Sample	Detection	Stationary phase	Mobile phase	Column dimensions	S-nitrosothiol	Cleavage	concentration	Ref
Rabbit	electrochemical detection (ECD) with a dual Au / Hg electrode set at both oxidizing (+0.15 V) and reducing (-0.15V) vs Ag / AgCl	C-18 reverse phase column	0.1M monochloroacetic acid, 0.125 M (EDTA), 1.25 mM sodium octylsulfate, and 1% (v / v) acetonitrile, pH 2.8		CySNO	No	0.221±0.259	[190]
Human	Optical density at 350 and 532 nm	Novapak C-18 column (Waters, Inc.)	24% solvent A (50% acetonitrile and 50% H ₂ O), 56% solvent B (H ₂ O) and 20% solvent C (0.1 M phosphoric acid, pH 3.0, with NH ₄ OH). Between 4 and 8 min, the gradient changed linearly to 56% solvent A, 24% solvent B and 20% solvent C	3.9 mm x 15 cm	RSNO	HgCl ₂	0.181±0.15	[302]
Human hepatic diseases	MS	fused-silica capillary column Optima	Helium (30 kPa) and methane (200 Pa) were used as the carrier and the reagent gas, respectively	(15 m x 0.25 mm I.D., 0.25 µm film thickness)	AlbSNO	HgCl ₂	0.161±0.274	[303]
Human	UV-Vis fluorescence	Nucleosil 100-5C18 material	7.5 vol% methanol and 92.5 vol% sodium acetate buffer (0.15 M, pH 7.0 or 10 mM NaH ₂ PO ₄ and 1-octansulfonic acid in acetonitrile (80 / 20, v / v) and the pH was adjusted to 2.0 by H ₃ PO ₄	250 x 4.6 mm i.d. packed with 5-µm particle size	GSNO	2-mercapto ethanol	<0.1 (LOD)	[262]
Human plasma	Spectrophotometry: 540 nm	Octadecyl silane C18 ultrasphere column	10 mM sodium acetate buffer (pH 5.5) containing 0.5 mM DTPA	a 250 x 4.6 mm, 5 µm	AlbSNO LMWSNO	HgCl ₂	0.062±0.024 <0.02 (LOD)	[304]
Human plasma	UV and MS-MS	Nucleodur C18	For UV detection: Water / acetonitrile 95 / 5, v / v, contained 10mM TBAHS as the ion pairing agent, 1 mM EDTA and 10 mM K ₂ HPO ₄ . pH 7 For MS-MS detection: Acetonitrile / water (70 / 30, v / v) and contained 20 mM ammonium formate, pH 7	For UV detection: 125mm x 4mm i.d., 3µm particle For MS detection: 5 different HILIC columns	GSNO	No	<2.8 nM (LOQ)	[26]
Plasma of rat and human	MS	Waters symmetry C18 column	90% of 0.1% formic acid, 10% methanol	7.8 x 150 mm	GSNO	No		[305]
Human plasma	fluorescence				HMW-RSNOs	HgCl ₂	0.02±0.05	[306]
Artificial samples	HPLC-UV 220 nm	C18 reversed phase column (Partisil ODS-3, Whatman, Hillsboro, OR)	0.05% trifluoroacetic acid and methanol (94:6)	5-µm particle size	GSSG and GSNO	No		[45]

Table 8: RSNO quantification using different techniques. Adapted from [3,26,30]

	Analytical method	Detected species	LOD	Main analytical characteristic	RSNO analyzed	Biological samples	Concentration reported
Direct Methods	Reactions with phosphines	Selective m/z GSNO adduct	~0.01 μM	Specificity	GSNO	Cell lysate	0.7-3.9 μM
	HPLC-MS	Selective m/z GSNO adduct	2.5 nM	Specificity	GSNO	Plasma	Not detected <LOD
	CE-UV	GSNO	10 μM	Specificity	GSNO	Plasma spiked with CySNO 0.2 mM	Endogenous <LOD
Indirect Methods	Spectrophotometry (Saville assay)	Azo dye	500 nM	Low sensitivity	LMW RSNO	Plasma	RSNO 5.9 μM
	Chemiluminescence	NO_2	pM	High sensitivity	LMW RSNO, AlbsNO	Arteries	0.05-2.5 μM
						Plasma	0.5 nM-7 μM
						Serum	35-930 nM
	Fluorimetry	Fluorescent marker	nM	Low selectivity	LMW RSNO, AlbsNO	Arterial blood	0.25-0.56 μM
	GC-MS	Derivatized NO_2^-	200 nM	Selectivity	AlbsNO	Plasma	0.2-9 μM
	HPLC-Saville's system	Azo dye (Griess reagent)	0.1 μM	Sensitivity	RSNO	Plasma	0.181 $\pm$ 0.15 μM
	HPLC with fluorimetric detection	Fluorescent	20 nM	Selectivity	LMW-RSNO	Plasma	GSNO 0.09-0.3 μM
	NO amperometric sensor	NO	10 nM	Selectivity	LMW-RSNO	Plasma	LMW-RSNO 0-3 μM
						Serum	LMW-RSNO $\approx$ 500 nM

III. Miniaturization and microfluidics

A.Introduction

The history of microfluidics comes back to the 1930's when Izmailove et al. [31] analyzed plant extracts by chromatography using microscope slides coated with several adsorbants and detected the different components after separation using fluorescence under UV light. Nine years later, Williams et al. made an advanced sandwich system based on Izmailove's method where a real microfluidic system starts to appear [307]. After this, several researchers developed a simplified methodology called micro planar chromatography for separation and quantitation of various molecules [308-310]. In 1975, the first miniaturized gas chromatography system was introduced, containing integrated circuit processing technology [311].

In the 1990's, the US military initiated a research program in microfluidic funded by the Defense Advanced Research Projects Agency (DARPA). This program had a significant effect on developments in this domain [312]. The military had a need to practice medicine in resource-limited areas, therefore they needed robust and easy transportable analytical systems. In addition, the advantages of distributing diagnostic devices in developing countries are: i) the access to new tools that permit faster and more accurate diagnostic, ii) more data about diseases allowing better epidemiological studies in order to ameliorate diagnostic, prevention and treatment of the diseases, iii) better use of minimally trained healthcare professionals and iv) better use of existing therapeutics [313].

Electrophoretic separations and electro osmotic flow (EOF) gave microfluidics an important advantage because they permit the non-mechanical control of fluid transport. When the surface of the microchannel wall is charged this induces the formation of a double layer of opposite charge when a buffer is introduced. This layer is constituted of an inner rigid layer (the so-called Stern layer) and an outer diffuse layer of counter ions. This electric double layer is the basis of EOF. When a voltage is applied, it causes the ions within the diffuse outer layer to move toward the electrode of opposite sign and drag solvent molecules in their movement.

B. RSNOs detection using microsystems

RSNOs separations have been developed using CE [89,300,92], but not in integrated microfluidic devices. Recently, Hunter et al. [40] proposed a microfluidic device for RSNOs. The decomposition of RSNOs was realized by visible light and their detection by amperometry. The sensitivity in PBS was 22.6, 25.5 and 5 pA / μM and LOD's of 60, 60 and 280 nM for GSNO, CySNO and AlbSNO, respectively. No separation step was performed and the decomposition duration was quite long (100 s). The use of a microfluidic device permitted more complete sample irradiation and thus higher conversion of RSNOs to NO after a specific time. Wang et al. [41] developed a capillary gel electrophoresis method coupled to laser induced fluorescence in order to detect nitrosylated proteins. A very low LOD was obtained (1.3 pM) by adding Dylight 488 maleimide to specifically label the thiol group derived from RSNO. This system was applied to monitor protein S-nitrosylation damage in menadione (MQ) mediated human colon adenocarcinoma cells (HT-29). This method was useful in quantifying S-nitrosylated proteins in a complex protein mixture but was not used for LMW-RSNOs. Wang et al. [42] have also developed a two dimensional CE in microchips (Sodium dodecyl sulphate micro-capillary gel electrophoresis (SDS m-CGE) and microemulsion electrokinetic chromatography (MEEKC)) separations of nitrosylated proteins from HT-29 and Alzheimer disease transgenic mice brain tissue. They performed profiling of several S-nitrosylated proteins that vary depending on the oxidative stress of the cells. The detection was performed by LIF and a LOD of $\approx 700 \text{ pM}$ was obtained.

Hulvey et al. [314] used MCE with amperometric detection to detect and separate peroxynitrite from other electroactive materials in $\approx 25\text{s}$. They obtained a limit of detection of $2.4 \mu\text{M}$ for ONOO^- . After this Gunasekara et al. [38] used microchip electrophoresis with amperometric detection for the study of the generation of NO by NONOate salts. They separated NO from nitrite and NONOate in less than 1 min, in high pH (pH 11) with reversed polarity and an EOF inverter. The LOD was not mentioned but we can estimate low sensitivity because solutions of $\approx 1\text{mM}$ DEANONOate were used for experiments. After this, Gunasekara et al. [39] separated and detected by MCE with amperometric detection several markers of cellular nitrosative stress (nitrite, azide (interference), iodide (internal standard), tyrosine, glutathione and hydrogen peroxide (neutral marker)) in less than 40s. They detected $100 \mu\text{M}$ of nitrite and $10 \mu\text{M}$ of iodide. Several researchers have detected NO in microfluidic devices using amperometric, colorimetric and fluorescence detection [315-320]. Most of these NO detections were done for

NO produced from cells cultured inside the microfluidic device. Only Hunter et al [315] have detected NO in a spiked wound fluid and in whole blood. These devices have several advantages such as the possibility to culture the cells on the microchip and studying the effect of some molecules in NO production stimulation, single cell analysis and simulating blood vessels conditions. A sensitivity of 1.4 pA / nM and a limit of detection of 840 pM were obtained. Recently Tu et al. [321] developed a glass MCE device to separate and detect NO, glutathione and cysteine by fluorescence labeling. The detection limits (LODs, $S / N = 3$) for NO, GSH and Cys were 7.0, 3.0 and 2.0 nM, respectively.

All these studies made a great part of the pieces of puzzle in the separation and detection of different RSNOs and of NO using microchips, but separation of HMW- and LMW-RSNOs and their quantification on microchips was not been accomplished yet. As described in the previous sections, it can be a good diagnostic factor for several pathologies. Other methods than derivatization of RSNOs for fluorescence detection could give better specificity and accuracy.

C.Materials for microfluidic devices

In this part we will discuss the materials used for fabrication of microchips and we will focus on microchip capillary electrophoresis (MCE). As shown in **Figure 56**, MCE system has three main zones: the injection zone, the separation zone and the detection zone.

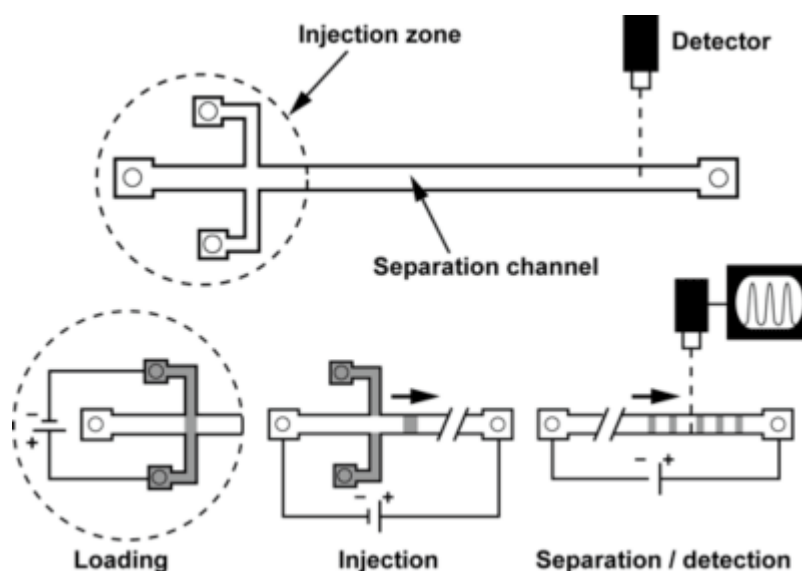


Figure 56: A typical microscale electrophoresis run begins by electrokinetically introducing a sample into the device, after which the voltage is switched so that a narrow band is injected into the separation channel. Different species migrate with different mobilities and separate into distinct zones that are detected downstream. Adapted from [322]

The material used for the fabrication of microfluidic systems plays an important role. Due to miniaturization, the properties of the surface are greatly amplified which can lead to consequences that are not present at the macro scale [323]. The desirable characteristics of a material for electrophoretic separation should be: 1) stable EOF, 2) good optical properties (in case of detection needing light), 3) accessibility to different fabrication methods, 4) compatibility with background electrolyte (BGE) and 5) good thermal and electrical properties (to minimize Joule's effect) [324]. Materials used for microfluidic devices can be divided into inorganic (silicon and glass), polymers and papers [325].

1) Silicon and glass

Silicon and glass were the first used to construct microfluidic chips ([326] and references therein). The fabrication of microchips based on these two substrates needs clean room facilities and equipment. It uses techniques borrowed from the semi-conductor industry such as wet and dry etching, photolithography and electron beam lithography ([326] and references therein). Silicon chemistry based on silanol is well known. It can be easily modified based on the application needed. It can not withstand the high voltages. It is transparent to infra-red but not to visible light, which is a problem for fluorescence detection. This problem can be solved by fabrication of hybrid devices with glass or polymer. The switching to glass was basically in order to be able to work with fluorescence. In addition glass has very similar surface properties to fused silica and it is not gas permeable. Silicon is chemically and thermally stable but expensive. For this reason, glass is preferred as it is less expensive, transparent and negatively charged (which permits good and reproducible EOF) ([307,327] and references therein).

2) Polymers

Later a new class of materials emerged for microfabrication, i.e polymers. Polymers are organic based long chain materials that are much less expensive to produce than glass, thus allowing disposable devices [327]. They can be as rigid and strong as glass or they can be soft and flexible. They can be subjected to mass production techniques (injection molding and hot embossing) and be modified chemically [325,328]. Polymers are often incompatible with organic solvents and high temperature, which limits their use in specific cases [328]. Channels in polymers can be formed by molding or by several soft lithographic processes. Sealing can be

done thermally or using adhesives. The surface chemistry of polymers is more complicated than that of silicon and glass.

Polymers can be divided into elastomers, thermosets and thermoplastics. Elastomers (PDMS, thermoset polyester and Teflon) are soft and deformable, they are reversibly extensible from 5-700% depending on the material, they come back to their original shape when tension force is elevated [329]. They are characterized by low cost rapid prototyping, possibility to highly integrate valves on chip which allows complicated and parallel fluid manipulation [323]. Thermosets (Su-8 and polyimide [323]) are stable at high temperature, resistant to most solvents and optically transparent. They are formed when thermoset molecules are exposed to light or heat thus provoking crosslinking, this step being irreversible. They cannot soften except if they are destroyed and cannot be reshaped [323]. Thermoplastics (polycarbonate (PC), Cyclic-olefin copolymer (COC), Poly(methyl methacrylate) (PMMA), Polyurethane (PU)) become pliable or moldable above a given temperature (glass transition temperature (T_g)) and solidify upon cooling, so they can be reshaped by heating [323], while remaining chemically and dimensionally stable over a wide range of operational temperature and pressures [330]. Thermoplastics are generally more durable, amenable to micromachining processes, optically clear, resistant to permeation of small molecules and stiffer than elastomers [325].

PDMS emerged as a very interesting polymer and it is the most used polymer in research laboratories due to its reasonable cost, rapid fabrication, low toxicity, optical transparency, reversible and irreversible sealing to a wide variety of materials, easily controlled surface chemistry and ease of implementation [328]. PDMS is cured in molds and they can be formed by multiple layers. PDMS is gas permeable which can be advantageous or problematic depending on the application. PDMS is hydrophobic, therefore susceptible to non-specific adsorption and permeation of hydrophobic molecules [323,331]. It can be more hydrophilic by exposition to plasma but it does not remain more than some hours.

Thermoset polyester is hydrophobic. It absorbs in UV but it is transparent over most of the visible light range. Teflon based materials such as Dyneon THV (a polymer of tetrafluoroethylene, hexafluoropropylene and vinylidene fluoride) are hydrophobic and oleophobic at the same time which gives them extreme inertness [325].

Polycarbonate is obtained by copolymerization of bisphenol A and phosgene. The softening temperature of polycarbonate is high ($\sim 145^\circ\text{C}$) which permits specific utilizations such as DNA

and thermal cycling [325]. PMMA is another thermoplastic that has interesting characteristics: gas impermeability, good optical clarity (UV and visible) [332], biological compatibility and ease of micromachining. COC has good transparency and therefore is compatible with many solvents and aqueous solutions. Its limitation is its hydrophobicity, surface treatment is necessary to have good EOF, biocompatibility and hydrophilicity and thus to promote water adsorption rather than protein and other solute adsorption [333]. Castor oil polyurethane represents a biodegradable polymer that is rapidly fabricated in less than 1h and at a very low cost [334]. SU-8 polymer (A negative photoresist that polymerize) is a very rigid polymer that can form well-defined structures. In addition to this it has excellent chemical and physical properties and is relatively inexpensive [335]. It is a negative tone epoxy photo-patternable resist, optically transparent in visible light, hydrophilic and chemically stable [336]. A combination of SU-8 with other compounds to form a hybrid microfluidic chip has already been done. Heyries et al. have developed a PDMS / SU-8 chip for the detection of allergen specific antibodies. The SU-8 was used as a cover layer. SU-8 / Pyrex device for electrochemical detection of neurotransmitters was developed by Alvarez et al [336]. The latter is sold now by Micrux technology®. SU-8 covering allows to avoid the problem of closing with other pyrex layer which is usually done by anodic bonding with a strong electric field and high temperature that can cause problems for metal integrated electrodes and damage them [335]. A summary of physical properties of some thermoplastics is shown in **Table 9** below.

Table 9: Summary of physical properties for common microfluidic thermoplastics. Adapted from [330]

Polymer	Acronym	T_g (°C)	T_m (°C)	CTE (10^{-6}°C^{-1})	Water absorption (%)	Solvent resistance	Acid/base resistance	Optical transmissivity	
								Visible	UV ^a
Cyclic olefin (co)polymer	COC/COP	70–155	190–320	60–80	0.01	Excellent	Good	Excellent	Excellent
Polymethylmethacrylate	PMMA	100–122	250–260	70–150	0.3–0.6	Good	Good	Excellent	Good
Polycarbonate	PC	145–148	260–270	60–70	0.12–0.34	Good	Good	Excellent	Poor
Polystyrene	PS	92–100	240–260	10–150	0.02–0.15	Poor	Good	Excellent	Poor
Polypropylene	PP	–20	160	18–185	0.10	Good	Good	Good	Fair
Polyetheretherketone	PEEK	147–158	340–350	47–54	0.1–0.5	Excellent	Good	Poor	Poor
Polyethylene terephthalate	PET	69–78	248–260	48–78	0.1–0.3	Excellent	Excellent	Good	Good
Polyethylene	PE	–30	120–130	180–230	0.01	Excellent	Excellent	Fair	Fair
Polyvinylidene chloride	PVDC	0	76	190	0.10	Good	Good	Good	Poor
Polyvinyl chloride	PVC	80	180–210	50	0.04–0.4	Good	Excellent	Good	Poor
Polysulfone	PSU	170–187	180–190	55–60	0.3–0.4	Fair	Good	Fair	Poor

T_m melting temperature, CTE coefficient of thermal expansion

^a high UV transmissivity often requires the selection of special polymer grades, e.g. without stabilizers or other additives

The characteristics of the material for MCE in terms of optical transparency, EOF and surface charge are summarized in **Table 10** the below. The choice of material can be based also on the degree of integration needed because of the complexity of the fabrication method [325].

Table 10: Summary of the physical properties of materials used in microchip electrophoresis. LTCC: Low-temperature cofired ceramic, TPE: Thermoset polyester, PFPE: polyfluoropolyether, PEGDA: poly(ethylene glycol)diacrylate, PEO: poly(ethylene oxide), FEP: fluorinated ethylene-propylene, PFA: perfluoroalkoxy polymer Adapted from [325]

material	optical clarity	UV transparency	EOF ($\times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$)	separation	surface chemistry	surface charge	refs
glass	good	>280 nm	3 (pH 7.0); 4.21 ± 0.18 (pH 9.2)	yes	silanol	negative	15, 33, 36
silicon	opaque	none	<i>a</i>	<i>a</i>	silicon/silanol	requires oxidation	20
LTCC	opaque	none	3.00 ± 0.05 (pH 7.0)	yes	aluminum oxide	negative	33
Ormocer	moderate	>300 nm	3.3 ± 0.3 (pH 10.0, lithography); 5.3 ± 0.1 (pH 10.0, UV-embossed)	yes	<i>a</i>	negative	36, 37
PDMS (native)	good	>220 nm	2.5 ± 0.5 (pH 6.0); 4.9 ± 0.9 (pH 9.0)	yes	silanol	negative	36, 79
PDMS (oxidized)	good	>220 nm	8.2 (pH 9.0)	yes	silanol	negative	49
TPE	moderate	>400 nm	2.2 (10 mM SDS); ~ 2.5 (pH 6.0); ~ 1.7 (pH 7.0); ~ 0.6 (pH 9.0); ~ 1.4 (pH 10.0); ~ 0.10 (pH 6–10, PEG-grafted); ~ 2.6 (oxidized)	yes	polyester	requires oxidation	49–52
PFPE	moderate	>350 nm	<i>a</i>	no	fluorocarbon	<i>a</i>	55, 56
PS	good	>300 nm	1.54 ± 0.03 (pH 9.2)	yes	phenyl	requires oxidation	15
PC	good	>360 nm	2.22 ± 0.09 (pH 9.2)	yes	phenyl/carbonate	requires oxidation	15
PMMA	good	>340 nm	2.07 ± 0.07 (pH 9.2)	yes	acrylate	negative	15, 49
PEGDA	good	>300 nm	<i>a</i>	yes	acrylate/PEG	negative	76
PEGDA/PMMA	good	>300 nm	0.63 ± 0.02 (pH 8.7)	yes	acrylate/PEG	negative	75
PDMS-co-PEO (5%)	moderate	>440 nm	2.5 ± 0.7 (pH 6.0); 5.7 ± 0.8 (pH 9.0)	yes	silanol/PEO	negative	79
PDMS-co-PEO (10%)	moderate	>440 nm	1.2 ± 0.3 (pH 6.0); 2.5 ± 0.3 (pH 9.0)	yes	silanol/PEO	negative	79
FEP/PFA	moderate	<i>a</i>	<i>a</i>	no	fluorocarbon	<i>a</i>	81
COC	good	>360 nm	3.08 ± 0.28 (TBE buffer)	yes	cyclic olefin	requires oxidation	82
thiol-ene	moderate	<i>a</i>	<i>a</i>	no	thiol/allyl	<i>a</i>	66–68
PU (castor oil)	moderate	<i>a</i>	2.6 (pH 7.0)	yes	carbamate/ricinoleic acid	negative	88
PU (tecotthane TT-1095A)	moderate	>300 nm	2.47 (pH 7.0)	yes	carbamate/phenyl	negative	89
zein	moderate	<i>a</i>	<i>a</i>	no	zein	<i>a</i>	91
SU-8	moderate	>360 nm	<i>a</i>	yes	epoxy/phenyl	negative	36
paper	opaque	none	<i>a</i>	<i>a</i>	cellulose	<i>a</i>	97, 98

^aInformation that was not reported in the literature.

There exist several methods to bind thermoplastics to form an enclosed microfluidic system. The most known methods are thermal fusion bonding and solvent bonding. Other methods exist also such as adhesive bonding, Welding using ultrasound or infra-red and surface treatment bonding. Each of these methods has pros and cons that are summarized in **Table 11** [330]

Table 11: Overview of thermoplastic bonding technique for microfluidic device. Adapted from[330]

Bonding method	Bonding strength	Bonding quality	Process complexity	Bonding time	Cost ^a	Advantage	Limitation	Suitable material
Adhesive printing bonding	High	Fair	Medium–high	Short–medium	Low–medium	Low temperature, high bond strength, low channel clogging, controllable adhesive thickness	Scarification channel (contact printing) or printing mask (screen printing) required	PMMA, COC
Adhesive interstitial bonding	High	Fair	Low	Short	Low	Simple, fast, low temperature, high bond strength, low channel clogging, low cost	Adhesive loading reservoirs or flush adhesive out of channel needed	PMMA
PDMS-interface bonding	Medium	Low	Medium	Long	Medium	Low temperature, compatible with PDMS microfluidics	PDMS layer may squeeze into channel	PMMA
Lamination film bonding	Medium	Fair	Low	Short	Low	Simple, fast, low cost, low temperature, no adhesive clogging	Lamination film may embedded inside channel	PMMA, PC, PS, PET
Thermal fusion bonding	Medium	Fair	Low	Long	Low–medium	Simple, homogeneous channel, compatible with various material	Channel deformation from un-optimized process condition	PMMA, PC, PS, nylon, COC, PSU
Solvent bonding	High	Fair	Low	Low	Low	Simple, fast, low temperature, high bond strength, low cost	Soften polymer surface may collapse channel from un-optimized process	PMMA, PC, COC
Solvent bonding with sacrificial material	High	Good	High	Medium	Low	High bond strength, low cost, low channel collapse and clogging	Sacrificial material need applied into channel before bonding and removed after bonding	PMMA
Localized welding	Medium	Fair	Medium–high	Medium	Medium	Low temperature, localized bonding	Energy director (ultrasonic welding) or metal layer (microwave welding) are required	PMMA, PEEK
Surface treatment bonding	Medium–High	Good	Medium	Medium	Medium–high	Low temperature bonding, low channel deformation	Surface chemistry changed after treatment	PMMA, PC, COC, PS, PET

3) Paper

Paper is the cheapest material used for microfluidic devices. It is biocompatible and biodegradable thus permitting a disposable device. Its porous structure allows filtration, flow and separation. The liquid flow is performed by capillary action, which is passive and does not need any external power. The paper can be chemically modified by surface chemistry functionalization. In comparison to other materials, papers have many cons: i) the channels are usually larger than 200 μm while a channel size of 20 μm can be attained with other materials, ii) liquids with low surface tension may not well be confined in the channels that are surrounded by hydrophobic material, iii) liquids evaporate as it is open channel format, iv) usually the sensitivity is not sufficient to detect the desired molecules in real samples [323].

4) Comparison

A comparison of properties between paper and other classical materials (glass, silicon, PDMS) for microfluidic devices is shown in **Table 12**.

Table 12: Comparison of different materials used for microfluidic devices. Adapted from [33]

Property	Material			
	Glass	Silicon	PDMS	Papers
Surface profile	Very low	Very low	Very low	Moderate
Flexibility	No	No	Yes	Yes
Structure	Solid	Solid	Solid, gas-permeable	Fibrous
Surface-to-volume ratio	Low	Low	Low	High
Fluid flow	Forced	Forced	Forced	Capillary action
Sensitivity to moisture	No	No	No	Yes
Biocompatibility	Yes	Yes	Yes	Yes
Disposability	No	No	No	Yes
Biodegradability	No	No	To some extent	Yes
High-throughput fabrication	Yes	Yes	No	Yes
Spatial resolution	High	Very high	High	Low to moderate
Homogeneity of the material	Yes	Yes	Yes	No
Price	Moderate	High	Moderate	Low
Initial investment	Moderate	High	Moderate	Low

The compatibility of these materials with CE and electrochemical detection are shown in

Table 13. Silicon and glass have excellent properties with CE. They have reproducible electro-osmotic flow, excellent optical clarity and similar surface chemistry to fused silica capillaries. Polymers are less compatible with CE in terms of optical clarity and reproducibility of EOF and surface properties than glass but they are cheaper, more resistant to fractures and easier to fabricate ([323] and references therein). Electrochemical detection is also widely used with glass chips and is commercialized due to the easy patterning of the metals (Au and Pt) on the glass substrate by lift-off.

Table 13: Comparison of different material compatibility with capillary electrophoresis and electrochemical techniques. Adapted from [54,55,323]

Applications	Silicon / glass	Elastomers	Thermosets	Thermoplastics	Paper
Capillary electrophoresis	Excellent	Moderate	Good	good	poor
Electrochemical detection	Good	Limited	Moderate	Moderate	Moderate

D.Separation on microfluidic devices

The main separation methods used with microfluidic devices are chromatographic and electrokinetic methods.

1) Microchip liquid chromatography

Conventional chromatographic separation (LC) is the main separation method used due to its outstanding separation power and versatility [337]. Until now it is less used than CE for on chip separation.

The first chromatography miniaturization trials were with gas chromatography and were performed at the end of the 70s [311,338] but the main problem was the bad performance of the miniaturized column due to the difficulty to introduce a homogeneous solid phase inside microchannel [337].

Liquid chromatography miniaturization presents several advantages over the conventional LC such as superior efficiency per time unit, simple positioning of a detection cell and low cost [337]. Both open (the stationary phase is bound to the wall of the column) and packed columns with particles or monolith layers (the stationary phase is bound to the particles or monolith) are used [339]. Open channel chromatography is very easy to prepare but the loading capacity of stationary phase is limited, therefore only small amount of analytes can be injected to avoid saturation and overloading, and a very sensitive detection method such as fluorescence is needed ([340] and references therein). The packed columns offer more surface area interaction with solutes but make the fabrication process more complicated (e.g. the frits to keep particles inside channel[340]). The columns can have non-homogeneous particle repartition thus decreasing the separation efficiency. Packed columns necessitate also a high pressure source. In order to solve this problem monolithic columns have been developed. They can be operated

with low pressure, but present a low reproducibility. Microfabricated pillar arrays are more reproducible [340]. The high pressure needed for miniaturized packed HPLC necessitates a very tight system. The conventional thermal bonding cannot support high pressure while the solvent bonding procedures that glue the polymer is more resistant [341]. Several stationary phases have been used: open channel material (glass or polymers that can be chemically modified or derivatized), chromatography resin, monolith and nanowires [339,342]. Pumping is achieved either by a pump in the case of packed columns or it can be by EOF in open columns [325,337].

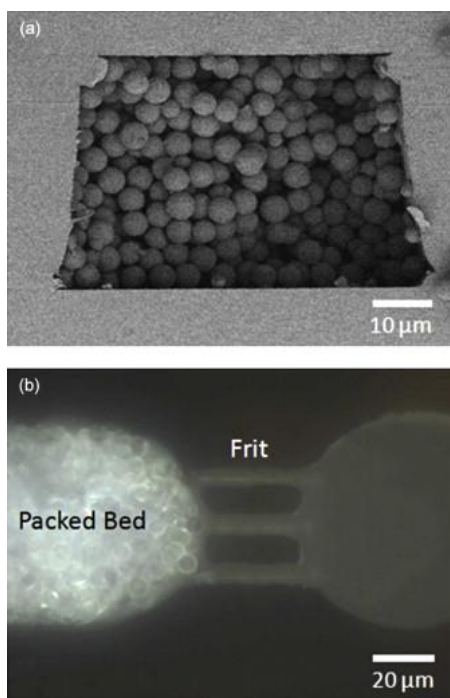


Figure 57: SEM of a polyimide microchannel with trapezoidal cross section packed with 5 μm C18 particles (upper panel); several smaller channels constitute a frit-like structure to contain the packed particles (lower panel). Adapted from [343]

2) Microchip capillary electrophoresis (MCE)

CE has several advantages over chromatographic separation such as high separation efficiency, simplicity, low sample and volume consumption and short analysis time [324]. MCE is a further simplification of CE. In MCE, the injection, separation and detection are done on the same platform which permits portability (**Figure 58**). The high separation efficiency is due to the homogeneous flow inside the channels due to the absence of packing [344]. Nowadays MCE is used for a wide variety of applications such as biomedical applications ([324,345] and references therein) (pharmaceuticals, genetic components (DNA, RNA, enzymes, aptamers...), proteomics [346], peptides [347], amino acids, antibodies, antigens, cells and their lysate), food

analysis [348], environmental [349], industrial, biological and life sciences [350-352]. The separation in MCE is similar to that of CE and is based on the difference in migration of analytes based on their effective ionic mobilities and EOF under an electric field. There exist different modes of electrophoresis based on the desired application. zone electrophoresis, electrochromatography (EC), micellar electrokinetic chromatography (MEKC), gel electrophoresis (GE), isoelectric focusing (IEF) and isotachopheresis (ITP).

In zone electrophoresis analytes are separated based on their charge to mass ratio. Positive small particles are the most rapid then big positive molecules. Neutral molecules are carried only by EOF only since they have no charge. Negative ions that have mobility smaller than EOF can migrate with EOF direction but slower. As the charge to mass ration of negative ions increase the ions migrate slower (**Figure 58**)

In MEKC analytes are separated based on their partition coefficient with the micelle (made from surfactant), thus allowing the separation of neutral molecules. Gel electrophoresis is used to separate mixtures of DNA, RNA and proteins based on their molecular mass. IEF is a technique that is used to separate molecules having different isoelectric points. ITP is a technique in analytical chemistry used for selective separation and concentration of ionic analytes. Charged analytes are separated based on their ionic mobility.

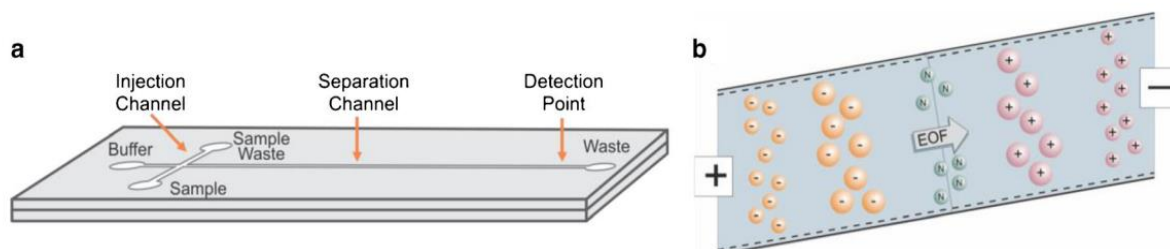


Figure 58: a) Schematic representation of microchip electrophoresis, b) migration order of ions ionic analytes based on their charge and mass. EOF: electrososmotic flow. Adapted from [353]

i. Injection techniques in MCE

Injection is one of the key elements in sample handling in microsystem. It can be performed either hydrodynamically or electrokinetically. Electrokinetic injection has some limitations such as electrokinetic bias (when one analyte is preferentially injected into the electrophoresis channel the analytes injected are not proportional to the analytes in the sample due to different mobilities of anaytes) and long injection times. It has however been mostly used as it is technically easier to generate an electrokinetically driven flow than pressure driven flow [354].

Hydrodynamic injection indeed needs micropumps that render the device more expensive and the operation process more difficult to control [355].

A good injection is an injection that:

1) Can be controlled in order to introduce enough analyte to be detected while providing a low height equivalent to a theoretical plate (HETP) or high efficiency. Remembering that

$$H = \frac{\omega_{sample}^2}{Kd_{id}}$$

where ω_{sample} is the length of sample plug (the desirable injected volume of sample

in the separation channel) along the separation channel axis, K is the injection profile factor (equal to 12 for a rectangular shaped plug), and d_{id} is the length of the separation channel between the injector and the detector [356].

2) It should have fixed-volume sample plug. Focusing of the sample or limitation of the sample diffusion in the separation channel outside this plug between the time of injection and separation in a way that it provide a precise shape can limit the phenomenon of dispersion (**Figure 59a** and **Figure 60**).

3) Limitation of sample leakage into the separation channel during separation, which can lead to band broadening, signal drift and decrease in signal to noise ratio [354,357,358] (**Figure 59c**).

4) The sample should not be diluted during injections and they should be reproducible. For this, sample pullback by back ground electrolyte (BGE) into the sample loading channel should be limited (**Figure 61**).

5) Matrix and mobility bias should be avoided in order to have a representative aliquot of the sample [359].

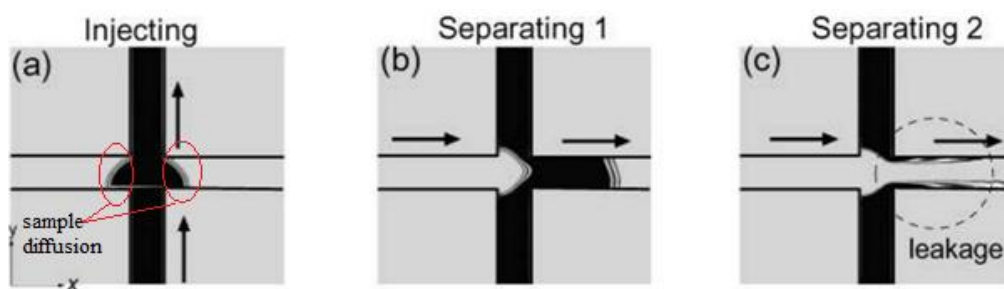


Figure 59: Simulation of steps of floating injection on a cross injection system. a) injection of sample (in black), b) and c) running of buffer. Adapted from [357]

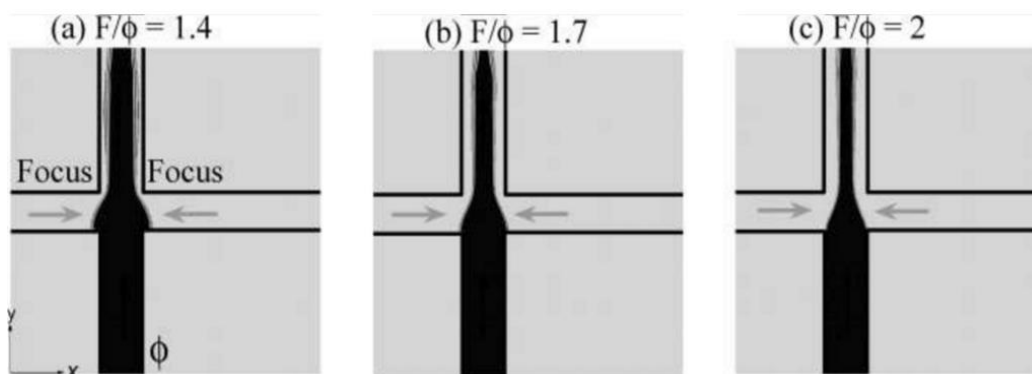


Figure 60: Simulation of loading step with different focusing ratios in cross form injection system. Adapted from [357]

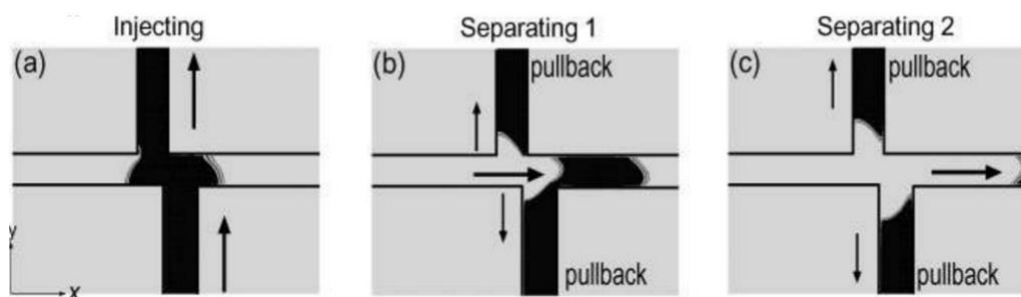


Figure 61: Simulation of steps of pullback floating injection on a cross injection system. A) sample (in black) injection, b) and c) running of buffer. Adapted from [357]

Scientists have worked on two ways to improve the injection conditions. The first way is the configuration of channels or injection design and the second is the injection mode.

Several channel configurations have been designed such as: T-type injection, cross or double-T injection, Multi-T injection form... The T type injection permits only floating injection where the sample is injected across the separation channel. The control of the volume of injected plug cannot be obtained. To solve this problem, the cross injection configuration has been made. This configuration permits other types of injection such as gated and pinched injections. In the cross channel system the volume of the injected analyte is determined by the geometry of the cross sectional area of the injection channel. In order to increase the injected plug double-T, triple-T and multi-T forms have been made [355] (**Figure 62**). In the double T configuration for example, the two branches of the injection channel can be separated by a specific distance where the sample will pass by the separation channel, to control the volume of the plug. A variation of the dimensions of the channels was also used to make injections [359].

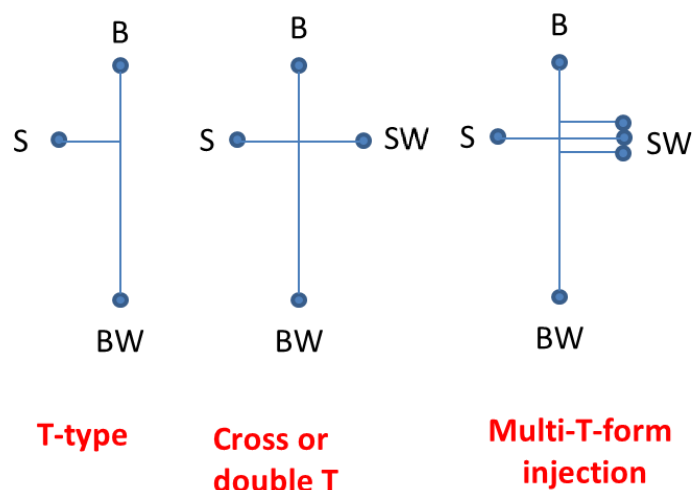


Figure 62: Different injection forms. S: sample reservoir, B: buffer reservoir, BW: buffer waste, SW: sample waste.

In the second axis, several modes have been applied such as: floating, pinched and gated modes.

a) Floating injection

In the floating mode the sample is driven from the sample reservoir to the sample waste reservoir (**Figure 63**: reservoir 1 and 3, respectively) in the cross, double-T, triple-T or multi-T-forms by the simple application of a potential difference leaving the other reservoirs grounded or floating (**Figure 63a**). Then, in the separation step, the buffer flows under the effect of an electric potential between the buffer and the buffer waste reservoirs (**Figure 63**: reservoir 2 and 4, respectively), taking with it into the separation channel the sample plug previously injected while the sample and sample waste reservoirs are floating (**Figure 63b**). The limitations of this process are the diffusion of the sample plug into the separation channel during the injection step and possible leakage during the separation step (**Figure 59**) [35]. In order to counteract the leakage during the separation step, sample pullback can be performed by applying potentials on reservoirs 1 and 3 in such a way that the buffer also enters into the injection channels (**Figure 63c**).

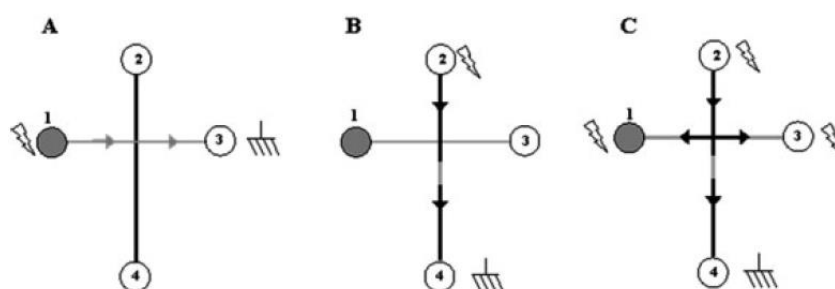


Figure 63: Floating injection. Sample introduction loading step (A) and dispensing step with (B) or without (C) sample pullback. Adapted from [354]

In order to escape from this vicious cycle, a control of the voltage in the different reservoirs at all moments should be done. This control is obtained through pinched and gated injections.

b) Pinched injection

With the pinched injection mode [360] the sample is focused into the injection channel during the loading step by applying potentials in both the buffer and buffer waste reservoirs (**Figure 64B1**), thus no sample diffusion occurs outside the desired plug and the sample plug is better controlled. As in the floating mode, a sample pullback methodology is implemented during the dispensing step in order to avoid sample leakage (**Figure 64B2**). Its limitation is the unsymmetrical distribution along the separation channel which makes dispersion increase in comparison to an ideal sample plug and decreases the number of theoretical plate.

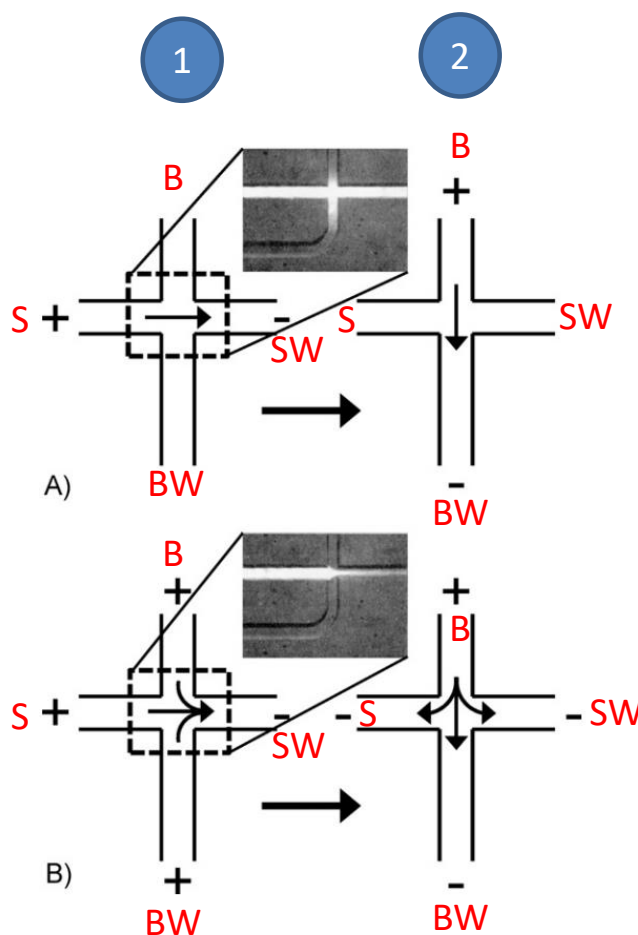


Figure 64: Comparison of floating (A) and pinched injections (B) at 1) injection and 2) separation steps. The white coloration is the sample and the colorless is the back ground electrolyte. No sample diffusion occurs in pinched one. S: sample reservoir, B: buffer reservoir, SW: sample waste reservoir, BW: buffer waste reservoir. Adapted from [35]

c) Gated injection

The gated injection [36] represents, along with pinched injection mode, the most frequently used methodology. With the gated injection mode, buffer and sample circulate continuously in laminar flow. Sample goes to sample waste reservoir and buffer to buffer waste reservoir and sample waste reservoir in such a manner that the sample cannot enter the separation channel during the loading step (**Figure 65b**). At the injection time, the electric potential is turned off in the buffer and sample waste reservoirs so that a plug of sample enters into the separation channel (**Figure 65c**). The length of the plug is proportional to the injection time and to the applied potential. The separation step is like the loading step in terms of potential distribution (**Figure 65**) [354]. In order to avoid leakage of the sample in the separation channel, the potential applied in the sample reservoir should be smaller than the one applied in the buffer reservoir.

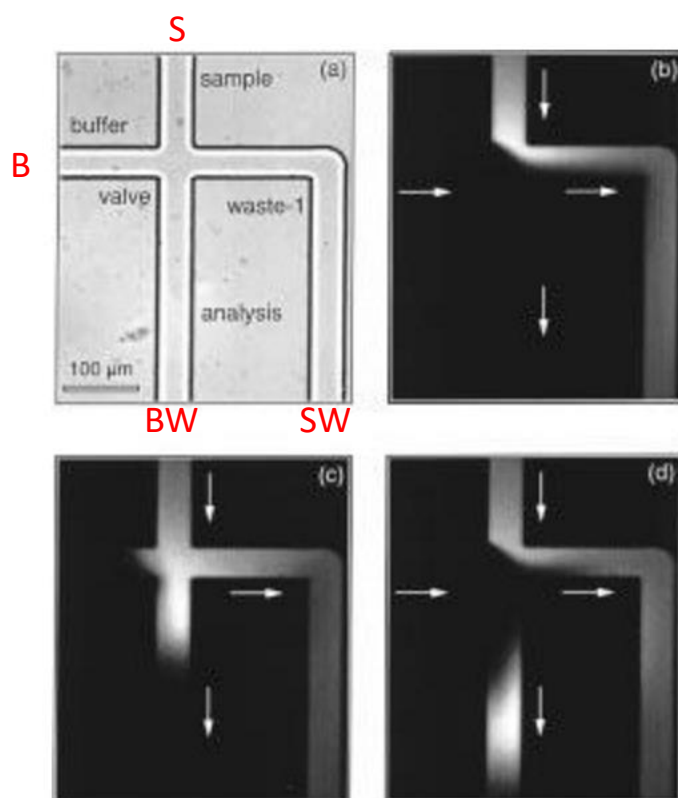


Figure 65: Illustration of gated injection. White image of microchip valves (a), and fluorescent image of gated injection loading step (b), injection step (c) and running step (d). adapted from [36].

In summary every injection type has its advantages and limitations, presented in **Table 14**.

Table 14: Advantages and limitations of different types of injections.

Injection type	Advantages	Limitations
Floating	- Easy to perform	-Diffusion of the sample during the loading step leads to peak broadening -Leakage of sample from the injection channel into the separation channel during separation -Electrokinetic bias
Pinched	-No sample diffusion during the loading step	-The injection plug is not ideal due to the unsymmetrical distribution along the channel -Electrokinetic bias
Gated	-The length of the plug can be controlled -No diffusion in the loading step -No leakage in the separation step	-Electrokinetic bias

ii. Detection techniques

Several detection methods can be implemented within microsystems (**Figure 58**). The most widely used are: optical (the most common), electrochemical and mass spectrometric ones.

a) Optical detection methods

Optical detection can be made by fluorescence, absorbance, refractive index detections, raman spectroscopy, surface plasmon resonance, chemiluminescence and electrochemiluminescence depending on the nature of the analyte ([37,326] and references therein). Laser induced fluorescence is the most widely used detection technique because of its very high sensitivity which is necessary in microfluidic device and for the ease of focusing the laser to the extremity of a very tiny separation channel [34,37]. Its limitation is the absence of fluorescence in most naturally occurring compounds and thus the need for derivatization as well as the commercial availability of only specific excitation wavelengths [37]. Derivatization can be performed before, during, or after on-chip separation ([34,326] and references therein). It also requires sophisticated experimental setup and may be expensive [349]. Despite its simplicity, absorbance detection necessitates large optical pathway in order to have good sensitivity which is not compatible with microchannel dimensions. Microfluidic channels have very small dimensions which limits the use of absorbance detection (LODs obtained for antimicrobial

metabolites were in the low mg / L, for 19 basic drugs were ≈ 2 mg / mL and for thiourea 167 μM ...[361]) Works in progress done in order to increase the optical path as well as to ameliorate sensor detection [361].

b) Mass spectrometry

Mass spectrometric detection is also used with MCE [362]. In comparison to conventional CE-MS, it offers a shorter analysis time and consumption of lower volumes of analytes [336] but needs sophisticated instrumentation and is not portable [363]. It gives complete analysis information on the mass of the analytes detected and their fragmentation pattern. It is usually employed in proteomics and for the detection of large biomolecules, providing quantitative and qualitative data ([327] and references therein). Matrix-assisted laser desorption / ionization (MALDI)(off-line) and electro spray ionization (ESI) (on-line) are two ionization methods for interfacing microchip with MS that have been extensively used ([362,327] and references therein).

c) Electrochemical detection methods

Electrochemical detection is widely used especially for inorganic ions detection [364]. It offers several advantages to the detection especially because of its remarkable sensitivity, facility of integration in miniaturized systems, miniaturization of detection devices in order to have a complete portable system, independence on the optical path and turbidity of the matrix, low cost and minimal power demand [37,365]. Electrochemical detection used with MCE are amperometry and conductimetry.

1. Amperometry

Amperometry, the most used electrochemical technique [37], detects the faradaic current provided by the oxidation or reduction of an electroactive molecule, by application of a constant potential at the working electrode. A major limitation of this method is the absence of detection of non electroactive molecules. The current is proportional to the number of moles of analyte oxidized or reduced at the surface of the working electrode [37]. A conventional three electrode system is usually used (working, reference and auxillary electrodes). Only two electrode systems are used in the case of some potentiostat configurations (such as wireless potentiostat provided by pinnacle technology) and in the case of micrometric working electrodes. The material for the working electrode is usually platinum, gold, or carbon (paste, ink, fiber or glassy

carbon) [365]. Adhesive layers of titanium and chromium usually are applied to permit the adhesion of platinum and gold to the surface of the chips [366].

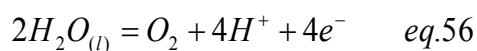
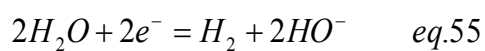
The position of the electrodes with respect to the separation channel has an important role in the amperometric detection. One problem comes from the interference between the currents generated during separation (in μA) with the detection currents (in nA) that can furthermore introduce a damage to the potentiostat. Thus, an electric isolation is achieved by three different configurations: End-channel detection (which has two alignment types: on-chip and off-chip), in-channel detection and off-channel detection **Figure 68**.

End-channel detection

With the end-channel detection, the electrode is positioned tens of micrometers away from the channel in the buffer reservoir, avoiding the high separation voltage because the electric field is eliminated directly at the end of the channel[367,336] (**Figure 68.1 and Figure 68.2**). This configuration is easy to obtain. The limitations are the shift of oxidizing or reducing potential of the analyte in comparison to these potentials without applying an electric field and lower in sensitivity in comparison to the other techniques. This lower sensitivity has two sources: the peak broadening at the end of the channel and the large background current due to interference with the electrophoretic current ([37] and references therein). Lunte et al. [368] have studied the effect of some parameters on the shift of the analyte half-wave potential for dopamine and catechol. A decrease in the distance between the working electrode and the end of the separation channel or an increase in separation voltage has led to positive shift of the half-wave potential. Thus, the reproducibility of results relies on the reproducibility of the experimental setup and the alignment of electrodes. Xu et al. [369] did not obtained reproducible results using a non-isolated potentiostat. So, voltammograms under a separation electric field should be analyzed depending on molecules and separating conditions. End-channel has two possible alignments: The off chip alignment which permits to adjust the distance of the electrode from the outlet and to change the electrode and polish it (**Figure 68.2**), and the on-chip alignment in which the electrode is integrated to the microchip, placed at the bottom of the buffer waste reservoir, that reduce the chip life-time depending on the electrode passivation state (**Figure 68.1**). In certain cases it is possible to renew the electrode electrochemically.

Off-channel detection

With the off-channel detection mode, the working electrode is integrated inside the separation channel, but the channel is grounded before the electrode by a decoupler (**Figure 68.3**). This induces a small area in the channel without electric field and analytes are pushed by the EOF generated prior to the decoupler in order to reach the working electrode. This can lead to a loss in efficiency [366] especially due to the change in flow value from electrokinetically driven to pressure-induced flow [370]. The pressure-induced flow provides a parabolic profile and the change in the flow leads to a back pressure from the length of the channel past the decoupler [370]. The advantage of this technique is to avoid the effect of alignment on the half-wave potential [370,371]. Several decouplers have been used such as palladium, cellulose acetate, PDMS-sugar [372]. At positive polarity, hydrogen gas is produced by hydrolysis of water due to the grounding of the electric field (**eq. 55**) [370]. The decoupler should absorb H₂ gas produced at the cathode thus preventing the formation of gas bubbles inside the separation channel [370,371]. While at negative polarity, O₂ is produced (**eq. 56**) that cannot be absorbed by it, so the in-channel detection becomes interesting [56].



In-channel detection

The in-channel detection mode allows to eliminate the band broadening occurring with the end-channel detection. In this case the working electrode is positioned inside the separation channel: it does not need grounding (and thus decoupler) before the working electrode but a special isolated potentiostat is necessary [56,373] (**Figure 68.2**). An example is a floating potentiostat working on a 9V battery, with no connection with ground so that it cannot be damaged by the electrophoretic current. Usually, it needs high detection voltage because of positive shift as shown in **Figure 66** where there is a 600 mV positive shift between of catechol when it passes from end channel to in-channel detection[369,373]. Martin et al. [373] have shown by a comparative study with end-channel detection that in-channel detection has led to 4.6 times increase in the number of theoretical plates and increase of the symmetry peak for catechol. **Figure 67** shows the difference in electropherograms between in-channel and end-channel detection where the peak in the in-channel mode (A) is more Gaussian and symmetric than the second one (B).

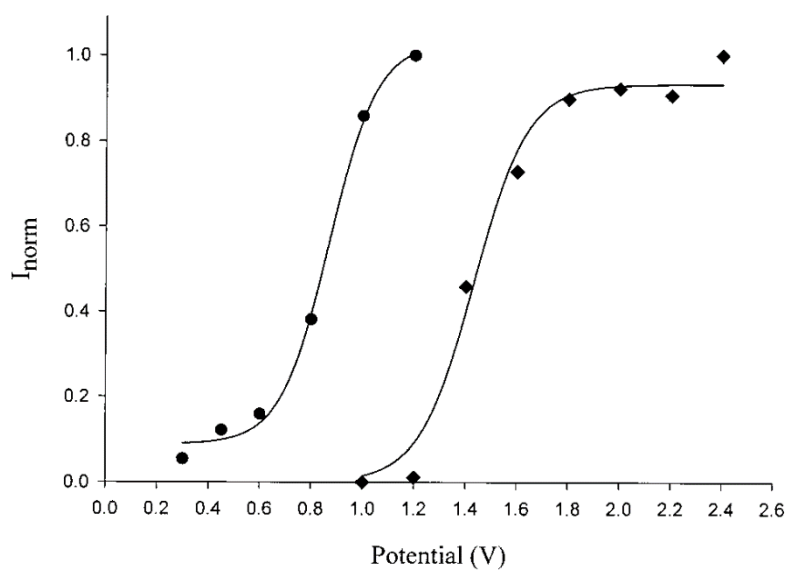
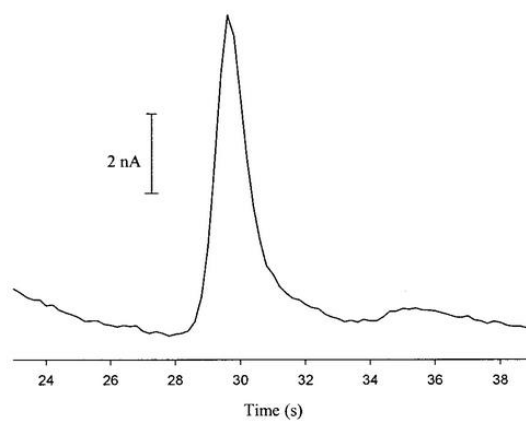


Figure 66: Hydrodynamic voltammograms recorded for catechol using in-channel (♦) and end-channel (●) EC detection. Conditions: 20 mM boric acid buffer, pH 9.2, $E_{\text{sep}} = 300 \text{ V / cm}$, potentials vs Ag / AgCl reference. Adapted from [373].

A



B

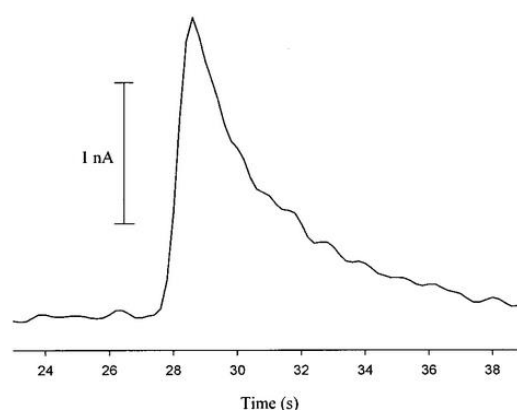
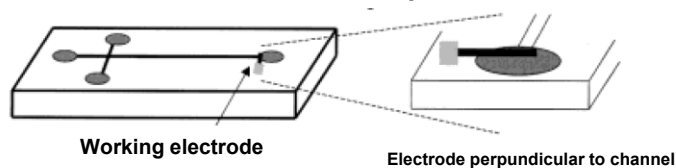
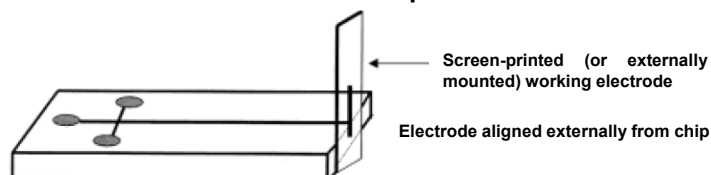


Figure 67: Electropherograms for catechol using (A) in-channel EC detection at +2.2 V and (B) end-channel. EC detection at +1.0 V. Conditions same as in Figure 66.

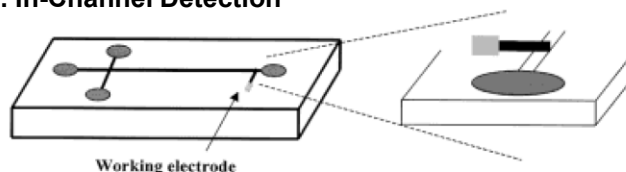
1a. End-Channel Detection: on chip



1a. End-Channel Detection: off chip



2. In-Channel Detection



3. Off-Channel Detection

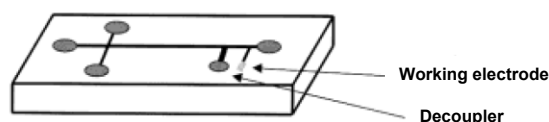


Figure 68: Modes of alignment of electrodes in amperometric detection. Adapter from [37]

2. Conductimetry

Conductimetry is a universal detection technique that is based on measuring the difference in conductance between the buffer and the analyte [364]. It requires simple experimental setup in two modes: either contact or contactless (C^4D) [349]. Even though contact conductivity is more sensitive, it has a lot of limitations such as: no selectivity, electrode fouling, water electrolysis at the electrodes which provokes bubble formation [374] and damage of the electronics in absence of special protection [349]. The distance between the electrodes as well as the thickness that separates the electrodes from the running solution play an important role in MCE with Capacitively coupled contactless conductivity detection (MCE- C^4D) [374]. The electrodes are placed outside the fluidic channels. They can be metallic [374] or pencil electrodes drawn on paper[375] (**Figure 69**). MCE- C^4D is in strong continuous growth and diverse new applications are reported [376]. Amperometric MCE as well as MCE- C^4D is commercialized [377].

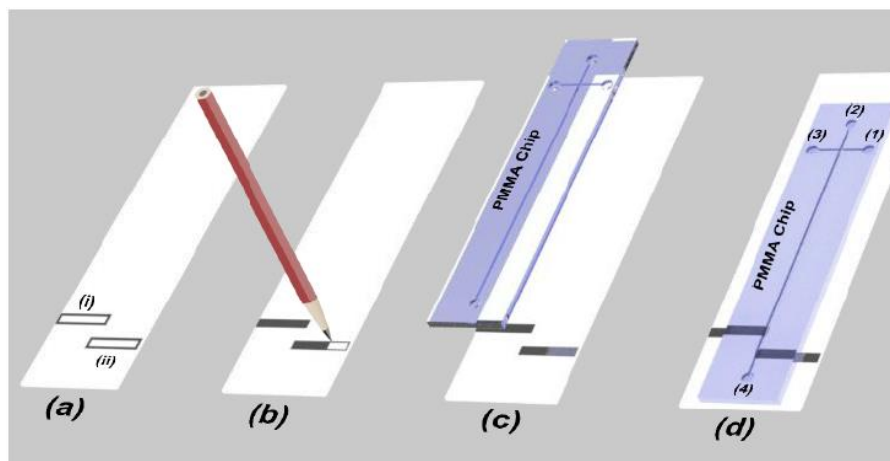


Figure 69: Microchip electrophoresis coupled to contactless conductivity detection. The electrodes were drawn with pencil. a, b, c and d represent the drawing and the association of the paper electrodes with the PMMA microchip. The labels 1, 2, 3 and 4 (in (d)) indicate the buffer, sample, sample waste and buffer waste reservoirs, respectively. Adapted from [375]

Summary

In this chapter we provided a state of the art about i) biosynthesis and biological activities of NO and RSNOs ii) the synthesis, reactivity, decomposition and analysis methods of RSNOs and iii) miniaturization: the materials and techniques used and what is achieved for RSNOs detection in microfluidic devices.

The second chapter focuses on two developed methods for the GSNO characterization and its decomposition products by capillary electrophoresis coupled to MS or to capacitively coupled contactless conductivity detection (C⁴D).

The third chapter is dedicated to the investigation of the decomposition pathways of GSNO using colorimetric and electrochemical detection ways.

Finally the last chapter describes the first results on the miniaturization of the diagnostics approaches. It demonstrates the application of a point of care device to detect RSNOs using microfluidic paper-based analytical devices, then the trials to integrate the previously studied separation, decomposition, and detection step inside a microfluidic device.

CHAPTER II: ANALYSIS OF GSNO DECOMPOSITION AND REACTIVITY BY CAPILLARY ELECTROPHORESIS: KINETICS AND DECOMPOSITION PRODUCTS IDENTIFICATION

As it was stated in the first chapter, S-nitrosothiols (RSNOs) can be employed as a diagnostic indicator or as a drug for several medical pathologies. The presence of RSNOs in some medical preparations necessitates the study of the decomposition products and impurities present in the sample that could have toxic effects. In addition to this, by separating RSNOs from their decomposition products, it is possible to identify the decomposition products, the decomposition pathway and the kinetics of decomposition. As the variation of RSNOs concentration in biological fluids can be also a diagnostic indicator, the separation of different RSNOs is rather interesting. This chapter is divided into two parts which present the development of two methodologies for characterizing and separating RSNOs by CE: the first one deals with the identification of GSNO decomposition products using CE coupled to MS detection. The second one describes the decomposition kinetics of GSNO and the transnitrosation reactions by CE coupled to capacitively coupled contactless conductivity (C⁴D).

I. EC and MS techniques for the analysis of decomposition products of GSNO at solid state

As stated earlier GSNO is considered as the most abundant low molecular weight (LMW) RSNO in human body [378], playing very important physiological and pathological roles depending on its concentration [301]. GSNO is a drug candidate for many diseases (cardiovascular diseases, cystic fibrosis, female sexual dysfunction) [301]. Even though GSNO is one of the most stable RSNOs, it is very sensitive to metabolism in human body [301], and to decomposition by light, heat, or trace metal ions [24]. However its decomposition pathways in aqueous solution and in biological systems are still under investigation. For now, the only reported GSNO decomposition products by light and heat are oxidized glutathione GSSG and NO [24]. It has been suggested that sulfonamide and glutathione sulfinic acid could be formed in aqueous solutions from the reaction between GSNO and GSH [379]. The oxidation products of cysteine-containing proteins such as cysteine sulfenic acids (Cys-SOH) have been observed in man and have been suggested as playing a role in enzymatic catalysis and in redox regulation [380]. In general sulfenic acid proteins are unstable and can produce further oxidation products such as cysteine sulfinic acid (Cys-SO₂H), and cysteine sulfonic acid (Cys-SO₃H), or form disulfide bond with GSH or other sulfhydryl, the last oxidation steps being irreversible.

Several techniques have been employed to identify the RSNOs decomposition products and mechanisms under different conditions, such as electron paramagnetic resonance [45], liquid chromatography coupled to UV-visible detection [45], MS [381-384], chemiluminescence [385], gas chromatography coupled to MS detection [379], capillary gel electrophoresis with laser induced fluorescence detection [386], CE coupled to UV detection [89,92]. CE is a very attractive separation method because of its low sample consumption, short analysis time, high separation efficiency, ease of operation and automation. The hyphenation of CE with MS (CE-MS) is nowadays accepted as a multidimensional analytical approach, complementary and / or competitive to chromatographic MS-hyphenated separation techniques. It is emerging as an essential analytical tool in the fields of life, environmental and forensic sciences. CE-MS combines the advantage of both techniques so that qualitative (migration times) and quantitative (peak areas) information, in combination with molecular masses and / or fragmentation patterns can be obtained in a single run [387].

In this chapter we describe, for the first time, a CE coupled to UV and MS method in order to determine the decomposition products in aged GSNO samples in solid state. From this CE-MS characterization, we aim at designing a global decomposition pathway which occurs at solid state during the storage of synthesized GSNO. This would also allow identifying all the decomposition products that could be formed while dissolving GSNO in aqueous solution. The determination of impurities and decomposition products of GSNO is of high importance for the safety of this potential drug.

A.Experimental

1) Chemicals

Reduced glutathione (GSH), oxidized glutathione (GSSG) sodium nitrite, N,N-dimethylformamide anhydrous (purity 99.8%) (DMF), acetic acid, ammonium carbonate for HPLC (30-33% NH₃ basis) and ammonium bicarbonate (purity 99.5%) were obtained from Sigma-Aldrich-Fluka (St-Quentin Fallavier, France). Glucose was purchased from Normapur®. Buffers were freshly prepared using Milli-Q water 18.2 MΩ.cm from a pure lab flex system from ELGA Labwater (Veolia water, France). Background electrolyte (BGE) was composed of 9.6 mM ammonium bicarbonate and 7.4 mM ammonium carbonate (pH 8.5, 20 mM ionic strength).

2) Sample synthesis

GSNO was synthesized as described elsewhere [232]. Briefly, in an ice bath, GSH was dissolved in 0.626 M HCl aqueous solution and equimolar sodium nitrite was added to the mixture under stirring for 40 min. The final solution was precipitated with cold acetone and stirred for another 20 min, then filtered and washed once with 80% acetone, twice with 100% acetone and three times with diethylether. GSNO, as a pink solid, was further freeze-dried and kept at -20°C. The solid GSNO was dissolved in BGE just before CE-MS separation. GSNO concentration was determined using absorbance at 333 nm (using 920 M⁻¹. cm⁻¹ as extinction coefficient) and Saville method [27].

3) CE apparatus and measurements

Electrophoretic measurements were performed with an HP^{3D}CE system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector. Data were handled by HP Chemstation software. Bare fused-silica capillaries of 75 μm i.d. (Polymicro Technologies, Phoenix, AZ, USA) with effective length of 22 cm (to DAD detector) and total length of 80 cm (to MS detector) were used. Successive hydrodynamic injections were performed in the following order: neutral marker (30 mbar, 2 s), BGE (50 mbar, 3 s), sample (50 mbar, 3 s), BGE (50 mbar, 2 s). Separations were performed under a 20 kV positive voltage (electric field, 250 V / cm) and the temperature of the capillary cartridge was set at 25°C, unless otherwise specified. The detection wavelength was 200 nm. Injections were repeated three times ($n = 3$) to evaluate data reproducibility.

New capillaries were conditioned by successive flushes with NaOH 1 M, NaOH 0.1 M, water, BGE under a pressure of 950 mbar for 15min each. Between runs capillaries were rinsed for 1 min using BGE. Capillaries when not used were rinsed with NaOH 1 M and water. Electro-osmotic flow (EOF) was measured with the neutral marker, being a mixture of DMF (0.02 % in BGE) for UV detection and glucose (5 mM in BGE) for MS detection.

4) MS detection

The principle of MS detection is presented in **annex 2**. An Agilent Series 1100 MSD single quadrupole mass spectrometer (Agilent Technologies) equipped with an orthogonal electrospray (ESI) source was used in the positive ionization mode. Nitrogen was used as nebulising (NG) and drying gas (DG). In the optimized conditions, the temperatures of NG and DG were set to 100 °C (pressure 68.95 kPa) and 250 °C (flow rate 6 L.min⁻¹), respectively. Optimized spray and skimmer voltages were 3000 V and 20 V, respectively.

CE was coupled to the ESI interface using an Agilent Technologies triple coaxial tube nebulizer held at ground potential. The optimized coaxial sheath liquid, composed of EtOH / H₂O (90:10, v / v) containing 2 % acetic acid, was delivered at a flow-rate of 6 $\mu\text{L}.\text{min}^{-1}$ by an 1100 series isocratic pump (Agilent) equipped with a splitter (1:100).

Signal acquisition was first performed in the scan mode (mass range of m/z : 150-3000 and 150-800). MS spectrum was then extracted under each peak for peak identification. Signal

acquisition was then performed in the single ion monitoring (SIM) mode at each identified and characteristic m/z value to improve signal sensitivity.

B. Results and discussion

CE coupled to UV detection has already been employed to characterize GSNO and its by-products [89,92,388]. In either acidic or neutral BGE conditions, GSNO and GSH were identified from their respective intensities and electrophoretic mobilities. One or two additional peaks were also present, with the hypothesis of the presence of GSSG for one of them (**Figure 70**).

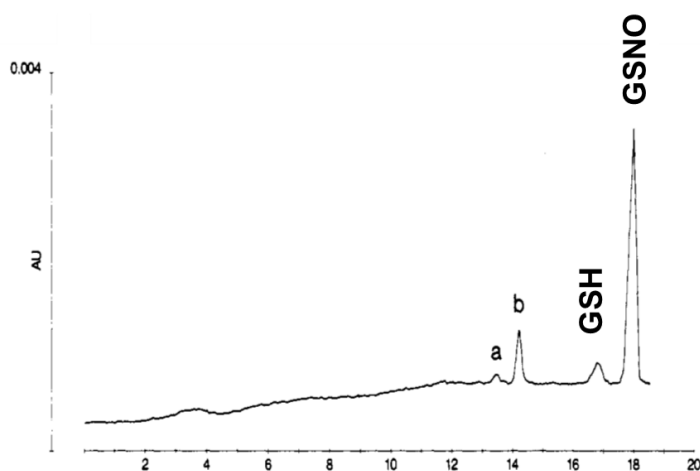


Figure 70: GSNO with 92 %conversion of 250 μ M GSH to GSNO (170 μ M) and to two additional unidentified peaks (peaks a and b), one of which is likely GSSG. Sample buffer 0.01 M sodium phosphate, 0.01 M HCL pH 2.3, positive polarity at 11 kV; absorbance 200 nm. Adapted from [89,92,388].

So as to go deeper in the understanding of GSNO decomposition, we aim at conceiving a new characterization methodology employing electrophoretic separations coupled to MS detection. We first optimized the experimental conditions, and particularly the BGE in terms of salt nature and pH, so as to be compatible with MS detection and to provide the best resolution. Sodium ions should be absent in order to avoid the excessive formation of adducts so an ammonium carbonate / ammonium bicarbonate BGE was used and the pH should not be acidic in order to avoid the artificial foration of GSNO starting from GSH and nitrite. Very basic pH can destroy the RS-NO bond so a slightly basic pH was chosen. **Figure 71** describes the optimized separation in BGE solution with both UV (**Figure 71a**) and MS (**Figure 71b**) detection of a six-months aged GSNO solid sample.

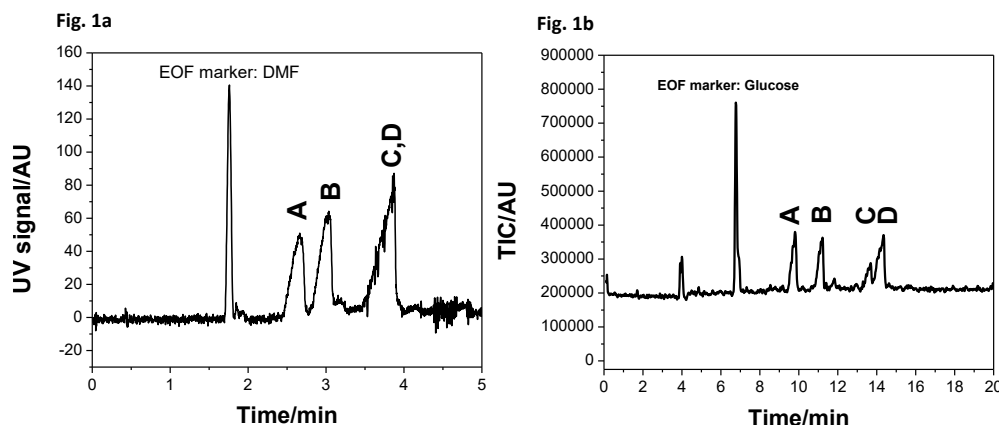


Figure 71: UV and mass spectrum electropherograms of 4.61 mM GSNO stored in solid state for 6 months, then dissolved in BGE just prior to analysis. BGE: ammonium carbonate buffer (20 mM, pH 8.5). Injection: neutral markers (30 mbar, 2 s), BGE (50 mbar, 3 s), sample (50 mbar, 3 s), BGE (50 mbar, 2 s). Capillary: 75 μ m id, total length 80 cm, length to UV detector 22 cm (Fig 1a) and to MS detector 80 cm (Fig 1b). Applied voltage: 20kV. MS in positive mode: see experimental part for details. Peak assignment: A. GSNO, B. GSSG, C. GSO₂H and D. GSO₃H. Neutral markers (DMF 0.02%, Glucose 5mM).

As expected from the respective lengths to detectors (22 cm and 80 cm for UV and MS, respectively), the migration time of neutral marker glucose is 3.85 times the one of DMF, indicating the robustness of the process during the entire analysis time.

The electroosmotic mobility was calculated using the following formula:

$$\mu_{eof} = \frac{L.l}{V.t} \quad eq.1$$

Where L is the total length of the capillary (in cm), l is the effective length to the detector (in cm), V is the applied voltage (in V) and t is the time of the peak of the EOF indicator (in s).

Under an “electroosmotic mobility” (including suction effect) of $77 \times 10^{-5} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$, the separation of a six month aged GSNO sample (stored in solid state), solubilized in BGE just before analysis presents two well resolved peaks and a wider peak in UV that seems to be composed of two non-resolved peaks in MS (in total ionic current mode) (**Figure 71**).

Thanks to the MS detection, a deeper characterization was performed by first providing a mass spectrum under each peak, followed by characterization in SIM mode at the corresponding characteristic m/z values.

Table 15: Mass spectrum assignment of ions with their % peak abundance identified by Total Ion Current (TIC). The data is related to Figure 72. X represents A, B, C, or D according to its position in table

Ion Assignment	A: GSNO		B: GSSG		C: GSO ₂ H		D: GSO ₃ H	
	<i>m/z</i>	% ab.	<i>m/z</i>	% ab.	<i>m/z</i>	% ab.	<i>m/z</i>	% ab.
[X+H] ⁺	337.1	100	613.1	100	340.1	100	356.2	100
[X+Na] ⁺	359.2	16	635.2	27	362.2	41	378.1	24
[2X+H] ⁺	673.1	16	-	-	679.1	6	711.1	13
[2X+Na] ⁺	695.2	10	-	-	701.0	7	733.0	7
[X+2Na-H] ⁺	-	-	657.1	4	384.1	12	400.1	5
[X+3Na-2H] ⁺	-	-	-	-	406.0	6	-	-
[(X+H)-NO] ⁺	307.1	8	-	-	-	-	-	-
[X+2H] ²⁺	-	-	307.1	61	-	-	-	-
[X+Na+H] ²⁺	-	-	318.2	6	-	-	-	-
[(X-O)+H] ⁺	-	-	-	-	324.2	8	-	-
[(X-O)+Na] ⁺	-	-	-	-	346.3	11	-	-

Figure 72A shows the mass spectrum corresponding to the first CE peak (noted as A). According to the *m / z* values and their abundances (**Table 15**), the peak was attributed to GSNO. This is in accordance with what was reported in literature [382] but conversely *m / z* 613 is not present in our study. In literature [382], the *m / z* 613 was interpreted as the loss of two NO molecules from two GSNO followed by dimerization into [(2A+H)-2NO]⁺, which corresponds to GSSG. However, thanks to the separation performed before the MS detection, these results shown here demonstrate that GSSG is not produced during the analytical process but is present in the initial sample. Indeed, GSSG is not present in the mass spectrum of the GSNO peak but under a subsequent peak as a decomposition product (see below for second peak interpretation). In summary, the five main *m / z* peaks shown in **Figure 72A** are:

- The parent peak of GSNO (symbolized as A) [A+H]⁺ at *m / z* 337.1
- A sodium adduct [A+Na]⁺, at *m / z* 359.15;
- Although ESI is a soft injection method, it leads to a minor loss of NO molecule resulting in the formation of a peak at *m / z* 307.1 [(A+H)-NO]⁺;
- Dimers of two GSNO molecules could be formed to give the peaks at 673.1 and 695.2 corresponding to [2A+H]⁺ and [2A+Na]⁺, respectively.

The mass spectrum of the second CE peak corresponds to GSSG (symbolized as B) (**Figure 72B, Table 15**):

- The parent peak at m/z 613.1 corresponding to $[B+H]^+$
- The peak corresponding to double charge $[B+2H]^+$ at m/z 307.1
- Three other peaks corresponding to sodium adducts at m/z 318.2, 635.2 and 657.1 corresponding to $[B+Na+H]^{2+}$, $[B+Na]^+$ and $[B+2Na-H]^+$ respectively.

The third peak (**Figure 72C, Table 15**) corresponds to GSO_2H (symbolized as C) with:

- The most intense one $[C+H]^+$ of m/z 340.1
- Peaks related to sodium adducts at 362.2, 384.1 and at 406.0 corresponding to $[C+Na]^+$, $[C+2Na-H]^+$ and $[C+3Na-2H]^+$, respectively.
- Dimerization peaks appear also at m/z 679.1 and 701.0 corresponding to $[2C+H]^+$ and $[2C+Na]^+$, respectively.
- Additional peaks appear at 324.2, 346.3 and 368.2 m/z values that can be related to a $GSOH$ entity and correspond to $[C-O+H]^+$, $[C-O+Na]^+$ and $[C-O+2Na-H]^+$, respectively.

This could be due to the co-elution of $GSOH$ and GSO_2H in these conditions of BGE. However, because of the known instability of sulfenic acid compounds we suggest that they are produced from decomposition of GSO_2H by the ESI process before entering the MS detector.

The fourth peak (**Figure 72D, Table 15**) with the lower apparent mobility corresponds to GSO_3H (symbolized as D) with:

- m/z 356.2 corresponding to $[D+H]^+$ [381]
- Sodium adducts at 378.1 and 400.1 corresponding to $[D+Na]^+$ and $[D+2Na-H]^+$.
- Dimerization of two molecules of GSO_3H is identified as well at m/z 711.1 and 733.0 corresponding to $[2D+H]^+$ and $[2D+Na]^+$, respectively.
- A non-identified peak was furthermore obtained at m/z 614.2.

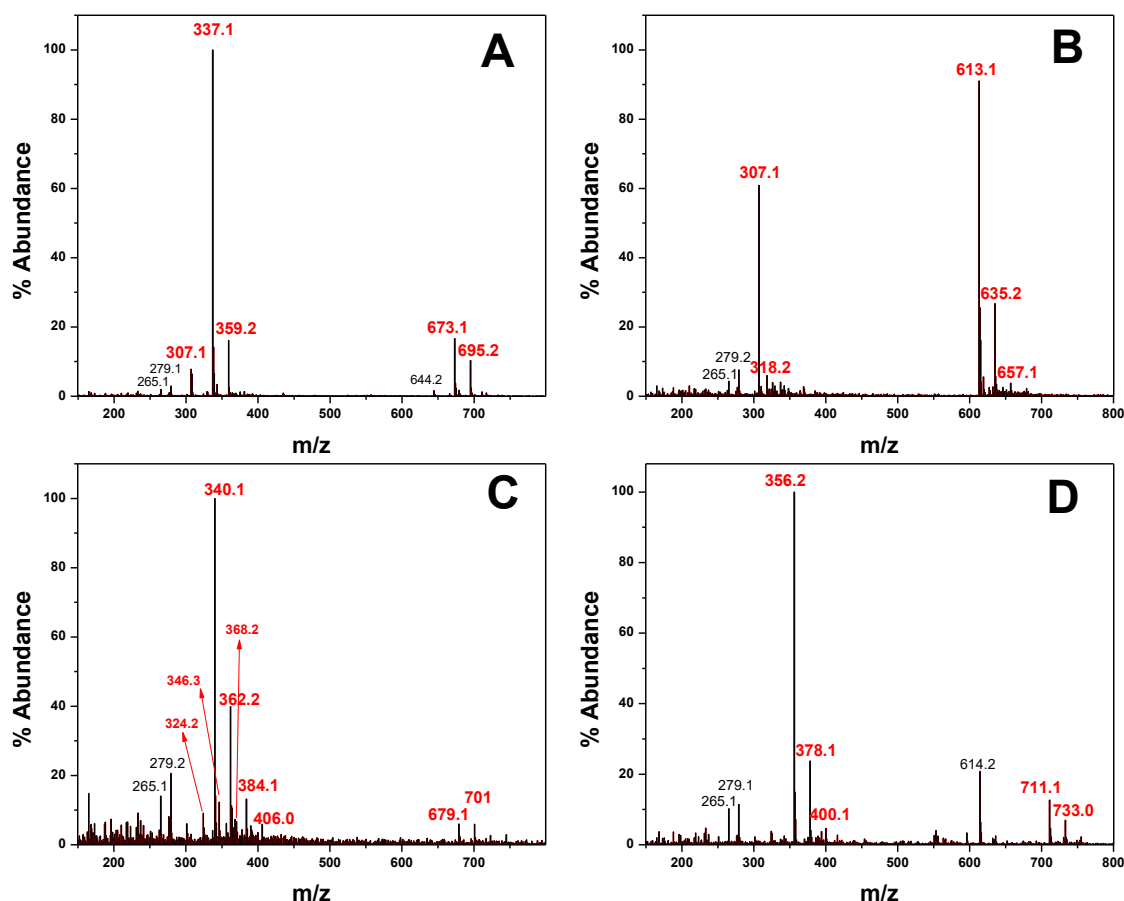


Figure 72: Mass spectrum extracted from the four peaks identified on Figure 71 by Total Ion Current (TIC). The letters A, B, C and D represent the successively obtained peaks (see Figure 71)

Concerning the electrophoretic migration order, as the separation of these anionic compounds is in positive mode, it appears that the electrophoretic mobility in absolute value (proportional to the charge over hydrodynamic volume ratio) is increasing going from GSNO, GSSG, GSO₂H, to GSO₃H (**Figure 73.I**). If one considers that no change in the amino and carboxylic acid groups pK_a values in the glutathione backbone occurs after dimerization, GSNO (336.3 g.mol⁻¹) should have a lower electrophoretic mobility (in absolute value) than GSSG (612.6 g.mol⁻¹), which is coherent with the separation order. On the other hand, GSO₂H and GSO₃H present a higher charge density than GSNO and GSSG, due to the presence of the additional acidic function of sulfinic and sulfonic groups (pK_a~2 [389] and ~-0.9 [390], respectively), they therefore have a higher electrophoretic mobility, as demonstrated in this study.

These experimental results along with previously published studies [391-394] allow us to establish a schematic and global decomposition pathway for aged GSNO in solid state at -20°C, and dissolved in a slightly basic medium (pH 8.5) as shown in **Figure 73.II**. We proved the formation of GSO₂H and GSO₃H during aging which could be of very important biological

consequences. They could inhibit glutathione transferase and glutathione transferase-like enzyme families [395], which are responsible for detoxification of many carcinogenic, mutagenic, toxic and pharmacologically active molecules.

Oxidation of GSNO in aqueous medium to GSO_2H is not well described in literature although it is vaguely mentioned [379]. Molecules arising from GSNO decomposition such as GS(O)SG and GS(O)NH_2 were described [379,381], but they were not identified in our case. This can be due to the mild alkaline separation conditions used for sample preparation that lead to their instability giving rise to GSSG and GSO_2H , respectively, or to the fact that the liquid samples examined in this study were freshly prepared and undergo no decomposition during separation [381].

Oxidation of sulfhydryl group in biological systems or by addition of hydrogen peroxide in aqueous solution to form sulfenic, sulfinic and sulfonic acids is well reported in the literature [393,394,396-399]. Indeed biological system oxidation to sulfenic acid is reversible while that to sulfinic and sulfonic acids is irreversible although some studies suggest that sulfinic acid could be reduced using enzymatic processes through a sulfiredoxin pathway [393,398,400,401]. Sulfenic acid is unstable [402] leading to GSSG (in the presence of GSH) or undergoing further oxidation till stable sulfinic and sulfonic acids (in the presence of hydrogen peroxide and other reactive oxygen species). Also GSH can produce sulfenic acids directly in the presence of reactive oxygen species (ROS) or reactive nitrogen species (RNS) as it is presented in **Figure 73.II**. In our conditions the reactive nitrogen species is the protonated form of nitrous acid H_2NO_2^+ (or NO^+) [403]. ROS present in biological medium could not be present in our solid sample so a simple oxidation by air oxygen is suggested. Also GSNO undergoes hydrolysis to produce sulfenic acid and HNO [393,404,405,384].

As the production of the oxidation products as GSSG, GSO_2H and GSO_3H starting from GSNO was shown here, the separation of freshly prepared solid GSNO or 6 months later (stored as a solid at -20°C) was performed so as to identify a possible kinetics of decomposition. By employing glucose as internal standard for quantitation, when going from the freshly prepared GSNO to the 6 months old GSNO, it appears that GSNO and GSO_2H concentrations decrease about three times and by 20%, respectively, whereas GSSG and GSO_3H concentrations increase by about 20% and 6 times, respectively. This is in correlation with the proposed decomposition pathway.

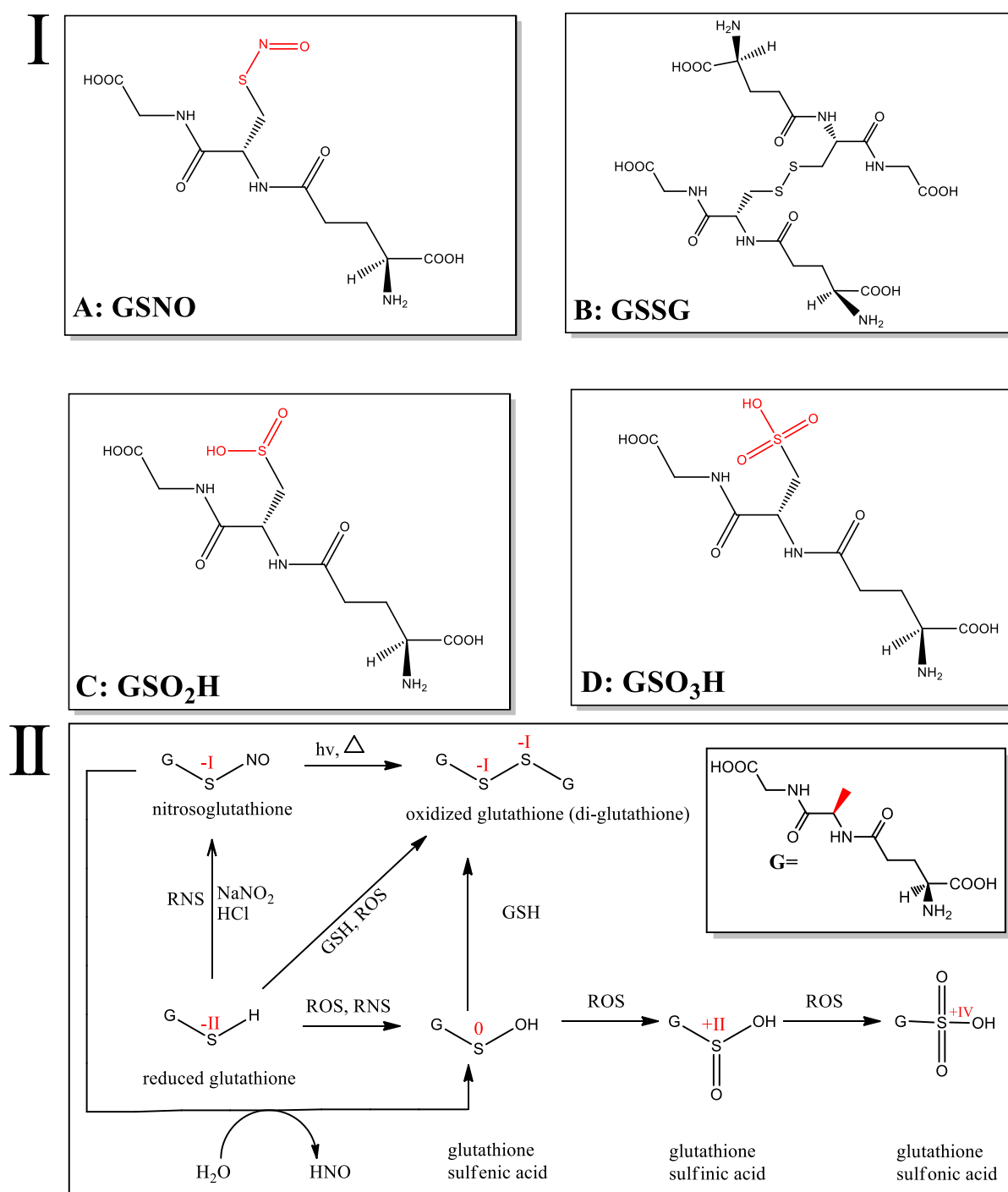


Figure 73: I) structures of the four identified compounds A, B, C, D. II) Decomposition pathways of GSNO, according to the CE-MS characterization of this study. Red numbers above the sulfur atoms represent the oxidation state of sulfur, S-nitrosoglutathione (GSNO), oxidized glutathione (GSSG), glutathione sulfinic acid (GSO₂H) and glutathione sulfonic acid (GSO₃H).

C. Conclusion

We have demonstrated for the first time that CE coupled to MS is a useful methodology to characterize the purity of and ageing effect on GSNO. We have also formally identified for the first time the presence of other decomposition products than GSSG, namely, GSO_2H and GSO_3H . From these results we have been able to construct a decomposition pathway describing the mechanisms leading to these products some of which have been suggested elsewhere in the literature. The determination of impurities and decomposition products of GSNO is of significance for the safety of this potential drug. As noted above, we have found evidence for the presence of GSO_2H and GSO_3H during aging and this may have important biological consequences. Such compounds may potentially inhibit glutathione S-transferase and glutathione transferase-like enzyme families [395], these being responsible for the detoxification of many xenobiotic compounds. This methodology could also be applied to physiological fluids to identify possible toxic products of GSNO when injected at high concentration or in some diseases.

II. EC and C^4D for the analysis of the decomposition of GSNO solution under light and heat

Capacitively Coupled Contactless Conductivity (C^4D) [406-408] has been widely used as detection method coupled to CE due to its good sensitivity, simple instrumentation and no need for derivatization steps. C^4D is a non-selective detection method suitable for all charged ions ; when coupled to CE its response is proportional to the difference in the mobility of the analyte and the co-ion of the background electrolyte (BGE) [407]. However, C^4D was not described since now as a detection method for the analysis of GSNO samples

Therefore, in this study we have demonstrated, for the first time, the separation and characterization of aged solid GSNO samples and some of its decomposition products using the coupling between CE and C^4D . We then demonstrated the powerfulness of this CE- C^4D for the study of the decomposition of GSNO by light and by heat with information about the kinetics

of these reactions through quantitation of GSNO and GSSG. Finally, we reported the transnitrosation reaction with cysteine using this new methodology.

A. Experimental

1) Samples, reagents and solutions

Reduced glutathione (GSH), oxidized glutathione (GSSG) and *dihexadecyl dimethyl ammonium bromide* (DDABr) were purchased from Sigma Aldrich. Sodium nitrite, sodium nitrate, sodium hydroxide, sodium monophosphate monobasic anhydrous were purchased from Synth (São Paulo, Brazil). Cyclohexyl-2-aminoethanesulfonic acid (purity 98% for biochemistry) was obtained from Acros Organics. GSNO was synthesized according to a procedure described elsewhere [232]. Briefly, equimolar amount of nitrite was added to equimolar amount of GSH and hydrochloric acid. The resulting pure solid was rinsed once with 80% acetone, twice with 100% acetone and three times with diethyl ether and then stored in the dark at -20°C during 4 months. Ultra-pure water (18.2M Ω) was obtained from a Direct-Q 3 UV Water Purification System (Millipore, Molsheim, France).

The background electrolyte (BGE) for CE separation was composed of 20 mM CHES (N-cyclohexyl-2-aminoethanesulfonic acid) adjusted to suitable pH (between 9 and 10 according to separations) using 1 M concentrated NaOH in positive polarity experiments and 20 mM CHES with DDAB 116 μ M adjusted to suitable pH (between 9 and 10 according to separations) using 1 M concentrated NaOH in negative polarity experiments. Samples were prepared in the 20 mM CHES BGE (at pH 9) unless specified.

2) Capillary Electrophoresis Instrumentation

The CE separations were performed in a homemade CE system equipped with C⁴D [409] (**annex 3**). The C⁴D principle is presented in **annex 4**. Bare fused-silica capillaries with 75 μ m id and 47 cm total length (37 cm effective length) or 50 cm total length (40 cm effective length) were used for CE separations. The separation voltage was 27 kV in positive or negative polarity (CZE2000, Spellman High Voltage Electronics Co., NY, USA) and was applied at the inlet capillary end, while the outlet capillary end remained grounded. The samples were injected hydrodynamically by applying positive pressure (11 kPa) at the sample reservoir for a period

of 3 s. The C⁴D operated at 600 kHz (sinusoidal signal) and 1.9 V_{pp} (peak-to-peak amplitude), with a data acquisition rate of 3.35 Hz. The C⁴D was positioned 10 cm from the capillary outlet end. All experiments were carried out at ambient temperature ranging from 20 to 25 °C.

Once a day, the fused-silica capillary was sequentially flushed with 0.1 M NaOH, water and BGE (5 min each). After each run, the capillary was flushed with BGE for 1 min. Standard curves were obtained by injecting (in triplicate) standard solutions containing pure GSH, GSSG or the synthesized GSNO. GSNO concentration was determined by absorbance at 336 nm ($\epsilon=920 \text{ M}^{-1}\text{cm}^{-1}$). A linear regression was performed on the standard curves using the least-squares method. Peak integration and statistical analysis were carried out with the software Origin 8.1 (OriginLab, Northampton, MA, USA).2.3 Decomposition and transnitrosation protocols

3) Decomposition and transnitrosation protocols

Light decomposition. GSNO decomposition was studied by exposing GSNO solution (1 mM, purity 66%) to a visible light lamp (Dolan-Jenner industries, Fiber-Lite® LMI-6000 LED fiber optic illuminator).The decomposition time was between 1 and 75 min.

Temperature decomposition. GSNO decomposition was studied by putting vials of $\approx 1.3 \text{ mL}$ each of GSNO solution (1 mM, purity 66%) in an oven heated previously at 80°C. The decomposition time was between 10 and 108 min.

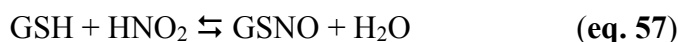
Transnitrosation protocol was achieved by mixing different solutions of cysteine (76, 152, 305, 381 and 495 μM) with GSNO (331 μM) just prior to analysis in the CE vial.

B. Results and discussion

This study is aimed at characterizing a 4-months aged solid GSNO samples in terms of purity and decomposition states and at studying transnitrosation processes. In this context, we employed CE-C⁴D, as it corresponds to a very efficient separation method providing a pertinent detection process. Indeed, C⁴D can detect all charged molecules and is easily coupled with separation by CE. Thus we performed this coupling for the separation and detection of all the impurities and decomposition products present in a GSNO sample.

As CE is coupled to C^4D , the best CE BGE should be compatible with C^4D detection, *i.e.*, have a low co-ion electrophoretic mobility and a high counter-ion electrophoretic mobility in comparison to the analyzed analyte in order to achieve best sensitivity [407].

GSNO can be synthesized and stocked at solid state by reacting nitrous acid with the thiol group of the reduced glutathione (GSH) [46] (eq. 57):



The chemical structures of GSNO, GSH and potential decomposition products of GSNO are presented in **Figure 74**. The pK_a values of GSH and GSSG moieties are of wide controversy especially the pK_a of thiol and of amino of the N-terminal of GSH (**Figure 74; Table 16**). The selected pH range was between 9 and 10 in order to be in the pK_a range of thiol groups (when present) and the amino groups of N-terminal of the tri-peptide GSH. CHES buffer (pK_a 9.41) was chosen because it fits the criteria of low mobility and has a pK_a in this selected pH range.

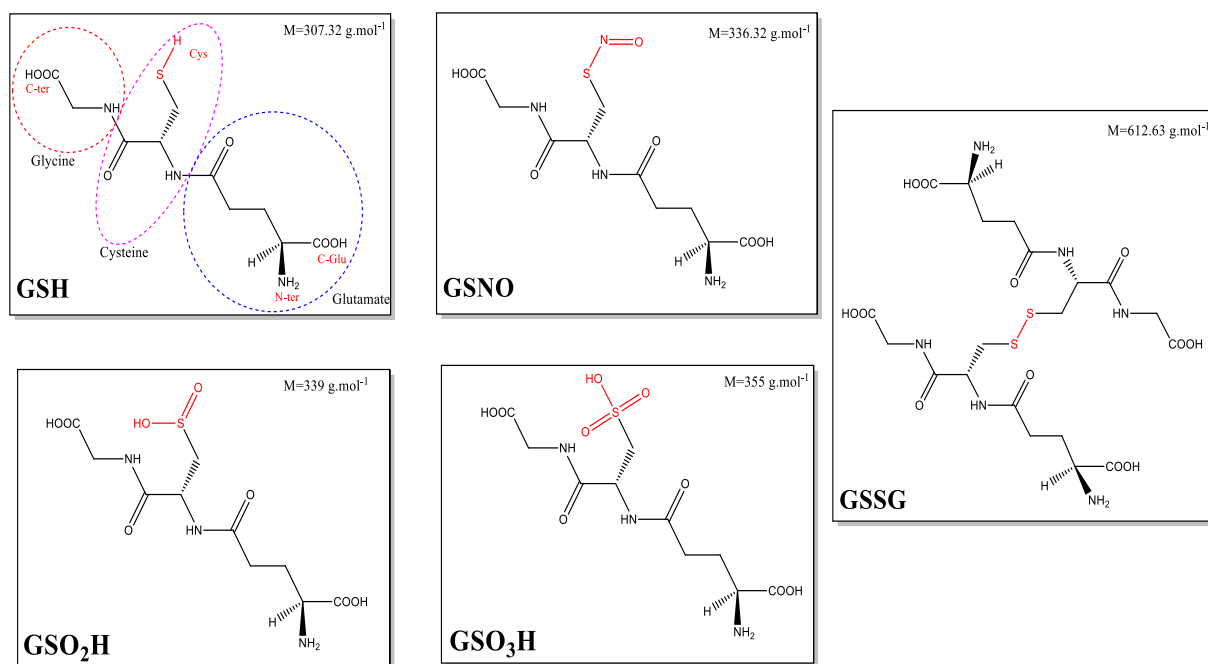


Figure 74: Structural formulas of reduced glutathione (GSH), S-nitrosoglutathione (GSNO), oxidized glutathione (GSSG), glutathione sulfinic acid (GSO₂H), glutathione sulfonic acid (GSO₃H). Structural formula of reduced glutathione GSH shows the amino acid composition of this tripeptide with the symbols of N- and C-terminals and cysteine SH. (see Table 16 for pK_a values).

Table 16: pKa values of the different carboxylic and amine moieties of reduced (GSH) and oxidized (GSSG) glutathione.

Reference	Residue			
	C-Glu	C-Ter	Cys	N-Ter
GSH				
[410]	2.69	3.68	8.88	9.46
[411]	2.12	3.53	8.66	9.62
[412]	-	3.59	8.75	9.65
[413]	2.05	3.40	8.72	9.62
[414]	2.13	3.51	8.74	9.66
[415]	2.12	3.53	9.12	8.66
[416]	2.12	3.59	9.65	8.75
GSSG				
[410]	2.73	3.80	-	9.51
[411]	2.3	3.7	-	9.2
[417]	2.09	3.49	-	9.18
[418]	1.96	3.50	-	9.18

1) Characterization of GSNO sample

The separation of GSNO samples (4 months-old and stored at solid state -20 °C) was performed by dissolution in 20 mM CHES (pH 9.0), and analysis by CE-C⁴D straightly after dissolution, without giving time for decomposition. **Figure 75** displays the electropherogram obtained after optimization of C⁴D maximal response (by variation of amplitude and frequency), and of

separating conditions (pH, Voltage and injection time). The electropherogram exhibits four distinct peaks (similar to those obtained in **Chapter 2. part 1**), corresponding to an analysis time of less than 2.5 min. The first peak with large intensity was attributed to GSNO. The second peak was attributed to GSSG with the use of an external standard (**Figure 75.1**). If we consider that no change in the amino pKa values in the glutathione backbone occurs after dimerization, GSNO should have a lower electrophoretic mobility than GSSG, therefore migrating before GSSG. The attribution of GSNO to the first peak was experimentally confirmed by decomposing GSNO using light (see next section). The electropherogram of GSH standard (**Figure 75.2**) proves that GSH present in sample is below the LOD of the method for GSH (11.8 μM). The height of the last two peaks does not vary during time upon aqueous decomposition of GSNO which eliminates the possibility that these two peaks result from aqueous decomposition of GSNO. Stamler et al. [92] have also studied the separation of GSNO sample (purity 92%) synthesized from GSH using CE-UV in acidic medium (pH 2.6) with an overall analysis time of 18 min. The authors showed four peaks which were attributed to GSNO, GSH (resulting from incomplete conversion of GSH during fabrication of GSNO) and two unidentified peaks (**Figure 70**). One of these two peaks was suggested to be due to GSSG. As their separation was performed in acidic medium, the determined electrophoretic mobilities of GSNO, GSH and GSSG were much smaller than ours.

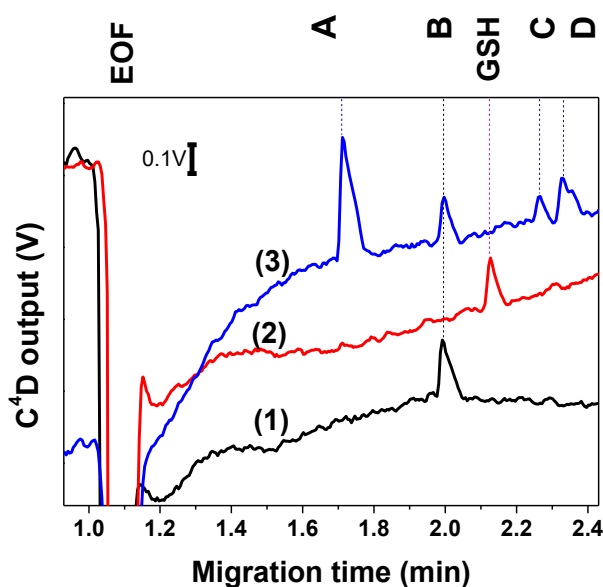


Figure 75: Electropherograms of GSSG, GSH standard solutions and a 4 months old GSNO sample solution. All samples were prepared in CHES (20 mM, adjusted to pH 9.0 with NaOH). Hydrodynamic injection: 3 s, 11 kPa; BGE: 20 mM CHES (adjusted to pH 10.0 with NaOH); Separation voltage: +27 kV, Capillary: 47 cm (37 cm effective), id 75 μm . Detection: 600 kHz, 1.9 V_{pp}. (1) GSSG 157 μM , (2) GSH 356 μM , (3) GSNO sample 1 mM (purity 66 % determined by colorimetric detection at 336 nm using $\epsilon=920 \text{ M}^{-1} \text{ cm}^{-1}$). Average capillary electric current was 36 μA . A. GSNO, B. GSSG, C. GSO₂H and D. GSO₃H.

Our results are in accordance with those obtained by CE-MS at pH 8.5 in a background electrolyte (BGE) compatible with MS. The attribution of the four peaks was performed by comparison to the previously performed MS spectra in similar CE conditions. The peaks appeared in the following order: GSNO, oxidized glutathione (GSSG), glutathione sulfinic acid (GSO_2H) and glutathione sulfonic acid (GSO_3H). As GSO_2H and GSO_3H possess a higher charge density than GSNO and GSSG, due to the presence of the additional acidic function of sulfinic and sulfonic groups ($\text{pK}_a \approx 2$ [389] and ≈ -0.9 [390], respectively), they therefore have a higher electrophoretic mobility.

For quantitative purpose, calibration curves were performed for GSNO, GSH and GSSG at pH 10 (**Figure 76**). This pH was chosen since it provides better sensitivity due to the higher electrophoretic mobility (higher negative charge) of the studied molecules. A rapid and complete separation of all molecules present in the sample was obtained in less than 2.5 min. The calibration curves have an excellent linearity ($R^2 > 0.99$) in the range 50-600 μM (**Figure 76**). The calculated limits of detection (using $S/N=3$) were 15.4 μM , 11.8 μM and 6.2 μM and those for electrophoretic mobilities were $-(34.6 \pm 0.27) \times 10^{-5}$, $-(46.29 \pm 0.31) \times 10^{-5}$ and $-(42.91 \pm 0.25) \times 10^{-5} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$ for GSNO, GSH and GSSG, respectively.

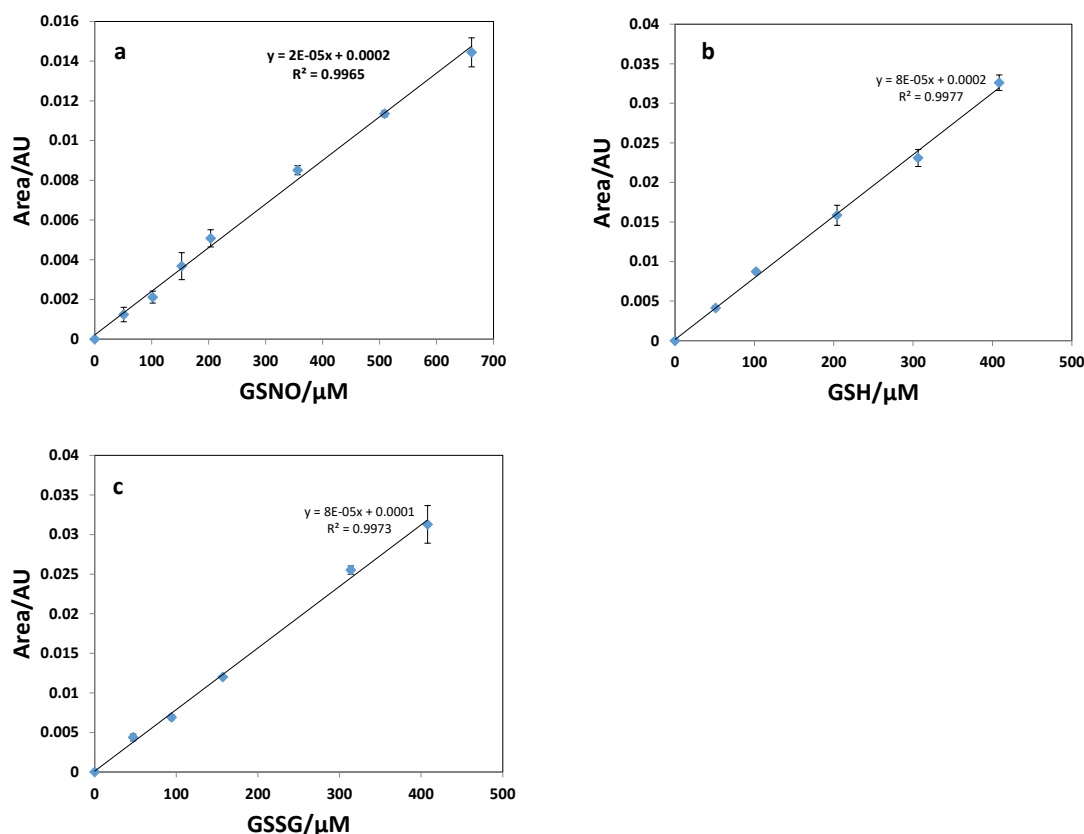


Figure 76; Calibration curves of a) GSNO, b) GSH and c) GSSG. Conditions same as Figure 75

In order to identify other decomposition products, the separation was performed in reversed electroosmosis by dynamically modifying the capillary inner wall with DDAB. The results showed an inverted migration order for GSNO, GSH and GSSG as expected. The presence of nitrite and nitrate was also detected, as nitrate can be formed from oxidation of nitrite during 4 months storage (**Figure 77**). Note that this oxidation is very slow in aqueous solution without the presence of oxidizing agent such as oxyhemoglobine, hydroxyl radical, or superoxide [70,419]. The last two peaks (which are attributed to GSO_2H and GSO_3H) appear in these conditions as a single peak.

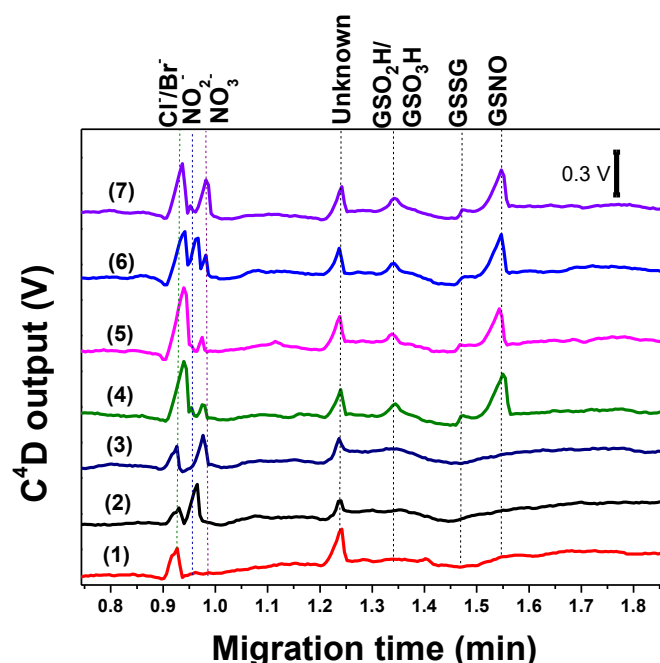
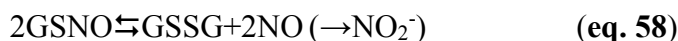


Figure 77: Electropherograms of GSNO (purity 66 %), different standard solutions of nitrite, nitrate and chloride and their mixtures. Samples were prepared in CHES (20 mM, adjusted to pH 9.0 with NaOH) + DDAB 116 μM . BGE: CHES (20 mM, adjusted to pH 10.0 with NaOH) + DDAB 116 μM . Hydrodynamic injection: 3 s, 11 kPa; Separation Voltage: -27 kV; Capillary: 50 cm (40 cm effective), id 75 μm . Detection: 600 kHz, 1.9 V_{pp} . (1) blank, (2) nitrite 125 μM , (3) nitrate 93 μM , (4) GSNO sample 0.9 mM, (5) GSNO sample 0.9 mM + chloride 120 μM , (6) GSNO sample 0.9 mM + nitrite 125 μM , (7) GSNO sample 0.9 mM + nitrate 93 μM . The EOF time was 2.7 min. Average capillary electric current was 33 μA .

2) Decomposition of GSNO using light.

GSNO can be decomposed using light [43,83-85] (UV, visible and infrared light), and the decomposition rate depends on the power of the radiation delivered (which depends on intensity and distance between source and sample). The final decomposition products described in literature are GSSG and nitrite (**eq. 58**) (**Chapitre 1. Section I.C.3**), but many radical species are formed during this process, among which NO [12,24]:



In this study, the decomposition of GSNO by visible light just before its analysis by CE-C⁴D induces a decrease in GSNO peak intensity, simultaneously with the increase in GSSG peak intensity (**Figure 78**). The concentration of GSO₂H and GSO₃H did not change during irradiation. By plotting the natural logarithm of GSNO peak areas as a function of illumination time (**Figure 78C**) the profile of the curve indicates that the kinetics of GSNO decomposition is first order. The slope of this curve corresponds to the apparent rate constant, equal to $(3.40 \pm 0.17) \times 10^{-3} \text{ s}^{-1}$. These results are in accordance with those reported by Sexton *et al.* [43] who described GSNO decomposition as an approximately first order process with $k_{\text{obs}} = (4.9 \pm 0.3) \times 10^{-7} \text{ s}^{-1}$. The rate constant obtained in our work is 10^4 higher than that reported in the literature. This difference can be attributed to the fact that the apparent decomposition rate constant depends on the light intensity and also on the light source.

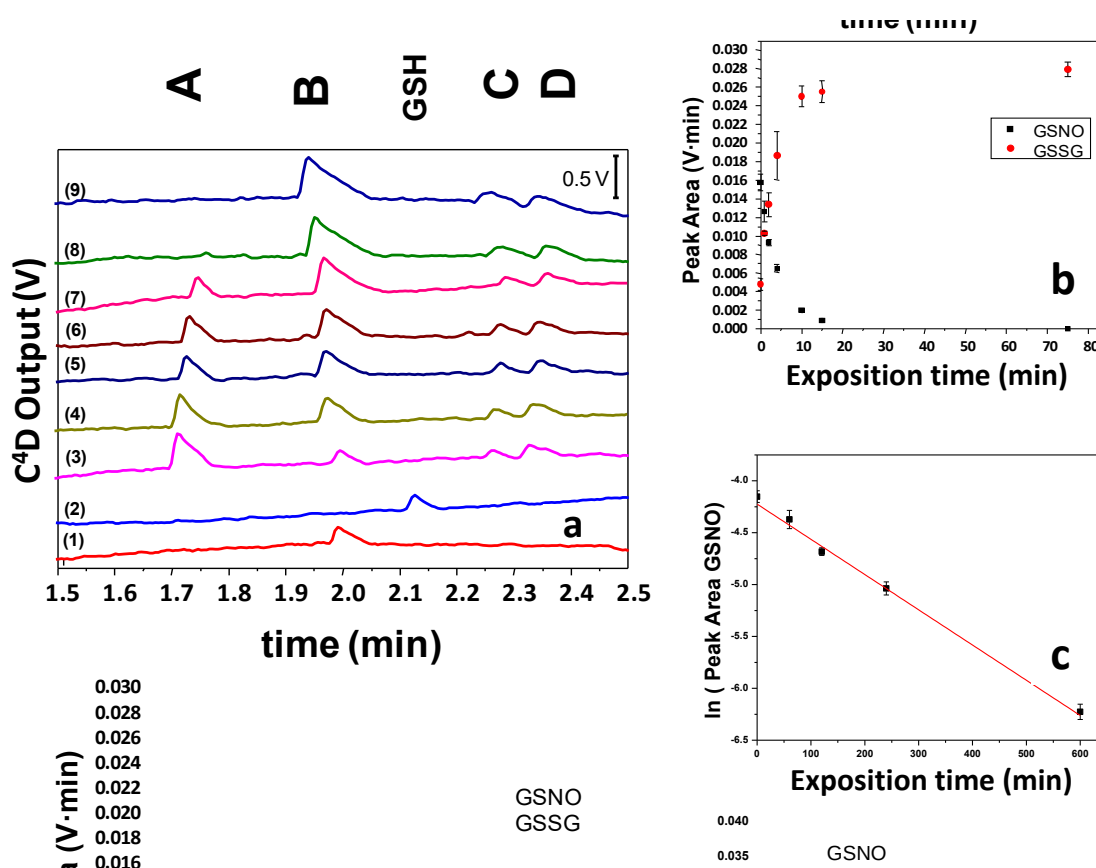


Figure 78: Study of decomposition of a GSNO sample induced by light. A) Electropherograms of (1) 102 μM GSSG, (2) 77 μM GSH, (3-9) 1 mM GSNO (purity 66 % determined by colorimetric detection at 336 nm using $\epsilon = 920 \text{ M}^{-1} \text{ cm}^{-1}$) after visible light decomposition for (3) 0 min, (4) 1 min, (5) 2 min, (6) 4 min, (7) 10 min, (8) 15 min and (9) 75 min. B) GSNO and GSSG peak areas as function of time of exposition. All samples were prepared in CHES (20 mM, adjusted to pH 9.0 with NaOH). C) natural logarithm of peak areas of GSNO as function of time of exposition. Hydrodynamic injection: 3s, 11 kPa; BGE: 20 mM CHES (adjusted to pH 10.0 with NaOH); Separation voltage: +27 kV, Capillary: 47 cm (37 cm effective), id 75 μm . Detection: 600 kHz, 1.9 V_{pp}. Average capillary electric current was 37 μA .

3) Decomposition by heat

GSNO is thermosensitive [24,44,224], but its decomposition is very slow at ambient temperature and in the dark (GSNO half-life ≈ 80 h [45]). We have studied its decomposition at 80°C, which is sufficient to accelerate the process while avoiding the artifact of concentrating the solution due to evaporation and boiling. The results show the decrease in GSNO peak intensity concomitant with the increase in GSSG peak intensity (**Figure 79**) while GSO₂H and GSO₃H concentrations are not altered.

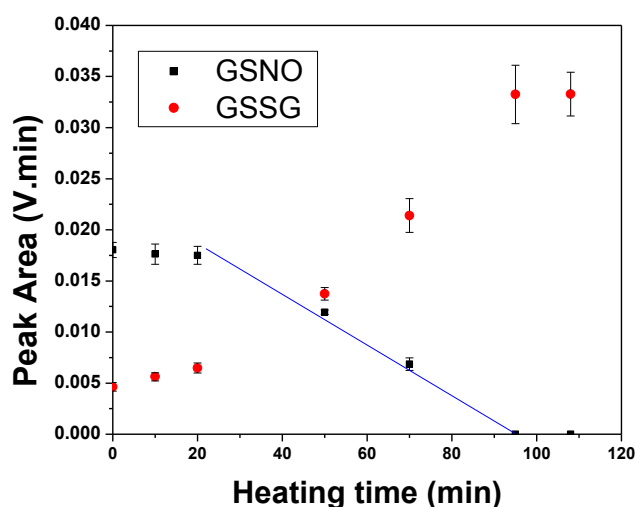


Figure 79: Study of heat decomposition of a GSNO sample (purity 66 %). GSNO and GSSG peak areas as function of time of heating. All samples were prepared in CHES (20 mM, pH 9). Other conditions as in Figure 2

By comparing **Figure 78C** and **Figure 79**, it is evident that the kinetics of the decomposition by heat is different from that using light. Indeed, during the first 20 min, almost no GSNO decomposition occurs. After 20 minutes, a linear decrease of GSNO concentration is observed, suggesting zero order kinetics where the rate is constant and independent of concentration. The apparent rate constant at 80°C deduced from the slope of the linear portion of curve **Figure 78B** is $k_{\text{obs}, 80^\circ\text{C}} = (4.34 \pm 0.14) \cdot 10^{-6} \text{ mol L}^{-1} \text{ s}^{-1}$. This could be explained by different mechanisms between light and heat decomposition. The mechanism of GSNO decomposition is still under study and is controversy. Indeed, De Oliveira *et al.* [44] have suggested a radicalar mechanism of decomposition of GSNO in water without the addition of chelating agent in which the initial rate depends on concentration of RSNO and, with formation of thiyl radical ($GS^\bullet$) that helps in the further decomposition of GSNO. Conversely, Singh *et al.* [45] reported that, in the absence of light, the decomposition does not involve any thiyl radical formation ($GS^\bullet$).

4) Transnitrosation reaction between GSNO and Cysteine

Transnitrosation reaction is a widely described chemical reaction [12]. It is thought that RSNOs act on functional proteins in human body by transnitrosation with these proteins and not by simple release of NO near these functional proteins [420]. The human body can be represented as a pool that contains many RSNOs in equilibrium nitrosating (giving NO) or denitrosating (taking NO) functional proteins, therefore [46,75,192]. The most probable mechanism of transnitrosation is a nucleophilic attack of the thiolate anion on the nitroso nitrogen of RSNOs resulting in nitroxyl (NO^+) transfer. Arnette *et al.* [421] have shown that the rate of transnitrosation is dependent on the pH and thus on the charge of thiol and on its nucleophilicity. Since the pKa of the thiol group varies with the chemical environment of the sulfur atom, each thiol containing molecule can have a different charge distribution which could affect the equilibrium.

Some researchers used to add an excess of cysteine into a GSNO solution in order to take out NO from GSNO and obtain nitrosocysteine (CysNO). They then added Cu^{2+} in order to release NO from cysteine and quantify it using chemiluminescence technique, so as to estimate the initial GSNO concentration in a sample. [2,422]. Park *et al.* [423] have studied transnitrosation reaction between GSNO and CysNO at pH 7.4 using HPLC-UV. Their results showed that the reaction is slow at this physiological pH, leading first to the formation GSH and CysNO with the concomitant presence of GSNO, while providing cystine (oxidized Cysteine), oxidized glutathione (GSSG) and Cysteine-glutathione Disulfide (CySSG) with almost no detectable GSNO at the end of the reaction.

In this study, we have added different amounts of cysteine to GSNO and made successive electropherograms as a function of reaction time (experiments conducted at room temperature of 25 ± 1 °C). **Figure 80** shows the obtained results. They clearly agree with the already previously described mechanism (eq. 59) [12], where, upon increasing cysteine concentration, the peak associated to GSNO decreases while that of GSH increases with the concomitant increase of two other peaks, one before GSNO and one after it.



The peak at a lower electrophoretic mobility than that of GSNO is supposed to be CysNO (due to absence of a charged thiol) while the peak at a higher electrophoretic mobility than that of

GSNO is attributed to cysteine by comparison with the electropherogram of cysteine standard solution.

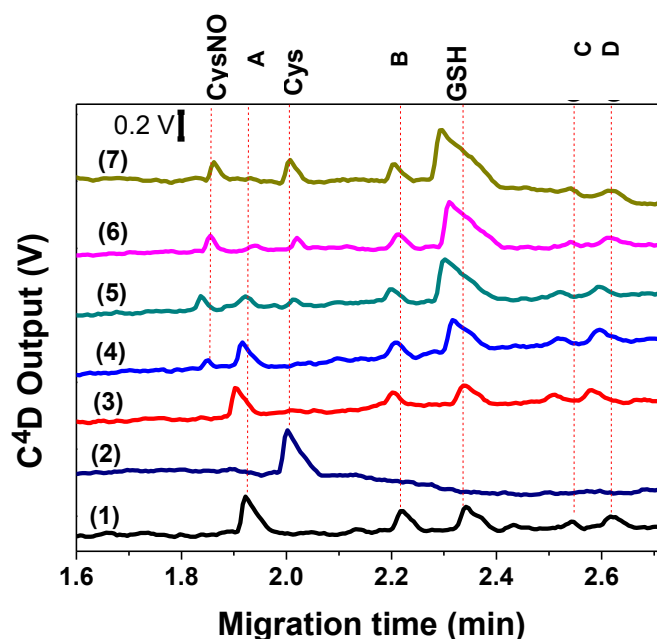


Figure 80: Study of transnitrosation between GSNO and cysteine. Electropherograms of solutions containing: (1) 331 μM GSNO + GSH 90 μM , (2) 495 μM cysteine, (3-7) 331 μM GSNO with increasing cysteine concentrations (3) 76 μM , (4) 152 μM , (5) 305 μM , (6) 381 μM and (7) 495 μM . All samples were prepared in CHES (20 mM, adjusted to pH 9.0 with NaOH). Other conditions as in Figure 75. The EOF time was 1.2 min. A. GSNO, B. GSSG, C. GSO₂H and D. GSO₃H.

C. Concluding remarks

We have demonstrated for the first time that CE coupled to C⁴D is a very useful method for RSNOs separation and quantitation, in terms of purity and decomposition kinetics under different physico-chemical conditions. Indeed, CE-C⁴D is a direct method that does not necessitate any derivatization, sample decomposition or purification. This method makes it possible to separate and quantify a complex mixture of GSNO and its impurities within few minutes (less than 2.5 minutes) benefiting from all the advantages of electrophoresis. CE-C⁴D was applied to the analysis of decomposition products of GSNO, when submitted to heat or light, with the evaluation of the decomposition rate constants. Finally, CE-C⁴D was applied to the analysis of some chemical reactions involving GSNO, especially transnitrosation. This analytical approach also permits the separation of LMW-RSNOs. After further optimization of detection sensitivity, this new methodology will present a great interest for diagnosis of many diseases during which the variation of the proportion of RSNOs plays an important role.

CHAPTER III: DECOMPOSITION OF S-NITROSOGLUTATHIONE BY Cu^{2+} / GSH AND BY GOLD NANOPARTICLES

After characterization and separation of GSNO from its decomposition products using CE- C^4D and CE-MS, we investigate here the methods of its assisted and / or catalyzed decomposition pathways.

GSNO is sensitive to light, heat, metal ions (Hg^{2+} , Cu^+ and Fe^{2+}) and gold nanoparticles (AuNPs). Hg^{2+} - catalyzed decomposition pathway was reported to yield nitrite [27] while Cu^+ and AuNPs – catalyzed ones yield NO [25,49,424-426]. GSNO decomposition using Cu^+ was thought to depend on the ratios between GSNO, GSH, and Cu^{2+} [49]. However, there is a need to optimize conditions to allow achieving the decomposition and quantification of RSNOs using Cu^+ . Indeed, several publications report different values of RSNOs using this approach. This can be due to the preliminary treatment of the sample or to the non-optimization of the decomposition method. RSNOs decomposition using AuNPs pathway was shown to occur but no analytical method to quantify RSNOs in biological medium was reported [424-426,25]. Thus, AuNPs could be used to quantify RSNOs in biological fluids but this was not reported in the literature.

Decomposition of GSNO using light and heat has been studied in **chapter II** using CE- C^4D detection. In the case of the decomposition using metallic ions and AuNPs, this type of detection is more difficult and complicated due to the need of addition of external reagent which changes the ionic force of solution. It should be noted that the detection limit obtained for GSNO using CE- C^4D (15.4 μM) is high and does not allow its detection in biological fluids whereas the detection limits for the electrochemical technique reported in the literature were very low (in nM range). In addition, the detection by CE- C^4D provides information about the products

obtained at the end of the reaction while the electrochemical one allows having real time information about the release of NO.

Two methods were used here: one based on the use of Cu^{2+} / GSH and one based on the use of AuNPs. Both of them were associated with electrochemical detection.

This chapter is divided into two parts. Firstly, we will discuss the re-investigation of decomposition of GSNO using Cu^{2+} / GSH and associated with the electrochemical detection of NO where the pronounced effect of GSH has been shown. In the second part the decomposition of GSNO by AuNPs associated with electrochemical detection and a tentative approach for its quantification in biological fluids.

I. Quantitation of S-nitrosogluthathione using Saville and electrochemical detection upon its Cu^{+} -catalyzed decomposition

Among the large variety of RSNOs, GSNO is the most abundant biological LMW-RSNOs, either commercially available or easily synthesized. As it was shown in **chapter 2**, the synthetic route of GSNO is most of time accompanied by several decomposition products or impurities such as glutathione in its oxidized (GSSG) and reduced (GSH) forms that can affect its metal-catalyzed decomposition. It was reported that Cu^{+} , produced by reduction of CuSO_4 , catalyzes the decomposition of GSNO to form NO and GSSG, and that the presence of ligands of Cu^{+} and / or Cu^{2+} such as EDTA (a complexing agent for Cu^{+} and Cu^{2+}) or neocuproine (a complexing agent for Cu^{+}) blocks the reaction [24,47,211,427]. It was also reported that the decomposition rate of GSNO increased linearly with the increase of CuSO_4 concentration [47]. The decomposition of GSNO in presence of CuSO_4 was explored using several reducing agents for CuSO_4 such as ascorbic acid [48], sodium hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$), sodium borohydrate (NaBH_4), cysteine [297,299,385] and GSH [49,428,429]. The ratio of CuSO_4 to reducing agents and to GSNO is thought to play an essential role in the kinetics and percentage of GSNO decomposition [49]. Also the GSNO decomposition by chloride-assisted copper metal corrosion (generating Cu^{+}) was described in association with an electrochemical

detection of NO operating with different configurations of dual electrodes (ring-disc, disc-disc, or band-band) of NO-sensor and copper metal sources [51]. Meyerhoff et al. developed NO-sensor polymeric materials containing immobilized metallic species (copper, selenium, tellurium) [292-295,430-433] aimed at decomposing RSNOs to NO. The authors showed the possibility of electrochemically detecting RSNOs by utilizing these materials as coating layer at the distal end of the amperometric NO-sensor in phosphate buffer solution (PBS) containing EDTA and reducing agents (GSH or ascorbate).

In this part we re-investigate the Cu^+ -catalyzed decomposition of GSNO to rationalize and improve its detection. For this purpose the different parameters (i.e. concentrations of CuSO_4 and its reducing agent, and their ratio) were optimized. The protocol was designed and adjusted for a better control of GSNO decomposition. GSH was selected as the reducing agent since it is naturally present in biological fluids or in aged or impure samples, therefore avoiding the introduction of additional compound that could lead to side-reactions. EDTA was added in the protocol as it can block any side reaction with metal-contaminants [245]. The decomposition reaction of GSNO was monitored *ex situ* using the indirect colorimetric modified Saville detection of nitrite (where nitrite reacts with N-ethylene diamine (NED) in acidic medium to give a colored azo-dye that absorbs at 540 nm see **Figure 49**) and *in situ* using the direct electrochemical detection of generated NO by a homemade selective NO-microsensor.

A.Experimental

1) Chemicals

NO donor compound, DEA-NONOate (diethylammonium (Z)-1-(N,N-diethylamino)diazene-1,2-diolate) was from Cayman Chemical (USA, www.caymanchem.com). Phenol, eugenol, GSH, Griess reagent, monosodiumphosphate (NaH_2PO_4) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Aldrich. All other chemicals were reagent grade and were used without further purification. All aqueous solutions were made using ultra-pure water with a resistivity of 18.2 M $\Omega\cdot\text{cm}$ from a Pure Lab Flex system (ELGA Labwater,

France). Phosphate buffer solution was prepared by mixing 78.2 mL of NaOH (0.1 mol L⁻¹) and 100 mL of NaH₂PO₄ (0.1 mol L⁻¹). DEA-NONOate and GSNO were kept at -20 °C.

GSNO was synthesized as described elsewhere [232]. Briefly equimolar amounts of GSH and nitrite were mixed in presence of equimolar amount of hydrochloric acid under agitation and Ar flux and in ice bath for 40 minutes. Solid GSNO appears as pink solid in the solution which was then filtered and rinsed once using acetone 80%, twice using pure acetone, and three times with di-ethyl ether. The final product was characterized by UV-visible spectrophotometry and also Ellman assay [434] to assess the amount of the remaining unreacted GSH.

2) Microsensor fabrication and NO detection

Commercial Pt UME (Uniscan, diameter 25 µm) was polished using polishing diamond dispersions with different granulation (1 and ¼ µm) and then rinsed with water. After that the NO sensor was prepared by coating the surface of the UME either with poly(eugenol) and poly(phenol) [51] or poly(eugenol) alone to ensure adequate selectivity. At first the poly(eugenol) layer was deposited by applying a potential of 150 mV vs Ag / AgCl for 15 min in a solution of eugenol (10 mM) + sodium hydroxide (0.1 M) (**Figure 81**). Then the poly(phenol) layer was deposited using cyclic voltammetry between 0 and 0.7 V vs Ag / AgCl at a rate 10 mV / s for 10 cycles in PBS (0.1 M ; pH 7.4) + phenol 0.5 M. In the case of poly(eugenol) alone the last step of polyphenol deposition was not made.

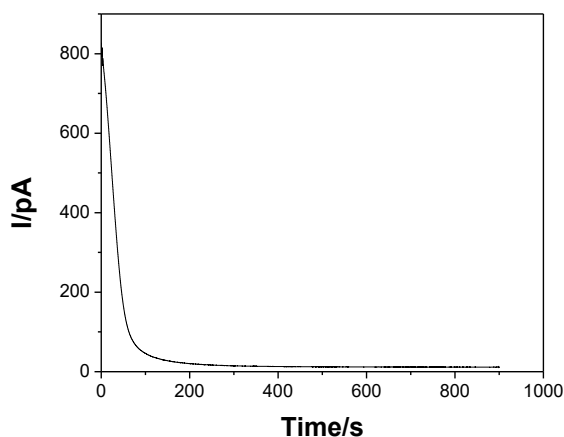


Figure 81: Amperogram of deposition of eugenol layer on 25 µm Pt-UME at E = 150 mV vs Ag / AgCl for 15 min in a solution of eugenol (10 mM) + sodium hydroxide (0.1 M).

NO detection was achieved using amperometric measurements at 0.8 V vs Ag / AgCl, with Quadstat (eDAQ Pty Ltd, Australia) and a three-electrode configuration (counter electrode and reference electrode were the same Ag / AgCl electrode due to very small current passing thanks to UME) at room temperature in PBS (0.1 M, pH 7.4) + EDTA (450 μ M) in aerobic conditions. A nitrite selectivity test is performed at beginning and from time to time in order to continuously monitor the electrode (**Figure 82**). When the selectivity factor (signal produced by NO over signal produced by equivalent amount of nitrite) decrease less than 20, a new membrane is fabricated. The electrochemical experiments were performed inside a Faraday cage to reduce the environmental electromagnetic interferences thus obtaining better limits of detection (LOD). The calibration range and other parameters of calibration will be discussed in details in the text.

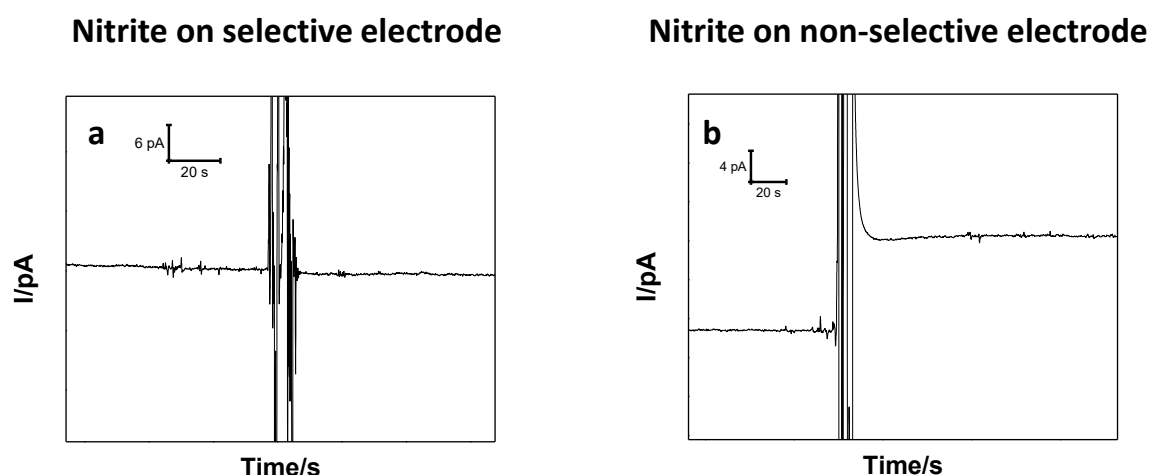


Figure 82: Addition of nitrite (100 μ M) in 0.1 M of PBS (pH 7.4) on a 25 μ m Pt-UME coated with polyeugenol-polyphenol a) directly after preparation and b) at the end of working day when it starts losing its selectivity.

3) Colometric assays

Saville assay [27] was performed by mixing equal volumes (500 μ L of each) of the solution containing decomposed GSNO (using HgCl₂ or CuSO₄) and commercial Griess reagent in eppendorf test tubes. The mixing with Griess reagent was done 30 or 5 minutes after the addition of CuSO₄ or HgCl₂, respectively. The obtained colored diazo product was detected at 540 nm using North Star scientific UVIKON XL spectrophotometer after 15 min of mixing. Calibration curves were performed with standard solutions of sodium nitrite in the range 1 to 80 μ M using the Griess reagent.

Ellman test for GSH was performed by mixing 2 / 1 (V / V) volumes of solution containing GSH and of solution of 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) prepared in 0,1 M PBS pH 7,4. The mixing was done for 10 minutes at ambient temperature in dark. The resulting 2-nitro-5-thiobenzoic acid (TNB) is a yellowish colored dye that has a maximum absorption at 410 nm. Calibration curves were done using standard GSH solutions in the range from 1 to 100 μ M.

B. Results and discussion

The Saville reaction is based on the decomposition of RSNOs using HgCl_2 which leads to quantitative transformation to NO^+ and a RS-Hg complex [27,435] (**chapter 1**). NO^+ is quickly oxidized to NO_2^- that is quantified using Griess reagent through the formation of a final colored azo dye [436]. **Figure 83** (curve a) shows the calibration curve for the quantitation of GSNO with the Saville protocol, in PBS solution. The calculated limit of detection is 1.5 μ M, for a signal / noise ratio of 3. **Figure 83** (curve b) shows the calibration curve for the quantification of nitrite with the Griess protocol in PBS for comparison. The slopes of both calibration curves are almost identical (only 2% of relative deviation) thus confirming that the decomposition of GSNO using HgCl_2 leads within 5 minutes to the quantitative formation of nitrite.

Decomposition of GSNO in presence of CuSO_4 was performed using GSH as a reducing agent to form the catalytically active Cu^+ . This reaction is named Saville-like because it quantify RSNOs by their decomposition by metallic ions other than Hg^{2+} . Indeed, GSH is present as an “impurity” in the commercially available or synthesized GSNO or naturally present in the biological fluids. The concentration of GSH inside cells is in millimolar range and distributed heterogeneously among cellular compartments [437], while that in extracellular medium is in low micromolar range ($<5\mu\text{M}$) [438]. So as to optimize the three main parameters of the Cu^+ -catalyzed GSNO decomposition reaction (i.e. CuSO_4 and reducing agent concentrations and their ratio), two experimental protocols were developed:

Protocol **1** consists in the decomposition of a given amount of GSNO ($\approx 40\mu\text{M}$) with increased amounts of CuSO_4 (from 0 to 1300 μM) for different concentration of reducing agent (GSH) (0.25 μM , 20 μM , 50 μM , and 100 μM).

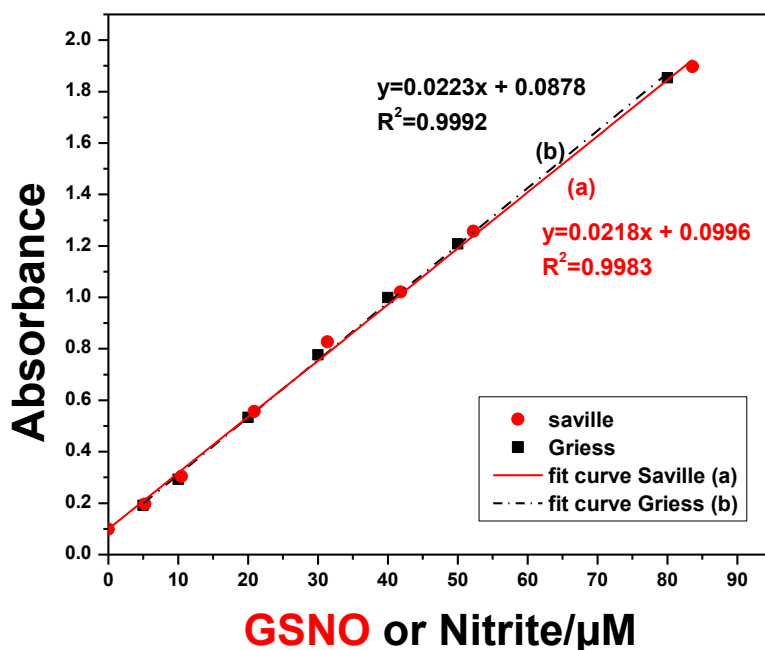


Figure 83: Calibration of Saville (a) and Griess (b) methods. (a) absorbance measurement at 540 nm of 500μL of PBS (0.1 M, pH 7.4) containing different GSNO concentrations + HgCl₂ (537 μM) + EDTA (450 μM) + 500 μL Griess reagent. (n=3); (b) absorbance measurement at 540 nm of 500 μL of PBS (0.1 M, pH 7.4) containing different nitrite concentrations + EDTA (450 μM) + 500 μL Griess reagent. The slope of Griess method is related to a $A = f([\text{nitrite}])$ graph and the slopes of Saville methods are related to $A = f([\text{GSNO}]_0)$ graphs.

Protocol 2 consists in the decomposition of different given amounts of GSNO ($\approx 20 \mu\text{M}$, $\approx 35 \mu\text{M}$, and $\approx 80 \mu\text{M}$) by varying the concentration of added GSH (0 to 200 μM) for a given concentrations of CuSO₄ (600 and 1000 μM). In all cases, EDTA (450 μM) was added to the initial GSNO solution as ligand for metal ions [12,245] in order to prevent the spontaneous decomposition catalyzed by metal ion impurities [245]. Furthermore, addition of EDTA permits to use with the same GSNO solution for 12h without significant spontaneous decomposition verified by spectrophotometry (data not shown). It is worthwhile to mention that addition of CuSO₄ leads to the presence of Cu(II) in different forms: CuSO₄, Cu(II)-EDTA and Cu(II)-GSH complexes.

Figure 84 shows the decomposition trend of GSNO (39 μM) using protocol 1 as a function of CuSO₄ concentration. The graph represents the normalized absorption with respect to the maximum of each curve. This helps in the comparison of the trend for different GSH concentrations because the value of the maximum will vary depending on the GSH concentration. It should be noted that the used GSNO solution contains

remaining unreacted GSH (0.6% w / w evaluated by Ellman assay) that acts as the reducing agent allowing the formation of catalytically active Cu^+ . In these conditions, only negligible amount of GSNO is decomposed ($< 2\%$) for CuSO_4 concentrations below $440\ \mu\text{M}$. Indeed, under these conditions, EDTA (at a concentration of $450\ \mu\text{M}$) complexes all the added CuSO_4 . For CuSO_4 concentrations above $560\ \mu\text{M}$, the decomposition of GSNO is almost constant. The same trend was observed when $20\ \mu\text{M}$ of GSH was added to the sample before the copper-catalyzed decomposition starts, but with a percentage of decomposition higher than without added GSH (normalized values are shown to facilitate comparison). Larger GSH concentrations induce slight modification of the profile of the evolution of the decomposition yield, with lower decomposition yield for CuSO_4 concentration above $600\ \mu\text{M}$. From these results, it appears that for amounts of added GSH $< 50\ \mu\text{M}$, the percentage of the GSNO decomposition is large and almost constant for CuSO_4 concentration above $700\ \mu\text{M}$. The concentration of CuSO_4 was thus fixed for further experiments at $600\ \mu\text{M}$ and $1000\ \mu\text{M}$.

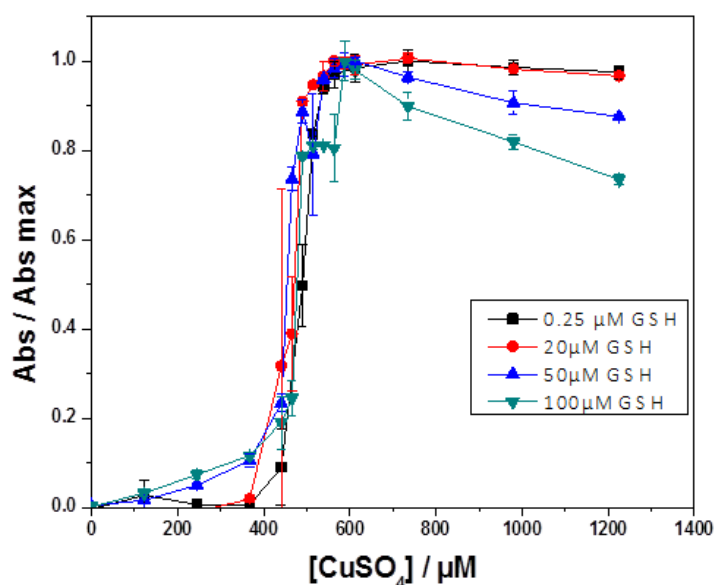


Figure 84: Normalized absorbances of Cu-catalyzed decomposition of GSNO solution treated with Griess reagent using colorimetric detection at 540 nm (each curve was normalized with respect to its maximum absorbance). Effect of the addition of increasing concentrations of CuSO_4 to a solution of GSNO ($39\ \mu\text{M}$) + EDTA ($450\ \mu\text{M}$) in PBS (0.1M ; pH 7.4) containing different amounts of GSH. ($n=3$)

Figure 85 shows the decomposition trend of GSNO when varying added amounts of GSH for three different concentrations of GSNO and using $600\ \mu\text{M}$ and $1000\ \mu\text{M}$ of CuSO_4 (protocol 2), with respect to the maximum of decomposition in presence of $20\ \mu\text{M}$ GSH. The highest decomposition yield of GSNO was obtained with $15\text{-}30\ \mu\text{M}$ of

added GSH whatever GSNO concentration. But it should be noted that the use of 600 μM of CuSO_4 leads to a profile of plateau for GSH concentration range 20-75 μM . For higher GSH concentrations, the decomposition yield decreased probably due to the complexation of Cu^+ by GSH to form a Cu^+ / GSH complex which is catalytically inactive [439]. The effect of GSH on the inhibition of GSNO decomposition is more pronounced, as the concentration of GSNO is lower. Thus the ratio of GSNO / GSH is as important as the ratio of $\text{CuSO}_4 / \text{GSH}$ on GSNO decomposition. It is worthwhile to mention that when using the colorimetric test there is a risk of highly underestimating GSNO levels when working with biological samples such as intracellular extracts which contain high concentrations of GSH (millimolar range) contrary to plasma where GSH concentration is low. The slope of calibration curves using CuSO_4 (600 and 1000 μM) + GSH (20 μM) was 89 % of that using Saville method (*Table 17*). This shows that the two concentrations give the same yield of decomposition of GSNO to nitrite. The remaining 11 % could be other nitrogen products like N_2O and NH_3 as demonstrated by Singh et al. when GSH was mixed with GSNO in millimolar range [429].

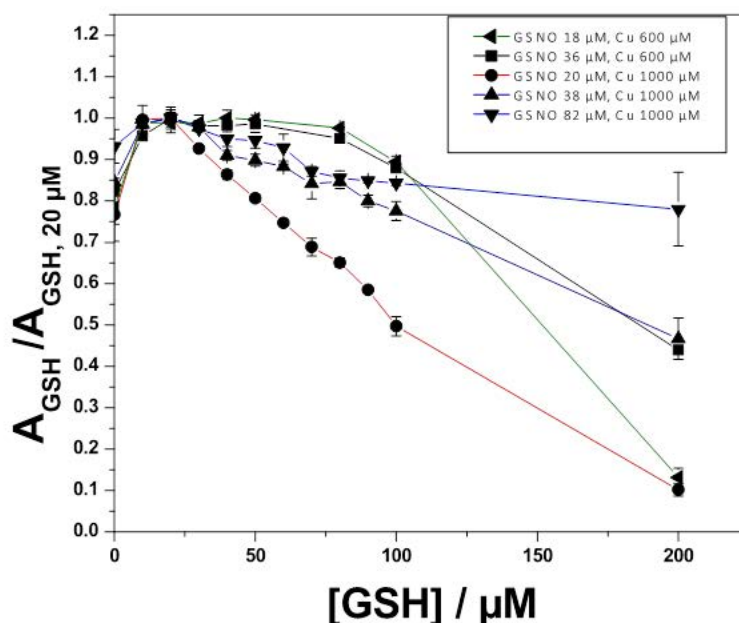


Figure 85: Effect of increasing GSH concentration on the normalized (with respect to the maximum of each curve at each different GSNO concentration) Cu^{2+} -catalyzed decomposition of GSNO solution treated with Griess reagent using colorimetric detection at 540 nm. 20, 38, and 82 μM of GSNO in PBS (0.1 M; pH 7.4) + EDTA (450 μM) + CuSO_4 (1000 μM) or 18 and 36 μM of GSNO in PBS (0.1 M; pH 7.4) + EDTA (450 μM) + CuSO_4 (600 μM) were used ($n=3$). The first points start at GSH present as impurity in GSNO solutions (0.6%)

The optimal CuSO_4 and GSH concentrations, 1000 μM and 20 μM , respectively, leading to a ratio of $\text{CuSO}_4 / \text{GSH}$ of 50, were then used to demonstrate the interest of the electrochemical detection of the released NO in real time for the quantitation of GSNO.

Detection of released NO was performed through its oxidation using the selective and miniaturized amperometric NO sensor at +0.8 V vs Ag / AgCl described in the experimental section. **Figure 86a** shows the experimental amperograms obtained for additions of different amounts of GSNO, varying from 4 to 290 μM , in PBS solution containing EDTA (450 μM), CuSO_4 (1000 μM) and GSH (20 μM). Whatever the GSNO concentration, a fast increase in current is observed after GSNO addition, up to a maximum, attributed to the oxidation of released NO, followed by a relatively rapid decrease in current, as soon as the oxidation of NO by molecular oxygen present in the aerated electrolytic solution dominates the overall reaction. It can be pointed out that the decrease of the current value down to the original baseline is indicative of the high selectivity of the home made sensor against nitrite, the main metabolite of NO which is accumulated in the solution due the reaction with dissolved oxygen. It is noticeable that, as it was previously reported for NO-donor moieties [52], the maximum of the amperograms varies in time and intensity according to GSNO concentration: the higher the concentration of GSNO, the shorter the time to reach maximal NO production and the higher the maximum intensity. **Figure 86b** shows the calibration curve deduced from the amperograms, by reporting the evolution of the maximal intensity of the current as a function of the concentration of added GSNO, displaying two distinct linear parts.

Table 17. Slopes of calibration curves of Griess (using nitrite standard solutions), Saville (decomposing GSNO by HgCl_2 to nitrite), and Saville-like (decomposing GSNO by CuSO_4 at 600 and 1000 μM with GSH 20 μM to NO which transforms to nitrite at the end). The slope of Griess method is related to a $A=f([\text{nitrite}])$ graph and the slopes of Saville methods are related to $A=f([\text{GSNO}])$ graphs. Absorbance measurement at 540 nm after mixing 500 μL of Griess reactive with 500 μL of increasing concentrations of nitrite or GSNO with HgCl_2 (537 μM) + EDTA (450 μM) in PBS (0.1M, pH 7.4). R^2 was always > 0.99. (n=3)

	Griess (nitrite)	Saville (HgCl_2)	Saville-like (Cu 1000, GSH 20 μM)	Saville-like (Cu 600 μM , GSH 20 μM)
Slope (μM^{-1})	0.0223	0.0218	0.0198	0.0198
% of slope / slope Griess	100	98	89	89

The slope of the first linear regression ($1.84 \pm 0.13 \text{ pA} / \mu\text{M}$) (n=3) is in correlation with the slope of the linear variation of the current as a function of NO concentration ($1.98 \text{ pA} / \mu\text{M}$) from amperometric measurements using decomposed DEA-NONOate (**Figure 87A**). NO concentration was calculated by simulation based on decomposition of DEA-NONOate at different concentrations [52]. This is indicative of a quantitative release of NO for GSNO concentrations below 25 μM . The lower slope value of the second linear

regression ($0.25 \text{ pA} / \mu\text{M}$) can be explained by the fact that, at higher GSNO concentrations, CuSO_4 / GSNO and GSH / GSNO ratios decrease to an extent that affects the efficiency of the decomposition of GSNO, as stated by colorimetry. In order to maintain these ratios constant, GSH and CuSO_4 concentrations were increased and the decomposition rate of GSNO ($100 \mu\text{M}$) was analyzed. The results show that 3 mM of CuSO_4 and $20 \mu\text{M}$ of GSH (ratio = 150) allow the highest decomposition rate. Thus, the calibration curve of the sensor was achieved using these conditions, for GSNO varying between 0 and $90 \mu\text{M}$ (**Figure 87B**) and the slope of the linear regression ($1.38 \text{ pA} / \mu\text{M}$) is lower than the one obtained with $1000 \mu\text{M}$ of CuSO_4 . This can be explained by the possible quenching of NO by copper ion at high concentrations. In these conditions, the limit of detection is $0.6 \mu\text{M}$. These data clearly show that the evolution of the ratio between GSH, CuSO_4 and GSNO should be adjusted to tune the highest decomposition rate of GSNO and the most efficient electrochemical detection of NO.

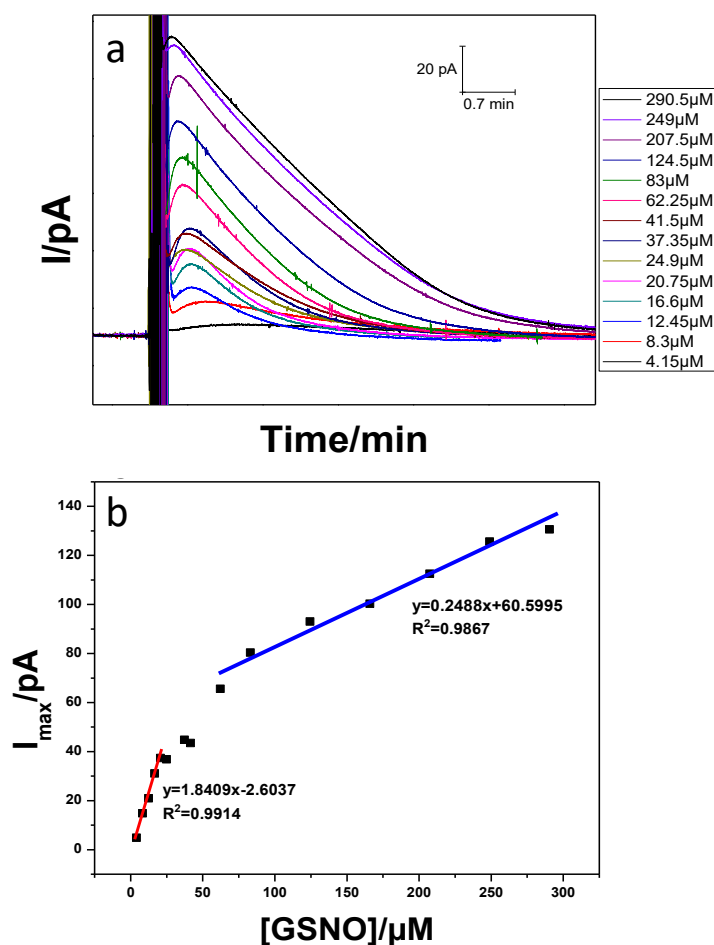


Figure 86: a) Evolution of the amperograms measured by NO-sensor upon addition of GSNO to PBS (0.1 M ; $\text{pH } 7.4$) + EDTA ($450 \mu\text{M}$) + CuSO_4 ($1000 \mu\text{M}$) + GSH ($20 \mu\text{M}$). Measurement made at 0.8 V vs Ag / AgCl; b) calibration curve obtained with data from Figure 86a.

In order to increase NO-sensor sensitivity and thus decrease its limit of detection, the electrode surface was modified with poly (eugenol) only. The performances of the electrode remained satisfactory since NO-sensor was still very selective versus nitrite and exhibits better LOD of 0.1 μM (vs 0.6 μM for poly(eugenol) / poly(phenol) modified electrode). The sensitivity was more than 2.5 times better (**Figure 87D**). The comparison of the slope of calibration of GSNO decomposition (4.86 pA / μM) was also in good correlation with the slope of the linear variation of the current as a function of NO concentration (4.58 pA / μM) from amperometric measurements using decomposed DEA-NONOate (**Figure 87C**). The calibration curve of GSNO with CuSO_4 (600 μM) + GSH (20 μM) gives a slope of 4.64 pA / μM that is very close to that obtained with Cu (1000 μM) + GSH (20 μM) (4.86 pA / μM) (**Figure 87D**).

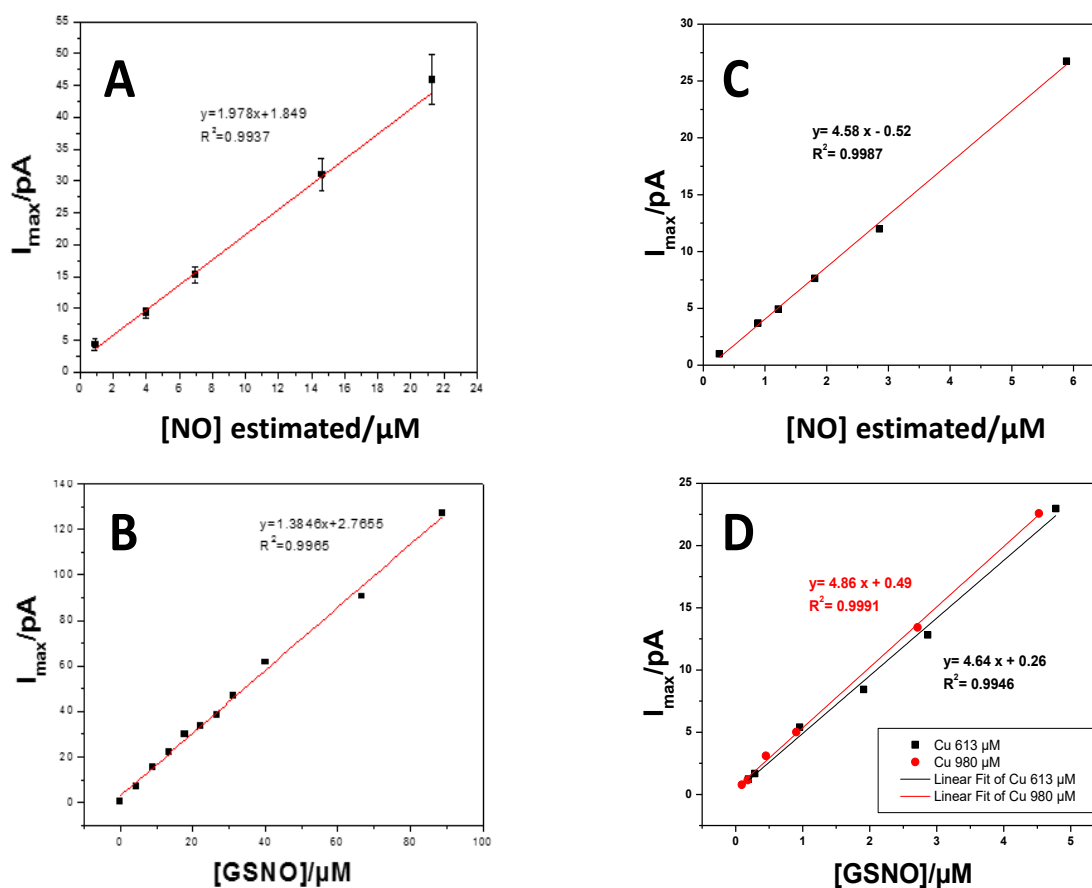


Figure 87: (A,C) Calibration curves obtained from measuring the maximum current intensities obtained by different additions of DEA-NONOate, as a function of the estimated concentration of NO released by DEA-NONOate at the maximum of the peak (PBS 0.1 M; pH = 7.4; E = 0.8 V vs. Ag / AgCl) (B, D) Calibration curve obtained from amperometric measurement of NO released from copper-catalyzed decomposition of GSNO in PBS (0.1 M; pH 7.4) + EDTA (450 μM) + CuSO_4 (3 mM) + GSH (20 μM). The NO-sensor was a Pt Ultramicroelectrode (25 μm diameter) coated by (A, B) polyeugenol-polyphenol membrane or by (C, D) polyeugenol and polarized at 0.8 V vs Ag / AgCl.

C. Conclusion

This study presents a deep insight into the study of copper-catalyzed decomposition of GSNO so as to optimize its quantitation by electrochemical detection using a miniaturized NO-sensor. To this aim, a simple colorimetric reaction was employed for the rapid screening of parameters influencing GSNO decomposition yield. The protocol was simplified by using GSH as copper reducing agent and rationalized by studying the influence of CuSO_4 and reducing agent concentrations and their ratio. The results show that GSH (naturally present in biological medium) is a very important factor in GSNO decomposition. Furthermore, the interest of electrochemical detection was demonstrated for quantitation and elaboration of a real time study of the decomposition of GSNO at a selective amperometric NO-sensor, with a limit of detection of $0.1 \mu\text{M}$ for optimized sensor. Thus, the results obtained here are of great importance for biologists as the existence of other thiols, which are normally present in biological fluids, could suppress the signal of NO leading to an underestimation of RSNOs. Our approach offers a sensitive and rapid method to detect GSNO electrochemically, mentioning the importance of the presence of thiols on signal suppression and suggesting a separation step to eliminate eventual excess of thiols. The time necessary to attain the max is 10-15 s, more rapid than the decomposition time using light (15 min) and heat (90 min at 80°C)

II. Quantification of GSNO using gold nanoparticles

Recently, AuNPs were shown to react with RSNOs in aqueous solution through a strong binding between the sulfur atoms of RSNOs and the AuNPs surface. In the presence of AuNPs, -S-NO bonds are homolytically broken resulting in the release of NO [25,424-426]. The extent of NO released in this reaction can be controlled by the amount of AuNPs surface available (i.e. number of binding sites). However, AuNPs are also highly reactive against free thiols leading to a difficulty in their use for the quantification of RSNOs in real biological systems such as plasma.

In the framework of the *COFECUB-CAPES* projects coordinated by the lab and that of *UNICAMP* (Pr. A. Fracassi and M. Ganzarolli de Oliveira from the *Institut of Chemistry*), V.

Baldir proposed the idea of using AuNP aqueous dispersions in the electrochemical detection of RSNOs in plasma. In this context, I worked in association with *V. Baldir* to assure repeatability of the experiments and I performed the experiments of detection of RSNOs in plasma. The obtained results have led to an article in *Anal. Chem.* (DOI: 10.1021/acs.analchem.5b04035). A summary of the experimental and results sections of this article is presented below.

A. Experimental section

1) Materials.

Reduced glutathione (GSH), bovine serum albumin, lyophilized human plasma, L-cysteine, sodium nitrite, chloroauric acid trihydrate, iodoacetic acid and eugenol were purchased from Sigma-Aldrich. Sodium hydroxide (NaOH), hydrochloric acid (HCl), trisodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$), nitric acid (HNO_3) were of analytical grade. All solutions were prepared with deionized H_2O ($18 \text{ M}\Omega \text{ cm}^{-1}$).

2) Preparation of gold nanoparticles.

AuNPs were prepared by reduction of HAuCl_4 with sodium citrate, also known as the Turkevitch method [440,441]. All glassware was cleaned with aqua regia ($\text{HCl} / \text{HNO}_3$, 3 / 1) and extensively rinsed with deionized H_2O before use. An aqueous solution of HAuCl_4 1.1 mM (250 mL) prepared by dilution of a stock solution (4 g / L) was heated under vigorous stirring until it started to boil. Next, 25 mL of aqueous $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ solution (44 mM) was added at once and the solution was maintained under heating and stirring during 10 min. After cooling down to room temperature, the dark red AuNPs dispersion was transferred to falcon tubes and stored at 4°C . **Figure 88** shows a representative UV-Vis spectrum of a sample of AuNPs dispersion. Particle size distribution and nanoparticle average diameter were measured by Dynamic Light Scattering (DLS) and Transmission Electronic Microscopy (TEM). The molar concentration of the dispersions was determined using **relation 1**, where d stands for the AuNPs average diameter (16 nm, inset of **Figure 88**), N for the Avogadro constant, M_{Au} for the molar mass of gold, ρ_{Au} for the density of gold (19.3 g/cm^3) and $A_{400\text{nm}}$ stands for absorption at 400 nm,

determined with a UV-Vis spectrophotometer [440]. These preparation conditions led to AuNPs dispersions whose concentrations were found to be in the nanomolar range, between 7-11 nM.

$$[AuNP] = \frac{6}{\pi} \frac{M_{Au}}{\rho_{Au} N} \frac{Abs_{400nm}}{d^3} 0.417 * 10^{-3}$$

relation 1

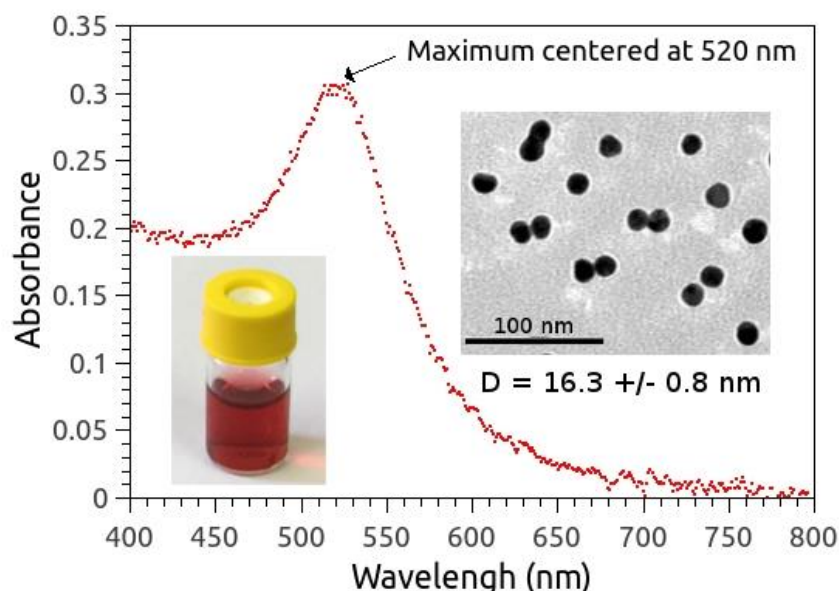


Figure 88: Representative UV-vis spectrum of a dispersion of AuNPs obtained by the Turkevitch method. Inset (right): TEM of the AuNPs showing average diameter. Inset (left): sample of gold nanoparticles dispersion.

3) S-nitrosoglutathione synthesis.

Equimolar amounts of GSH and NaNO_2 were mixed in the presence of 0.5 M HCl under stirring and argon flux in an ice bath for 40 min. Pink solid GSNO was filtered and rinsed once with acetone 80%, twice with pure acetone, and three times with diethyl ether, freeze-dried for 24 h and stored at -20°C , protected from light until use [442]. Purity of solid GSNO was accessed spectrophotometrically through the absorption of GSNO aqueous solution at 336 nm ($\epsilon = 835.6 \text{ M}^{-1} \text{ cm}^{-1}$). The remaining unreacted GSH was quantified by Ellman's assay [434].

4) Reconstituted human and mice plasma manipulation.

Reconstituted human plasma was prepared by dissolving commercial lyophilized human plasma in 1 mL of a 10 mM iodoacetic acid and 0.5 mM EDTA aqueous solution. Mice plasma was isolated from balb / c blood. Human plasma was ready to be used after 2 h of reaction with

the blocking agent, iodoacetic acid [246-248]. Control RSNOs-free plasma was prepared by adding HgCl_2 to the reconstituted human plasma up to 30 mM.

5) Preparation of the NO selective Pt ultramicroelectrode (UME).

Same as the preparation of NO sensor in part one (page 148) except that only polyeugenol membrane was used.

6) Amperometric detection of NO.

The Pt / PE UME was connected to a potentiostat (eDAQ Pty Ltd, Australia) and immersed in 2 mL of AuNPs dispersion (0-10 nM) placed inside a well of a 24-well cell plate, set to +0.8 V vs Ag / AgCl. The measurements were performed after baseline stabilization for c.a. 15 min. For the quantification of GSNO, aliquots of 0-20 μL of 1 mM GSNO solution were added to the wells of the plate (each one containing 2 mL of 9 nM AuNPs dispersion), while the current against time was recorded.

B. Results and discussion

1) Effect of AuNPs on the GSNO quantification.

GSNO reacts readily with AuNPs (**Figure 89**), producing NO and NPs coated with glutathiolate (GS-AuNP) (**eq. 60**).



The extent of GSNO consumption depends on the total available gold surface [426], which in turn depends on the concentration, size and shape of the AuNPs. If AuNPs are not the limiting reagent, quantitative GSNO consumption is expected. The NO released was measured amperometrically employing a NO selective Pt / PE sensor, as described in the experimental section. Briefly, NO diffuses through the PE membrane and is oxidized to the nitrosonium cation (NO^+), as shown in reaction **eq. 61**, at the surface of the Pt UME, leading to an increase in the measured electrochemical current. The magnitude of the change in the electrochemical current is directly proportional to the initial GSNO concentration.

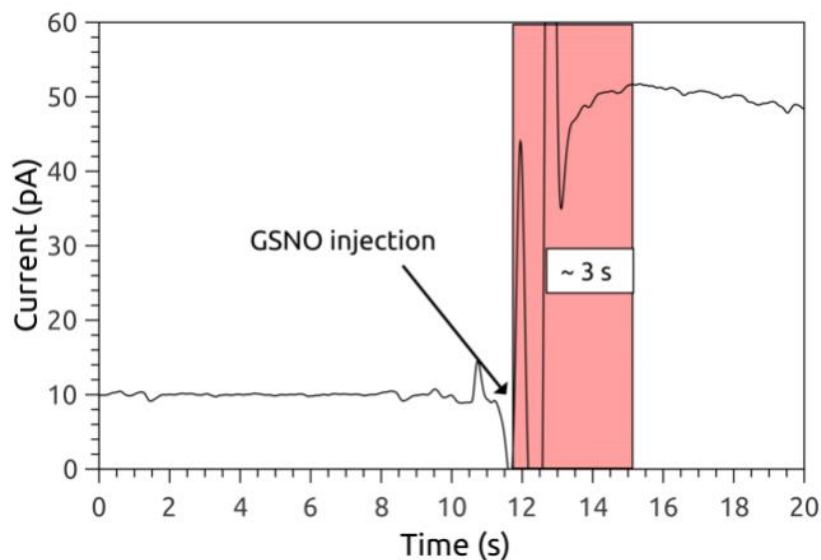
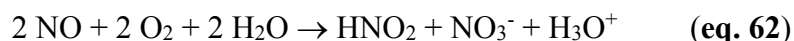


Figure 89: Electrochemical current change after a GSNO injection in a well of a 24-well cell plate containing a dispersion of AuNPs. Maximum is reached in c.a. 3 s.

Figure 90 shows a representative sharp increase in the electrochemical current observed after GSNO addition (to a final concentration of 30 μM) to a 9 nM of AuNPs dispersion. The sharp increase in the current reflects the fast release of NO in the reaction between GSNO and AuNPs. After peaking, the electrochemical signal starts decreasing as NO concentration diminishes over time due to its oxidation by oxygen [24] (**eq. 62**).



It must be noted that the decay profile of **Figure 90** is a characteristic profile of aerated solutions and that similar decay curves for NO electro-oxidation in the presence of O_2 measured with selective amperometric sensors, were reported by *Griveau et al.* [52]. On the other hand, in deaerated NO solutions, the current decay over time is much more slower, as reported by *Lantoine et al.* [443].

After ca. 3 min, a subsequent addition of the same GSNO amount did not lead to any change in the amperometric signal. This indicates that the amount of GSNO introduced in the first addition was enough to completely saturate the entire AuNPs surface. The saturation of the AuNPs surface with glutathiolate is represented in the first line of Scheme 1. In this situation (i.e. excess of GSNO), there is a linear dependence of the electrochemical current with the AuNPs

concentration (0 to 5.8 nM) after additions of GSNO solution to the same final concentration (Figure 91).

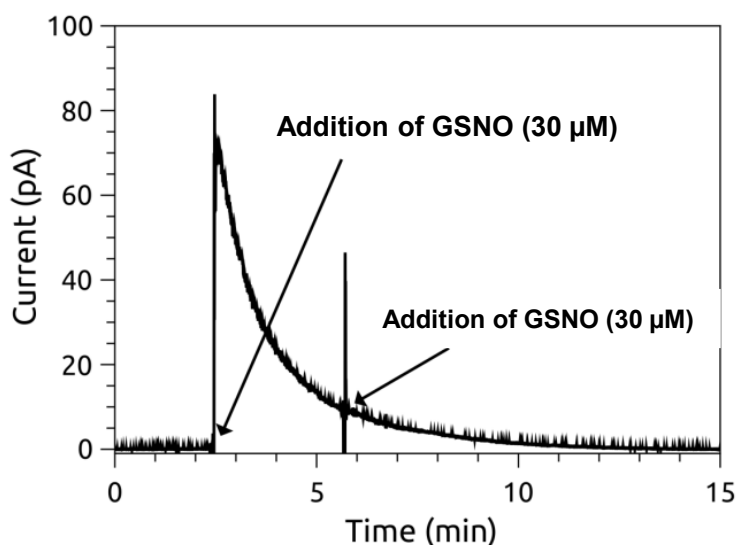


Figure 90. Current change observed after a GSNO addition to a final concentration of 30 μM on 9 nM AuNPs dispersion. A second addition of the same GSNO amount did not lead to any change in the current.

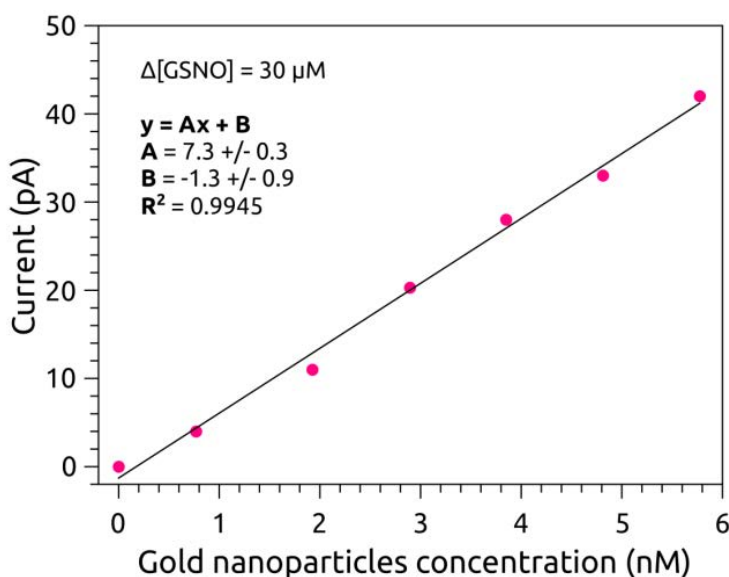
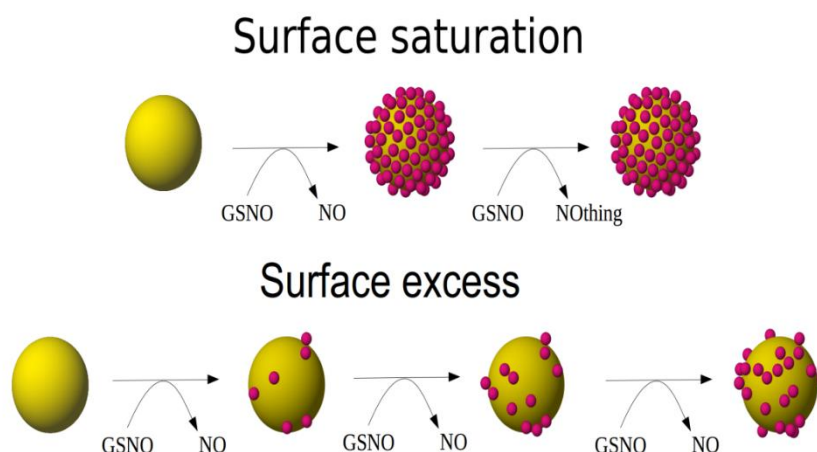


Figure 91. Linear dependence of the electrochemical current with the AuNPs concentration (0 to 5.8 nM) measured with a NO selective amperometric sensor after additions of GSNO solution to a final concentration of 30 μM .

Figure 92 shows the current measured during three consecutive additions of GSNO (to a final concentration of 1.0 μM) in a dispersion of AuNPs (9 nM). The equal current changes observed (c.a. 7 pA) show that, in this condition, GSNO is the limiting reagent and the excess of AuNPs surface allows its quantification (bottom line of Scheme 1).



Scheme 1. Reactions between GSNO and AuNPs in two different conditions: in GSNO excess relative to the total AuNPs surface available, leading to the saturation of the surface (above) and in AuNPs excess, relative to GSNO, leading to the quantitative decomposition of GSNO (below).

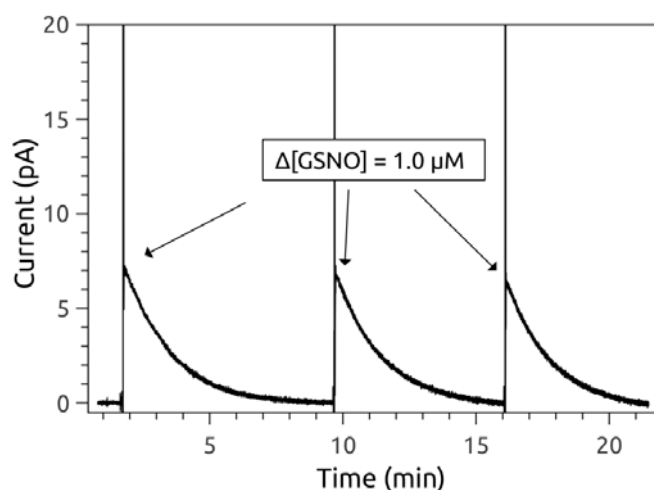


Figure 92: Current changes observed after GSNO additions to final concentrations of 1.0 μM each on a 9 nM AuNPs dispersion.

Figure 93 shows the maximum value of current change against GSNO concentration in the range of 0.5 to 35 μM , for a 10.9 nM of AuNPs dispersion. The amount of NO released is linearly proportional to the GSNO concentration, and can be used as a calibration curve up to a detection limit of c.a. 100 nM. The linearity of the correlation between current and concentration of GSNO is lost as the system approaches the region of lack of available gold surface and at high GSNO concentrations a plateau is formed.

2) Effect of plasma thiols on RSNOs quantification.

Human plasma contains high concentrations of several sulfhydrylated molecules such as albumin (500 μM), GSH (5-30 μM) and cysteine (15 μM), as well as their RSNOs derivatives [444].

The effect of thiols of different molecular sizes, namely GSH and albumin, on the quantification of GSNO using AuNPs was evaluated. AuNPs dispersions with fixed and known amounts were mixed with GSH or albumin in a broad range of concentrations, leading to dispersions of Au-SR. Then, a concentrated solution of GSNO was added to each of these dispersions and the current was measured by the NO selective sensor. When GSNO is in excess relative to the total AuNPs surface available, a current change proportional to the amount of available surface was measured after the release of NO according to **eq. 60 (Figure 94)**. The intersection between the curves gives the maximum [GSH / AuNPs] and [albumin] / [AuNPs] ratios. This ratio is larger for GSH (1500) than for albumin, (18) due to the much lower molecular volume of GSH compared to albumin. These ratios have the same order of magnitude of those reported by Taladriz-Blanco *et al.* for GSH, 1430 (calculated) and 619 (experimental) [25], and by Huang *et al* for albumin, 21 (experimental) [445]. These values allow estimating the plasma volume that saturates a particular AuNPs dispersion. For example, 2 mL of a 7.5 nM of AuNPs dispersion would be saturated by less than 1 μ L of plasma containing 500 μ M of albumin. Also, these ratios can be used to properly choose the most suitable concentration of AuNPs dispersion for the quantification of a particular RSNO based on its previously known thiol / AuNPs ratio.

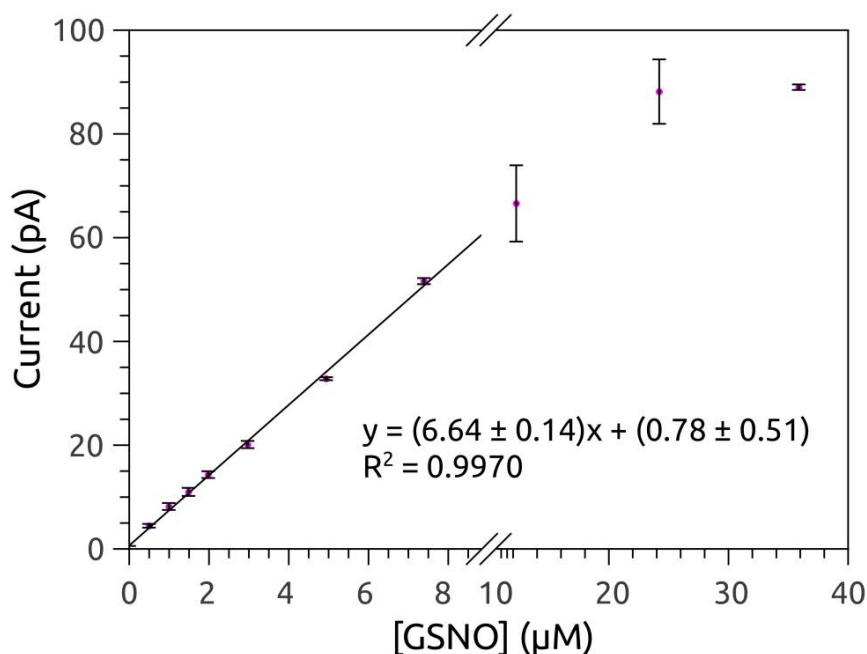
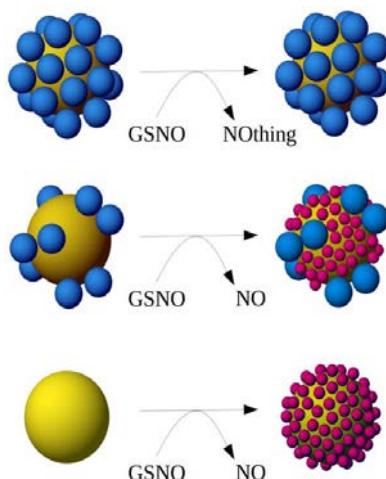


Figure 93. Calibration curve showing the current maxima obtained in the addition of GSNO (in water / EDTA 0.5mM / IAA 10mM) to different final concentrations on a 10.9 nM AuNPs dispersion which corresponds to an excess of Au surface relative to the GSNO amount necessary for the complete coating of the Au surface.



Scheme 2. The yellow sphere represents an AuNPs and the blue and red spheres represent surface-bound albumin and glutathiolate, respectively. The first line shows a saturation of the AuNPs surface with bound albumin, leaving no space for GSNO reaction. The second line shows a gold particle partially coated with albumin, allowing part of the GSNO molecules to react, but not in a quantitative fashion. In the third line, albumin-free AuNPs allow a quantitative GSNO reaction.

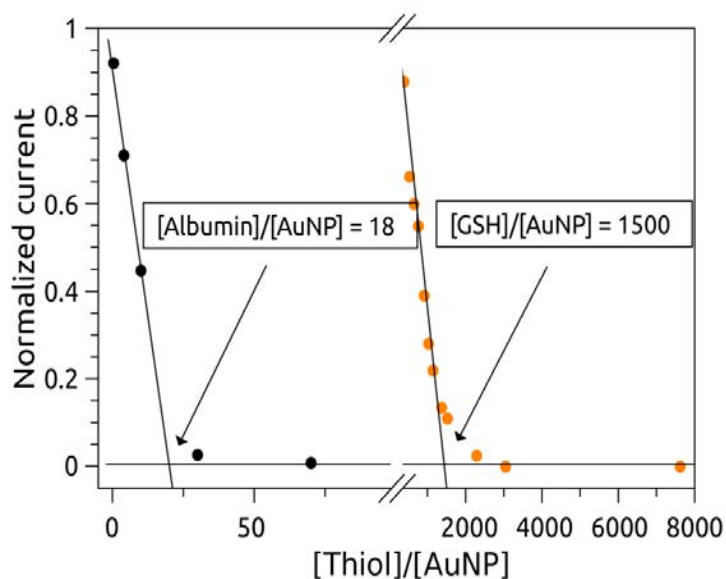


Figure 94. Current maxima measured after the addition of GSNO to a final concentration of 30 μM on AuNPs dispersions with different [albumin] / [AuNP] (black dots) and [GSH] / [AuNP] (orange dots) ratios.

Figure 95 shows the current change after addition of GSNO (30 μM) to 2 mL of a 7.5 nM of AuNPs dispersion previously reacted with different volumes of RSNOs-free plasma. This high concentration of GSNO was used in order to ensure that its reaction with the plasma thiols bound AuNPs was quantitative. These data show that 1.5 μL of plasma was enough to completely saturate the entire AuNPs surface, in accordance with the previously estimated value.

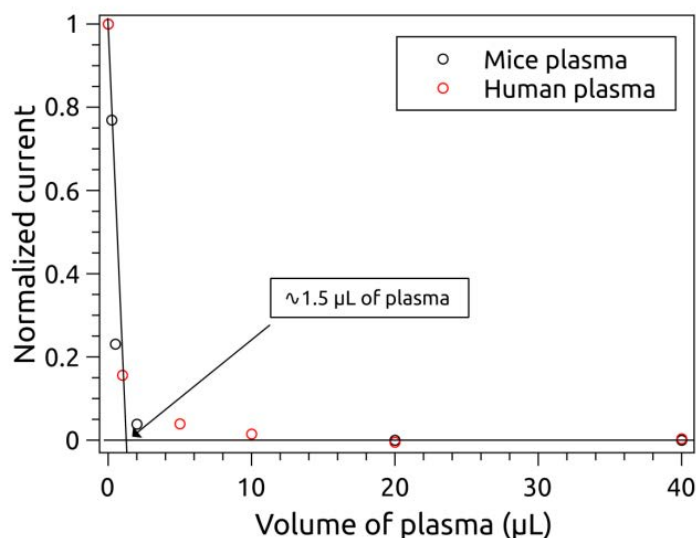


Figure 95. Current maxima after addition of GSNO (final concentration of 30 μM) in 2 mL of a 7.5 nM of AuNPs dispersions previously reacted with different volumes of RSNOs-free plasma.

3) Detection of total RSNOs in plasma.

The reaction of AuNPs with plasma thiols can be avoided by specifically blocking the thiol groups with iodoacetic acid, forming the carboxymethyl derivative of the cysteine residues [248] (**eq. 63**) before the addition of plasma to AuNPs dispersion. This strategy has been employed frequently for gas chromatography and MS experiments, among others, and several other thiol blocking agents have been employed for the same purpose [247]. Therefore, after the blocking procedure, RSNOs groups are the sole species that can react with the AuNPs.

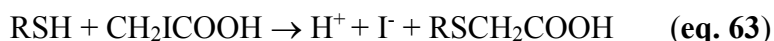


Figure 96 shows that the addition of 40 μL of sulphydryl blocked human plasma to 2 mL (dilution factor = 50) of a dispersion of 7.5 nM of AuNPs led to a current variation similar to that obtained for the reaction with GSNO only (**Figure 93**). Moreover, a subsequent addition of pure GSNO led to a second current variation, indicating that the surface of AuNPs was not completely saturated. The maximum current was reached after 4 min, probably due to the hindering caused by physisorbed molecules from the plasma at the surface of the AuNPs. Injections of volumes of plasma ranging from 10 to 75 μL to 2 mL of 10.9 nM AuNPs dispersions led to changes in current, whose values were linearly proportional to the injected volume (**Figure 97**). This shows that AuNPs are able to totally decompose RSNOs in plasma and that the analysis was conducted in the region of excess of surface that enabled their reliable

detection. Since the slopes correlating RSNOs concentration and current are not known for each of those present in plasma, it was not possible to quantify each of them.

These results show that the present approach, based on AuNPs dispersion, can be used as a novel strategy for the quantification of RSNOs in plasma. The LOD (100 nM) obtained for GSNO is in accordance with the values reported in the literature for plasma RSNOs analysis. Total RSNOs in plasma of healthy humans have been reported in the range of tens of nM to 7 μ M [2,85] and several techniques have been employed in such analysis. These include: HPLC (20 – 100 nM) [179,256]; chemiluminescence (1 – 50 nM) [446,447]; GC-MS (200 nM) [3], among others summarized by Giustarini *et al.* [2]. Electrochemical detection of plasma RSNOs with Pt UMEs have been used with different RSNOs decomposition strategies such as: membrane embed catalysts [295,448,294] or glutathione peroxidase (20-200 nM) [449]; photolysis (240-2660 nM) [40] and Cu^+ in the presence of GSH (100 nM) [450], among others reviewed by Griveau and Bedioui [3].

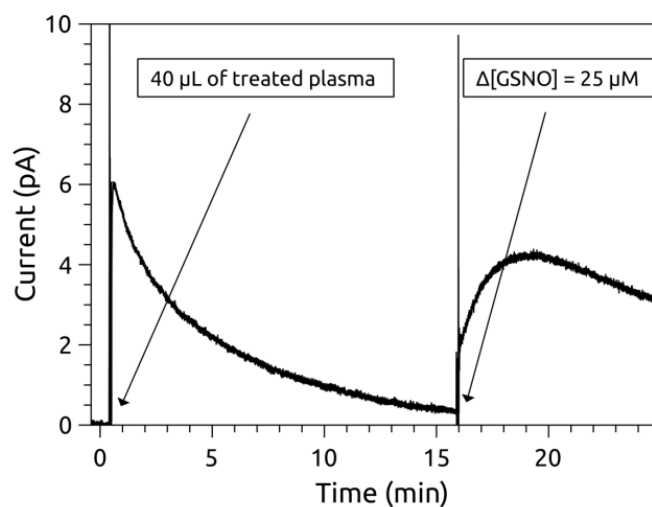


Figure 96. Current changes after injection of 40 μ L of sulfhydryl blocked human plasma to 2 mL of a 7.5 nM of AuNPs dispersion. After 15 min, an addition of GSNO (final concentration of 25 μ M) led to a second change in the measured current.

C. Conclusion of part II

Dispersions of AuNPs, in a condition of total surface excess, can be used for the amperometric quantification of aqueous GSNOs with a detection limit of ca. 100 nM. Each electrode is unique which necessitate a preamble calibration curve. RSNOs present in plasma were also detected provided that free thiols present in the sample are previously blocked. The response was

proportional to the volume of plasma added. Different kinetics of liberation of NO from different RSNOs makes it more difficult to quantify total RSNOs which necessitate preliminary separation step of different RSNOs.

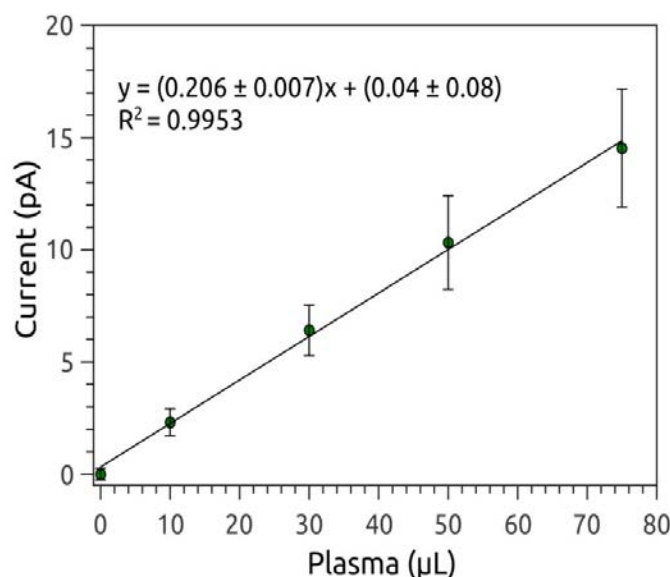


Figure 97. Current maxima after injection of different volumes of sulfhydryl blocked human plasma to 2 mL of a 10.9 nM AuNPs dispersion.

III. Conclusion of chapter III

Two decomposition methods of GSNO using Cu^{2+} / GSH and AuNPs were developed. The Cu^{2+} / GSH method permitted us to find the best conditions to decompose GSNO quantitatively and it showed the importance of GSH concentration in the decomposition of GSNO. The AuNPs method was useful to decompose GSNO and to detect RSNOs in plasma after the blocking of plasma thiols. Electrochemical detection used was used in both cases. It allowed the real-time measurement of NO and it gave better LOD than colorimetric and CE- C^4D (0.1 vs 2 and 15 μM) techniques. The GSNO decomposition by AuNPs is more rapid than that of Cu^{2+} / GSH (5s vs 10-15 s), but it necessitates large volume (2 mL vs μL). This limits its use for microchip analysis. A concentration step of AuNPs should be performed in order to make this miniaturization feasible.

CHAPTER IV: MINIATURIZATION

Miniaturization is the trend to fabricate ever smaller mechanical, optical, and electronic products and devices [451]. In the domain of chemistry it offers several advantages such as small volumes, rapid analysis, shorter reaction time, integration of various steps within one microchannel, low cost, small footprint of the devices and portability [312,326,342]. Several groups tried to make microfluidic devices to analyze RSNOs. These analyses were based on reacting a fluorescent dye with RSNOs or on analyzing the decomposition of RSNOs by light with an amperometric detection [40,41]. Fluorescence detection has drawbacks such as the stability of RSNOs (that is linked to a fluorescent dye) during the pretreatment step, the necessity of sophisticated experimental setup, and its high cost [349]. In the first part of this chapter we will introduce a new method for the determination of RSNOs using microfluidic Paper-based Analytical Device (μ PAD) using a simple colorimetric detection. In other terms it relies on the miniaturization of the spectrophotometric method developed in **chapter 3** but the recipients are papers instead of quartz cuvettes and the detector is a scanner (but could also be a camera or a cell phone) instead of a conventional UV-Vis spectrophotometer. In a second part, we will describe preliminary results obtained on glass electrophoretic microchips with integrated micro band electrodes for amperometric detection in order to quantify different RSNOs in artificial and biological media. This development will benefit from all the previous optimization steps (separation, decomposition, and electrochemical detection of RSNOs) to undergo the integration of the whole analytical chain on a microchip.

I. Colorimetric analysis of S-nitrosothiols decomposition on paper-based microfluidic devices

This first part was done in Brazil in the laboratory of Pr. Wendell Coltro (Federal University of Goias, Brazil). It is a collaborative work that was done with Marillya Oliveira (pharmacy student). Both of us brought new ideas and performed the experiments together through

intensive collaboration between the two groups. The obtained results led to a manuscript that was submitted for publication in the analyst (manuscript ID: AN-ART-06-2016-001439)

In this manuscript we describe for the first time the simultaneous decomposition of different RSNOs on wax-printed μ PADs using LEDs (UV, visible, and IR) as well as mercuric ions. LEDs were positioned at a given distance from the μ PAD using a 3D printed polymer device. Colorimetric detection was based on pixel intensity analysis from images digitalized by a scanner. As a proof-of-concept, the detection of RSNOs and nitrite in plasma samples was successfully demonstrated.

A. Experimental

1) Chemicals and materials

Bovine serum albumine (BSA), cysteine, reduced glutathione (GSH), nitrite, hydrochloric acid, citric acid, sulfanilamide, N-naphthylethylenediamine dihydrochloride, mercuric (II) chloride, monosodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), ethylenediaminetetraacetic acid (EDTA), sodium hydroxide, acetonitrile, acetone, di-ethyl ether were purchased from Sigma-Aldrich (St. Louis, MO, USA). Five samples of human plasma were donated by a local University Hospital. Ultrapure water was obtained from a water purification system (Direct-Q® 3, Milipore, Darmstadt, Germany) with resistivity equal to 18.2 M Ω .cm. All reagents were of analytical grade and used as received. Chromatography papers (grade 1 CHR) were received from Whatman® (Maidson Kent, UK). A scanner (model Scanjet G4050) was purchased from Hewlett-Packard (Palo Alto, CA, USA) to perform colorimetric measurements. Ultraviolet (UV), visible (Vis) and infrared (IR) LEDs were purchased from Eletrônica Santos Dumont (Goiânia, GO, Brazil). A centrifuge (IKA mini G, Staufen, Germany) was used in order to separate the proteins from the plasma.

2) S-nitrosothiols synthesis

GSNO was synthesized as described in **chapter 2** [232]

CysNO was synthesized as described by Peterson and co-workers [452]. Briefly, solutions of 5 mM CysNO were freshly prepared by making cysteine react with equimolar concentration of nitrite in acidic medium (0.1 M HCl) in a dark flask to avoid light decomposition. After 5 min

more than 90% of cysteine were converted to CysNO. The solution was neutralized by 0.1 M PBS buffer (pH 7.4) containing 0.5 mM EDTA in order to prevent decomposition by trace metal cation contaminants. Another method of CysNO preparation was also used [453] involving equimolar concentrations of cysteine and nitrite reacted in 0.1 M PBS (pH 1.5) + 0.5 mM EDTA.

AlbSNO was synthesized by addition of nitrite and albumin in equimolar concentrations (the amount of free thiol groups in albumin was measured equal to 30% of total albumin using Ellman test [434]). Albumin was dissolved in water and then acidified by a 1 M HCl solution. The reaction mixture was left in the refrigerator (4°C) for 1h. This solution was used when AlbSNO was studied under acidic conditions. Otherwise, the solution was neutralized by NaOH and then buffered by a 0.1 M PBS (pH 7.4) solution containing 0.5 mM EDTA.

3) Fabrication of μ PADs

μ PADs were fabricated by wax printing technology [454]. The device design was drawn using the graphical software Corel Draw[®] X7 and printed on chromatographic paper through a wax printer model ColorQube 8570 from Xerox[®] (Connecticut, USA). μ PADs were designed in a geometry containing eight circular detection zones for decomposition and posterior detection of analytes interconnected by microfluidic channels and one central sample inlet zone. All channels were wax created with a 10-mm length and 2-mm width. The diameter values for detection and central zones were 6 and 7 mm, respectively. The final dimensions of μ PADs were 45 mm $\times$ 45 mm (**Figure 98a**). After the printing process, μ PADs were passed in a hotplate laminator model 230C from Excentrix (São Paulo, BR) heated at 150°C in order to melt the wax allowing its penetration through the cellulosic porous structure to form hydrophobic barriers on the paper. Lastly, μ PADs were laminated with a polyester film coated with a thermosensitive layer (250 μ m thickness) acquired from Globo (Goiânia, GO, Brazil) in order to provide better rigidity and to make their surface planar ensuring lateral flow with homogeneous velocity [455].

4) Fabrication of a 3D printed holder

A polymeric device designed to hold lamps for the decomposition procedure was fabricated through the use of a low cost 3D printer model PRUSA Movtech from Movtech Comercial Tecnologia (São Bernardo do Campo, SP, Brazil) [456]. The 3D

holder was printed using a polymer composed of poly(lactic acid) (PLA) acquired from Movtech Comercial Tecnologia (São Bernardo do Campo, SP, Brazil). This piece was manufactured in a ring-shaped format with outside and inside diameters of 44 and 20 mm, respectively. In addition, eight holes with 4-mm diameter were printed in this holder to allow the coupling with LEDs. The distance between them was similar to detection zones configuration. The 3D printed device thickness was 6 mm. The printed holder was positioned on the μ PAD to allow the coupling with light sources, as displayed in **Figure 98b**. The holder was lightened by an USB power delivery system connected to the computer (**Figure 99**).

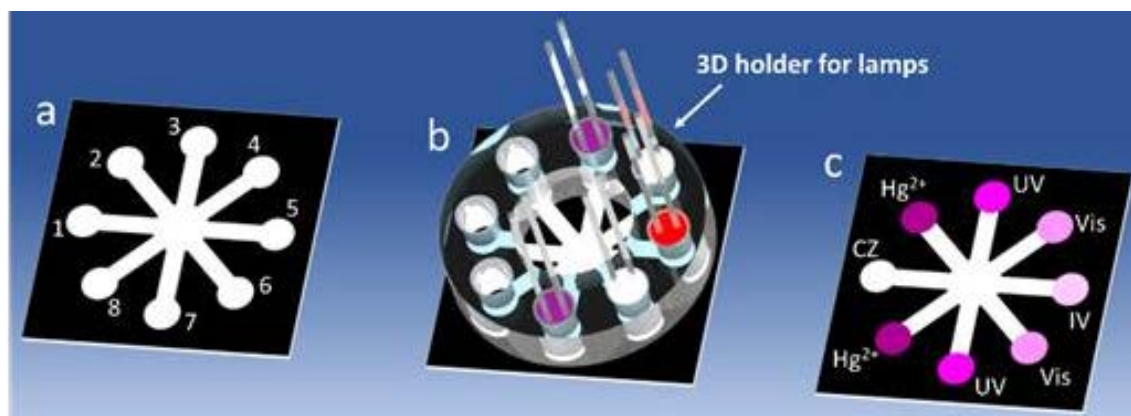


Figure 98: Presentation of (a) paper microfluidic device layout containing eight zones (1-8), (b) coupling of a 3D printed polymer piece for holding UV, Vis and IR lamps and (c) resulting device showing colored zones after colorimetric assays through the decomposition with Hg²⁺, UV, Vis and IR light. In (c), the label CZ means control zone.



Figure 99: Image of the device connected to laptop by a USB drive which assures lightening. A camera could be connected also to directly analyze the results.

5) Lateral flow procedure and colorimetric analysis

20 μL of RSNOs solution was added into the central zone of the μPAD . The solution flows laterally through the hydrophilic channels bordered by hydrophobic wax towards the decomposition / detection zones. Then, lights were turned on for 25 min in case of GSNO and 5 min in case of CysNO (these reaction times were optimized for each RSNO). A 1.5 μL -aliquot of Griess reagent mixed with water or Hg^{2+} (0.0295 M) (2 / 1, v / v) was then added inside detection zones for quantitation of nitrite produced after decomposition through light sources and mercuric ion, respectively. Afterwards, μPAD was allowed to dry for 10 min and an additional 1.5 μL -aliquot of Griess (with or without Hg^{2+}) solution was added inside detection zones as described above. Ten minutes after the color development (**Figure 98c**) μPADs were scanned and analyzed using Corel Photo-Paint® software. **Figure 100** displays an optical image showing the resulting color development after an assay with real samples. The color intensity was recorded using the magenta channel filter (after converting the scanned image to CMYK format). The intensity of magenta coloration is proportional to the amount of nitrite present and thus to the amount of RSNOs decomposed using each specific procedure.

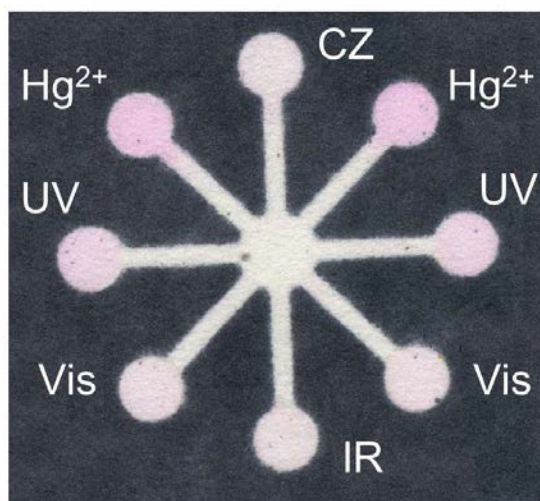


Figure 100: A real figure of paper microfluidic device containing human serum showing colored zones after colorimetric assays through the decomposition with Hg^{2+} , UV, Vis and IR light. The label CZ means control zone.

6) Plasma RSNOs detection

In order to have a translucent sample that can be detected using Griess reagent, a deproteinization method was used. Briefly, 200 μL of human plasma was placed in two eppendorfs and then the total volume was divided into two parts. Equivalent amount of

acetonitrile was added on each part. One part (100 μL) was treated using HgCl_2 (0.0295 M) to allow the decomposition of RSNOs, the other part was treated only with water. Then, the solutions were centrifuged for 10 min at 6000 rpm. A 20- μL -aliquot of the previously Hg^{2+} treated solution was added into the central zone of the μPAD to detect nitrite, LMW-RSNOs, and HMW-RSNOs after the addition of Griess reagent. The untreated plasma sample was used to detect nitrite or the sum of nitrite and LMW-RSNOs using the Griess reaction without and with Hg^{2+} , respectively. The treated part serves to find the sum of nitrite, LMW- and HMW-RSNOs.

B. Results and discussion

Experimental procedure involving the sample volume and the addition mode of the colorimetric reagent was firstly optimized. This was performed using Griess reagent and nitrite standard solutions. The nitrite calibration curve exhibited a linear behavior for the concentration range between 10 and 100 μM with a sensitivity of $0.36 \pm 0.01 \text{ AU} \cdot \mu\text{M}^{-1}$ (**Figure 101**). This serves as a reference for all subsequent decomposition curves since one RSNO can at most give only one nitrite. The LOD ($3 \text{ S} / \text{N}$) achieved for nitrite was 3 μM . The obtained value represents an important improvement in comparison with literature associated with nitrite detection on μPADs [457,458]. This could be partially due to the fact that Griess reagent was added twice to the detection zone, which helped in obtaining better homogeneity and higher color intensity.

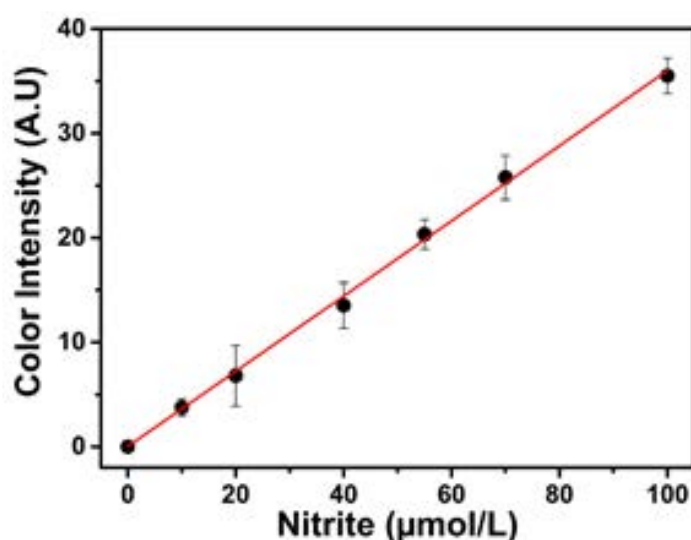


Figure 101: Calibration curve for nitrite dissolved in 0.1 M PBS (pH 7.4) containing 0.5 mM EDTA.

Decomposition of GSNO, CySNO and AlbsNO was achieved using different methods involving Hg^{2+} , UV, Vis and IR lights at physiological pH values. It should be pointed that whatever the RSNO studied, there is proportionality between the intensity of the color obtained

and the initial concentration of the RSNOs. This proportionality along with the calibration curve obtained for nitrite was used to evaluate the efficiency of the decomposition within the time scale of each approach. For this purpose, the data recorded for GSNO, CySNO and AlbSNO (presented in **Figure 103** were compared to the results achieved for nitrite (**Figure 101**). The calibration curve for the decomposition product of GSNO with Hg^{2+} (**Figure 103a**) exhibited a slope similar to that obtained with nitrite standard solutions ($0.34 \pm 0.01 \text{ AU} \cdot \mu\text{M}^{-1}$ vs $0.36 \pm 0.01 \text{ AU} \cdot \mu\text{M}^{-1}$) (displayed in **Figure 101**). This indicates a total and instantaneous decomposition of GSNO to give nitrite. UV decomposition carried out during 25 min of illumination was less effective (slope of $0.22 \pm 0.01 \text{ AU} \cdot \mu\text{M}^{-1}$) indicating that only 60% of GSNO was decomposed to nitrite (**Figure 103a**). It should be noted that an additional experiment, made using UV lamps in test tubes, revealed that the decomposition of GSNO to nitrite is indeed not complete (maximum of 53% of decomposition to nitrite (**Figure 102**). This also indicates that the decomposition of GSNO in test tubes and on paper follows similar patterns and no bias in the decomposition reaction was observed upon miniaturization. Finally, the percentage (36%) of decomposition into nitrite using Vis light for 25 min of illumination was lower than that obtained by Hg^{2+} and UV (**Figure 103a**). The sensitivity of the detection was $0.13 \pm 0.01 \text{ AU} \cdot \mu\text{M}^{-1}$. The LODs values found for GSNO decomposed by Hg^{2+} , UV and Vis lights were 4, 6 and 11 μM , respectively.

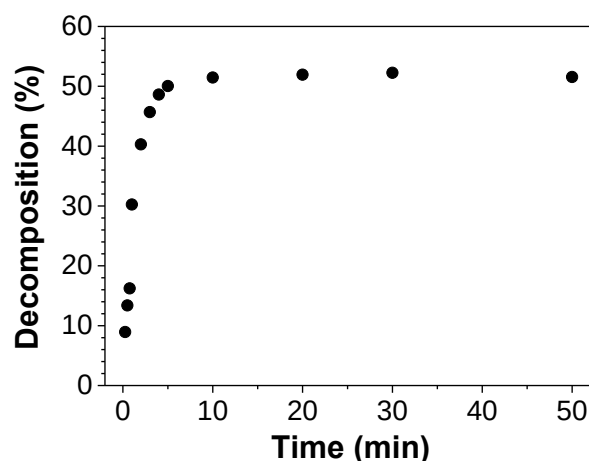


Figure 102: Graphical representation of the percentage of GSNO decomposition by UV light using test tubes. Percentage calculation were based on Griess and Saville reactions. $\lambda=540\text{nm}$

Similar experiments were performed with CySNO and it was found that Hg^{2+} decomposition is the most efficient process (slope of $0.35 \pm 0.01 \text{ AU} \cdot \mu\text{M}^{-1}$; **Figure 103b**), leading to the total decomposition of CysNO similarly to what was observed for GSNO. It should be noted that the light decomposition was conducted for 5 min only

because CySNO is poorly stable and decomposes very quickly. Calibration curve obtained for UV decomposition revealed a slope of $0.32 \pm 0.01 \text{ AU} \cdot \mu\text{M}^{-1}$, higher than that for GSNO ($0.22 \pm 0.01 \text{ AU} \cdot \mu\text{M}^{-1}$). This confirms that these two RSNOs behave differently under UV light (**Figure 103b**). Calibration curve of CySNO decomposed by Vis light also showed higher slope than for GSNO ($0.22 \pm 0.01 \text{ AU} \cdot \mu\text{M}^{-1}$ vs $0.13 \pm 0.01 \text{ AU} \cdot \mu\text{M}^{-1}$) (61% vs 36% decomposition percentage to nitrite) (**Figure 103**). These results are in accordance with those reported by Hunter *et al* [40], who observed the same trend when RSNOs were decomposed inside a microchannel using Vis green light ($\lambda=530 \text{ nm}$) amperometric detection to quantify NO. Riccio *et al.* [84] reported low efficiency for the decomposition of RSNOs into NO in bulk solution using visible green light ($\lambda=500\text{-}550 \text{ nm}$). The found values for CySNO, GSNO and AlbsNO were 11, 7 and 1.3%, respectively

Lastly, decomposition of CysNO and GSNO was also performed using IR light on μPAD . For CysNO, no coloration was detected even with concentrations up to 0.5 mM , indicating that such pathway is not efficient.

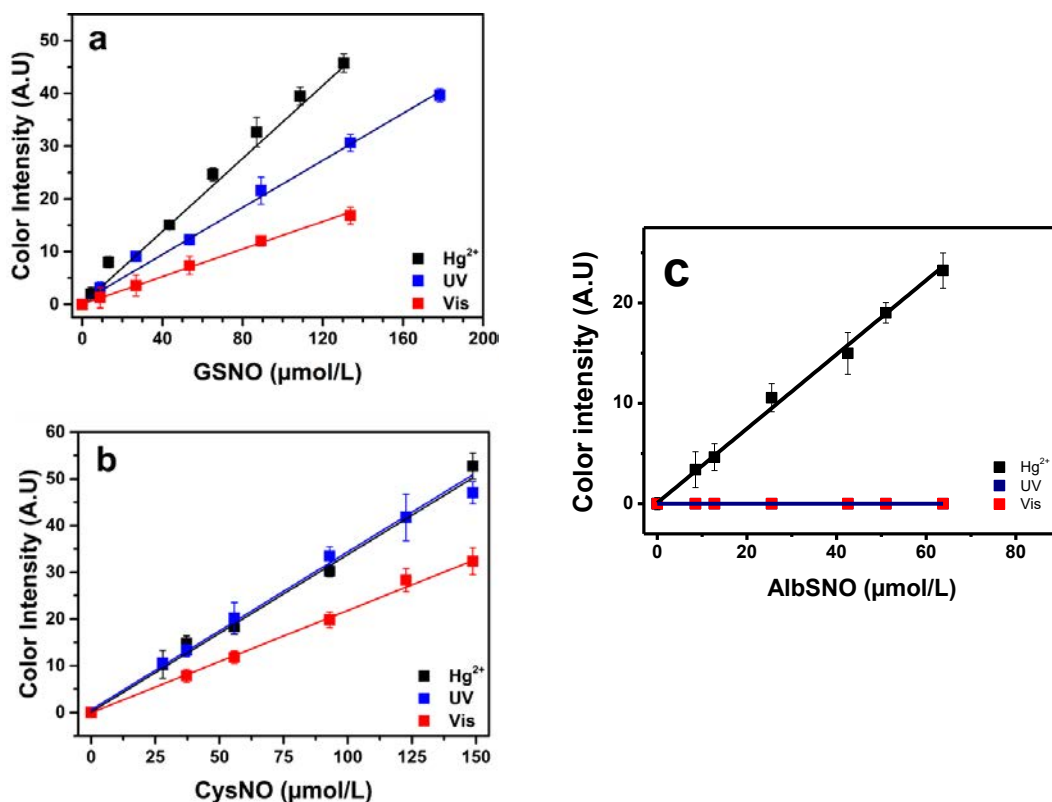


Figure 103: Calibration curves for GSNO (a), CysNO (b), and AlbsNO (c) dissolved in 0.1 M PBS ($\text{pH } 7.4$) containing 0.5 mM EDTA. Colorimetric measurements were recorded after decomposition promoted by 10 mM Hg^{2+} (black squares), UV (blue squares), and Visible lights (red squares). For mercuric decomposition, Griess reagent was mixed with Hg^{2+} at the decomposition time. For light-mediated decomposition, Griess reagent was added after the decomposition reaction occurred.

On the other hand, as it can be seen in **Figure 104a**, the IR decomposition of GSNO provided the lowest sensitivity ($0.06 \pm 0.01 \text{ AU} \cdot \mu\text{M}^{-1}$) when compared to decomposition pathways promoted by Hg^{2+} , UV and Vis lights (**Figure 103a**). Finally, mercuric decomposition of AlbSNO presented a slope of $0.36 \pm 0.01 \text{ AU} \cdot \mu\text{M}^{-1}$ indicating a complete decomposition (**Figure 103c**), while no decomposition was obtained using light sources. Based on these results and those reported by Hunter *et al.* [40] a correlation between the molecular weight of the RSNO and its sensitivity through the decomposition by light can be suggested. LMW-RSNOs are more prone to decompose than HMW-RSNOs. This correlation has already been reported[24,60,459,460] and was attributed to the stability of the RSNO bond. Indeed, AlbSNO contains only one reduced cysteine (CySH), present in the hydrophobic pocket of albumin^{57, 58}, where NO could bind to.

The decomposition of CySNO in acidic PBS (0.1 M; pH 1.5) was carried out and the obtained results showed that only the use of Hg^{2+} leads to a total decomposition with a slope of $0.36 \pm 0.01 \text{ AU} \cdot \mu\text{M}^{-1}$. The illumination with different light sources (UV, Vis, and IR) did not yield any decomposition (data not shown), even after 2h illumination.

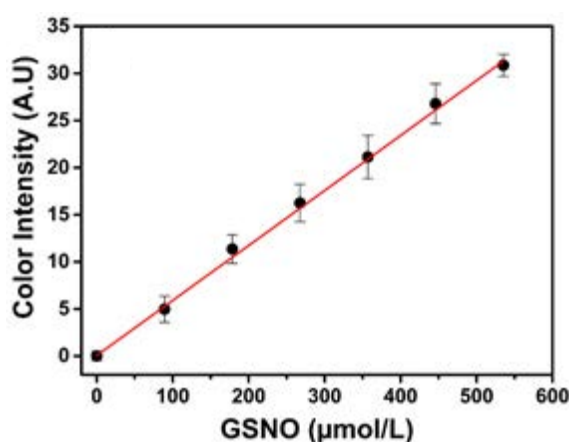


Figure 104: Calibration curves for GSNO dissolved in 0.1 M PBS (pH 7,4) containing 0.5 mM EDTA decomposed by IR light during 25 min and

This can be explained by different decomposition pathways: Hg^{2+} decomposition leads to nitrosonium and then nitrite, while light decomposition gives NO which can in turn react with thiols to give back RSNOs before being transformed into nitrite. The explanation justifies the absence of pink coloration detected after the addition of Griess reagent. It should be noted that when Griess reagent was mixed with CySNO prior to light decomposition, a pink coloration appeared which could be explained by a competition between Griess reagent and thiols.

The detection of RSNOs in human plasma was then performed in order to show the feasibility of the proposed device for real sample analysis. A deproteinization step was necessary because the Griess reaction was masked by the yellow color of HMW plasma protein. Upon deproteinization HMW-RSNOs are separated from the solution containing LMW-RSNOs and nitrite. There was no difference in coloration when Griess reagent was used with or without Hg^{2+} in plasma after deprotonization. This indicates that LMW-RSNOs levels were below the achieved LOD for the method compared to nitrite and this is in accordance with the nanomolar reported values of LMW-RSNOs [2,85]. The decomposition of the sum of LMW- and HMW-RSNOs (before deproteinization step) using HgCl_2 has led to the changes in the color intensities. Based on the data extracted from the analytical curves, the concentration levels ranged from 5 to 16 μM depending on the plasma sample, as displayed in **Table 18**. This variation corresponds to HMW-RSNOs, principally AlbsNO as it is the main HMW protein in plasma (88 % of plasma proteins [461]). These values are not very far from those obtained by Stamler et al. [53] and others ([2] and references therein. The nitrite concentration was between 37 and 58 μM (**Table 18**). These values were determined by the following procedure: the control assay (where no decomposition occurs) alone expresses the nitrite levels already present in the sample. The sample decomposed by Hg^{2+} after deproteinization indicates the sum of LMW-RSNOs and nitrite (because HMW-RSNOs are deproteinized before the decomposition step). In addition, the sample decomposed before deproteinization displays the sum of nitrite, LMW-RSNOs and HMW-RSNOs. By subtracting these values, it is thus possible to calculate the concentrations of nitrite, LMW- and HMW- RSNOs. The data presented in **Table 18** shows the potentiality of the proposed method to estimate HMW-RSNOs in the concentration range above 4 μM . The proof-of-concept of the proposed device was successfully demonstrated using Hg^{2+} as a standard protocol. However, the use of UV LEDs has provided similar performance, which enables their use for replacing the reference approach allowing consequently on-site applications without toxic effects. In comparison with other metal-based decomposition protocols, like Cu^+ , the use of LEDs is simpler to be implemented in a portable device and faster due to their operational and experimental simplicity.

Table 18: Concentrations of nitrite, LMW- and HMW-RSNOs in human plasma samples (n=5).

Sample	Nitrite (μM)	HMW-RSNOs (μM)	LMW-RSNOs (μM)
1	49,9 $\pm$ 2,3	14,0 $\pm$ 2,9	< LOD
2	49,5 $\pm$ 3,8	4,5 $\pm$ 2,4	< LOD
3	37,5 $\pm$ 3,3	5,7 $\pm$ 2,7	< LOD
4	58,5 $\pm$ 3,4	16,2 $\pm$ 3,6	< LOD

C. Conclusions

The analytical method presented in this study represents the first colorimetric assay on μ PADs to detect RSNOs. Decomposition of LMW- and HMW-RSNOs was performed on paper substrate using using different light sources (UV, Vis, IR) as well as Hg^{2+} as a reference procedure. Hg^{2+} decomposition was total for GSNO and CysNO. For each RSNO, UV decomposition was more efficient than visible light decomposition, the latter remaining more efficient than IR decomposition. RSNOs did not show any color development when decomposition was carried by light in acidic solution, while they showed total decomposition into nitrite using Hg^{2+} ion. The LOD achieved for nitrite was 3 μM , which represents one of the lowest values already reported in literature using colorimetric measurements and μ PADs. The enhanced analytical performance has also provided relatively good detectability levels for GSNO. When decomposed by Hg^{2+} , UV and Vis LEDs, the obtained LODs were 4, 6 and 11 μM , respectively.

Due to the portability and compatibility to be powered by a USB port, the proposed platform emerges as simple POCT device. When compared to the standard decomposition procedure (Hg^{2+}), the use of UV LED has provided similar performance. Since it may be also powered by battery or even USP port, the coupling of this simple light source with μ PADs represents a good alternative to be used in a truly portable device. HMW-RSNOs were differentiated from LMW-RSNOs by subtracting the color intensities recorded after and before deproteinization by Hg^{2+} . Detection of nitrite and RSNOs in plasma samples was performed after a preliminary sample treatment. HMW-RSNOs and nitrite were detected while LMW-RSNOs were below the LOD. On-chip microfiltration could be promising to replace deproteinization. Its coupling to on-chip electrokinetic separation of different RSNOs before decomposition could provide a powerful integrated lab-on-a-chip for their simultaneous quantitation. The separations on microchips can be quite attractive in order to achieve the concentrations of different RSNOs.

II. Electrochemical detection of RSNOs in electrophoretic micro device: preliminary studies

As we have seen in the previous part of this chapter with μ PADs (Part I), in order to quantify the different RSNOs a separation step is necessary before decomposition and detection, otherwise they cannot be differentiated. The electrokinetic separations are performed more easily with polymer and glass microsystems than with paper. Few papers were reported on electrokinetic separation with paper device, which presented difficulties in the injection of the sample, in the quality of the separation and in the robustness of the method [54,55]. The integration of electrochemical detection in μ PAD is furthermore less efficient compared to microsystems with glass or polymer. Based on this and in order to perform all the steps required for the quantitation of RSNOs (electrokinetic separation of RSNOs and by-products or impurities, decomposition of RSNOs and electrochemical detection of NO) in an integrated microfluidic device first experiments were performed with a, home-made polymethyl(methacrylate) (PMMA) system with a 25 μ m Pt UME similar to the one used in **chapter III** for electrochemical detection. Off-chip end-channel configuration was used. In order to solve problems encountered with sensitivity and repeatability, a commercial glass / Su-8 (epoxy-based negative photoresist) device from Micrux Technologies (Oviedo, Spain) that contains integrated platinum electrodes was used later on with a wireless potentiostat. This system needs a holder which is opaque. In order to optimise the injection method, a commercial system of cyclic olefin copolymer (COC) (Topas, from ChipShop (Jena, Germany)) which has dimensions of cross section between the ones of the PMMA and the glass systems, was used. Subsequently, the online decomposition of GSNO and consequent detection of nitrite using the glass / Su-8 were discussed.

A.Experimental

1) Microchip configuration

i. PMMA microchip fabrication

The CO₂-laser (gravograph LS100) was used to cut PMMA into the desired shapes. It uses infrared laser to cut and trace on PMMA. The depth and width of the channel depend on three

parameters: energy, speed, and number of passages of the laser. These parameters were optimized in order to have channel geometry of $100\mu\text{m}$ width, $80\mu\text{m}$ depth and 25 mm length. The two microfabricated pieces (the bottom and the top) were sonicated in alcohol and then in pure distilled water for 5 min each. Then the two PMMA pieces were put over each other and placed in an oven (120°C , 23 min) so they are thermally glued. This thermal method presents the advantage over chemical gluing (chloroform) to avoid channel clogging (**Figure 105**). Eventually, a colored dye was injected for leakage control in order to assess the gluing quality.

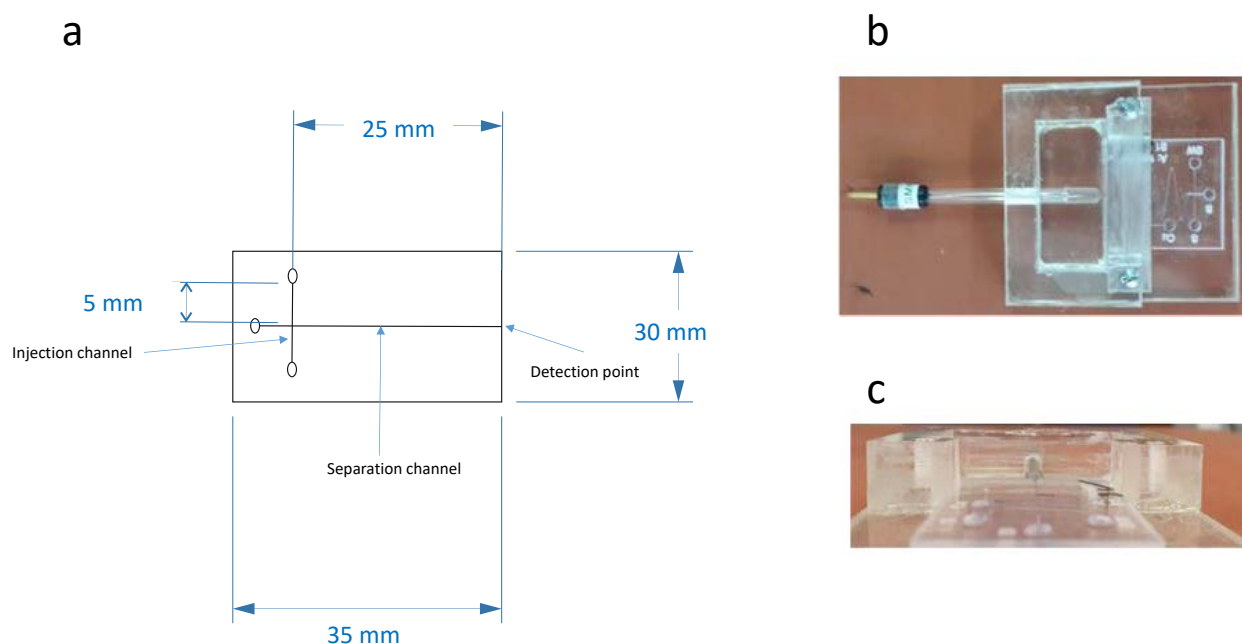


Figure 105: (a) Schematic representation of microfabricated PMMA chip. Top (b) and front (c) view of the PMMA microchip with working and reference electrodes situated in off-chip end-channel configuration.

ii. Commercial microchips

Two commercial microchips were studied for the proof of concept:

- A commercial polymer COC (Topas) microchip from Microfluidic ChipShop (Jena, Germany). The COC microchip geometry is of $5\mu\text{m}$, $50\mu\text{m}$, and 87 mm channel width, depth, and length, respectively. The channels are separated from the crossing point by 5 and 6 mm (**Figure 106**).

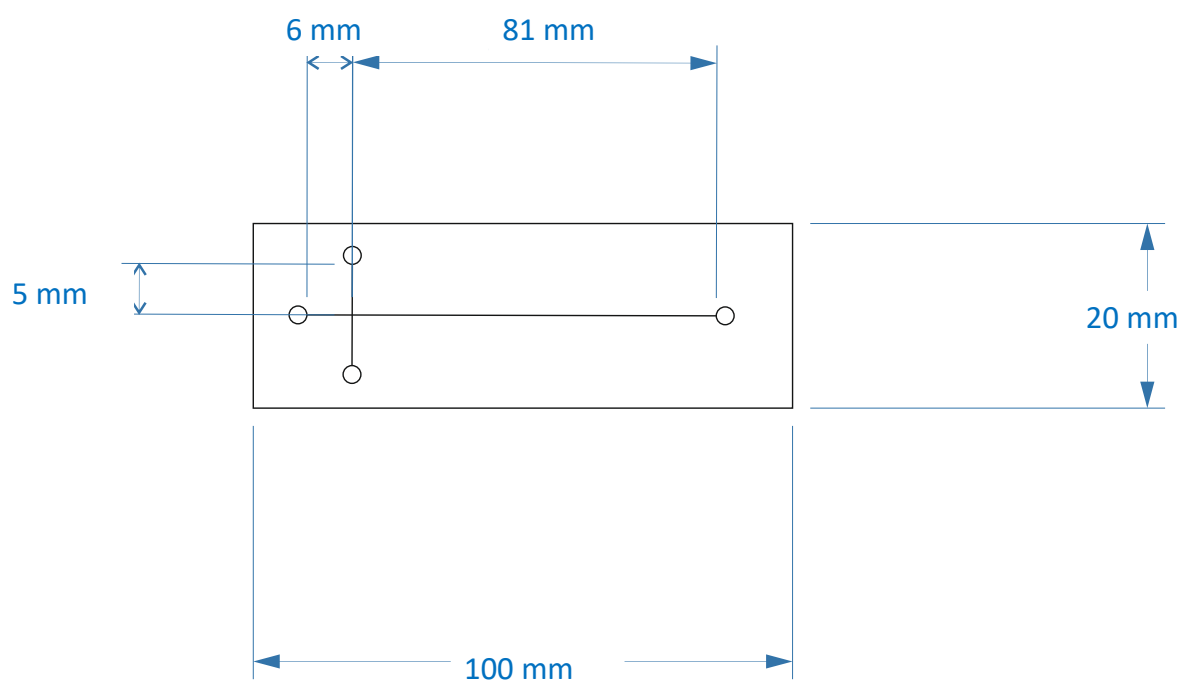


Figure 106: Schematic representation of a commercial COC microchip from Microfluidic ChipShop (Jena, Germany)

- A commercial glass microchip with integrated micro band electrodes at the outlet end of the separation channel from Micrux Technologies (Oviedo, Spain). The dimensions and the configuration of this microchip are shown in **Figure 107**. The channel depth and width are smaller than those of the PMMA microfabricated chip which permitted us to work with higher potentials in gated injection mode.

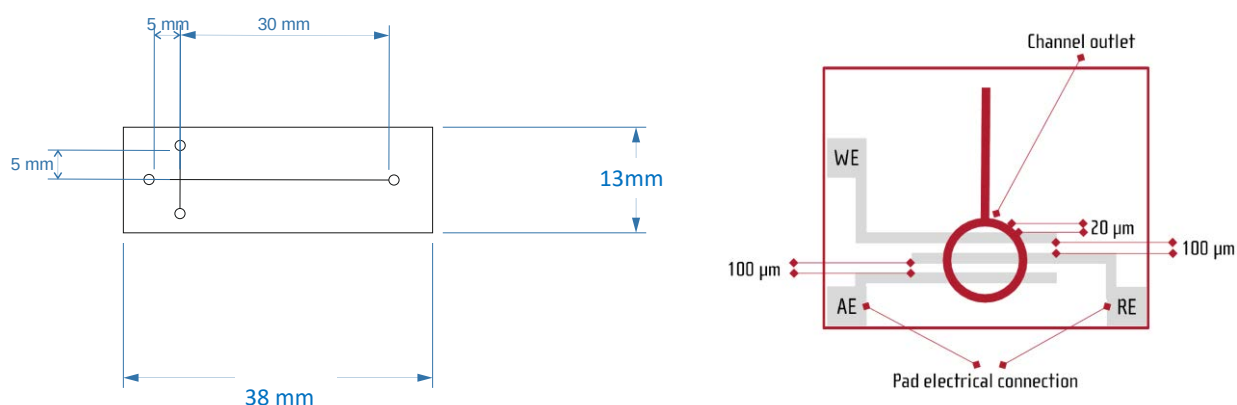


Figure 107: Schematic representation of a commercial glass / Su-8 from Micrux Technologies (Oviedo, Spain)

2) Operating conditions for the microchip electrophoresis of GSNO

i. PMMA and COC microchip

a- For **injection purpose**, either floating or gated injection modes were evaluated:

Floating injection 1000 V was applied for 10 s in the transversal channel, then 1000 V were applied in the separation channel. (**Figure 64**)

Gated injection: Different V_1 and V_2 (250-300 V and 400-500 V on PMMA and COC, respectively) on sample and buffer reservoirs were applied in such a manner that V_2 was always higher than V_1 (**Figure 64**)

- b - For **separation purpose** high voltage potentiostats were used, either home-made or a commercial one (Lab smith HVS 448 6000D). The home-made had 2 channels and did not monitor the current while the commercial one had 8 channels and afforded to control the current.
- c - For **detection purpose**, either optical or electrochemical methodologies were applied:

In the case of an amperometric detection, the working electrode was brought near to the outlet end of the micro-channel at a distance equal to tenth of micrometers to prevent any electric interference with the high voltage applied for separation (**Figure 105b**). The working electrode was connected to a PalmSens potentiostat in a three-electrodes configuration in which the auxiliary electrode was made of platinum and the reference was an Ag / AgCl electrode. Detection voltage was 0.8V.

In the case of a fluorescence detection: an inverted fluorescence microscope system (Axio Observer A1, Zeiss) equipped with a 100 W metal halide lamp and filter set 75 HE was used. A CDD digital camera was combined with HIRIS software (RD vision) for detection processing. Objective of 20 times magnification was used. The acquisition parameters of the CCD camera were kept constant, with a 25 ms exposition time, a zero gain and no binding.

ii. Commercial glass microchip

A chip holder purchased from Micrux technologies (Oviedo, Spain) was used to connect the high voltage potentiostat (Lab Smith HVS 448 6000 D) for separation and the handheld wireless potentiostat (Pinnacle Technology, Lawrence, KS, USA) for detection to the desired electrodes on the microchip. A two-electrode configuration was used for detection purpose because the

wireless potentiostat only has this configuration possibility. The detection voltage was always 1 V vs Pt pseudo reference. Gated injection was used in positive and negative mode depending on the molecules to be detected. BGE used was a 10 mM arginin adjusted to pH 5.6 by acetic acid.

3) Chemicals and GSNO synthesis

Coumarin 334 obtained from Sigma-Aldrich was dissolved first in ethanol and then diluted in 20 mM PBS (pH 7.4) / ethanol (99 / 1 v / v) to the desired concentration.

Others, see part 1.

B. Results and discussions

1) Preliminary study

The goal was to make a totally home-made microsystem for the online separation, decomposition and detection of RSNOs. To do so, the preliminary studies were performed in PMMA microchips with working and reference electrodes situated in an off-chip end-channel configuration, but this system was changed after some time in order to ameliorate the performance and to eliminate some encountered problems. The chosen neutral marker was paracetamol ($pK_a=9.5$). Below pH 8.5 (pK_a-1) paracetamol is not ionized and thus has no electrophoretic mobility. Corresponding electropherograms are displayed **Figure 108**, employing the floating injection mode, and sequential paracetamol injection. EOF was measured equal to $2.5 \times 10^{-5} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$ in 20 mM PBS (pH 7.4) in PMMA channels, which can be considered as quite low. We also evidenced a loss in sensitivity when the ground of the high voltage separation system was placed in the outlet reservoir, due to an interaction of the separation electric field with the potentiostat. Placing the detection electrode far from the outlet end of the separation channel or setting the separation voltage at 0 V did not help in eliminating the noise. Such a low sensitivity (which was alike for nitrite) was not compatible with the analysis of RSNOs in biological medium which are present at nanomolar and low micromolar concentrations.

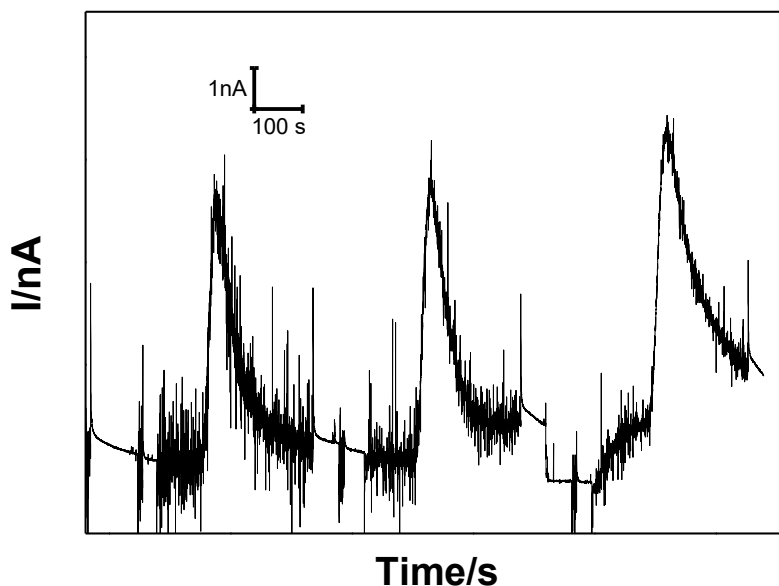


Figure 108: Electropherogram corresponding to the successive floating injections (1kV for 10 s) of a paracetamol solution (5.23 mM) in a PMMA microfluidic system. Operating conditions: see experimental part.

In order to improve the performance of the system we went further in this study according to three ways:

- Employing a wireless potentiostat for detection which has the role of eliminating the interferences coming from the high voltage potentiostat used for separation (whether it comes from the ground or from the separation voltage)
- Optimizing the injection protocol, from the floating to the gated mode
- Using microchip with integrated Pt micro band electrodes with fixed and controlled interspacing.

i. Employment of wireless potentiostat

A modified model 8151BP 2-channel wireless, electrically isolated potentiostat (Pinnacle Technology, Lawrence, KS, USA) operating in a two-electrode format at 5 Hz sampling rate (gain=5 000 000 V / A, resolution=30 fA) was used. Sirena acquisition software was used for all data acquisition. The data acquisition was performed by wireless data transmission from the potentiostat. This wireless potentiostat is not grounded which permits to avoid interferences from the high voltage power supply system. It permits in-channel detection with a low noise comparable to end-channel detection. Without the wireless potentiostat, in-channel detection could not be achieved because the conventional potentiostats would be damaged [56].

ii. Optimization of the injection mode

There exist several kinds of injection: floating, pinched and gated as was discussed in **chapter 1**. Each one has its own advantages and disadvantages. Floating injection was used at first due to technical issues (the Lab Smith high voltage was not present at laboratory) and because of its simplicity (no need to optimize injection voltages, only one is applied at a time). Because of the broad peaks obtained we decided to try the gated injection mode that allows controlling injected amounts and avoids sample diffusion and leakage phenomena. The Lab Smith high voltage potentiostat and the microchips PMMA were used at the beginning. These microchips permit fluorescence detection in contrast to the micrux microchips that will be used further. The formation of air bubbles was evidenced at high voltages especially because the electrodes in the sample and BGE reservoirs are very near to the ground electrode in the sample waste reservoir which results in a higher electric field for the same applied voltage. Therefore injection voltages (V_1 and V_2 at sample and BGE reservoirs, respectively) were decreased below 250 and 300 V, respectively. Another system made of COC polymer was used. This system also permitted fluorescence detection but it has smaller cross section so we tested the hypothesis that we can apply higher voltages. The cross sections in the COC system were $50 \times 50 \mu\text{m}$ in comparison to $100 \times 80 \mu\text{m}$ in the PMMA system. Maximum voltages that could be applied to the system without formation of air bubbles were 400 V and 500 V for V_1 and V_2 , respectively. Our hypothesis was validated and we could imagine higher voltages could be applied with the glass / Su-8 system because it has even smaller cross section dimensions ($20 \times 50 \mu\text{m}$). Fluorescence microscopy permitted to image the different steps of the gated injection mode in real time as shown on **Figure 109**. For this purpose, coumarin 334, a neutral fluorescent marker, was injected.

3 steps can be identified:

-The first step is the loading upon which coumarin 334 is injected between the sample (S) and the sample waste (SW) reservoirs while the BGE is injected between the BGE reservoir (BGE) and both the sample waste (SW) and buffer waste (BW) reservoirs. The amount of liquid that passes is proportional to the voltage applied so V_2 should be higher than V_1 in order to prevent the sample from going into the separation channel.

-The second step is the injection step during which the voltage in the BGE reservoir is turned off so the sample leaks toward the BGE, the sample waste, and the buffer waste reservoirs. This step is ultrashort (less than three seconds) and permits the injection of a sample plug which is

proportional to the injection time and applied voltage. The approximate volume of the injection plug compared to the volume of the channel was $\approx 0.1\%$.

-The last step is the running step which has the same voltage program as the first step. Upon this step the injected plug is electrophoretically moved toward the detection zone.

Using the gated injection permitted us to obtain more repeatable injections without any sample leakage upon running.

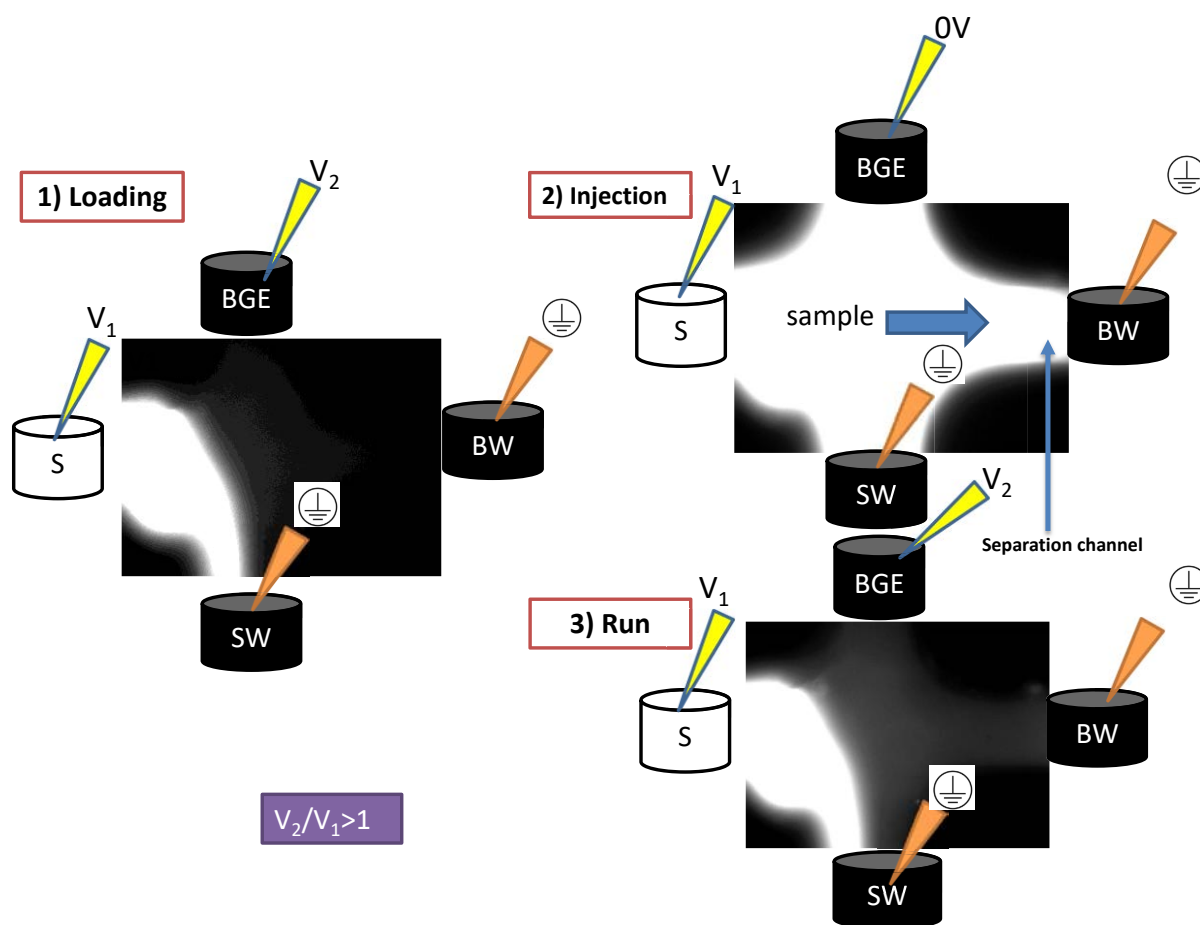


Figure 109: Gated injection on COC micro-chip. The fluorescent molecule coumarine 334 (5 mg / L prepared in BGE / EtOh 99 / 01 (v / v)) appears in white. The BGE (PBS (C= 20mM, pH 7.4)) appears in black. S: sample reservoir, SW: sample waste, BGE: back ground electrolyte, BW: buffer waste.

iii. microchip with integrated electrodes

A commercial microchip from Micrux[®] made of glass / SU-8 and integrating patterned platinum electrodes was used instead of the PMMA microfabricated microchip. The first step was to determine the EOF, then to test model molecules such as nitrite. The EOF determined in 20 mM arginine solution adjusted at pH 5.6 with acetic acid after gated injection of paracetamol as neutral marker ($V_1 = 1000$ V and $V_2 = 1200$ V, SW and BW grounded) was $(9.5 \pm 0.12) \times 10^{-4}$ m/s.

$5 \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$ (**Figure 110**). The results show that for a 1 mM concentration of paracetamol the S / N is around 100 which is a very big improvement compared to results obtained in previous home-made PMMA systems with a conventional potentiostat (S / N of ≈ 4 for a concentration of 5 mM). It is well-known that the EOF is lower in microfluidic materials than in conventional silica capillaries [325] and that is consistent with our experimental results measuring EOF always less than $10^{-4} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$ in all tested microsystems.

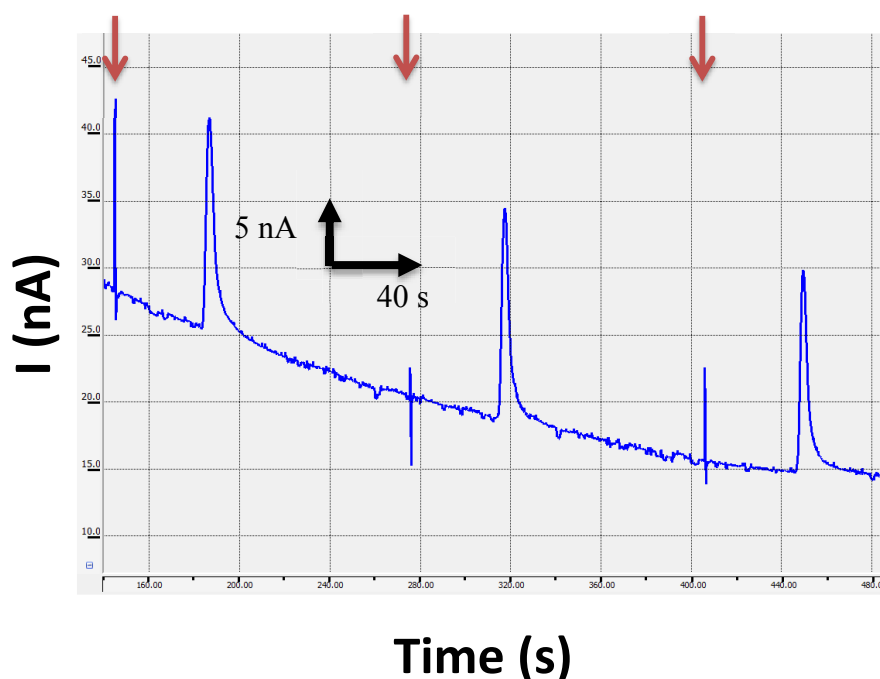


Figure 110: Electropherogram corresponding to the electrophoretic profile of 1 mM paracetamol in glass / Su-8 microchip. Gated injection $V_1 = 1000 \text{ V}$, $V_2 = 1200 \text{ V}$, injection time 0.5 s, successive injections: 120s, detection 1V vs Pt. BGE: 20 mM arginine solution adjusted at pH 5.6 with acetic acid.

After detection of paracetamol we have decided to test a standard stable molecule, nitrite which has a rather highly negative electrophoretic mobility (about $30 \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$ at pH 3.8 [462]) due to its small size. In a glass microchip, the electroosmotic flow is directed toward the cathode, thus only cations and "slow"-migrating anions will be detected at the outlet end of the separation channel in a normal polarity (positive polarity at the inlet end of the microchannel). As nitrate is expected to migrate counter-electroosmotically (that is, with an electrophoretic velocity higher than the electroosmosis, in absolute value) it should not be dragged toward the detector if a positive polarity is applied at the inlet end of the microchannel. We therefore imposed a negative polarity at the inlet end so as to detect nitrite, as evidenced experimentally (**Figure 111**). Under these conditions, the electrophoretic mobility of nitrite was estimated equal to $-(27.8 \pm 0.2) \times 10^{-5} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$. Concentrations down to $50 \text{ } \mu\text{M}$ could be detected with S / N around 9. This LOQ is good but still insufficient to detect NO at biological concentrations keeping in mind that NO sensitivity is lower than nitrite sensitivity.

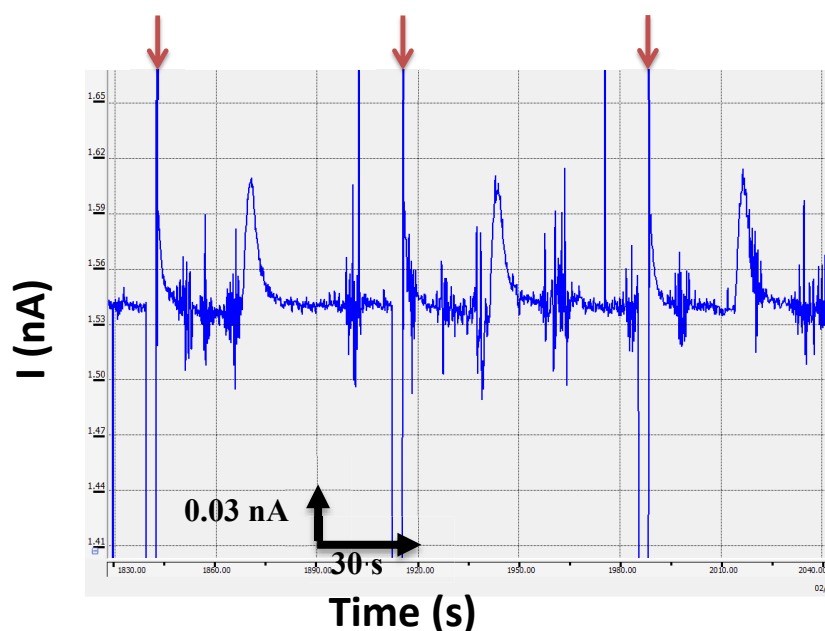


Figure 111: Electropherogram corresponding to the electrophoretic profile of a 50 μM nitrite in glass / Su-8 microchip. Gated injection $V_1 = -800$ V, $V_2 = -1000$ V, injection time 3 s, successive injections: 70s, detection 1V vs Pt. BGE: 20 mM arginin solution adjusted at pH 5.6 with acetic acid.

In conclusion, the use of wireless potentiostat and integrated microfluidic system permitted us to detect low concentrations of nitrite (LOQ (S / N=9) as low as 50 μM using integrated glass / Su-8 μchips with wireless potentiostat vs 5 mM with normal potentiostat and homemade PMMA μchips . The use of the gated injection mode permitted us to obtain repeatable injections without leakage of the sample upon running. The development of this analytical system has permitted us to solve problems due to non-precise positioning of the electrodes at the outlet end of the channel (which resulted in noisy and non-repeatable results) and to avoid eventual problems due to NO diffusion through the μsystem material (polymers are more permeable to gases than glass).

2) Application to the separation and quantitation of RSNOs

As discussed in **Chapter I part IIIB**, detection of RSNOs in microfluidic devices was already reported in microsystems (**Chapter 1**). Briefly, RSNOs (GSNO, CySNO and AlbSNO) were characterized without separation process, after light decomposition and electrochemical detection, or nitrosylated proteins were separated by MCE and detected with laser induced fluorescence (LIF) [40,41] after derivatization. Separation of LMW- and HMW-RSNOs was not reported before. To detect RSNOs online, the decomposition process should be faster than the separation one. Moreover the degradation product that will be further detected and

quantified should be stable through the analysis time and under operating conditions. According to previous chapters, decomposition products vary depending on the agent used to decompose RSNOs. If Cu^+ or light is used, NO is produced, while if Hg^{2+} is used, nitrite is produced. NO is unstable and it reacts with oxygen to produce nitrite which is stable in solution at neutral and basic pH. Since decomposition with Hg^{2+} is complete, spontaneous, and produce nitrite which is stable, we chose to decompose RSNOs using Hg^{2+} , and then to detect the nitrite produced.

In a first attempt GSNO analysis was performed. Hg^{2+} was placed in the BW reservoir. When applying a normal polarity, Hg^{2+} remains in BW reservoir while positive molecules and negative molecules which have electrophoretic mobility less than the EOF will reach the detection zone where Hg^{2+} is present. However, GSNO could not be detected in these conditions. We therefore applied a reversed polarity, leading to the entry of Hg^{2+} in the separation channel and its migration toward the BGE reservoir.

Results are summarized below:

- By applying normal polarity in carbonate buffer at pH 8.5 (same conditions as that for CE-MS analysis in **chapter II**), no signal was observed for GSNO meaning that its electrophoretic mobility is equal or higher than the EOF in absolute value. This is not consistent with what was obtained on **chapter II** where GSNO and its derivatives were detected by CE-MS in normal polarity (see **chapter II**). This can be explained by the difference in EOF (9.5×10^{-5} instead of $75 \times 10^{-5} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$). Decreasing the pH in MCE from 8.5 to 5.6 was made in order to decrease the electrophoretic mobility of nitrite and increase EOF but still no signal was observed
- By reversing the polarity, a peak was detected at a mobility similar to the nitrite samples but with a far lower intensity (**Figure 112a**).
- Decomposing GSNO by mixing with Hg^{2+} directly in the sample reservoir before injection and separating the resulting mixture by applying reversed polarity also resulted in a peak having similar sensitivity and mobility as nitrite standard solution peak. This suggests that the problem of sensitivity was mostly due to the decomposition of small amounts of RSNOs into nitrite inside the microfluidic channels
- Replacing Hg^{2+} by BGE in the BW reservoir, injecting GSNO, and leaving the separation under high voltage until GSNO is supposed to reach the working electrode. At this point high voltages are turned off and Hg^{2+} is added to BW reservoir and the electrode is left to stabilize in this new solution, before high voltage is turned on again.

This resulted in a peak at the same migration time as before. (**Figure 112b and Figure 112a**)

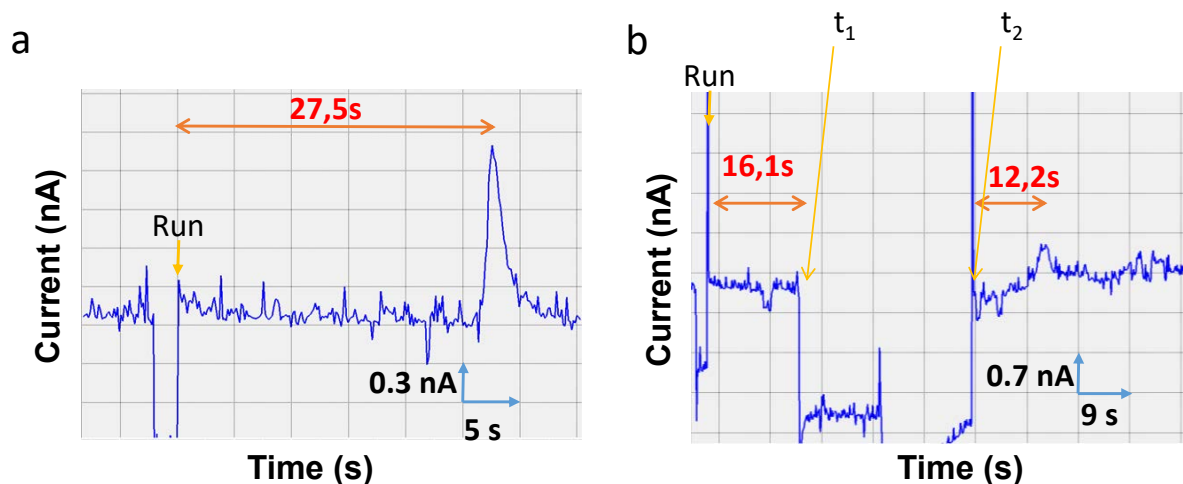


Figure 112: Electrophoretic profile of 3 mM GSNO injected in « gated mode » (-1000, -800 V) for 1s. a) Hg^{2+} is already put in the reservoir. b) t_1 : stopping the electric field, injection of Hg^{2+} , and stabilization of the electrode of detection 16.1 s after the beginning of the separation of GSNO. t_2 : application of a new electric field.

These results permitted us to conclude that GSNO and nitrite have similar mobilities under these conditions (BGE: 20 mM arginine adjusted to pH 5.6 with acetic acid). So either the experimental conditions should be changed in order to permit these two molecules to have different mobilities and to be separated, or the analysis should be done in two times: the first without Hg^{2+} detects nitrite and the second with Hg^{2+} detects the sum of GSNO with nitrite. GSNO concentration can be deduced from the difference between the two values.

The objective of this work was to quantify different RSNOs after their separation. Therefore, RSNOs should be separated from each other before decomposition and detection. So, Hg^{2+} should decompose RSNOs after their efficient separation. For this, separation and decomposition steps must be set up subsequently. Two hypotheses can be done:

- Separation is performed and then before arriving to the detection zone Hg^{2+} is added so that decomposition will occur at the nearest possible position to the detection.

- Another channel for mercury is added. Separation is done between S, SW, BGE and BW. Before the sample reaches the introduced Hg^{2+} channel, the electric field is shifted to the Hg^{2+} reservoir instead of the BW, thus Hg^{2+} enters after the separation step and decomposes RSNOs into nitrite, that continues to the Hg^{2+} reservoir where the detection electrode is present. (**Figure 113**).

The first approach is the one used above but Hg^{2+} ions when introduced induce big modifications in the amperogram. Thus they needed a specific time in order to let the electrode stabilize in the new solution, thus the electrophoresis should be stopped during this time and this is not practical in addition to an increase in the dispersion of the peaks in-channel due to diffusion. For this we think the second approach is more adapted and it should be done in a future work.



Figure 113: schema of the proposed microchip where an Hg^{2+} reservoir is added. Separation occurs between S, SW, BGE, and BW reservoirs. Then, potential is applied between S, SW, BGE, and Hg^{2+} so the RSNOs decompose to nitrite and we detected the different nitrites coming from different RSNOs.

C. Conclusion

In this chapter we showed for the first time a μPAD system that can be developed as point of care test to diagnose some diseases (in case of RSNOs concentration more than $4\text{ }\mu\text{M}$). It is non-expensive, disposable, and an easy to use device. Its limitation for real samples is the high LOD (more than ...), which is not compatible with the detection of some RSNOs in biological fluids that exist at nano-molar and lower micro molar concentrations. Other limitations are the multistep process required here to ameliorate the overall method LOD and the toxicity of Hg^{2+} .

A microchip electrophoresis coupled to electrochemical detection has been developed. The best conditions involved an isolated potentiostat with patterned printed electrodes that have fixed positioning. As expected, glass had higher EOF than other polymeric microsystem materials. Floating and gated injection were optimized using a fluorescent probe. The gated injection mode proved to be more efficient as it avoids sample diffusion during injection and sample leakage during separation. It was shown that the maximum voltages that are possible to apply with an amperometric detection depended on the channels dimensions. Decomposition of GSNO inside the channels using Hg^{2+} was done which permitted the detection of the nascent nitrite. Optimization of the separation before the decomposition step is to be continued.

GENERAL CONCLUSION

The main objective of the thesis was the elaboration of devices for bioanalysis, and more specifically that of S-nitrosothiols (RSNOs) in biological fluids. Therefore, it was important to consider all the physico-chemical particularities of RSNOs and assess the possibility of separation and simultaneous quantification of high molecular weight (HMW) and low molecular weight (LMW) RSNOs in biological fluids using electrokinetic separation, UV visible, MS and amperometric detections. For this purpose, the work was divided into four main steps: separation of RSNOs from their spontaneous decomposition products and / or impurities, decomposition of RSNOs to allow their detection through their two main metabolites NO and nitrite, detection and quantitation of the released NO and nitrite and finally miniaturization of an analytical device integrating separation, decomposition and detection, to reach the point of care goal.

The first step was dedicated to the optimization of the separation of nitrosoglutathione (GSNO) from its contaminants, decomposition products and other RSNO, nitrosocysteine (CySNO). Indeed, it was important to develop a method to separate and identify the impurities present upon synthesis and the decomposition products upon storage of GSNO. GSNO was a good model compound as it is the most common LMW-RSNO. We have established for the first time that CE coupled to MS is a useful method to characterize the purity of and effect of ageing on GSNO. We have also formally identified for the first time the presence of decomposition products other than GSSG, namely GSO_2H and GSO_3H . From these results we were able to construct a decomposition pathway describing the mechanisms leading to these products, some of which have been suggested elsewhere in the literature. The determination of impurities and decomposition products of GSNO is of importance for the safety of this potential drug. As noted above, we have found evidence for the presence of GSO_2H and GSO_3H during aging and this may have important biological consequences. Such compounds may potentially inhibit glutathione S-transferase and glutathione transferase-like enzyme families, which are responsible for the detoxification of many xenobiotic compounds. This method could also be applied to physiological fluids, to identify possible mitigation of toxic effects by GSNO at high concentration or for some diseases. Then we have demonstrated for the first time that CE

coupled to C^4D is a promising method for RSNOs separation and quantitation, in terms of purity and decomposition kinetics under different physico-chemical conditions. Indeed, CE- C^4D is a direct method that does not necessitate any derivatization, sample decomposition or purification. This method also allows to separate and quantify a complex mixture of GSNO and its impurities within few minutes (less than 2.5 minutes) benefiting from all the advantages of electrokinetic separations. CE- C^4D was applied to the analysis of the decomposition products of GSNO, when submitted to heat or light, with the evaluation of the decomposition rate constants. Finally, CE- C^4D was applied to the analysis of some chemical reactions involving GSNO, especially transnitrosation. This analytical approach also permits the separation of LMW-RSNOs

The second step was dedicated to the optimization of the decomposition of RSNOs. It was essential to determine more precisely the kinetics of decomposition as this step had to be then integrated in further application in the separation channel of the final microsystem before the detection step. The previous CE- C^4D served for the analysis of decomposition of GSNO in solution by light and heat as these procedures don't require the addition of external reagents that can affect the ionic strength and the separation process. Results showed that light and heat (at 80 °C) decompositions have different kinetic orders and both are not rapid enough for our purpose (15 min and 90 min, for light and heat, respectively). In order to study the decomposition by Hg^{2+} , a spectrophotometric method (so-called Saville's reaction) was used. This method is based on the decomposition of RSNOs by Hg^{2+} followed by the addition of the Griess reagent that usually allows the detection of nitrites through the formation of a pink colored azo-dye that absorbs spectrophotometrically at 540 nm. Calibration was done using the Griess reagent. Then the decomposition by Cu^{2+} / GSH (a reductor of Cu^{2+} to Cu^+) was compared to the one with Hg^{2+} . It should be reminded that decomposition by Hg^{2+} produces nitrite while all other decomposition pathways produce NO that transforms furtherly into nitrite. The results show that GSH (naturally present in biological medium) is a very important factor in GSNO decomposition. Thus, the results obtained here are of great importance for biologists as the existence of other thiols, which are normally present in biological fluids, could suppress the signal of NO leading to an underestimation of RSNOs. Our approach offers a cheap, sensitive and rapid method to detect GSNO electrochemically, mentioning the importance of the presence of thiols on signal suppression and suggesting a separation step to eliminate eventual excess of thiols.

The optimization of the electrochemical detection step was beneficial to continue the study of the decomposition step by Cu^{2+} / GSH. The kinetics of the decomposition was studied from the resulting amperograms obtained by electrochemical NO-sensor. The sensor was manufactured by coating a Pt ultramicroelectrode with different membranes: polyeugenol / polyphenols or polyeugenol alone. Calibration of GSNO using the second membrane showed around three times more sensitivity than using the first one (4.6 vs 1.4 pA / μM). Quantification of GSNO was also possible by electrochemistry with an LOD much better than by spectrophotometry (100 nM vs 2 μM). In addition electrochemistry permitted real time monitoring of NO. The time needed to have maximal NO liberation was around 15s still not totally ideal for microchip electrophoretic separation where all the process should be finished within 1 min.

Another new method using AuNP-catalyzed decomposition method was developed associating electrochemical detection in order to quantify RSNOs. Results were interesting in terms of having better sensitivity than the Cu^{2+} / GSH method (6.64 vs 4.6 pA / μM). In addition to this, the time to attain the maximum is 5s instead of 15s. This is a great amelioration due to the difference in NO liberation mechanism between the two pathways. Plasma RSNOs were also detected after having blocked the thiols initially present in the fluid using a blocking agent. These results highlighted the necessity of a separation step in order to quantify different RSNOs. The main restriction of this approach for integration in a microsystem is the AuNPs volume (2 mL) necessary, which needs to be optimized according to microfluidic volumes.

The last step of this work was dedicated to the miniaturization. This was made through two axis: the miniaturization of the spectrophotometric “Saville” method using microfluidic paper-based analytical devices (μPADs) for point of care (POC) use and the development of a glass / polymer microfluidic system for electrokinetic separation and electrochemical RSNOs detection. In the first axis, the first colorimetric assay on μPADs to detect RSNOs was developed. Decomposition of LMW- and HMW-RSNOs was performed on paper substrate using different light sources (UV, Vis, IR) as well as Hg^{2+} as a reference procedure. Hg^{2+} decomposition was total for GSNO and CysNO. For each RSNO, UV decomposition was more efficient than visible light decomposition, the latter remaining more efficient than IR decomposition. RSNOs showed total decomposition into nitrite using Hg^{2+} ion. The LOD achieved for nitrite was 3 μM , which represents one of the lowest values already reported in literature using colorimetric measurements and μPADs . The enhanced analytical performance has also provided relatively good detectability levels for GSNO. When decomposed by Hg^{2+} , UV and Vis LEDs, the obtained LODs were 4, 6 and 11 μM , respectively. Detection of nitrite

and RSNOs in plasma samples could be performed after a sample treatment involving deprotonization. HMW-RSNOs were differentiated from LMW-RSNOs by subtracting the color intensities recorded after and before deproteinization by Hg^{2+} . HMW-RSNOs and nitrite were detected while LMW-RSNOs were below the LOD. Due to the portability and compatibility to be powered by a USB port, the proposed platform emerges as simple POC device. When compared to the standard decomposition procedure (Hg^{2+}), the use of UV LED has provided similar performance. Since it may be also powered by battery or even USP port, the coupling of this simple light source with μPADs represents a good alternative to be used in a truly portable device. The separations on microchips can be quite attractive in order to discriminate the concentrations of different RSNOs.

On the second axis as monitored from the preceding μPAD , MCE in polymer or glass devices is needed for the separation of different RSNOs since the use of CE on μPADs is still not well developed. Several improvements were done when passing from a home-made PMMA microchip to a commercial glass / Su-8 system. Nitrite and GSNO were detected by MCE coupled to end-channel amperometric detection. The best conditions were employing an isolated potentiostat with printed electrodes that have a given positioning. There were limitations in controlling the position of GSNO decomposition by Hg^{2+} and in LOD values that do not permit real samples RSNOs detection. A new configuration was suggested that could optimize the timing between separation and decomposition, so as to profit from the maximum of the channel length for separation.

This work allowed describing a methodology aiming at the establishment of an analytical procedure for the analysis of the RSNOs. It has the merit to have considered quite efficiently the steps necessary for the considered approach. This work must be naturally pursued and in the sight of my experience in this domain, a particular attention must be given to the following points in order to make the analysis of RSNOs in biological fluids within an integrated micro-analytical device for a simple and ready to use in reality:

- Considering the timing between the separation and decomposition steps: a side positioned channel containing the catalyst (Hg^{2+} or Cu^{2+}) needed for the decomposition of RSNOs could be interesting to consider in making the separation and the decomposition steps successive thus possibility of detection of different RSNOs after their separation and in-channel decomposition by the Hg^{2+} or Cu^{2+} .

- On the decomposition level: i) optimization of the decomposition step within micro volumes that correspond to the microchip channels dimensions should be done in order to better understand phenomena and optimize in-chip decomposition, ii) concentration of AuNPs should be adapted to small volumes seems interesting, and their positioning in the microchannel should be controlled, and iii) other decomposition procedures, such as the decomposition by an integrated fixed organometallic membranes that will act as catalyst inside the channels, should be tested as well.
- Considering the integration of a sample pretreatment step for a micro-total analysis system: on-chip microfiltration could be promising to replace deproteinization in μ PAD system. Its coupling to on-chip electrokinetic separation of different RSNOs before decomposition could provide a powerful integrated lab-on a chip for their simultaneous quantitation on μ PADs.
- Considering the sensitivity of the detection: i) using a pre-concentration step within the microchannel such as isotachopheresis or stacking effects could be helpful to detect lower concentrations of RSNOs, ii) technical ameliorations in the sensitivity of potentiostats would revolutionize this domain of coupling MCE to amperometry, iii) coupling electrochemical detection with fluorescence detection could give complementary results that could help in sample analysis, and iv) utilizing and coupling with other electrochemical detection methods such as C^4D looks very interesting.

ANNEX

I. Annex 1

Table 19: Values of k for the reaction of GSNO with ascorbic acid over a wide pH range. Adapted from [220]

pH	K ($10^{-3} \text{ dm}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$)
3.6	0.15
4.6	0.37
5.6	0.58
6.5	1.98
8.5	183
9.7	1020
10.1	2810
10.8	9830
11.5	56000
12.5	177000
13.7	323000

II. Annex 2

MS is a qualitative and quantitative analytical technique that identifies chemical species based on their mass to charge ratio (m/z). By analyzing the obtained mass spectrum (relative abundance of each m/z value obtained), one can deduce several informations concerning molecules such as their isotopic signature, their molar mass and their structural formula. Chemical species should be ionized at first. This can lead to the fragmentation of the molecule to several smaller ones. These fragments vary according to the method of ionization and according to condition used using the same method. After that these fragments are separated

from each other by accelerating them using magnetic or electric field, the ions with same m/z ratio undergoing the same deflection. Finally, ions are detected by a mechanism that can detect charged particles such as electron multiplier and identified by comparing their molar mass obtained with the known one or from the fragmentation pattern [463].

The mass spectrometer is composed of the ion source, the mass analyzer, and the detector (**Figure 114**). The role of ion source is to produce gaseous ionized chemical species starting from a solid, liquid or gaseous sample. These ions will be analyzed by the mass analyzer. The most known ion sources are electro spray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI). There exist several types of mass analyzer that differ in term of resolution. The most known mass analyzers are time of flight (TOF), orbitrap, quadropole and Fourier transform ion cyclotron resonance.

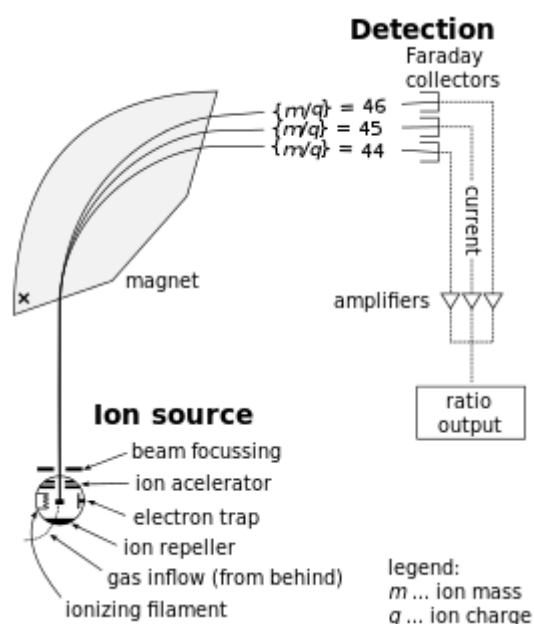


Figure 114: Schematics of a simple mass spectrometer with sector type mass analyzer. Adapted from [463]

MS is a technique that can be coupled to other separative techniques such as liquid chromatography, gas chromatography and CE. CE is a powerful technique to separate GSNO from its contamination products and from CySNO. For this we have interest to couple it to MS in order to identify formally the products present. CE delivers very low flux (in $\text{nL} \cdot \text{min}^{-1}$ range) and there is necessity to close the electric circuit at its end. These two factors constitute the principle challenges to couple CE with MS. Thanks to ESI, they have been resolved by coupling through an interface. This interface should keep the electrical contact for the two parts causing no or little band broadening and provide good ionization conditions ideally without the

interference of BGE. In order to achieve this, a sheath liquid is added at the interface which helps in obtaining a good ionization at low flow rates, limits the influence of the BGE composition on the ionization and permits to obtain stable and favorable ionization conditions by optimizing its composition. The only limitation of the use of sheath liquid is the loss in sensitivity due to dilution at the end of the capillary [464]. A nebulizing gas is also used in order to have droplets of liquid. Fine droplets are then obtained by the influence of a strong electric field. These droplets gain charge due to the electric field which triggers electrochemical reactions, then the liquid evaporates more and an excess of charge is obtained in each droplet which leads to electrostatic repulsion that overcomes the surface tension of the droplets after certain time. A cascade of coulombs fission continue to occur until we obtain a gaseous ion molecules [464].

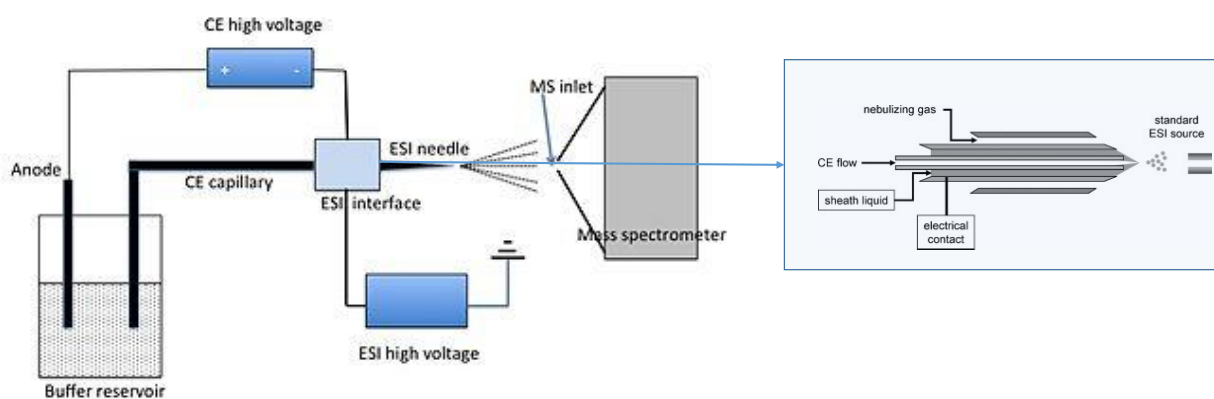


Figure 115: sheath flow CE-MS analyzer. Adapted from [464,465]

III. Annex 3

Capacitively coupled contactless conductivity (C^4D) is a type of conductivity-based detection where the electrodes are not directly in contact with the sample solution. Its first use in combination with electrokinetic methods was with the use of the isotachophoretic mode. Later Zeman et al. [466] and Fracassi da Silva et al. [409] Integrated it with CE. This method of detection is universal since it detects each molecule that has a different conductivity than the support electrolyte. If sample the conductivity is higher, the response will be a normal peak and if the sample conductivity is lower, an inverted peak will appear. The principle of C^4D is

summarized in **Figure 116**. At first a transmitter electrode sends high frequency and amplitude signal, this signal crosses the capillary wall two times and the solution inside the capillary in order to reach the receiver electrode (**Figure 116a**). The attenuation of the signal varies depending on the wall of the capillary and on the solution passing through (**Figure 116b**). The electrical circuit is presented in **Figure 117** where the capillary walls stand as capacitor and the solution between them as a resistor. Depending on the conductivity of the solution the resistance varies. The other capacitor presented in the figure stands for the stray capacitor and depends on the geometry and positioning of the electrodes as well as the dielectrics in which they are immersed. At end there is conversion of the amplitude of the AC signal to a DC analog voltage signal (**Figure 116c**) [467]. The sensitivity has a maximum that depends on the operating frequency. Generally the higher the difference between the mobility of the analyte and the co-ion of the electrolyte, the higher the sensitivity and the higher the counter ion mobility, the higher the sensitivity. For this, a common practice is to use a low mobility co-ion to analyse analytes of high mobility and vice versa [409].

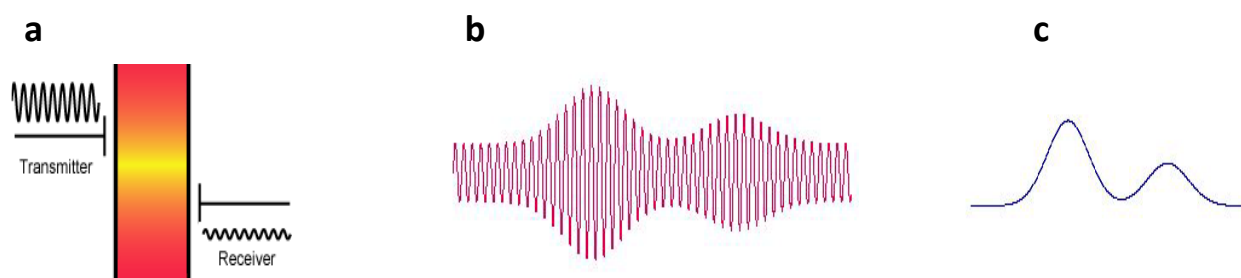


Figure 116: C⁴D detection principle.a) the transmitter electrode send a high frequency and large amplitude AC The receiver electrode receive the attenuated signal, b) the AC received signal is affected by the conductivity of the sample, c) DC analog voltage signal

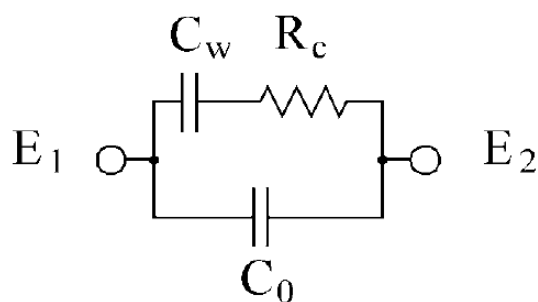


Figure 117: equivalent circuit of C4D with stray capacitance. C_w stands for the total capacitance due to the silica wall. R_c stands for the solution resistance between the electrodes. C₀ stands for the total stray capacitance between both electrodes. Adapted from [409]

IV. Annex 4

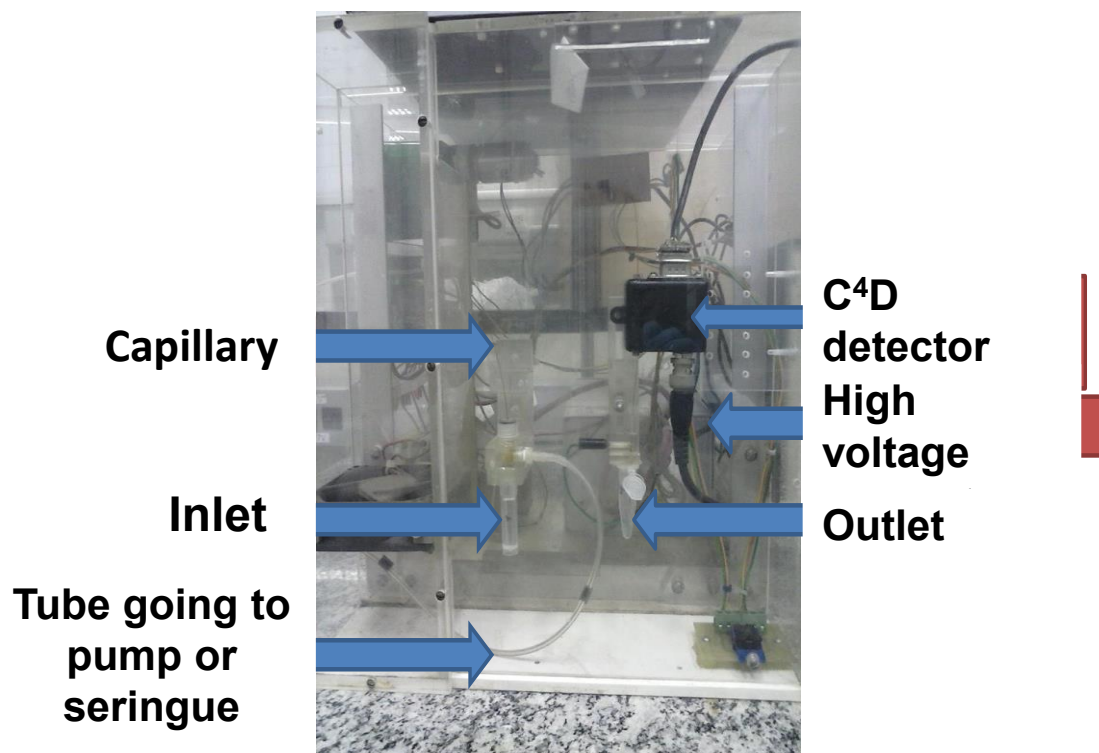


Figure 118: photo of the home-made CE-C⁴D showing a capillary connecting two vials (inlet and outlet).

The inlet, where the electrokinetic process starts, is connected to a pump that makes hydrodynamic injection. In addition to this inlet and outlet are connected to a high voltage power supply by platinized electrodes. The C⁴D detector encapsulate the capillary just before the outlet vial. The entire CE-C⁴D is put in a home-made PMMA container.

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