A kinetic approach for the estimation of intracellular concentrations of nitrosative species in cells challenged by nitric oxide

by

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Submitted to the Department of Chemical Engineering in Partial Fulfillment of the Requirements for the Degree of
DOCTOR OF PHILOSOPHY IN CHEMICAL ENGINEERING

at the
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

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ABSTRACT

Our understanding of how nitric oxide (NO) contributes to the initiation and progression of diseases such as cancer may benefit from determining intracellular concentrations of its reactive derivatives, for which experimental estimates are largely unavailable. During exposure of inflamed tissues to NO under physiologically relevant levels of oxygen, the potent nitrosative agent nitrous anhydride (N$_2$O$_3$) is formed, responsible for deamination of nucleobases in various forms of nucleic acids – yielding xanthine (X) and oxanine (O) from guanine, hypoxanthine (I) from adenine and uracil (U) from cytosine – and thus playing a multifaceted role in the pathology of chronic inflammation with implications for altered genetic content. Due to its reactivity, direct detection of the short-lived nitrosative intermediate is impossible in vitro or in vivo but, in this study, RNA nucleoside deamination products were chosen as surrogate markers for N$_2$O$_3$-induced damage based on the wider cellular distribution and solvent accessibility of RNA compared to genomic DNA. The objective of this thesis was to estimate the steady-state cytosolic N$_2$O$_3$ concentration by measuring formation rates for deamination lesions in RNA of mammalian cells stressed by NO. To establish a frame of reference in kinetic terms, purified total RNA from human lymphoblastoid TK6 cells was exposed to NO and O$_2$ at constant steady-state levels of 1.7 µM and 210 µM, respectively, in a novel NO-delivery device. Deaminated purine nucleosides were then quantified using sensitive HPLC-coupled mass spectrometry methods and analysis of the kinetics of nitrosative deamination of the RNA revealed that adenine was significantly less reactive than guanine leading to concurrent formation of inosine, oxanine and xanthosine with rate constants of 3.3 × 10$^4$, 4.8 × 10$^5$ and 1.0 × 10$^6$ M$^{-1}$s$^{-1}$, respectively. After assessing the reliability of the delivery device under conditions approximating exposure of cell cultures to NO, similar kinetic studies were performed by exposing mammalian TK6 cells to comparable levels of nitrosative stress. Smaller but measurable increases in deamination products were detected in total RNA extracted from intact cells which, when applied to expressions of the rate law in combination with the aforementioned rate constants, provided estimates for the intracellular N$_2$O$_3$ concentrations ranging between 0.2 and 70 fM. Parallel sets of experiments showed that a roughly 100-fold drug-induced depletion of glutathione (GSH) levels in cells prior to being challenged in the delivery device did not significantly alter the accumulation rate of rI, rX and rO, standing in contrast with previous kinetic analyses predicting GSH to be a dominant cellular scavenger of N$_2$O$_3$. Further calculations point to a more complicated protection against NO-induced deamination occurring in the cellular milieu with multiple scavengers likely to play an important role in mitigating the damaging effects of nitrosating species in the cytosol.
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I am also very grateful to my thesis committee members, Professors Kristala L. Jones Prather and Steve R. Tannenbaum, for sharing their time and knowledge with me, for all the stimulating discussions we had during our meetings and for reminding me to always use critical thinking to the best of my ability to deal with the different experimental hurdles that appeared in the course of my studies.

I would like to thank Professor Gerald N. Wogan for being a great mentor, always welcoming me to his laboratory as well as for our collaboration on characterizing the operation of the NO delivery device. I would also like to acknowledge Professor Wan Simon Chan for the studies we performed together investigating the formation of abasic sites in NO-damaged RNA.

I am indebted to present and past members of the Dedon, Deen and Wogan Groups for offering me their help, advice and scientific expertise on a great variety of issues and for cultivating a comforting sense of camaraderie. In particular, I would like to acknowledge the help of Dr. Michael Demott, Dr. Erin Prestwich and Brandon Russell for taking the time to proofread parts of this thesis. I cannot forget Olga Parkin, Kristine Recchia Marzilli, Jackie
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I would like to dedicate this thesis to the victims of the events surrounding the 2013 Boston Marathon.
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Chapter 1
Introduction and Background

1.1 Introduction

The goal of the research presented in this thesis is to estimate the concentration of the potent and short-lived nitrosating agent nitrous anhydride (N₂O₃) in the cytosol of mammalian cells subjected to doses of nitric oxide (NO) similar to those expected in inflamed tissues. A key player in inflammation, NO is one among several reactive species secreted by inflammatory cells recruited at sites of injury or infection and aimed at combating the foreign threat. Diffusion of those chemical mediators of inflammation with subsequent reactions among them or other targets can affect the surrounding healthy tissue while damaging cellular proteins, lipids, carbohydrates and nucleic acids. The accumulated lesions can cause cell death or aberrant growth and accelerate malignant transformation, especially if these pathological conditions are maintained for extended periods. To determine levels of generated N₂O₃ – previously estimated only via mathematical modeling – nucleobase deamination products in RNA were chosen as surrogate markers for the damage inflicted in the intracellular milieu. This study aims to infer the N₂O₃ abundance in the cytosol by using the appropriate expression of the rate law while applying information derived from a) kinetic analyses of NO-induced deamination in solutions of purified RNA and b) in vivo experiments determining the formation rate of deamination lesions in RNA extracted from intact cells under constant nitrosative stress, using the same well-defined exposure vessel in both cases.
In this chapter, the general concepts describing the inflammatory process are first introduced, while associations with different diseases, including cancer, are reviewed next. A brief overview of specific aspects of neutrophil and macrophage biology that lead to generation of reactive nitrogen (RNS) and oxygen species (ROS) is addressed, followed by a comprehensive discussion of the chemistry related to key mediators of inflammation, focusing in more detail on how NO-mediated nitrosative stress damages cellular targets. The concepts of kinetic models are then reviewed and their role in the challenge of understanding the chemical microenvironment at sites of inflammation is illustrated. Finally, a more detailed description of the scope of this research is presented.

1.2 Inflammation and disease

1.2.1 General characteristics of inflammation

Starting with the biological process responsible for the nitrosative environment examined in this work, inflammation is the response of vascular tissue to insult or injury, a way for the body to manage challenges related to pathogenic infections, chemical irritants, physical trauma, or other damage to host cells. More detailed studies on macroscopic manifestations associated with inflammation, like redness, swelling and heat [1], have revealed intricate biochemical processes that involve increased local flux of red and white blood cells, changes in permeability of blood vessels and lymphatic flow, increased cellular metabolism and diffusion of soluble agents [2-4], among others. This critical response orchestrated mainly by elements of the innate immune system [5,6] is viewed as an immediate self-defense mechanism of the organism. The inflammatory process is a rapid local reaction and remains activated for hours or days until the
threat has been removed, with resolution enabling damage repair and local healing with varying mechanisms in different tissues [7-9].

The inflammatory response triggers cellular processes in components of both innate and acquired immunity, with those in the former category predominating during the initial stages of the insult and also being more relevant to the focus of this thesis. Once neutrophils and different classes of macrophages – discussed in more detail below – infiltrate the microenvironment where the stimulus is located, foreign bodies, cell debris and infectious agents are targeted by conserved molecular patterns on their surface [10] and subsequently internalized by the leukocyte cell membranes into phagosomes [11]. Phagocytosis ensues with degradation of the ingested material as the final outcome. The inflammatory process is maintained as long as the injurious elements persist, but is followed by tissue regeneration that involves cell proliferation upon resolution of the insult [9].

Acute inflammation is a critical facet of the body’s natural defense and essential to the organism’s survival against opportunistic infections. In certain cases, however, inflammation can contribute to pathology when it escalates from acute to chronic (lasting for months or years) if the source of stimulation persists [12]. Prolonged inflammation is believed to be an aberrant condition that injures host tissues while components of the innate immune system remain activated [13], occasionally with equally harmful effects compared to the stimulus itself [14]. Besides persistent microbes, chronic inflammation can also occur due to continuous or recurring damage caused by autoimmune disorders [15] or undegradable foreign bodies. The list of diseases that are linked to long-term inflammation is constantly expanding, with only a fraction shown in Table 1.1. With this overview of inflammation, and before examining in more detail
**Table 1.1.** Examples of inflammatory disorders (adapted from [4]).

<table>
<thead>
<tr>
<th>Disorders in which an important pathogenic role is assigned to inflammation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Alzheimer’s disease</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>Asthma</td>
<td>Psoriasis</td>
</tr>
<tr>
<td>Atherosclerosis</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>Atopic dermatitis</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td>Type I diabetes mellitus</td>
</tr>
<tr>
<td>Gout</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>Xenograft rejection</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diseases of infectious origin in which inflammation may contribute as much to pathology as does microbial toxicity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial dysentery</td>
<td>Influenza virus pneumonia</td>
</tr>
<tr>
<td>Cystic fibrosis pneumonitis</td>
<td>Pneumococcal meningitis</td>
</tr>
<tr>
<td><em>Helicobacter pylori</em> gastritis</td>
<td>Sepsis syndrome</td>
</tr>
<tr>
<td>Hepatitis C</td>
<td>Tuberculosis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diseases of diverse origin in which post inflammatory fibrosis is a principal cause of pathology</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleomycin-induced pulmonary fibrosis</td>
<td>Hepatic cirrhosis (post-viral/ alcoholic)</td>
</tr>
<tr>
<td>Idiopathic pulmonary fibrosis</td>
<td>Schistosomiasis</td>
</tr>
</tbody>
</table>
the specific cellular and chemical components of the inflammatory response, we will first shed some light on how these features connect to human disease and specifically cancer.

1.2.2 Diseases associated with inflammation – link with cancer

When the inflammatory response transitions from physiological to pathological, a number of outcomes that interfere with precisely regulated cell growth and function – including damage to nucleic acids, one of the key themes of this work – seem to prompt the emergence of disease. Cancer, for example, appears to be strongly linked to inflammation despite its apparent heterogeneity. Epidemiological evidence suggests that inflammation contributes significantly to the development of cancer: 15-25% of all cancers are due to chronic infection or other types of chronic inflammation [16,17] with both endogenous (e.g. genetic diseases) and exogenous (e.g. infections, irritants) inducers of inflammation involved, as seen in Table 1.2 [18]. For instance, inflammatory bowel diseases, like Crohn's and ulcerative colitis, are associated with increased rates of colon adenocarcinoma [19]. Alcohol-induced chronic pancreatitis as well as hereditary pancreatitis contribute to a 50-fold increased risk of pancreatic cancer [20]. When it comes to microbial, viral or parasitic infections, hepatitis B and C lead to inflammation of the liver and are responsible for the majority of hepatocellular carcinomas worldwide [21], while colonization of the stomach by Helicobacter pylori causes chronic gastritis and is linked to most gastric cancers [22]. Clinical studies show the longer the inflammation persists, and the more severe it is, the higher the risk for carcinogenesis [23]. Another indicator supporting a strong correlation between inflammation and cancer is that certain anti-inflammatory drugs reduce the risk of developing various cancers: long-term use of non-steroidal anti-inflammatory drugs (NSAIDS) has been associated with reduced risk of several types of malignancies [24].
Table 1.2. Chronic inflammation or infection increases cancer risk (adapted from [18]).

<table>
<thead>
<tr>
<th>Disease</th>
<th>Type of cancer</th>
<th>Increased risk</th>
</tr>
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<tbody>
<tr>
<td>Auto-inflammatory/non-infectious</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crohn's disease</td>
<td>Colon cancer</td>
<td>3</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>Colon cancer</td>
<td>6</td>
</tr>
<tr>
<td>Chronic pancreatitis</td>
<td>Pancreatic cancer</td>
<td>2–50</td>
</tr>
<tr>
<td>Endometriosis</td>
<td>Endometrial cancer</td>
<td>1.4</td>
</tr>
<tr>
<td>Hemochromatosis</td>
<td>Liver cancer</td>
<td>219</td>
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<td>Thyroiditis</td>
<td>Thyroid cancer</td>
<td>3</td>
</tr>
<tr>
<td>α-1-Anti-trypsin deficiency</td>
<td>Liver cancer</td>
<td>20</td>
</tr>
<tr>
<td>Acquired</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viral</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>Liver cancer</td>
<td>88</td>
</tr>
<tr>
<td>Hepatitis C</td>
<td>Liver cancer</td>
<td>30</td>
</tr>
<tr>
<td>Epstein–Barr virus</td>
<td>Hodkin's and Burkitt's lymphoma</td>
<td>4</td>
</tr>
<tr>
<td>Bacterial</td>
<td></td>
<td></td>
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<tr>
<td><em>Helicobacter pylori</em></td>
<td>Gastric cancer</td>
<td>11</td>
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<td>Pelvic inflammatory disease</td>
<td>Ovarian cancer</td>
<td>3</td>
</tr>
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<td>Chronic prostatitis</td>
<td>Prostate cancer</td>
<td>2–3</td>
</tr>
<tr>
<td>Parasitic</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Schistosoma hematobium</em></td>
<td>Bladder cancer</td>
<td>2–14</td>
</tr>
<tr>
<td><em>Schistosoma japonicum</em></td>
<td>Colon cancer</td>
<td>2–6</td>
</tr>
<tr>
<td>Liver fluke</td>
<td>Cholangiocarcinoma and liver cancer</td>
<td>14</td>
</tr>
<tr>
<td>Chemical/physical/metabolic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol</td>
<td>Liver, pancreas, head, neck cancers</td>
<td>2–7</td>
</tr>
<tr>
<td>Asbestos</td>
<td>Mesothelioma</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Obesity</td>
<td>Multiple cancers</td>
<td>1.3–6.5</td>
</tr>
<tr>
<td>Tobacco smoke</td>
<td>Lung cancer</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Gastric reflux, Barrett's esophagus</td>
<td>Esophageal cancer</td>
<td>50–100</td>
</tr>
</tbody>
</table>
Common to the spectrum of diseases mentioned above, a wide range of locally generated mediators of inflammation including cytokines, chemokines, free radicals, growth factors, transcription, and signaling molecules may lead to cellular conditions favorable to tumor progression. While many of the underlying mechanism(s) and cellular targets are still unknown, there is a growing body of evidence that reactive oxygen and nitrogen intermediates are responsible for inducing altered cellular growth and transformation [25,26]. Elevated levels of these intermediates sustained for extended periods can lead to direct or indirect DNA damage, as shown in mouse models [27-29], to post-translational modification of various proteins [29], and to increased metastatic growth potential [30]. In the following sections, more will be revealed on the spectrum of chemical mediators of inflammation that are generated by specialized components of the immune system and how some of those species are converted into second-generation intermediates capable of inducing cellular damage on multiple levels.

1.3 Cellular components of the inflammatory process

Some types of cancer, among other inflammation-associated diseases, are thought to be caused in part by the harmful conditions localized to certain tissues or organs whether the initial stimuli are based on infection, genetics or otherwise. The observed patterns are mostly a consequence of the selectivity that the immune response can exhibit. To not only sequester and isolate the foreign threat but also to immerse it in an offensive chemical microenvironment involving RNS and ROS, the inflammatory response engages both branches of the immune system: a rapid initial response by more non-specific components and a subsequent secondary phase orchestrated by the adapted components. More relevant to the current work, a key feature of the early induced innate immunity is the recruitment of phagocytic cells through pathogen-associated molecular patterns (PAMPS; conserved motifs on pathogens that are not found on
higher eukaryotes) binding to pattern-recognition receptors (PRR) [11,31]. The most prominent of these phagocytic defense cells are neutrophils, eosinophils, monocytes and circulating or resident macrophages [10], described briefly below.

1.3.1 Neutrophils

Neutrophils (also known as polymorphonuclear leukocytes, PMN) constitute more than half of the total circulating leukocytes in the body and are considered the most immediate defense against infectious threats or “non-self” agents entering the host organism [32]. They are formed in the adult bone marrow from differentiated pluripotent hematopoietic precursor cells and subsequently released into circulation under tight chemokine-related control. The human body normally maintains a pool of cells available to be released while the number of neutrophils drastically increases during infection and other diseases [33].

The role of neutrophils is multifaceted and involves a) release of anti-microbial peptides and proteins from intracellular granules, b) generation of reactive oxygen species (ROS) in a process called the respiratory burst and c) receptor-mediated processes during which particles are internalized by the cell membrane into phagosomes. These strategies are combined to combat microbes either inside the cytoplasm of neutrophils via internalization or in the extracellular space [34]. During the respiratory burst, however, an abundance of reactive intermediates derived mainly from the reduction of molecular oxygen (such as superoxide, hydrogen peroxide, hydroxyl radicals [35] and hypohalous acids [36]; outlined in Figure 1.1 and reviewed in more detail shortly) are thought to diffuse into the surrounding normal tissue and promote cytotoxicity, apoptosis and necrosis in host cells while assisting in eliminating persistent pathogens. These reactive species have been shown to damage a multitude of biomolecules, which again poses
Figure 1.1. Schematic of the respiratory burst in neutrophils.

Microbicidal products arise from specialized organelles in the cytoplasm of the neutrophil (BPI, bactericidal permeability increasing protein; MMP, matrix metalloproteinase; Phox, phagocyte oxidase; adapted from [37]).
the question of whether exposure to such conditions over prolonged periods could be responsible for the pathology observed in diseases associated with inflammation.

Neutrophils circulate for only approximately 6-8 h and are among the shortest-lived cells in the human body [33]. During their lifetime they undergo intense transcriptional activity that results in the synthesis of various signaling molecules to attract other components of the non-specific immune system [38]. The immediate recruitment of neutrophils at inflammatory sites is followed by local proliferation of monocytes/macrophages, whose function is described below, as the insult persists [37].

1.3.2 Monocytes and macrophages

Monocytes – the precursors of macrophages – are derived from hematopoietic cells in the bone marrow, reside undifferentiated in reservoirs predominantly located at the spleen [39] and can be mobilized by signals originating from tissues responding to injury or infection. Released chemokines and cytokines from sites of inflammation activate the differentiation of monocytes to form macrophages [40] and modulate their transport through the endothelium (extravasation) to locate the source of the threat. Alternatively, circulating monocytes eventually enter lymphoid and nonlymphoid tissue where they reside for longer periods and are believed to be involved in homeostasis [41]. Macrophages are equipped with a broad range of pathogen-recognition receptors that both assist in phagocytosis and induce synthesis of inflammatory cytokines [42], similar to those in neutrophils.

Macrophages are divided into subpopulations with specialized tissue-resident cells found in the bone, lung, gut, liver and other organs (depicted in Figure 1.2). Macrophages from lymph nodes, for example, clear viruses and initiate antiviral humoral immune responses [43] while a
Figure 1.2. Distribution of tissue-specific macrophages (adapted from [44]).
subset of macrophages patrolling the brain and eyes are believed to ingest foreign particles and recruit additional circulating macrophages during infection or injury [44].

The apparent heterogeneity in localization and function gives the non-specific immune system a degree of flexibility when responding to a variety of injurious stimuli and allows macrophages to adopt different roles. Studies have shown that macrophages switch from one functional phenotype to another in response to the transient signals of the local microenvironment [45]. Once the source of insult is located, macrophage activation follows the initial monocyte recruitment to affected sites and subsequent macrophage proliferation is initiated in the presence of interleukin IL-4 (mediated by T helper 2 lymphocytes) [46]. Interleukin IL-12 induced production of interferon-γ (IFNγ) by T helper 1 cells also stimulates macrophages by signaling the appropriate cellular processes to initiate phagocytosis and secretion of a battery of reactive species [47]. Following the molecular signals linked to a physical insult or infection, activated macrophages produce inflammatory mediators such as interleukin IL-1, tumor necrosis factor (TNF), free gaseous radicals and other oxidants [48,49]. In particular, the synthesized reactive oxygen and nitrogen intermediates – such as nitric oxide (NO) and superoxide described in detail below – are believed to be highly toxic for microorganisms but also disruptive to host cells adjacent to affected sites, potentially leading to collateral tissue damage if the insult persists over prolonged periods [50]. Along with the epithelial cells of the affected tissue, the cellular components of inflammation are responsible for generation of reactive oxygen, halogen and nitrogen species that cause mutation and cell death as part of the pathology of inflammation-associated disease.
1.4 Chemistry of inflammation

Parsing out the role of small reactive molecules from the complex array of mediators of immunity secreted by differentiated leukocytes and examining their effects on human cells will increase our understanding of the inflammatory response. The current work aims at contributing toward that process. As already discussed, inflammation induces various types of immune cells which subsequently up-regulate oxidant-generating enzymes to produce a battery of reactive agents: reactive oxygen, halogen and nitrogen species are among the most prominent molecules formed endogenously to combat invading pathogens and complement phagocytosis. Nitric oxide, specifically, plays a key role in the formation of secondary reactive nitrogen intermediates, which when combined with other species, may have a synergistic bactericidal ability by attacking an array of targets in vivo.

1.4.1 Reactive oxygen species

During the respiratory (or oxidative) burst of neutrophils and macrophages, an increased consumption of oxygen is observed mostly in the vicinity of the phagocytic cell membranes, where electrons from NADPH are transferred to molecular oxygen with the help of NADPH-oxidase, rapidly producing superoxide anion (O$_2^-$). The formed O$_2^-$ is then metabolized to the less toxic hydrogen peroxide (H$_2$O$_2$) by superoxide dismutase (SOD) [35]. H$_2$O$_2$ is further broken down to H$_2$O and O$_2$ by catalase enzymes or, alternatively, reacts with divalent metals (such as Fe$^{2+}$) to form highly reactive hydroxyl radicals (HO’) and HO$^-$ as predicted by Fenton chemistry [51]. In terms of reactivity, O$_2^-$ is not a strong oxidant and reacts slowly with biological targets compared to its rate of decay in the presence of SOD (the concomitantly formed H$_2$O$_2$ is also a relatively weak oxidant [52]), whereas HO’ is a non-selective extremely powerful oxidant [53]
that attacks most molecules at diffusion-controlled rates [54]. Singlet oxygen ($^{1}\text{O}_2$), another excited form of molecular oxygen, is thought to be generated in stimulated phagocytes during the respiratory burst and reacts with a wide range of biological targets including DNA, RNA, proteins, and lipids [55].

1.4.2 Reactive halogen species

High levels of myeloperoxidase (MPO) in the primary granules of neutrophils are responsible for enzymatically converting H$_2$O$_2$ – released during the respiratory burst – to hypohalous acid in the presence of halide anions. With Cl$^-$ being the most abundant anion, hypochlorous acid is thought to be the main product formed, however, other mammalian peroxidases can oxidize bromide (e.g. in eosinophils) and iodide in a similar manner [56,57]. A fraction of the produced hypochlorous acid (HOCl) reacts again with Cl$^-$ to form Cl$_2$ gas [58]. The reactive oxidant HOCl has antiseptic properties shown to deplete energy transduction in bacteria [59], induce apoptosis in human endothelial cells [60], and damage host proteins at sites of inflammation \textit{in vivo} [61]. In addition, activated human neutrophils have been shown to convert nitrite (NO$_2^-$) into the inflammatory oxidant NO$_2$Cl through an MPO-dependent pathway, which suggests that NO$_2^-$ (the end-product of NO oxidation in aqueous solutions, discussed shortly) may also play a role in phagocyte-mediated oxidative reactions at sites of inflammation [62]. \textit{In vivo} oxidation or halogenation, resulting to the aforementioned species may affect a spectrum of cellular components including nucleic acids, carbohydrates and polyunsaturated fatty acids [63].
1.4.3 Reactive nitrogen species

Endogenous nitrate and nitrite production, extensively investigated in the early 1980’s by Tannenbaum and coworkers [64], were associated to an intermediate derived from activated macrophages [65] which was shown to be NO [66,67]. At the same time NO was also independently linked to vascular smooth muscle relaxation [68] and found to be generated by a family of enzymes called nitric oxide synthases (NOS) using L-arginine and O₂ as substrates, with NADPH as an electron donor and heme, FMN, FAD and tetrahydrobiopterin (H₄B) as cofactors [69], generating L-citrulline as a final product. At least three isoforms of NOS have been identified: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS), the first two being soluble and the latter membrane-bound [70]. The endothelial and neuronal isoforms are generally considered to be constitutively expressed in cells (although there is recent evidence of another level of dynamic regulation in eNOS [71]), whereas the inducible type is activated by cytokines or bacterial infection in a number of cells types, including endothelium, hepatocytes and phagocytes [72], of particular interest with respect to inflammation [73]. The presence of NOS in a spectrum of tissues supports the argument that NO is an important messenger molecule that regulates vasodilation in multiple organs (e.g. kidneys, intestines) and a physiological neurotransmitter [74], while dysregulation of NO signaling may be associated with several disease states [75,76].

Besides its critical involvement in numerous signaling pathways, NO is also very important due to its reactions with other free radicals, halides and transition metals – among others – and it is the generation of corresponding reactive nitrogen species that is most relevant to the work presented here. Reactions of NO expected to occur in vivo include the formation of nitroso adducts with the metal centers of proteins such as Fe²⁺ in hemoglobin [74], with various
organic radicals [77], and oxygen to form the oxidant nitrogen dioxide (NO₂) which in turn reacts with NO to producing the nitrosative agent nitrous anhydride (N₂O₃) [78] to which we will refer repeatedly in the following chapters. NO₂ is thought to react with many classes of molecules such as lipids, significant antioxidants like ascorbic acid and α-tocopherol [79], and glutathione and urate [80]. On the other hand, evidence suggests that N₂O₃ is the predominant nitrosating species in an aqueous environment [81] where it can readily nitrosate amines [82] and thiols [83], while also being responsible for nucleobase deamination [70,84]; in the absence of any other species N₂O₃ is hydrolyzed to nitrite [85]. When NO is produced simultaneously with O₂⁻ (e.g. at the primary granules of neutrophils [62,86]), the two radicals can recombine at a near diffusion-controlled rate to produce peroxinitrite (ONOO⁻) [87]. In equilibrium with its protonated form peroxynitrous acid (ONOOH; pKₐ = 6.8), ONOO⁻ reacts with CO₂ to form nitrosoperoxycarbonate ONOOCO₂⁻ [88] which then decomposes into the potent oxidant carbonate radical (CO₃²⁻) and NO₂ [89]. Conversely, ONOOH can undergo homolysis to HO⁻ and NO₂ or react directly with organic moieties [90]. By attacking biomolecules in vivo, any of the aforementioned strong oxidants is capable of giving rise to a new radical which in turn can react with NO₂ resulting in a range of nitration products [91], a possibility supported by experimental findings of inflammation-associated protein damage [92]. Table 1.3 summarizes some of the chemical mediators of inflammation discussed above and lists a fraction of the molecular targets that each species may disrupt in vivo.

The reactions mentioned above – i.e. oxidation, nitration, halogenation and nitrosation – all leading to multiple possible products, illustrate the chemical complexity inherently linked to the inflammatory response and raises the question of which damaging pathway, if any, predominates in vivo and forces cells towards a state of unregulated proliferation. Focusing on a
Table 1.3. Properties of chemical mediators of inflammation (adapted from [29]).

<table>
<thead>
<tr>
<th>species</th>
<th>reactivity</th>
<th>Reaction</th>
<th>cellular targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO(^{-})</td>
<td>oxidant</td>
<td>(\text{OH}^{-} + \text{H}^{+} + e^{-} \rightarrow \text{H}_{2}\text{O})</td>
<td>most protein functional groups, alkenes</td>
</tr>
<tr>
<td>(\text{CO}_3^{2-})</td>
<td>oxidant</td>
<td>(\text{CO}_3^{2-} + \text{H}^{+} + e^{-} \rightarrow \text{HCO}_3^{-})</td>
<td>most protein functional groups, nucleic acids</td>
</tr>
<tr>
<td>HOCl</td>
<td>oxidant</td>
<td>(2\text{HOCl} + 2\text{H}^{+} + 2e^{-} \rightarrow \text{Cl}_2 + 2\text{H}_2\text{O})</td>
<td>thiol, amines, nucleic acids (guanine), alkenes</td>
</tr>
<tr>
<td>HOBr</td>
<td>oxidant</td>
<td>(2\text{HOBr} + 2\text{H}^{+} + 2e^{-} \rightarrow \text{Br}_2 + 2\text{H}_2\text{O})</td>
<td>thiol, amines, nucleic acids (guanine), alkenes</td>
</tr>
<tr>
<td>ONOO(^{-})</td>
<td>oxidant</td>
<td>(\text{ONOO}^{-} + 2\text{H}^{+} + e^{-} \rightarrow \text{NO}_2^{\cdot} + \text{H}_2\text{O})</td>
<td>sulfydryl groups, metalloproteins</td>
</tr>
<tr>
<td>NO(_2)</td>
<td>oxidant</td>
<td>(\text{NO}_2^{\cdot} + e^{-} \rightarrow \text{NO}_2^{-})</td>
<td>sulfydryl groups, phenols, alkenes</td>
</tr>
<tr>
<td>(\text{N}_2\text{O}_3)</td>
<td>electrophile</td>
<td>(\text{Nuc-H} + \text{N}_2\text{O}_3 \rightarrow \text{Nuc-NO} + \text{NO}_2^{-} + \text{H}^{+})</td>
<td>thiols, amines, nucleic acids</td>
</tr>
</tbody>
</table>
type of chemistry and systematically studying its effects in biological systems under controlled conditions is the most straightforward approach to elucidate its relative significance in inflammation-related diseases, such as cancer. Based on that strategy, the goal of the present work is to examine aspects of NO-mediated nitrosation and deamination (last entry in Table 1.3) occurring in vivo. A brief review of our current understanding of how N₂O₃ damages biological targets is presented next.

1.5 Damage caused by NO-mediated nitrosative chemistry

1.5.1 Deamination of nucleic acids

The multitude of products derived from reactions in which NO is involved likely affect several aspects of the cell architecture however, the most significant in terms of cancer development is damage to nucleic acids and alteration of genetic information, an idea explored extensively in the current work. Soon after its discovery as a free radical secreted from phagocytic cells, a plethora of evidence showed the nitrosating effect that NO can have on nucleic acids (in the presence of O₂) and the associated mutagenic potential [93,94]. Its genotoxicity was observed when human cells were exposed to NO [84,95] while subsequent analysis demonstrated that the nitrosative effects are mediated by the formed intermediate N₂O₃ [85]. In detail, N-nitrosation of a primary amine by N₂O₃ creates a nitrosamine, which in an aqueous environment is quickly transformed to a diazonium ion intermediate (Figure 1.3) and that ion is then promptly hydrolyzed [96]. The end result is the net replacement of the attacked amino group by an oxygen.
Figure 1.3. Nitrosative deamination of 2-deoxyguanosine (adapted from [97]).

The nitrosation-mediated replacement of the exocyclic amino group with a carbonyl oxygen is demonstrated following the intermediate steps of the formation of 2-deoxyxanthosine from 2-deoxyguanosine.
Any base with an exocyclic amino group can undergo deamination as a result of its reaction with \( \text{N}_2\text{O}_3 \). Hence, nitrosation of exocyclic amines in DNA or RNA nucleobases result in the conversion of guanine to xanthine and oxanine, adenine to hypoxanthine, cytosine to uracil (or methylcytosine to thymine), as shown in Figure 1.4. Abasic sites are also thought to be direct products of nitrosation, in particular when \( \text{N}_2\text{O}_3 \) attacks secondary amine groups on nucleobases (e.g. \( \text{N}^7 \) position of guanine, \( \text{N}^7 \) or \( \text{N}^3 \) position of adenine) and forms a cationic base that is proposed to destabilize the glycosidic bond and lead to depurination [98]. The aforementioned lesions are of major interest in this thesis and therefore their rate of formation in nucleic acids – both in purified solutions and in cells – are explored in the next chapters.

The observation of another type of damage – single strand breaks in cellular DNA following exposure to NO – was initially attributed to depurination of deoxyxanthosine (dX) [95], but from subsequent analysis demonstrating the relative stability of dX in physiological pH [99] it was deduced that a different type of chemistry was likely responsible for the detected breaks (e.g. ONOO\(^-\) oxidation). Cross-linking of adjacent guanines was also attributed to the presence of \( \text{N}_2\text{O}_3 \) forming during NO exposures [100].

Formation of deamination products was observed when purified DNA was exposed to constant steady-state levels of \( \text{N}_2\text{O}_3 \) \textit{in vitro} [101]. However, much smaller increases of dI, dX, and dU were measured in the genomic DNA of human cells that were subjected to similar conditions [102]. The protective intracellular environment, including nuclear histones protecting DNA and glutathione (GSH) scavenging \( \text{N}_2\text{O}_3 \) (as will be discussed shortly), coupled with efficient DNA repair were thought to be some of the reasons why deamination appeared to have a modest mutagenic effect \textit{in vivo}, a conclusion also supported by results in mouse models of
Figure 1.4. Products of nucleobase N-nitrosation in DNA (adapted from [51]).
NO overproduction [28]. It is likely that N\textsubscript{2}O\textsubscript{3}-mediated deamination occurring at sites of inflammation may contribute to carcinogenic processes in more ways than solely altering the genomic content in cells, a possibility supported by findings in the recent work by Dedon and collaborators in which higher levels of deamination products were detected in RNA compared to DNA [103]. Information in coding or non-coding RNA altered by nitrosative deamination could have severe implications for protein transcription and translation (e.g. cell cycle checkpoint, differentiation and other key processes in controlled growth) while disruption of small regulatory RNA species in many pathways could lead to cancerous phenotypes and other diseases [104]. Nitrosative chemistry affecting nucleobases in small signaling RNAs could lead to dysregulated cellular growth, constituting one of the underlying factors that accelerate the metastatic activity of transformed cells [105] and reinforcing the emerging link between inflammation and tumor progression.

1.5.2 Nitrosation of thiols, biological ions and amino acids

Other nucleophiles that react with N\textsubscript{2}O\textsubscript{3}, thus potentially competing with amines as nitrosation targets \textit{in vivo}, include glutathione and sulphydryl groups of various proteins [83] which give rise to S-nitrosothiols. S-nitrosation as a post translational protein modification process has been implicated in controlling O\textsubscript{2} delivery to tissues, modulating the function or activity of transcription factors, enzymes, membrane receptors and ion channels [106]. Another mechanism that is gaining increasingly more attention and may be affecting the steady-state cellular levels of nitrosated thiols is the intermolecular transfer of the NO group from one protein to another or from nitrosated glutathione to proteins (i.e. transnitrosation reactions) believed to be linked to signal transduction cascades in cells [107]. In addition to thiols, a number of inorganic anions such as chloride, phosphate [82] and bicarbonate [108] as well as the potent
antioxidant ascorbate [109] seem to react fast with N\textsubscript{2}O\textsubscript{3}, effectively acting as scavengers and reducing the rate with which N\textsubscript{2}O\textsubscript{3} attacks other targets in solution. The same can be stated for certain amino acids with nucleophilic residues – tryptophan being the most thoroughly investigated among those – that participate in N-nitrosation pathways whose \textit{in vivo} significance is not yet fully understood [110,111]. The scavenging effect of the aforementioned species has been quantified in terms of nitrosation rate constants which can be helpful in predicting the fate of N\textsubscript{2}O\textsubscript{3} (among other reactive nitrogen species) \textit{in vivo} by simulating the cellular or extracellular environment via kinetic models, as addressed below.

\subsection*{1.6 Application of kinetic models to understand inflammation chemistry}

The spectrum of nitrosative damage resulting from the generation of NO and N\textsubscript{2}O\textsubscript{3} illustrates the complexity of the inflammation-associated chemistry and the biological implications linked to some of the most abundant products (e.g. lesions in nucleic acids). Part of the ongoing challenge is to determine the extent to which reaction rates observed \textit{in vitro} or in precisely controlled biological systems represent the processes occurring at inflamed tissues, which is one of the major goals addressed in this thesis. A critical step in elucidating the role of chemical stress in disease and carcinogenesis is isolating each underlying mechanism and examining it individually. However, a frequent problem in this approach is that the concentrations of most reactive species are too low and their half-lives too short to directly measure in cells or culture media. The analysis becomes even more complicated when we consider the microenvironment at sites of inflammation in animal models (or humans) which is technically impossible to assess, thus making the process of validating our predictions very challenging.
The difficulty in making direct measurements motivated the development of mathematical models that take into account what is already known about the kinetic rates of the anticipated reactions. To that end, Deen and coworkers have published detailed models describing the spatial distributions of reactive nitrogen species generated by macrophages attached to carrier beads in suspension [112], or by macrophages grown in plates [113,114].

Briefly, the NO concentration in the medium surrounding the cells was predicted to be constant whereas $O_2^-$ fluxes (from estimated rates of production in the cytosol and mitochondria, competing with SOD scavenging) led to a highly localized extracellular formation of $\text{ONOO}^-$ affecting only the immediate vicinity of RNS secreting cells. Further modeling showed that rates of diffusion are fast enough to cause the intracellular concentrations for NO, $O_2$ and $CO_2$ to each closely approximate those in the adjacent extracellular fluid: the levels of those gases within the cell are assumed to be no different than the levels imposed by the surroundings [115]. In an attempt to assess the microenvironment expected to form at sites of inflammation, models were developed to simulate inflamed colonic crypts [116] taking into consideration spatial distribution and reactive substrates for the various RNS and ROS. Predictions from that model indicated that NO was expected to reach a maximum level of 0.3 $\mu$M which was comparable to the 1 $\mu$M value derived from previous kinetic studies simulating the environment in the vicinity of activated macrophage cultures [117].

After estimating levels for NO and its derivatives at the vicinity of activated macrophages, the next challenge was to determine intracellular levels of inflammatory mediators that develop in cells affected by nitrosative stress. To that end, comprehensive models were proposed to investigate NO-related oxidation, nitrosation, and nitration pathways occurring in the cytosol, describing both time-dependent responses to the sudden introduction of key reactants.
[118] and processes in equilibrium, where rates of formation and consumption of various chemical species are in continuous balance [80]. The most recent model predicted steady-state concentrations for RNS including the effects of cellular antioxidants (e.g. glutathione, ascorbate), amino acids, proteins and lipids [80]. A two-phase analysis was considered (representing the cytosol and the cell membrane), although in retrospect membrane reactions were found to have a negligible effect on RNS concentrations. In addition, Deen and coworkers were the first to report order-of-magnitude estimates for the intracellular concentration of N_2O_3 (~1-60 fM) in algebraic expressions. Such predictions played an important role in the work presented here and can be ultimately tested by measuring levels of stable end products relevant to the examined chemistry, validating or possibly expanding the network of significant reactions on which the intracellular kinetic model is based.

1.7 Research perspective

The overarching objective of this thesis is to use nucleobase deamination products in RNA as surrogates for the short-lived N_2O_3 and employ kinetic information from in vitro and in vivo nitrosation studies to estimate intracellular levels of N_2O_3 forming in human cells challenged by NO. The very low levels of N_2O_3 expected to form render its direct quantification in NO-stressed cells nearly impossible, thus measuring the nitrosative damage it causes by its interaction with other components in the cytosol is the easiest approach to assess its abundance in biological systems.

The objective in Chapter 2 is to investigate the performance of a silicone tubing-based system of NO delivery, initially developed by Wang and Deen [119], that is essential in providing the necessary kinetic profiles for deamination of nucleic acids addressed in the
following chapters. Dissolved NO concentrations were measured to determine the effect of cell culture media components on the function of the device and whether levels of NO can be predicted with confidence using an established mass transfer model [119] when more complicated fluids – rather than simple biomolecule solutions – are treated under the same conditions.

The main aim in Chapter 3 is to quantify the rate at which purine nucleobases in purified RNA react with N$_2$O$_3$ under controlled experimental conditions approximating those expected to form at sites of inflammation. Using the delivery device mentioned above, deamination rate constants were calculated based on a second order reaction between purines and N$_2$O$_3$; the resulting values were compared to reveal differences in susceptibility towards nitrosation from nucleobases in RNA, genomic DNA and free nucleosides. In addition, rigorous measures were taken to avoid artifacts while examining the formation of oxanine as a naturally occurring deamination product in nucleic acids.

Chapter 4 addresses the questions of whether N$_2$O$_3$-mediated RNA damage leads to depurination (i.e. formation of abasic sites) in addition to the observed rise in deamination products examined in Chapter 3 and how the rates of the two processes compare in magnitude.

The goal in Chapter 5 is to study the rate of RNA deamination in human cells under nitrosative stress and combine the resulting kinetics with information derived in previous chapters to estimate the apparent N$_2$O$_3$ intracellular concentration. The controlled and predictable conditions of the NO-delivery system (Chapter 2) were employed to expose TK6 cell cultures to increasing NO doses and measure the accumulation of deamination products in RNA extracted from intact treated cells. The rates observed in NO-challenged cells and the rate constants
determined from \textit{in vitro} deamination experiments (Chapter 3) were applied to an expression of the rate law to calculate the effective total intracellular $\text{N}_2\text{O}_3$ concentration to which cell constituents are exposed in the cytoplasm and compare the experimentally-derived value with prior mathematical model predictions.

Finally, in Chapter 6, we conclude by summarizing the information gained from investigating the kinetics of nitrosative RNA damage and propose future experiments that will improve our insight on the significance of those reactions \textit{in vivo}.

The results of Chapter 2 have been published [120] and two manuscripts are currently in preparation from the work presented in Chapters 3 and 5.
1.8 References


Chapter 2

A system for exposing molecules and cells to biologically relevant and accurately controlled steady-state concentrations of nitric oxide and oxygen

2.1 Introduction

Nitric oxide (NO) is a free-radical gas involved in diverse biological processes, such as apoptosis, neurotransmission, blood pressure control and innate immunity [1]. Of critical importance to NO function is its steady-state concentration in tissues, with biologically relevant concentrations ranging over three orders of magnitude. On the basis of literature estimates, Thomas et al. proposed concentration categories for NO function, ranging from cGMP-mediated signaling processes at ~1-30 nM, modulation of kinase and transcription factor activity at ~30-400 nM (e.g., Akt, HIF-1α, p53) and pathological nitrosative and oxidative stresses above ~500 nM [2]. The balance between low and high concentrations may thus dictate cell decision-making processes such as survival and proliferation as opposed to growth arrest and cell death. While the functions of NO are controlled in part by the rate of generation by NO synthases, numerous chemical reactions affect its concentration by consuming NO, such as reactions with superoxide (O$_2^\cdot$) to form peroxynitrite (ONOO$^-$), and with molecular oxygen to form the oxidizing and S-nitrosating nitrogen dioxide radical (NO$_2$) and the S- and N-nitrosating species, nitrous anhydride (N$_2$O$_3$). Glutathione and other cellular reductants and electrophiles further intervene in reactions with these NO derivatives (e.g. ref. [3]). Activated macrophages are the major source of pathologically high levels of NO [4], producing local steady-state concentrations approaching 1 μM [5]. Simultaneous generation of O$_2^\cdot$ leads to ONOO$^-$, while reactions of NO with O$_2$ yield
N₂O₃ and NO₂. These reactive species damage all types of cellular biomolecules and thus contribute to the mechanistic link between inflammation and cancer [5,6].

Evidence for the concentration-dependence of NO function highlights the need for delivery of predictable and biologically relevant steady-state levels of NO and O₂ in vitro to mimic the biological environment. NO can be introduced into a solution by several methods. One approach to NO delivery in vitro involves the use of “NONOates” that release NO with predictable kinetics to provide transient, non-uniform levels that must be averaged over time to define exposure [7]. Garthwaite and coworkers were able to obtain constant steady-state levels of NO over several minutes by balancing NO release from a NONOate with consumption by a scavenger [8]. The difficulty of controlling the steady-state concentration of NO with NONOates over longer periods of exposure [7,9] is compounded by the unknown effects of the chemical species generated upon release of NO [7], the generation of nitroxy [10], and the fact that different NO-donor compounds with different half-lives must be used to span a range of exposure times [11]. Alternative methods of NO delivery include addition of aliquots of NO-saturated aqueous solutions, which produce local NO “hotspots” [12], and co-culture of NO-producing macrophages with target cells, which involves poor control of NO levels and complicates interpretation of results with multiple cell lines [13,14].

Over the past decade, we have developed NO delivery systems for controlled steady-state concentrations of NO (0.1-1 μM) and O₂ (50-200 μM) to mimic biological environments such as inflammation [15,16]. The best characterized and most widely applicable of the systems employs NO- and O₂-permeable polydimethylsiloxane (Silastic) tubing to deliver the gases at constant and predictable levels. Mass transfer models were developed to calculate the concentrations of NO and O₂ in the bulk liquid, given any combination of tubing length and gas feed composition.
[16], as well as rates of gas consumption by cells [17]. The device was designed primarily for cultured cells and was used to elucidate threshold effects in NO-induced toxicity and mutagenicity in human cells [17,18]. However, the mass transfer model was validated only for NO and O₂ delivery in phosphate buffers and was not tested with the more complex case of cell culture media, components of which could consume NO, such as riboflavin-generated superoxide that may react with NO [19,20]. Although partial oxidation of NO within the permeable Silastic tubing motivated the development of a Teflon-membrane delivery apparatus for chemical kinetic experiments [21], the Silastic tubing system has proven utility for long-term cell culture studies [16,22].

Here we describe the fabrication of the NO/O₂ delivery system from commercially available components, and the operation and calibration of the system. We then describe an application of the system for delivery of NO and O₂ into cell culture media, with a comparison of experimental results with a mass transfer model that predicts the steady-state levels of various NO-derived reactive species. We also determined that photo-sensitive reactions taking place in cell culture media, such as riboflavin-derived O₂⁻ synthesis, do not affect steady-state NO or O₂ levels. Finally, we illustrate use of the delivery system with different cell types and explore the range of cumulative NO-dosing and its effect on cell survival. This system provides critical control of NO delivery for in vitro models of NO biology and chemistry and offers great potential for exploring reactions between nitrogen oxides and biomolecules or cellular components.

2.2 Materials and methods

2.2.1 Fabrication of the delivery system
The NO/O₂ delivery system consists of three major components: a vessel for exposing cells (the delivery apparatus), a system for delivering NO and O₂ to the exposure vessel (the peripheral tubing system) and accessories for temperature control and stirring. The delivery apparatus is comprised of a Teflon screw-capped jar with a lid fitted with luer adapters for connecting external gas tubing to the internal Silastic tubing for diffusion of gases into solution, as described previously [16]. The lid can also be modified with ports for probes to monitor solution levels of NO and O₂ (Figure 2.1). An alternative configuration was conceived for exposure of adherent cells (Figure 2.2). Gases are delivered at controlled flow rates by the peripheral tubing system (Figure 2.3), which consists of gas tanks, tubing and flow controllers to deliver two gas mixtures to the delivery apparatus: NO in argon (pure argon for controls) and O₂ in a mixture of N₂ and CO₂. Tables A.1 and A.2 in Appendix A contain a complete parts list required to fabricate the system (assembly procedure described in Appendix A). Temperature control is achieved by immersing the vessel in a water bath mounted on a magnetic stirrer (set at 100 rpm). To avoid operator exposure to NO gas, the system must be operated in a fume hood (see Appendix A). Gas leak alarms can be installed in the vicinity to minimize the potential for accidental exposure (available online: http://www.certifiedairsafety.com/bw-technologies-gas-detectors-honewell-gasalertmax-gaslaertmicro5/bw-technologies-by-honeywell-gasalert-extreme).

2.2.2 Dissolved NO and O₂ measurement in the apparatus

Levels of NO and O₂ in the bulk liquid were measured using a modified apparatus where a stainless steel fitting (Swagelok; Table A.2 in Appendix A) was threaded through a drilled hole centered on the device’s cap to firmly suspend a dissolved O₂ meter Orion 810 A-plus (Thermo...
Figure 2.1. Schematic of probes attached to the NO/O\textsubscript{2} delivery apparatus.

The arrangement of fittings before insertion of the NO and O\textsubscript{2} electrodes on the modified cap is shown. Relative positioning of additional holes on the modified screw cap required to support the probe-bearing fittings (the 2 loops of tubing yield the same results whether arranged in a crossover pattern or in parallel, as long as contact with the probes is avoided). All parts required to fabricate the apparatus are listed in Table A.2 (Appendix A).
**Figure 2.2.** Schematic of the NO/O₂ delivery apparatus modified for adherent cell cultures.

The arrangement of fittings, impeller assembly and impeller shaft that enable stirring in the delivery device to expose adherent cell cultures are shown. The bottom part of the impeller assembly (not engaged with the glass shaft) was shaved off to avoid contact with the cell dish resting in the container while still allowing for the magnet to be attached and rotate freely. Relative positioning of the additional hole on the screw cap required to support the impeller assembly/shaft (the 2 loops of tubing need to be arranged in parallel to avoid contact with the rotating impeller). All parts required to fabricate the apparatus are listed in Table A.2 (Appendix A).
Figure 2.3. Schematic of the peripheral tubing system providing NO and O₂ gas mixtures to the delivery apparatus.

Upper right inset illustrates Swagelok steel fittings for connecting Teflon tubing to adapters. All parts required to construct the peripheral tubing system are listed in Tables A.1 and A.2 (Appendix A).
Electron). A female luer bulkhead fitting (Cole-Parmer) with its barbed hose filed away and its inner diameter increased to ~4 mm was threaded in a different hole drilled in the cap (Figure 2.1); appropriately sized rubber gaskets (McMaster-Carr) were placed between the fittings and the cap to seal the connections. The 4 mm margin was large enough to allow insertion of the 2 mm ISO-NOP Nitric Oxide probe (World Precision Instruments, Inc.) without disturbing its tip membrane. A rubber gasket that when fitted on the probe exceeded a 4 mm diameter was used to securely immobilize it on the filed luer adapter. To minimize the risk of touching the membrane and destabilizing the signal, the NO electrode remained semi-permanently attached; the O₂ probe on the other hand was robust enough to be inserted anew before each exposure. After sealing the container, the tips from both electrodes were immersed in the liquid and protected from any movement or contact with the stirring bar.

2.2.3 Calibration of NO and O₂ probes

Both NO and O₂ probes were pre-calibrated daily before use. NO electrode calibration involved immersing the tip of the probe in 0.1 M potassium iodide and 0.1 M sulfuric acid while known amounts of a potassium nitrite standard solution were added to the solution, generating an equimolar amount of NO [23]. Linear calibration curves were obtained over the range of 0-2 μM at both 23 and 37 °C. The calibration procedure for the O₂ electrode entailed water-saturated air according to the manufacturer’s instructions; the probe operated in a range of concentrations of dissolved O₂ between 100 and 300 μM.

2.2.4 Exposure of water, RPMI-1640 and DMEM to NO and O₂

Two loops of Silastic tubing (Dow Corning; Table A.2 in Appendix A) were attached to the female barbed hose luer fittings fixed on the modified apparatus (Figure 2.4), one for NO
Figure 2.4. Schematic of the NO/O₂ delivery apparatus.

(A) Side view of the 120 mL Teflon container with hose-barb luer fittings threaded into holes in the screw cap with rubber gaskets to seal the connections. (B) Top view of the screw cap showing a typical arrangement of luer fittings and how they connect to external gas feeds. (C) Cross-section of the cap demonstrating connection of the U-shaped loop of Silastic tubing to two luer fittings for gas transit. All parts required to fabricate the apparatus are listed in Table A.2 (Appendix A).
and the other for O₂ (corresponding lengths in Table 2.1). After pre-calibration, both electrodes were attached and the apparatus was filled with ~115-120 mL of either de-ionized water, RPMI-1640 or DMEM cell medium (BioWhittaker-Lonza). The device was sealed and placed in the water bath at 23 or 37 (±1) °C. Simultaneous initiation of gas flows followed (procedure described in Appendix A), employing a 10% NO in argon mixture and a 50% O₂:45% N₂:5% CO₂ mixture (Airgas). Exposures lasted for 40-50 min and experiments were repeated at least three times.

2.2.5 Nitrite measurements

For exposures involving pure water, nitrite (NO₂⁻) concentration was monitored by UV absorbance at 210 nm (ε = 5200 M⁻¹ cm⁻¹) [24] in 100 μL samples withdrawn from the device at regular intervals.

2.2.6 NO aqueous oxidation reaction scheme and mass transfer model

In aqueous media at physiological pH, NO reacts with oxygen giving rise to the stable end product nitrite (NO₂⁻), as illustrated by the following reactions:

\[2\text{NO} + \text{O}_2 \xrightarrow{k_1} 2\text{NO}_2\]  \hspace{1cm} (1)

\[\text{NO} + \text{NO}_2 \xleftrightarrow{k_2} \text{N}_2\text{O}_3\] \hspace{1cm} (2)

\[\text{N}_2\text{O}_3 + \text{H}_2\text{O} \xrightarrow{k_3} 2\text{NO}_2 + 2\text{H}^+\] \hspace{1cm} (3)
Table 2.1. NO and O₂ gas flow parameters for operation of the delivery system with water and culture media.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>NO gas line</th>
<th></th>
<th>O₂ gas line</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tubing length (cm)</td>
<td>Flow rate (sccm)</td>
<td>NO mole fraction (%)</td>
<td>Tubing length (cm)</td>
</tr>
<tr>
<td>23</td>
<td>7.0</td>
<td>100</td>
<td>10%</td>
<td>5.3</td>
</tr>
<tr>
<td>37</td>
<td>7.0</td>
<td>100</td>
<td>10%</td>
<td>6.3</td>
</tr>
</tbody>
</table>
When NO is the only nitrogen oxide added to the system, intermediates NO₂ and N₂O₃ are present only in trace quantities [25], justifying pseudo-steady state approximations for their concentrations, while the rate-limiting step is Equation (1). In that case, Equations (1) - (3) can simply be combined into the overall reaction:

\[ 4\text{NO} + \text{O}_2 + \text{H}_2\text{O} \rightarrow 4\text{NO}^- + 4\text{H}^+ \] (4)

Employing those approximations to eliminate the concentrations \( C_j \) of the intermediate species as independent variables from the volumetric rate of formation \( R_j \) for species \( j = \text{NO}, \text{O}_2 \) and \( \text{NO}_2^- \) gives, according to Equation (4):

\[ R_{\text{NO}} = -4k_1C_{\text{NO}}^2C_{\text{O}_2} \]
\[ R_{\text{O}_2} = -k_1C_{\text{NO}}^2C_{\text{O}_2} \]
\[ R_{\text{NO}_2} = 4k_1C_{\text{NO}}^2C_{\text{O}_2} \]

The mass balance equations for NO, O₂ and NO₂⁻, the dominant species during a typical exposure, are given by the following differential equations, with the bulk solution in the delivery device being the control volume [16]:

\[
\frac{dC_{\text{NO}}}{dt} = \frac{k_1^{(\text{NO})} A_{\text{NO}}}{V} \left[ \alpha_{\text{NO}} P_{\text{NO}}^{(\text{NO})} - C_{\text{NO}} \right] + \frac{k_1^{(\text{O}_2)} A_{\text{O}_2}}{V} \left[ \alpha_{\text{NO}} P_{\text{NO}}^{(\text{O}_2)} - C_{\text{NO}} \right] - 4k_1 C_{\text{NO}}^2 C_{\text{O}_2} \] (a)

\[
\frac{dC_{\text{O}_2}}{dt} = \frac{k_1^{(\text{NO})} A_{\text{NO}}}{V} \left[ \alpha_{\text{O}_2} P_{\text{O}_2}^{(\text{NO})} - C_{\text{O}_2} \right] + \frac{k_1^{(\text{O}_2)} A_{\text{O}_2}}{V} \left[ \alpha_{\text{O}_2} P_{\text{O}_2}^{(\text{O}_2)} - C_{\text{O}_2} \right] - k_1 C_{\text{NO}}^2 C_{\text{O}_2} \] (b)
\[
\frac{dC_{\text{NO}_2}}{dt} = \frac{A_{\text{NO}} q}{V} + 4k_1 C_{\text{NO}} C_{\text{O}_2} 
\]  

(c)

where \( C_j \) is the time dependent concentration for species \( j \), \( A_{\text{NO}} \) and \( A_{\text{O}_2} \) are the outer surface areas of the cylindrical tubing loops through which NO and O\(_2\) are supplied, \( V \) is the total liquid volume filling the device, \( k_1 \) is the rate constant from Equation (4), \( \alpha_j \) is the aqueous solubility for gas \( j \), \( k_j^{(i)} \) is the liquid phase mass transfer coefficient for gas \( j \) inside the tubing supplying gas \( i \), \( P_j^{(i)} \) is the partial pressure of gas \( j \) within the tubing loop supplying gas \( i \), and \( q \) is the extraneous flux of \( \text{NO}_2^- \), a pseudo-heterogeneous reaction taking place in a micron thick layer next to the outer surface of the NO-supplying tubing loop (thought to be caused by intramembrane oxidation of NO to \( \text{NO}_2 \), with essentially all the produced \( \text{NO}_2 \) converted to \( \text{N}_2\text{O}_3 \) and subsequently to \( \text{NO}_2^- \) at the interface [16]). The first two terms in the right hand side of Equations (a) and (b) represent mass transfer of the species in and out of the gas flows through the two tubing loops, whereas the third terms describe the net volumetric rates (consumption) of the dissolved gases in the bulk volume. The first term on the right hand side of Equation (c) represents the extra \( \text{NO}_2^- \) source term added to the mass transfer model to account for the experimentally observed higher rate of \( \text{NO}_2^- \) appearance during exposures, and the second term shows the rate of \( \text{NO}_2^- \) formation from the reaction taking place in the bulk volume.

The system of equations can be simplified taking into account the following observations:
1) Cross contamination of NO from O\textsubscript{2}, and vice versa, inside tubing loops can safely be assumed to be negligible, so \( P_{NO}^{(O_2)} = P_{O_2}^{(NO)} = 0 \)

2) NO loss through the tubing loop that supplies O\textsubscript{2}, represented in Equation (a), is insignificant, since the term \( \frac{k_{NO}^{(O_2)} A_{O_2}}{V} C_{NO} \) was shown to always be much smaller compared to the term \( \frac{k_{NO}^{(NO)} A_{NO}}{V} \left[ \alpha_{NO} p_{NO}^{(NO)} - C_{NO} \right] \) and thus can be ignored altogether, eliminating the need to input a value for the mass transfer coefficient \( k_{NO}^{(O_2)} \) in Equation (a) [16].

Using the mass transfer coefficients and physicochemical parameters displayed in Table 2.2, the dimensions of the delivery apparatus (filled with approximately 115 mL of fluid) and Silastic tubing loops (0.196 cm outer diameter), as well as experimentally determined pressure inside the tubing based on the given flow rates (~1.0 atm; ref. [16]) and initial values for the species concentrations at the beginning of each exposure (O\textsubscript{2} is the only species with non-zero initial concentration), the differential equations of the mass transfer model can be solved numerically to predict the time course of NO, O\textsubscript{2} and NO\textsubscript{2}─ concentrations within the device employing MATLAB 7.11 (MathWorks, Natick, MA). Because of previous work that has shown strong effects of O\textsubscript{2} on the NO-related mass transfer coefficients and the conditions under which those values were estimated [16], it is important to use the model in cases where solutions and suspensions will be at nearly air-saturated levels to maximize the model’s accuracy on predicting concentration profiles. To illustrate, Table 2.3 provides combinations of different tubing lengths and NO gas composition and the resulting calculated steady-state NO and O\textsubscript{2} levels expected to occur in the device during simulated exposures using water or buffers. As noted below, the
Table 2.2. Input parameters for the mass transfer model.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>10% NO</th>
<th>99% NO</th>
<th>1% NO</th>
<th>10% NO</th>
<th>99% NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{NO}^{(NO)} (10^{-5} \text{ m/s})$</td>
<td>0.40</td>
<td>1.07</td>
<td>2.06</td>
<td>1.11</td>
<td>1.84</td>
</tr>
<tr>
<td>$k_{O_2}^{(NO)} (10^{-5} \text{ m/s})$</td>
<td>2.60</td>
<td>4.39</td>
<td>3.50</td>
<td>3.69</td>
<td>5.22</td>
</tr>
<tr>
<td>$q , (\mu\text{mol m}^{-2} \text{s}^{-1})$</td>
<td>5.70</td>
<td>33.8</td>
<td>N.A.</td>
<td>6.79</td>
<td>39.4</td>
</tr>
<tr>
<td>$k_1 (10^6 \text{ M}^{-2} \text{s}^{-1})$</td>
<td>2.1</td>
<td></td>
<td>2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{O_2}^{(O_2)} (10^{-5} \text{ m s}^{-1})$</td>
<td>2.01</td>
<td></td>
<td>2.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha_{NO} (10^{-8} \text{ M Pa}^{-1})$</td>
<td>1.8</td>
<td></td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha_{O_2} (10^{-8} \text{ M Pa}^{-1})$</td>
<td>1.2</td>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Symbols: $k_{NO}^{(NO)}$, liquid phase mass transfer coefficient for NO and, $k_{O_2}^{(NO)}$, liquid phase mass transfer coefficient for O$_2$ at the tubing supplying NO; $q$, extraneous flux of NO$_2$ at the outer surface of the NO-supplying tubing loop; $k_1$, oxidation rate constant; $k_{O_2}^{(O_2)}$, liquid phase mass transfer coefficient for O$_2$ at the tubing supplying O$_2$; $\alpha_{NO}$, $\alpha_{O_2}$, aqueous solubility for NO, O$_2$, respectively. Values are reproduced from earlier studies [16,17]. No mass transfer coefficients available in the case of delivery of 1% NO at 23 °C.
Table 2.3. Predicted steady state NO and O\(_2\) concentrations in simulated exposures.

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>Composition of NO stream</th>
<th>NO tubing length (cm)</th>
<th>O(_2) tubing length (cm)</th>
<th>Steady-state [NO] (μM)</th>
<th>Steady-state [O(_2)] (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>10% NO in Ar</td>
<td>7.0</td>
<td>7.4</td>
<td>1.1</td>
<td>270</td>
</tr>
<tr>
<td></td>
<td>99% NO</td>
<td>5.0</td>
<td>12.3</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>1% NO in Ar</td>
<td>6.0</td>
<td>5.7</td>
<td>0.7</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td>10% NO in Ar</td>
<td>7.0</td>
<td>7.3</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>99% NO</td>
<td>5.0</td>
<td>11.5</td>
<td>6.0</td>
<td></td>
</tr>
</tbody>
</table>

Composition of the O\(_2\) stream is the same in every case (50% O\(_2\) in N\(_2\) with 5% CO\(_2\)). The O\(_2\) concentration of the solution filling the apparatus before the start of every simulated exposure was arbitrarily selected to be that of air-saturated water (270 μM at 23 °C and 210 μM at 37 °C) and remained essentially stable during the simulation using the tubing length combinations displayed. Model parameters were not available for predictions in the case of 1% NO at 23 °C.
model can also be used to validate and benchmark the operation of the system based on electrode measurements of NO and O_2.

2.2.7 Exposing cell cultures (in collaboration with L. J. Trudel and Dr. G. N. Wogan)

TK6 human lymphoblastoid cells [26,27] were obtained from ATCC, NH32 (an isogenic derivative of TK6; ref. [28]) and HCT116 human colon carcinoma cells [29,30] were provided by C. C. Harris (National Cancer Institute). TK6 and NH32 cells were kept in exponentially growing suspension cultures in RPMI-1640 medium supplemented with 10% heat-inactivated donor calf serum and HCT116 cells in McCoy’s 5A medium supplemented with 10% heat-inactivated donor horse serum, at 37 °C in a humidified 5% CO_2 atmosphere; 100 units/mL penicillin, 100 μM/mL streptomycin and 2 mM L-glutamine were also added in both types of media. Cell suspensions were transferred at a density of 5 x 10^5 cells/mL in 115 mL of supplemented RPMI-1640 medium to sterile delivery devices (autoclaved at 120 °C for 20 min, with Silastic tubing loops of appropriate length already attached) immediately prior to treatment. Adherent cells were plated in 60 mm dishes (10^6 cells per dish) 24 h prior to exposure, existing medium was aspirated immediately before the exposure, and dishes were positioned at the bottom of autoclaved delivery devices (Figure 2.2). The inner and outer surfaces of the dishes carrying the HCT116 cells needed to be sterile before insertion into the device, so handling was performed at all times with disinfected forceps within larger (150-mm) sterile dishes. Cell-carrying dishes without their caps were anchored for stability (using a small sterilized wedge made of Tygon tubing) to the bottom of the devices, and 110 mL of fresh supplemented McCoy’s 5A medium were gently added immediately before the exposure begun. Tubing loops of suitable lengths and gas tanks of appropriate compositions were selected so that a steady state NO concentration of 0.6 μM was achieved in the case of cell suspensions and 1.8 μM in the case
of adherent cells, with liquid O\textsubscript{2} levels maintained near air saturation. The total NO dose delivered was dictated by the exposure time and was quantified in units of \(\mu\)M min. Parallel exposures using Argon (instead of NO) were used as negative controls in all experiments. After the exposure, cell suspensions were collected by centrifugation and re-suspended in fresh supplemented RPMI-1640 medium, whereas fresh supplemented McCoy’s 5A medium was added to adherent cell cultures after being washed once with phosphate buffered saline. Subsequently, all cultures remained at 37 °C in a humidified 5% CO\textsubscript{2} atmosphere until further processing.

2.2.8 Cell viability analysis (performed by Laura J. Trudel)

TK6 and NH32 cell viability was determined 48 h after NO/O\textsubscript{2} treatment in the delivery device, whereas HCT116 cell viability was determined 24 h post exposure using the MTT assay (Roche Diagnostics, Indianapolis, IN) and following the manufacturer’s instructions. Per cent survival was expressed relative to Argon-exposed negative controls at each treatment dose.

2.3 Results

2.3.1 Validation of NO and O\textsubscript{2} delivery in water

The basic operation of the system is illustrated with the simple scenario of NO and O\textsubscript{2} delivery into water in the absence of other chemical species that could react with NO or its derivatives. A modified delivery apparatus with NO and O\textsubscript{2} electrodes inserted through the vessel lid (Figure 2.1) was used to measure dissolved gases in air-equilibrated de-ionized water. This allowed validation of the mass transfer model predictions of NO and O\textsubscript{2} steady-state levels achieved using the Silastic tubing of lengths, gas composition and gas flow conditions shown in Table 2.1. Measured levels of NO and O\textsubscript{2} in water agree well with model predictions at both 23
and 37 °C (Figure 2.5), with measured steady-state levels of 1.2 and 1.7 μM for NO and 240 and 210 μM for O₂, respectively.

The device can also be used to deliver predictable, well-controlled concentrations of NO-derived secondary species such as NO₂ and N₂O₃. Though these species are too reactive to be measured directly, their mutual degradation product, NO₂⁻, can be easily and accurately quantified [31]. Formation of NO₂⁻ in the system operating with water deviated from the model’s prediction by a factor of 1.6-2 (Figure 2.6), with correction for the model’s extra NO₂⁻ source q, ranging from 11 to 11.5 μmol m⁻² s⁻¹, providing a good fit of the data. (The origin of this extra NO₂⁻ is discussed later.) This demonstrates the empirical nature of the mass transfer model and the need to calibrate the delivery system to account for batch-to-batch variations in Silastic tubing, which is important since there is a significant conversion of NO to NO₂ within the tubing wall [22]. Table 2.4 summarizes optimized parameters for the model, estimated by minimizing sums of squared differences between measured and predicted concentrations of NO, O₂ and NO₂⁻ at all time points (relative errors of concentration difference divided by measured concentration were used). Aside from the q values, the updated estimates of the mass transfer coefficients were in agreement with those previously reported (Table 2.2) [16].

The formation of NO₂⁻ demonstrates an important factor in the operation of the delivery system. NO₂⁻ generation is coupled with an equimolar accumulation of H⁺, which necessitates the use of buffers to maintain a uniform pH within the device. For example, we observed that a solution of 50 mM phosphate buffer sustains a pH of 7.4 for over 12 h under the conditions defined in Figure 2.6 (NO₂⁻ accumulation at 160 μM hour⁻¹), with accurate simulation of NO and O₂ levels by the mass transfer model [16,22]. Note that nitrate (NO₃⁻) formation was not detected in previous studies [16] and was thus not measured here. The fact that model parameters
Figure 2.5. Time course of measured and predicted bulk liquid NO and O$_2$ concentrations.

NO (circles, left axis) and O$_2$ (squares, right axis) concentrations are shown using water at 23 °C (A) and 37 °C (B). Data from two experiments (open and closed symbols) and predictions using the mass transfer model (solid curves) are shown. Exposure conditions: 10% NO, 100 sccm, 7.0 cm Silastic tubing loop; 50% O$_2$, 200 sccm, 5.3 cm tubing loop for 23 °C, or 6.3 cm tubing loop for 37 °C.
Figure 2.6. Time course for measured and predicted nitrite (NO$_2^-$) formation.

Discrete symbols represent experimental data (open circles from data at 37 °C, closed circles at 23 °C, triplicate measurements). The lines were generated by the mass transfer model: dotted line (37 °C) and solid line (23 °C) with $q = 11.03$ and $q = 11.54$ μmol m$^{-2}$ s$^{-1}$, respectively ($q$ values derived from recent data); dot-dashed line (37 °C) and dashed line (23 °C) with $q = 6.79$ and $q = 5.70$ μmol m$^{-2}$ s$^{-1}$, respectively (values from Table 2.2). Exposure conditions: 10% NO, 100 sccm, 7.0 cm Silastic tubing loop; 50% O$_2$, 200 sccm, 5.3 cm tubing loop for 23 °C, or 6.3 cm tubing loop for 37 °C.
Table 2.4. Mass transfer coefficients and boundary layer nitrite (NO$_2^-$) correction at the NO supplying tubing for operation of the delivery system with water.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>$k_{NO}^{(NO)}$ (10$^{-5}$ m/s)</th>
<th>$k_{O_2}^{(NO)}$ (10$^{-5}$ m/s)</th>
<th>$q$ (μmol m$^{-2}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>0.44 ± 0.03</td>
<td>2.3 ± 0.2</td>
<td>11.0 ± 0.95</td>
</tr>
<tr>
<td>37</td>
<td>1.0 ± 0.05</td>
<td>3.2 ± 0.35</td>
<td>11.5 ± 0.91</td>
</tr>
</tbody>
</table>

Updated mass transfer coefficients for NO, O$_2$ and boundary layer NO$_2^-$ source were based on data acquired with water (Figure 2.5 and Figure 2.6). Values for $k_{O_2}^{(O_2)}$ were taken from Table 2.2, assuming a negligible effect on O$_2$ delivery through the O$_2$ supplying tubing at the NO concentrations used (ref. [16]).
describe levels of NO and O\(_2\) equally well in water and phosphate buffers indicates that the presence of salts does not significantly affect delivery of NO or O\(_2\) in the device.

### 2.3.2 NO and O\(_2\) delivery in cell culture media

NO and O\(_2\) delivery into cell culture systems presents a more complex scenario, with cell culture media typically containing nutrients and salts that could react with NO and its derivatives. For example, riboflavin in many media preparations has been shown to cause photo-induced generation of O\(_2^-\) under standard lighting conditions [19,20]. The rapid reaction of O\(_2^-\) and NO to form ONOO\(^-\) could thus act as a sink for NO and potentially interfere with the predicted steady-state NO concentrations in the device. We assessed this problem with two commonly used mammalian cell culture media: RPMI-1640 and Dulbecco’s modified Eagle’s medium (DMEM). The apparatus was filled with medium and delivery was initiated with the operational parameters used for water (Table 2.1). As shown in Figure 2.7A, the resulting concentration profiles for NO and O\(_2\) in RPMI-1640 at both 23 and 37 °C were in good agreement with predictions from the mass transfer model. To assess the effects of riboflavin and ambient light on NO levels, the steady-state NO concentration was allowed to plateau in RPMI-1640 medium, at which point ambient lights were turned off (arrow in Figure 2.7A) to place the delivery device in complete darkness. As shown in Figure 2.7A, NO levels were identical in the presence or absence of ambient light, which ruled out substantial production of O\(_2^-\) that would interfere with steady-state NO levels in RPMI-1640. Identical results were obtained with DMEM (Figure 2.7B), which contains twice the concentration of riboflavin as RPMI-1640 (1 vs. 0.5 \(\mu\)M, respectively). Consistent with the conclusion that the agreement observed between experimental
Figure 2.7. Time course of measured and predicted bulk liquid NO and O₂ in cell culture media. NO (circles, left axis) and O₂ (squares, right axis) concentrations shown in (A) RPMI-1640 and (B) DMEM cell culture media at 23 °C (open symbols) or 37 °C (closed symbols). Data from two sets of experiments (discrete symbols) and predictions using the mass transfer model (solid curves) are shown. Arrows indicate the times at which ambient lighting was extinguished. Exposure conditions: 10% NO, 100 sccm, 7.0 cm Silastic tubing loop; 50% O₂, 200 sccm, 5.3 cm tubing loop for 23 °C, or 6.3 cm tubing loop for 37 °C.
measurements and model-generated curves is medium-independent, we observed that supplementation of RPMI medium with 10% heat-inactivated donor horse serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine also did not have an effect on the measured levels of NO at 37 °C, within the limits of accuracy of the electrodes (Figure 2.8). It should be noted here that the vessel, connectors and Silastic tubing can be sterilized by autoclaving for work with cell cultures over prolonged periods, with minimal effect on performance. However, the system should be validated at least once following autoclaving to ensure that the specific batch of tubing delivers NO and O₂ as expected.

### 2.3.3 Exposure of cell cultures to NO and O₂

The full potential of the NO/O₂ delivery device is realized with exposure of cultured cells. As described above, the mass transfer model was used to determine exposure parameters (Silastic tubing lengths, gas composition) and calculate the steady-state gas levels before the device was used to generate the survival curves shown in Figure 2.9. Cultures of human lymphoblastoid TK6 cells and the p53-deficient derivative NH32 cells were then exposed to calculated levels of 0.64 µM NO and ~200 µM O₂ for up to 24 h, resulting in a cumulative total NO dose of 920 µM min and a dose-dependent pattern of cell death. Also evident in Figure 2.9, NH32 cells were more resistant to NO toxicity than TK6 cells, with an apparent threshold for NO-induced cytotoxicity at ~300 µM min vs. 150 µM min for TK6 cells. Interestingly, when either cell type was exposed to doses lower than its respective threshold, no significant cell death occurred [18]. The HCT116 adherent colon tumor cell line proved to be even more resistant to NO-induced cytotoxicity (Figure 2.9), with a threshold of ~1000 µM min. That different killing curves were derived from widely used cell lines demonstrates the utility of the delivery device
Profiles of measured and predicted bulk liquid NO concentration shown during exposure of RPMI-1640 cell culture media, with and without serum, in the delivery device at 37 °C. Circles represent an experiment with regular RPMI-1640 medium while squares and triangles represent experiments where RPMI-1640 was supplemented with 10% heat-inactivated donor horse serum, 100 units/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine. Discrete symbols represent experimental data while the solid curve was generated by the mass transfer model. Exposures involved delivery of 10% NO at 100 sccm through a 7.0 cm long Silastic tubing loop and 50% O₂ at 200 sccm through a 6.3 cm long tubing loop at 37 °C for all data shown.
**Figure 2.9.** Cell viability in TK6, NH32 and HCT116 cells after exposure to NO.

Cell viability was determined by MTT assay, as described in Materials and Methods, for TK6 and NH32 at 48 h, and HCT116 cells at 24 h after exposure to NO and O₂ or argon at 37 °C. Data represent mean ± SD for 2-4 experiments; SD values for TK6 and NH32 cells were <8% and are not shown.
for studying mechanisms of NO function and toxicity. Exposure of control cell cultures to argon/O_2 had little or no effect on cell proliferation, which indicates minimal effects of turbulence caused by stirring in the reactor.

2.4 Discussion

There is an emerging recognition of the critical role of steady-state concentration in the biological function of NO and the enormous range of NO concentrations that distinguish physiological from pathological activity. However, most current NO delivery systems do not provide precise, accurate or long-term control of NO concentration [7,9]. Recent studies have led to interesting results, for example, on the response of an array of proteins to stratified levels of NO, with conclusions concerning anti- or pro-growth pathways related to the tumor microenvironment [11,32,33]. However, the means of NO delivery and estimation of total NO dosage in those studies do not avoid the common caveats that accompany the use of NO-donor compounds. Further, typical extant NO delivery systems rely on the relatively hyperoxic conditions in aqueous solutions exposed to ambient air. We have overcome these limitations with a system to deliver well-controlled and biologically relevant steady-state concentrations of NO and O_2 to biomolecules and cells. The fact that O_2 is delivered separately from NO resolves the issue of depletion of the former through cellular respiration or its reaction with the latter, a technicality that affected previous attempts to develop a similar delivery system [25,34]. We have presented here a detailed step-by-step assembly and operation guide for the NO/O_2 delivery system, along with the means to validate the system. This provides the inexperienced user with sufficient information to fabricate the device from commercially available components, install it in a typical laboratory setting, and operate it safely and effectively. The system has broad utility
in all types of in vitro studies of NO biology and chemistry, ranging from small molecule damage to exposures of suspension and mono-layer cell cultures to NO and its derivatives.

Based on our results, the mathematical model of mass transfer allows for accurate predictions of the NO and O\(_2\) levels in both simple aqueous solutions and in cell media. By keeping steady operational parameters, such as gas flow-rates, stirring speed and temperature, and by varying NO composition (1%, 10% or 99% NO) and Silastic tubing loop length, the model computes the time-dependent NO, O\(_2\) and NO\(_2^-\) concentrations in the apparatus, provided that an experimental estimate of the initial O\(_2\) concentration (before gas diffusion begins) is available. This can greatly facilitate experimental design, since changes in the configuration of the delivery apparatus (e.g., NO gas composition, tubing length) can be translated into predictable levels of NO, even before the actual gas delivery takes place. Further, the model allows fine-tuning of NO delivery by varying the lengths of the gas diffusion tubing.

The mass transfer model is readily modified to account for a variety of applications of the delivery device. For example, the initial formulation of the model assumed that cellular consumption of NO and O\(_2\) would be negligible. Thus, all the predictions apply to cell-free culture media (or solutions) as was the case with some of the exposures presented here. However, recent studies revealed measurable rates of NO and O\(_2\) consumption by several cell lines [17,20,35]. This allows a variation of the initial model in which the mass balance equation for each gas (differential equations delineating the net rate of change of NO and O\(_2\) liquid concentrations, described in Section 2.2.6) includes an additional term representing the net specific cellular consumption rate for each gas multiplied by the cell density [17]. For instance, NO and O\(_2\) consumption rates for TK6 cells (37 °C) were found to be 0.05 and 0.77 nmol min\(^{-1}\) (10\(^6\) cells\(^{-1}\)), respectively, in a typical cell density of 5 × 10\(^5\) cells mL\(^{-1}\) (e.g. ref. [18]). By
incorporating these new conditions into the model, we can predict changes in NO and O\textsubscript{2} concentrations caused by the cells (Figure 2.10). Due to the order-of-magnitude difference in consumption rates, we observe a much less pronounced effect on NO concentration compared to O\textsubscript{2} levels, when computation extends from cell-free solutions to cell cultures. Consumption rates are available for a variety of cell lines, so employing accurate estimates of those terms in the model increases its predictive power. These kinds of customizations are relatively straightforward and add to the precision and accuracy of NO and O\textsubscript{2} delivery provided by the system.

The presence of nutrients in cell culture media such as salts, vitamins, amino acids, sugars, and glutathione did not measurably affect the diffusion of NO and O\textsubscript{2} through the Silastic membrane or their consumption in solution, with similar performance when gases were delivered in water. The lack of any molecular sinks for NO under normal lighting conditions or in a dark environment, even with serum-supplemented media, reinforces the expectation that the device will operate as predicted by the mass transfer model during treatments of cell cultures. The lack of any apparent NO consumption when lights are activated in the vicinity of the device comes in contrast to previous studies [19,20] that attributed such trends to generation of O\textsubscript{2}\textsuperscript{-} presumed to originate from light-activated riboflavin in cell media. Although Keynes and coworkers did not describe their vessel characteristics in sufficient detail, Nalwaya and Deen used what was essentially a modified transparent polystyrene 60 mm culture dish to monitor reactions in media. On the other hand, the current delivery system is a translucent 4 mm-thick Perfluoroalkoxy container that may filter out wavelengths of laboratory light that could potentially excite riboflavin. Alternatively, O\textsubscript{2}\textsuperscript{-} formation inside the delivery device under the conditions
Figure 2.10. Effect of cellular consumption on predicted NO and O₂ levels.

Model-based predictions of bulk liquid NO and O₂ concentrations as a function of time during simulated exposures of a cell-free solution (solid curves) and a TK6 cell culture (dashed curves) in the delivery device at 37 °C. The difference between the two sets of curves represents the effect that adding cellular consumption rates for NO and O₂ have on how the model predicts gas levels in the apparatus. For both exposures, hypothetical initial O₂ concentration was arbitrarily set at 191 μM, NO feed composition at 10% NO, Silastic tubing NO-loop length at 7.0 cm, O₂ feed composition at 50% O₂, O₂-loop length at 6.3 cm.
describing the exposures may be smaller than the sensitivity of the probe that was used to measure NO levels.

The confidence that the mass transfer model gives in terms of steady delivery of NO and O2 motivated a series of experiments to explore cellular responses to NO exposure [17,18,36,37]. Those studies revealed the existence of NO toxicity thresholds (sub-lethal vs. lethal) as well as repercussions in a diverse range of endpoints such as glutathione depletion, DNA damage, and cell growth arrest. This characteristic pattern of dose-dependent survival is likely to be true for all types of cells, offering opportunities for the device to be employed to monitor molecular damage and killing caused by NO, and to systematically study how sub-threshold NO affects cellular components and signaling pathways without simultaneously causing apoptosis or disturbing their growth cycle. The NO/O2 delivery device is a versatile vessel that can accommodate cell cultures without itself affecting their survival. It is able to provide the gases of interest in a controllable and “clean” fashion, avoiding secondary effects that other delivery methods may cause.

A microscopic model developed to complement the general mass transfer model predicts that the apparatus has a 1 μm thick boundary layer adjacent to the NO tubing loop where the concentrations of oxidation intermediates NO2 and N2O3 greatly exceed those in the bulk liquid [22]. This “hot spot” appears to result from intramembrane oxidation of NO to NO2. The almost complete conversion of NO2 to N2O3 very close to the tubing surface explains the unexpectedly high rate of N2O3-derived NO2⁻ formation. Most molecules are small enough to enter the boundary layer and be exposed to high concentrations of NO2 and N2O3, which complicates the analysis when values of those concentrations are required for studies of chemical kinetics. An approach to estimate “hot spot” contributions to the total concentration compares reaction rates.
of target analytes to rates of another substrate with a known rate constant (e.g., morpholine nitrosation) [22,34,38]. However, the uncertainties introduced by this methodology motivated the development of a variation of the system in which NO is delivered through a porous Teflon membrane, instead of a Silastic tubing loop. Another modification is that the NO gas stream is fed through stainless steel (instead of Teflon) tubes, excluding any possible contamination by O₂ before oxidation takes place in the vessel [21]. The new design avoids adventitious NO₂⁻ formation, so it is thought to eradicate the “hot spot” phenomenon. However, because the “hot spot” should not affect cells (10-20 µm diameter) due to their inability to enter the micron-thin boundary layer [22,39], and the Silastic-based apparatus is easier to fabricate and sterilize than the Teflon-based one, the Silastic apparatus remains the more attractive option for exposing cells.

As mentioned above, the extraneous NO₂⁻ formation was postulated to be caused by intramembrane oxidation of NO to NO₂. Previous work has reported a rate constant (4.4 × 10⁵ M⁻² s⁻¹) [16] for this conversion occurring within the Silastic tubing loop which was correlated to the rate at which NO₂⁻ is formed in the bulk volume. Our results show a 1.6- to 2-fold increase in NO₂⁻ accumulation during exposures to de-ionized water and a much smaller dependence on temperature (Figure 2.6). In other words, if the intramembrane oxidation rate constant follows an Arrhenius-type behavior then our recent findings would point to a greater pre-exponential factor and lower activation energy, perhaps due to variations in the physical properties of the Silastic tubing. Accordingly, it is advisable for a new user to validate the system and ascertain that the NO and O₂ delivery parameters are as expected. Alternatively, the observed increase of NO₂⁻ formation could be related to incomplete exclusion of O₂ from the Teflon-based NO gas line as constructed in the current peripheral tubing system (noted by Skinn et al. [21]), which may differ
from the original experimental setup that was used to determine the boundary layer contribution to NO$_2^-$ synthesis [16].
2.5 References


Chapter 3
Nitric oxide-linked deamination kinetics of nucleic acids and nucleosides

3.1 Introduction

In recent decades, a growing body of evidence demonstrates the multifaceted role of the physiological radical, nitric oxide (NO), in the cardiovascular, nervous and immune systems [1,2]. However, pathological overproduction of NO for prolonged periods at sites of inflammation [3,4] is believed to be linked with initiation and progression of diseases such as cancer [5,6]. The cytotoxic and cytostatic effects of NO at sufficiently high concentrations are thought to be due to its reactive derivatives, namely peroxynitrite (ONOO−), the nitrogen dioxide radical (NO2) and nitrous anhydride (N2O3), formed from the reaction of NO with superoxide (O2−) and molecular oxygen, respectively [7]. In particular, N2O3 is a strongly nitrosating species that can attack the amino groups in heterocyclic bases of nucleic acids [8,9], among other nucleophilic targets [10,11], and give rise to deaminated nucleobases, abasic sites and DNA crosslinks (reviewed in [12]). It is likely that this type of nitrosative stress burdens host cells and tissues with an additional level of chemical damage, one that may overwhelm cellular defense and repair mechanisms, and eventually interfere with normal function and decision-making processes, such as survival and proliferation versus growth arrest and cell death.

Nitrosative deamination alters the primary structure of nucleic acids by giving rise to modified base products such as xanthine (X) and oxanine (O) derived from guanine (G), hypoxanthine (I) from adenine (A), uracil (U) from cytosine, and thymine (T) from 5-methyl-
cytosine [12]. Deaminated nucleobases, detected in both nitrous acid solutions and oxygenated NO solutions [13-15], are formed via an aryl diazonium intermediate following the reaction of native bases with N₂O₃ in physiological pH [16], the end result being the replacement of the primary amino group on the base moiety by an oxygen. A purine ring-opening and -closing mechanism, proposed to support experimental findings of oxanine formation in acidic nitrite environments [17], seemed consistent with results from other studies on nucleoside and DNA deamination using the transnitrosating agent 1-nitroso-indole-3-acetonitrile [18,19]. However this mechanism was not corroborated by previous research by Dedon and coworkers, in which 2-deoxyoxanosine (dO) was not detected in DNA from human TK6 cells exposed to controlled levels of NO and O₂ [20] and from tissues from a mouse model of NO overproduction [21]. Based on our recent data however, we now believe the reported inability to detect dO was caused by an artifactual reaction during sample processing and by the limited sensitivity of the analytical instruments used in previous studies, as discussed shortly. In addition to deamination of exocyclic amines, nitrosation is responsible for other nucleobase lesions including 2-deoxyguanosine (dG) intrastrand cross-links [22] and depurination sites, whose formation is attributed to the destabilization of the glycosidic bond in cationic bases formed by nitrosation of the N⁷ in deoxyguanosine and N⁷ or N³ position in deoxyadenosine [18,19]. Nitrosation-independent mechanisms leading to the accumulation of deaminated nucleobases include hydrolysis at high temperatures [23,24], enzymatic editing of RNA and DNA [25], bisulfate reactions with 2-deoxycytosine [26], mutations in key purine metabolism enzymes [27], or deaminase contamination of commercial enzyme preparations [20,28].

Approximating the microenvironment of inflamed tissues in a laboratory setting with the goal of assessing the extent of damage to nucleic acids caused by nitrosative deamination, among
others, has given rise to a number of NO-delivery strategies. Earlier quantitative studies showed that activated macrophages are a source of pathologically high levels of NO [4], producing local steady-state concentrations approaching 1 µM [12,29]. This level of NO can be reached in a solution by several means. “NONOates” release NO with predictable kinetics to provide transient, non-uniform levels that must be averaged over time to define exposure [30]. Although balancing NO release from NONOates with consumption by scavengers can lead to constant steady-state levels of NO over several minutes [31], the difficulty of controlling the steady-state concentration of NO with NONOates over longer periods of exposure [30,32] is compounded by the unknown effects of the chemical species generated upon release of NO [30] and the fact that different NO-donor compounds with different half-lives must be used to span a range of exposure times [33]. Alternative methods of NO delivery include addition of aliquots of NO-saturated aqueous solutions [34] or syringe bolus injection of NO gas into the reaction mixture [15], which produce local NO “hotspots”, and co-culturing of NO-producing macrophages with target cells, which involves poor control of NO levels and complicates interpretation of results with multiple cell lines [35,36]. Perhaps the best characterized and most broadly applicable system that mimics the nitrosative environment at sites of inflammation was developed by Deen and coworkers using NO- and O2-permeable polydimethylsiloxane (Silastic) tubing to deliver the gases at constant and predictable levels [28,37,38]. An accompanying mass transfer model was developed to predict the concentrations of NO and O2 in the system given the values of certain operational parameters [37], and a subsequent kinetic analysis estimated the levels of N2O3 formed in the solution [28], making the tubing-based delivery apparatus ideal for kinetic studies of nitrosative deamination.
The effects of NO-induced deamination on DNA have been explored in numerous in vitro studies [8,28,39]. Modest increases in deaminated DNA products reported in cells and tissues under nitrosative stress [20,21] suggest that underlying factors (such as a reducing environment in cells, efficient DNA repair, chromatin-associated protection, etc.) could lead to low steady-state levels of nitrosative DNA damage in vivo. Those findings and others [40] indicate that nucleobases in nucleic acids other than DNA may prove to be better indices of nitrosative stress, and more particularly RNA which is less amenable to cellular repair. The significance of surveying RNA modifications becomes even greater if one considers that N₂O₃-induced changes in the sequence of coding and non-coding RNA species may have consequences in gene expression, potentially affect the function of the translational machinery [41,42], interfere with post-transcriptional RNA editing [25], or disrupt regulating elements strongly correlated with the progression of different types of cancer [43-45].

The objective of the present work was to provide a rigorous estimation of the rate at which nucleobase damage from NO-induced deamination is expected to occur in nucleic acids and nucleosides under well controlled conditions representing the microenvironment of inflamed tissues in vivo, especially after further limiting factors that affect the stability of accumulated modifications. The juxtaposition of hypoxanthine, xanthine and oxanine levels measured in purified RNA, genomic DNA and free nucleosides throughout the exposures was intended to elucidate the susceptibility of nucleobases with varying degrees of solvent exposure to deamination.

3.2 Materials and methods

3.2.1 Chemicals
Na₂HPO₄, NaH₂PO₄ and ammonium acetate were obtained from American Bioanalytical, LC-MS grade acetonitrile from EMD, ethanol from Koptec. Glacial acetic acid, morpholine (Mor), adenosine (rA), 2-deoxyadenosine (dA), guanosine (rG), 2-deoxyguanosine monohydrate (dG), inosine (rI), 2-deoxyinosine (dI) and xanthosine (rX) were from Sigma (98% or higher purity) whereas 2-deoxyxanthosine (dX) was synthesized as described in previous work [28]. Water purified through a Milli-Q system (Millipore) was used throughout the study.

3.2.2 Human cell cultures for total RNA and genomic DNA extraction

TK6 human lymphoblastoid cells obtained from ATCC were grown in large cultures for the purpose of harvesting purified RNA and DNA. Cells were maintained in exponentially growing suspension cultures at 37°C in a humidified atmosphere of 95% air and 5% CO₂ in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated horse serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Stock cell cultures were routinely passaged to maintain an optimal growth density (~1.0 × 10⁶ cells/mL) in 150 mm dishes. Cell pellets harvested by centrifugation were washed with phosphate buffer saline (PBS) and processed using Qiagen’s RNeasy Maxi Kits and Genomic-tip 500/G following the manufacturer’s instructions. Extracted total RNA (2-4 mg per pellet containing ~600 × 10⁶ cells) and genomic DNA (0.5-0.7 mg per pellet containing ~100 × 10⁶ cells) concentrations were quantified by UV spectroscopy and stored at -80 °C until further use. RPMI-1640, L-glutamine, streptomycin /penicillin and PBS were obtained from Lonza, horse serum from Atlanta Biologicals, and trypan blue (4% in saline) for cell counting from Sigma.

3.2.3 Synthesis of isotopically labeled internal nucleoside standards
Uniformly $^{15}$N-labeled internal standards of the DNA and RNA deamination products dI, dX, dO, rI, rX and rO, were synthesized as described previously [40,46] from uniformly $^{15}$N-labeled deoxyribonucleosides (dA, dG) and ribonucleosides (rA, rG) as starting materials (Cambridge Isotope Laboratories). Standards were purified by HPLC, characterized by LC-MS, and quantified by UV absorbance using published extinction coefficients [46].

### 3.2.4 NO delivery system

A delivery system described in full detail in Chapter 2 [47] was employed to expose purified nucleic acids and nucleosides to accurately controlled constant levels of NO and O$_2$. Briefly, two distinct gas streams (one for NO the other for O$_2$) were fed to reactors (modified 120 mL PFA screw-top jars from Cole Parmer) containing solutions of nucleosides, DNA and RNA, with NO and O$_2$ diffusing separately into the liquid through gas permeable Silastic tubing loops (6-7 cm long, 1.96 mm o.d., 1.47 mm i.d. from Dow Corning). The flow rates were 100 standard cubic centimeters per minute (sccm) for the NO-feeding stream and 200 sccm for the O$_2$- feeding stream, for every reactor used. Gas flow rates were regulated via mass flow controllers obtained from Porter Instruments, Parker-Hannifin. The contents of each reactor were kept stirred using a magnetic stirrer plate from IKA (stirring speed at 100 rpm; stir-bar placed in every reactor) and maintained at 37 °C within a water bath. Mixtures of 10% NO in argon, 1% NO in argon, 50% O$_2$ in N$_2$ (and 5% CO$_2$) and pure argon (for controls) were obtained from Airgas.

### 3.2.5 Exposing nucleosides, RNA, DNA and morpholine to NO

The delivery system described above was used to estimate the rate at which deamination products form when individual nucleosides and nucleic acids were exposed to NO. Solutions of rA, rG, dA and dG (6 μM each) in 50 mM sodium phosphate (Na-PO$_4$) buffer pH 7.4 – prepared
from Na$_2$HPO$_4$, NaH$_2$PO$_4$ as described in ref. [48] – were separately exposed in reactors (~115 mL solution per reactor). A mass transfer model that simulates the system’s performance [37,47] was used to determine lengths of tubing loops (in combination with a particular NO gas stream content) necessary to achieve constant physiological levels of the dissolved gases in each reactor. Two sets of conditions were employed leading to constant steady-state NO concentrations of: a) ~1.7 μM, by passing 10% NO in argon through a 7.0 cm Silastic tubing loop and 50% O$_2$ in N$_2$ (with 5% CO$_2$) through a 6.3 cm loop, and b) ~0.7 μM, by passing 1% NO in argon through a 6.0 cm loop and 50% O$_2$ in N$_2$ (with 5% CO$_2$) through another 6.0 cm loop (flow rates in both cases were maintained as defined above). Oxygen levels were predicted to remain constant throughout at ~210 μM in both cases. Solutions of rA, dA and dG were exposed in the former conditions (set a) whereas rG was exposed in the latter (set b), to examine whether different NO/N$_2$O$_3$ levels have an effect on deamination rate constants. At various time points after initiating gas flows, 0.5 mL aliquots were withdrawn from each reactor and replaced with an identical volume of fresh solution.

Stock solutions of total RNA, generated as described above, were washed twice with purified water using spin filters (NanoSep 10K Omega, Pall) before being treated in the delivery system. Solutions of washed RNA (5 μg/mL) in 50 mM Na-PO$_4$ buffer pH 7.4 were then transferred to reactors (~115 mL in every reactor) with the proper combination of tubing loops to achieve a steady-state NO concentration of ~1.7 μM (employing 10% NO; see above). All buffers and reactors were autoclaved to completely neutralize the activity of remaining RNases and avert RNA degradation. During exposures, 2.5 mL aliquots were withdrawn from each reactor and replaced with an identical volume of fresh solution.
Exposure of genomic DNA solutions (20 μg/mL in 50 mM Na-PO₄ buffer pH 7.4) were performed similarly to RNA experiments, however stock solutions were not washed and buffer or reactors were not autoclaved before experiments.

The rates of nucleobase deamination in nucleosides and nucleic acids were compared to rates of Mor nitrosation for accuracy in the calculation of rate constants, discussed in more detail below. Solutions of 2 mM Mor in 50 mM Na-PO₄ buffer pH 7.4 were transferred to reactors (~115 mL per reactor) and exposed to both set of conditions listed above (i.e. 1% NO and 10% NO). Aliquots (0.1 mL) were withdrawn at regular intervals and subsequently replaced with an identical volume of fresh solution.

### 3.2.6 Enzymatic hydrolysis of DNA and RNA

DNA samples (~50 μg) collected during NO exposures were maximally concentrated using NanoSep 10K spin filters, resuspended in 100 μL water and quantified by UV spectroscopy before hydrolysis to nucleosides. A combination of nuclease P1 (Sigma), DNase I (Sigma), phosphodiesterase I (USB) and alkaline phosphatase (Sigma) were employed in the presence of deaminase inhibitors (coformycin from DTP/NCI, tetrahydrouridine from Calbiochem) and the antioxidant butylated hydroxytoluene (Sigma), as described previously [46]. Deferoxamine was omitted due to its effect on oxanine stability demonstrated in the current work. The following average amounts of internal standards were added to the digestion mixture per sample: 11 pmol ¹⁵N-dI, 11 pmol ¹⁵N-dX and 9 pmol ¹⁵N-dO.

Collected RNA samples (~13 μg) during exposures were concentrated, resuspended in purified water and quantified similarly to DNA samples. RNA integrity was confirmed using Bioanalyzer RNA Nano 6000 on-chip electrophoresis kits (Agilent), showing a predominance of
ribosomal RNA. Enzymatic hydrolysis of RNA, a variation of the method used for DNA, was based on a recently developed protocol [40] with the only difference being the exclusion of deferoxamine from the reaction mixture. Average amounts of internal standards added per sample: 1.2 pmol $^{15}$N-rI, 0.6 pmol $^{15}$N-rX and 0.5 pmol $^{15}$N-rO.

In both cases, enzymes were removed at the end of hydrolytic digestion processes by NanoSep 10K spin filters and filtrates were concentrated under vacuum.

3.2.7 HPLC prepurification of DNA and RNA deamination products

Hydrolyzed DNA and RNA preparations were reconstituted with purified water to a total volume of 100 μL and individual nucleosides were resolved and collected using a Agilent 1100 series HPLC system at empirically determined retention times adapted from previous work [40,46]. Different chromatographic methods were used for DNA- and RNA-derived samples, however both protocols entailed resolution of nucleosides on a Phenomenex Synergi C18 reversed-phase column (250 x 4.6 mm, 4 μm particle size, 80 Å pore size) with elutions performed at a 0.5-1 mL/min flow rate of 8 mM ammonium acetate solvent (A) with a stepwise increasing gradient of pure acetonitrile (B); details are given in Table B.1 (Appendix B). Elution times for the native and target nucleosides are shown in Figure B.1 (Appendix B). Blind collected fractions containing the target nucleosides (rI, rX, rO, dI, dX, dO) were desiccated under vacuum, redissolved in 50 μL water and analyzed by LC-MS/MS.

3.2.8 LC-MS/MS quantification of deamination products in DNA, RNA and nucleosides

Levels of deamination products in NO-treated and enzymatically hydrolyzed DNA and RNA samples were quantified by liquid chromatography-coupled triple quadrupole mass spectrometry (LC-MS/MS) using an Agilent 1100 series HPLC system interfaced with an AB
Sciex API 3000 tandem quadrupole MS with a turbo ion spray source. Resolution of target nucleosides was achieved on a Thermo Fisher Hypersil Gold aQ C18 reverse-phase column (150 x 2.1 mm, 3 μm particle size) using isocratic conditions of 0.1% acetic acid (A) and 0.7-3% v/v acetonitrile (B), depending on the target nucleoside, at a 0.2 mL/min flow rate; elution times, content of solvent B and column temperature for each of the target nucleosides are listed in Table B.2 (Appendix B). The MS was operated in positive ion mode with the first and third quadrupoles fixed to unit resolution. Multiple reaction monitoring (MRM) mode was used for sample detection with dwell times set to 200 ms. Mass spectrometric parameters were optimized for maximal sensitivity and are displayed in Table B.3 (Appendix B).

Deamination products forming in NO-treated nucleoside solutions were quantified directly (no hydrolysis or prepurification took place) using an Agilent 6410 triple quadrupole mass spectrometer interfaced with an Agilent 1100 series HPLC system and the Hypersil Gold aQ column mentioned above along with the same solvents (A: 0.1% acetic acid, B: acetonitrile) to resolve the target nucleosides. The effluent from the LC system was diverted to waste when not detecting nucleosides to minimize contamination of the electrospray ionization source (details of the chromatographic runs in Tables B.4 and B.5, Appendix B). The MS was operated in positive ion MRM mode with dwell times set to 200 ms. Mass spectrometric parameters were optimized for maximal sensitivity (displayed in Table B.6, Appendix B). The following average amounts of internal standards were individually added immediately prior to MS analysis depending on which deamination product was measured: 30 fmol $^{15}$N-dI, 65 fmol $^{15}$N-dX, 15 fmol $^{15}$N-dO, 70 fmol $^{15}$N-rI, 50 fmol $^{15}$N-rX and 20 fmol $^{15}$N-rO.

Linear calibration curves ($r^2 > 0.98$) for the isotope-labeled and unlabeled forms of each of the deamination products were generated for both MS systems. The two MS systems proved
to be equivalent since measurements of deamination products formed in RNA using both strategies led to coinciding kinetic profiles.

### 3.2.9 Detection of morpholine nitrosation

In morpholine (Mor) nitrosation experiments, the formed product $N$-nitroso-morpholine (NMor) was quantified by UV absorbance ($\varepsilon = 5500 \text{ M}^{-1} \text{ cm}^{-1}$ at 250 nm [49]) in 0.1 mL aliquots withdrawn from the reactors at regular intervals.

### 3.2.10 Kinetics of oxanine degradation

Stock solutions of dO and rO were generated by bubbling 100% NO gas through saturated dG and rG solutions in purified water, respectively, at room temperature while deamination products were isolated using the HPLC prepurification methods described above. The identity of dO and rO was confirmed by their characteristic UV absorbance signature (Figure B.2 in Appendix B; in agreement with previous reports in literature [50,51]) and elution times based on the aforementioned LC protocols; stock solutions were quantified by UV absorbance using published extinction coefficients [46]. Known amounts of dO and rO (6 μM) in 30 mM sodium acetate buffer pH 7.8 were incubated in 25 °C or 37 °C with varying concentrations of deferoxamine (Sigma). The reaction mixtures were resolved via HPLC and levels of unaffected dO, rO were monitored by quantifying their corresponding elution peaks based on UV absorbance.

### 3.2.11 Reaction scheme for NO-induced nitrosation and deamination

In aqueous media at physiological pH, NO reacts with oxygen, as illustrated in the following reactions:
\[
2\text{NO} + \text{O}_2 \xrightarrow{k_1} 2\text{NO}_2 \quad (1)
\]

\[
\text{NO} + \text{NO}_2 \xrightarrow[k_2]{k_{-2}} \text{N}_2\text{O}_3 \quad (2)
\]

\[
\text{N}_2\text{O}_3 + \text{H}_2\text{O} \xrightarrow{k_3} 2\text{NO}_2 + 2\text{H}^+ \quad (3)
\]

When NO is the only nitrogen oxide added to the system, intermediates NO\(_2\) and N\(_2\)O\(_3\) are present only in trace quantities [16], which justifies pseudo-steady state approximations for their concentrations, and N\(_2\)O\(_3\) is the principal nitrosating agent in the solution [10]. The majority of N\(_2\)O\(_3\) is directly hydrolyzed to the stable end product nitrite (NO\(_2^-\)) as shown in reaction 3. However, hydrolysis is accelerated in the presence of certain anions that act as ‘scavengers’, overall decreasing N\(_2\)O\(_3\) levels available to react with other targets, e.g. primary and secondary amines. Reaction 4 represents the additional hydrolysis pathway occurring due to inorganic phosphate (P\(_i\)):

\[
\text{N}_2\text{O}_3 + \text{P}_i + \text{H}_2\text{O} \xrightarrow{k_4} 2\text{NO}_2^- + \text{P}_i + 2\text{H}^+ \quad (4)
\]

In solutions of 50 mM Na-PO\(_4\) used for all exposures, reaction 4 is calculated to be ~25-fold faster than reaction 3, illustrating the catalytic effect of phosphate buffer in hydrolyzing N\(_2\)O\(_3\) and reducing its steady-state levels.

The small fraction of N\(_2\)O\(_3\) that is not consumed by (anion-assisted) hydrolysis can lead to other reactions, depending on which substrates are added to the mixture. In experiments where
Mor was also present in the buffer, a reaction that takes place in addition to those listed above is the nitrosation of $\text{Mor}^o$ (unprotonated Mor) to produce NMor:

$$k_5$$

$$\text{N}_2\text{O}_3 + \text{Mor}^o \rightarrow \text{NMor} + \text{NO}_2^- + \text{H}^+ \quad (5)$$

Alternatively, when exposing nucleosides to NO (without any Mor present), the following deamination reactions are considered in lieu of Mor nitrosation:

$$k_6$$

$$\text{N}_2\text{O}_3 + \text{rA} \rightarrow \text{rI} + \text{NO}_2^- + \text{H}^+ \quad (6)$$

$$k_7$$

$$\text{N}_2\text{O}_3 + \text{rG} \rightarrow \text{rX} + \text{NO}_2^- + \text{H}^+ \quad (7)$$

$$k_8$$

$$\text{N}_2\text{O}_3 + \text{rG} \rightarrow \text{rO} + \text{NO}_2^- + \text{H}^+ \quad (8)$$

$$k_9$$

$$\text{N}_2\text{O}_3 + \text{dA} \rightarrow \text{dI} + \text{NO}_2^- + \text{H}^+ \quad (9)$$

$$k_{10}$$

$$\text{N}_2\text{O}_3 + \text{dG} \rightarrow \text{dX} + \text{NO}_2^- + \text{H}^+ \quad (10)$$

$$k_{11}$$

$$\text{N}_2\text{O}_3 + \text{dG} \rightarrow \text{dO} + \text{NO}_2^- + \text{H}^+ \quad (11)$$

Each of the nucleosides rA, rG, dA and dG were individually exposed to NO (i.e. in separate reactors), however rX and rO formed simultaneously as products of reactions 7 and 8 in the same vessel(s) containing rG; similarly reactions 10 and 11 occurred in parallel when dG was treated. The reaction between $\text{N}_2\text{O}_3$ and cytidine was not considered since the corresponding
deamination product, uridine, is a naturally abundant nucleobase in RNA, making detection of small amounts generated by deamination very challenging (uridine and thymidine do not have exocyclic amino groups in their nucleobase to react with N2O3).

For experiments involving nucleic acids, two sets of rate constants were defined, mirroring reactions 6-11 displayed above: \( k_{12}, k_{13}, k_{14} \) for rI, rX and rO, respectively, forming concurrently when N2O3 reacted with RNA and \( k_{15}, k_{16}, k_{17} \) for dI, dX and dO produced in the case of NO-exposed DNA. Rate constants for reactions 1-5 are known (listed in Table 3.1) whereas those for reactions 6-17 were derived from comparing observed rates for Mor nitrosation and deamination, as will be discussed below.

### 3.2.12 Kinetics of deamination and nitrosation – estimation of concentrations and rate constants

Values of reactant concentrations are essential to calculating the unknown rate constants stated above, but there is no direct way to determine trace levels of N2O3 (as opposed to dissolved NO and O2 by electrodes [47]). On the other hand, since the kinetics of Mor nitrosation by N2O3 are known [10], the rate of NMor formation can be used as a measure of the N2O3 concentration achieved in Mor exposures; the same N2O3 levels are expected to form in experiments with nucleosides and nucleic acids in which buffer and exposure conditions were identical to those with Mor exposures. This approach is especially useful since earlier work has shown that, besides the well-stirred bulk liquid where the aforementioned intermediates are present only in trace levels, the exposure reactor develops a \(~1 \mu m\) thick boundary layer adjacent to the NO-feeding Silastic tubing loop where the concentrations of NO2 and N2O3 greatly exceed those in the bulk liquid [37]. Although this boundary layer is localized virtually on the surface of
Table 3.1. Reaction rate constant values.

<table>
<thead>
<tr>
<th>rate constant</th>
<th>value (37 °C)</th>
<th>units</th>
<th>ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1$</td>
<td>$2.4 \times 10^6$</td>
<td>M$^{-2}$ s$^{-1}$</td>
<td>[16]</td>
</tr>
<tr>
<td>$k_2$</td>
<td>$1.1 \times 10^9$</td>
<td>M$^{-1}$ s$^{-1}$</td>
<td>[52]</td>
</tr>
<tr>
<td>$k_2$</td>
<td>$8.0 \times 10^4$</td>
<td>s$^{-1}$</td>
<td>[52]</td>
</tr>
<tr>
<td>$k_3$</td>
<td>$2.1 \times 10^3$</td>
<td>s$^{-1}$</td>
<td>[53]</td>
</tr>
<tr>
<td>$k_4$</td>
<td>$1.1 \times 10^6$</td>
<td>M$^{-1}$ s$^{-1}$</td>
<td>[10]</td>
</tr>
<tr>
<td>$k_5$</td>
<td>$6.5 \times 10^7$</td>
<td>M$^{-1}$ s$^{-1}$</td>
<td>[10]</td>
</tr>
</tbody>
</table>

Values for $k_2$, $k_2$ reported in 20 °C; $k_3$ estimation at 37 °C based on activation energy also reported in ref [53]; $k_4$, $k_5$ calculated based on $k_3$ values as indicated in ref [10].
the tubing, most molecules are small enough to enter it and consequently be exposed to its local concentrations. Substrates in the reactor are affected not only from the bulk liquid but also from the boundary layer region, which despite its much smaller volume was found to contribute significantly towards nitrosation/deamination.

Focusing just on the bulk liquid region in experiments involving Mor, the quasi-steady state approximation in kinetics is valid and applicable to both NO₂ and N₂O₃. Combining the corresponding expressions for both species (considering all terms of formation vs. consumption in reactions 1-5) and solving for the N₂O₃ concentration yields:

\[
[N₂O₃] = \frac{2k_1[NO]^2[O₂]}{k_3 + k_4[P₁] + k_5[Mor₀]}
\]  
(12)

Given that a) [NO] and [O₂] are fixed in the delivery reactor, b) the low yield of NMor formation did not significantly affect Mor₀ levels (up to ~5 μM vs. ~270 μM, respectively), and c) values for the relevant rate constants are available (Table 3.1), the steady-state N₂O₃ concentration forming in the reactor is constant (independent of time) and can be readily calculated. To estimate how the boundary layer contributes to nitrosation, the rate law expression derived from reaction 5 is employed:

\[
\frac{d[NMor]}{dt} = k₅[Mor₀][(N₂O₃) + (N₂O₃)]
\]  
(13)

where the apparent increment in the bulk N₂O₃ concentration to which Mor is exposed, denoted as \([N₂O₃]\), is attributed to the boundary layer region. Using experimentally measured rates of
NMor formation and calculated values of [N₂O₃] discussed above, equation 13 reveals the degree to which the boundary layer adds to the overall rate of reaction.

Similar contributions to deamination from the two aforementioned liquid regions are expected to appear also when solutions of nucleosides (or nucleic acids) are treated in the reactor. In that case, no Mor nitrosation takes place while total hydrolysis illustrated in reactions 3 and 4 is assumed to be much faster, and thus more significant, than any deamination reaction occurring in the system (as will be shown). Hence the quasi-steady state approximation gives the resulting expression for the bulk N₂O₃ concentration, taking into account reactions 1-4:

\[
[N₂O₃]_D = \frac{2k_1[NO]^2[O₂]}{k_3 + k_4[P_i]} 
\]

(14)

The ratio of time independent bulk steady-state N₂O₃ concentrations shown in equations 12 and 14 is also a constant, denoted as \(\alpha\):

\[
\frac{[N₂O₃]}{[N₂O₃]_D} = \frac{k_1 + k_4[P_i]}{k_3 + k_4[P_i] + k_5[\text{Mor}^\text{r}]} = \alpha 
\]

(15)

shown to be arithmetically equal to the ratio of boundary layer contributions to N₂O₃ concentrations in nitrosation and deamination experiments (derivation discussed in the Supplementary material of ref [28]):

\[
\frac{[N₂O₃]}{[N₂O₃]_D} = \alpha 
\]

(16)

Applying the rate law to reaction 6 (deamination of rA), as an example, gives:
\[
\frac{d[rI]}{dt} = k_6 [rA][\left(N_2O_3\right)_D + [N_2O_3]_D] 
\] (17)

and dividing the previous expression with the corresponding rate law derived from Mor nitrosation experiments yields:

\[
\frac{d[rI]}{dt} = \frac{k_6 [rA][\left(N_2O_3\right)_D + [N_2O_3]_D]}{k_2 [Mor^o][\left(N_2O_3\right)_D + [N_2O_3]_D]} \quad (18)
\]

Combining equations 15, 16 and 18 provides the following formula:

\[
k_6 = \alpha k_5 \frac{[Mor^o]}{[rA]} \frac{d[rI]}{dt} \frac{d[NMor]}{dt} \quad (19)
\]

Equation 19 is used to calculate the particular rate constant for rA deamination, as long as slopes of rI and NMor formation are experimentally available, and the deamination/nitrosation products do not affect the corresponding reactant concentrations (which was the case as will be shown). This method can be extended to all other products in experiments involving nucleosides and nucleic acids mentioned in the sections above.

### 3.3 Results

#### 3.3.1 Degradation of oxanine in the presence of amines

Addition of deferoxamine (Def) as a chelating agent and antioxidant to nucleic acid hydrolysis protocols was found to have detrimental effects on the integrity of oxanine
nucleosides. As shown in Figure 3.1, in the presence of 2.5 mM Def at 23 °C (30 mM sodium acetate buffer pH 7.8) more than 99% of 6 μM dO decomposes in less than 8 h, with dO depletion accelerated by higher Def concentrations and temperatures. After comparing these kinetic profiles with conditions reported in a recent hydrolysis protocol [46] (incubation with 1.5-2.5 mM Def at 37 °C for at least 15 h), it becomes evident that the use of this antioxidant during dO analysis interferes with the detection of dO that may have formed in nucleic acids. Similar degradation kinetics were observed when the reaction was repeated with rO in 10 mM Def at 23 °C (in 30 mM sodium acetate buffer pH 7.8) as depicted in Figure 3.2, suggesting that the sugar on the nucleoside does not significantly affect the observed decomposition rates. The basis for Def degradation of dO likely involves a reaction of the primary amine of Def with the carbonyl group of dO, as observed in studies of the reaction of dO with glycine [54]. This is illustrated in Figure 3.3.

Interestingly, we observed formation of dX under the experimental conditions that yielded the fastest dO depletion (i.e., 10 mM Def, 37 °C in Figure 3.1) as shown in Figure 3.4, albeit at a much slower rate than the dO decomposition. These results point to a previously unidentified amine-catalyzed conversion of dO to dX.

The same pattern was observed when dO (~0.7 mM in 30 mM sodium acetate buffer pH 7.8) was incubated at 60 °C in the absence of Def, as shown in Figure 3.5 (incubation at 37 °C did not show any appreciable dO degradation). In this case, it is likely that the primary exocyclic amine of the dO nucleobase attacks the C-6 position of another dO, causing ring-opening and conversion to dX. These results suggest that the rate of the dO decomposition and dX formation reactions is not only affected by the concentration of total primary amine in the mixture but also by the nature of the attacking amine itself. Def-induced dO degradation taking place at a pH ~6.
Figure 3.1. Degradation kinetics of 2-deoxyxanosine (dO).

Depletion of dO in varying deferoxamine concentrations and temperatures (data represent mean ± S.D. for n = 3).
Figure 3.2. Comparison of oxanosine (rO) and 2-deoxyoxanosine (dO) degradation kinetics.

Time course of rO and dO degradation in the presence of 10 mM deferoxamine at 23 °C (data represent mean ± S.D. for n = 3).
Figure 3.3. Amine-induced ring-opening mechanism depleting oxanine nucleobases.

The ring-opened adduct is derived from the nucleophilic attack of primary amines at the electrophilic C-6 position in oxanine.
Figure 3.4. Formation of 2-deoxyxanthosine (dX) from 2-deoxyxanosine (dO) in the presence of deferoxamine.

Time course of dX formation during degradation of 6 μM dO in the presence of 10 mM Def at 37 °C (data represent mean ± S.D. for n = 3).
Figure 3.5. Formation of 2-deoxyxanthosine (dX) from 2-deoxyxanosine (dO) in the absence of amine.

Time course of dO degradation and concurrent dX formation during incubation at 60 °C (data represent mean ± deviation about the mean for n = 2).
(in 30 mM sodium acetate buffer) revealed a more than 5-fold decrease in the observed depletion rate compared to the same buffer at a pH 7.8 (consistent with a decrease in levels of unprotonated Def); the integrity of hypoxanthine- and xanthine-containing nucleosides was not affected by the presence of deferoxamine (data not shown).

### 3.3.2 Determination of N₂O₃ concentration in the NO-delivery system

Reliable estimates of N₂O₃ levels to which nucleosides and nucleic acids are exposed in the NO-delivery reactor are necessary to elucidate the deamination kinetics pertaining to each species. Experiments involving exposure of Mor to NO were instrumental in determining the relative effect of the boundary layer region discussed in the Methods section. Only the unprotonated form of Mor (Mor⁰) reacts with N₂O₃ as shown in reaction 5. Thus, in solutions of 2 mM Mor (in 50 mM phosphate buffer, pH 7.4), [Mor⁰] is calculated to be 274 μM (pKₐ of Mor is 8.2 at 37 °C [55]). Based on those values, along with the steady NO (1.7 μM) and O₂ (210 μM) levels maintained in the reactor, equation 12 predicts that [N₂O₃] – i.e. the steady-state concentration in the bulk liquid region – is 39 fM. However, a production rate of N-nitrosomorpholine (NMor) measured at 0.105 ± 0.008 μM/min (Figure 3.6; [Mor⁰] virtually unaffected by NMor formation) reveals a significant contribution stemming from the boundary layer despite its much smaller volume: solving equation 13 for the apparent N₂O₃ concentration increment and using the experimental and calculated values mentioned above yields \( [\text{N}_2\text{O}_3] = 59 \) fM. In other words, the boundary layer-associated term cannot be ignored since it increases the apparent N₂O₃ concentration in the reactor by ~150% (compared to the value calculated solely in the bulk region). In experiments with nucleosides and nucleic acids – where buffer, temperature, and exposure conditions were identical to those involving Mor – equations 14, 15
Figure 3.6. Time course of N-Nitrosomorpholine (NMor) formation from exposure of Morpholine (Mor) to NO/N2O3.

Formation of NMor in 2 mM Mor solutions (50 mM Na-PO4 pH = 7.4) at 37 °C during delivery of 1% and 10% NO gas corresponding to 0.7 μM and 1.7 μM steady-state dissolved NO levels, respectively (data represent mean ± S.D. for n = 3).
and 16 can be used to calculate the corresponding N₂O₃ concentrations. In particular, the calculated constant \( \alpha = 0.76 \) indicates that relatively higher N₂O₃ levels form in both liquid regions during all the deamination experiments, compared to those involving nitrosation (the absence of millimolar levels of Mor decreases the total consumption of N₂O₃). For the subset of experiments taking place at 0.7 \( \mu \)M NO, similar calculations led to the following values:

\[
d[\text{NMor}]/dt = 0.016 \pm 0.004 \mu\text{M/min (Figure 3.6)}, [\text{N₂O₃}] = 6 \text{ fM}, [\text{N₂O₃}] = 9 \text{ fM}, \alpha = 0.76.\]

The steady-state NO concentrations examined in this work are comparable with those measured in the vicinity of activated macrophages [4] or those predicted in inflamed tissues by kinetic models [29], hence the results displayed below are expected to be relevant to the chemical microenvironment at sites of inflammation.

### 3.3.3 Deamination kinetics of nucleosides

Exposing nucleosides to the well-controlled levels of NO in the delivery system provides an effective platform for calculating deamination rate constants. Solutions of ribonucleosides rA and rG in 50 mM Na-PO₄ pH 7.4 buffer were exposed in separate reactors to 1.7 and 0.7 \( \mu \)M NO, respectively, with aliquots withdrawn at regular intervals (different NO levels were used based on early indications that rA is less susceptible to deamination than rG). The resulting deamination products were analyzed by LC-MS/MS, as discussed in the Methods section (no further sample processing), with the results showing time-dependent increases of rI from rA, and both rX and rO from rG, as depicted in Figure 3.7. Although product accumulation was too small to significantly alter initial concentrations of rA and rG, it still provided the necessary slopes – derived from a linear least squares fit on each of the data sets displayed in Figure 3.7 – to calculate the corresponding rate constants. Taking rI formation as an example, the fitted slope
Figure 3.7. Time course for the formation of rI, rX and rO from rA and rG exposed to NO and O₂.

Deamination products rI, rX and rO formed during exposure of rA (6 μM) and rG (6 μM) to 1.7 μM and 0.7 μM NO, respectively, and 210 μM O₂ in both cases (data represent mean ± S.D. for n = 3-4). Data were subjected to linear regression analysis and resulting fitted slopes were used in rate constant calculations.
(d[rI]/dt) was determined to be 0.9 (± 0.3) nM/h. Based on that value and other inputs ($a$, $d[NMor]/dt$, $[Mor^0]$ calculated above; $k_5$ listed in Table 3.1; rA remained nearly constant at 6 μM) equation 19 yields: $k_6 = 3.4 (± 1.0) \times 10^5$ M$^{-1}$s$^{-1}$. Extending the same strategy in the case of rX and rO, an adapted version of equation 19 yields the following rate constants: $k_7 = 7.1 (± 2.0) \times 10^6$ M$^{-1}$s$^{-1}$ and $k_8 = 1.7 (± 0.4) \times 10^6$ M$^{-1}$s$^{-1}$. This is the first time that kinetics of oxanine accumulation were determined when rG was exposed to precisely controlled levels of NO in vitro, and it is the first evidence for different levels of susceptibility to deamination for rA and rG. Closely similar rate constants – shown in Table 3.2 – were derived from data obtained when dA and dG were subsequently exposed in delivery reactors (Figure 3.8), which demonstrates that the sugar moiety of the nucleosides does not affect the deamination rates. The terms $k_6[rA]$, $k_7[rG]$ and $k_8[rG]$ are all much smaller than $k_4[P_i]$ which validates the simplification made in the denominator of equation 14; the same is true for terms $k_9[dA]$, $k_{10}[dG]$ and $k_{11}[dG]$.

### 3.3.4 Deamination kinetics in RNA and DNA

The rate of formation of deamination products in nucleic acids exposed to accurately defined N$_2$O$_3$ concentrations revealed a significant protective effect of macromolecular structure, in comparison to free nucleosides. Employing again the predictable conditions maintained in the NO-delivery reactor, pure RNA (5 μg/mL) and DNA (20 μg/mL) in Na-PO$_4$ buffer were separately exposed to constant femtomolar levels of N$_2$O$_3$ for several hours while 2.5 mL aliquots were withdrawn from the reaction vessels at regular intervals. Collected samples were subsequently hydrolyzed to mixtures of nucleosides with a combination of enzymes discussed in the Methods section, while the target lesions – rI, rX, rO in RNA experiments and dI, dX, dO in DNA samples – were resolved using LC pre-purification strategies followed by accurate quantification through LC-MS/MS. The time course of deaminated nucleobases accumulating in
Table 3.2. Deamination rate constants resulting from the reaction of N$_2$O$_3$ with free nucleosides at 37 °C. Data represent mean ± S.D. for n = 3.

<table>
<thead>
<tr>
<th>rate constant</th>
<th>Value (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ribonucleosides</strong></td>
<td></td>
</tr>
<tr>
<td>$k_6$ (rI)</td>
<td>$3.4 (± 1.0) \times 10^5$</td>
</tr>
<tr>
<td>$k_7$ (rX)</td>
<td>$7.1 (± 2.0) \times 10^6$</td>
</tr>
<tr>
<td>$k_8$ (rO)</td>
<td>$1.7 (± 0.4) \times 10^6$</td>
</tr>
<tr>
<td><strong>2-deoxyribonucleosides</strong></td>
<td></td>
</tr>
<tr>
<td>$k_9$ (dI)</td>
<td>$3.6 (± 0.8) \times 10^5$</td>
</tr>
<tr>
<td>$k_{10}$ (dX)</td>
<td>$9.7 (± 2.5) \times 10^6$</td>
</tr>
<tr>
<td>$k_{11}$ (dO)</td>
<td>$3.0 (± 0.9) \times 10^6$</td>
</tr>
</tbody>
</table>
Deamination products dI, dX and dO formed during exposure of dA (6 μM) and dG (6 μM) to 1.7 μM and 210 μM O₂ (data represent mean ± S.D. for n = 2-3).

**Figure 3.8.** Time course for the formation of dI, dX and dO from dA and dG exposed to NO and O₂.
N$_2$O$_3$-treated RNA and DNA throughout exposures is summarized in Figure 3.9. As observed for nucleosides, guanine is relatively more susceptible to nitrosative deamination than adenine. Examining the fitted slopes in Figure 3.9 also indicates that the reactivity of RNA nucleobases is comparable to those in DNA, hence the single- or double-stranded structure (i.e. differences in the degree of base pairing) does not seem to significantly affect the rate at which nucleobases in nucleic acids are attacked by N$_2$O$_3$. The rate constants for the reaction between N$_2$O$_3$ and purines in RNA and DNA can be calculated from variations of equation 19. Focusing on rI formation in RNA, for instance, the least squares fit on the product accumulation rate was estimated at 8.2 (± 0.7) × 10$^{-7}$ μM/min (Figure 3.9) while the initial concentration of the parent nucleoside, [rA], at approximately 3.7 μM based on the total amount of RNA in solution. Taking into account measurements of Mor nitrosation performed in identical conditions discussed previously, the rate constant for the reaction between N$_2$O$_3$ and adenosine in RNA was found to be $k_{12} = 2.9$ (± 0.2) × 10$^4$ M$^{-1}$s$^{-1}$. A similar set of calculations in the case of rX and rO formed in RNA, as well as for dI, dX and dO in DNA, led to the rate constants listed in Table 3.3 which facilitates a more quantitative comparison of nucleobase reactivity in polymeric nucleic acids and their corresponding monomers (see Table 3.2).

### 3.4 Discussion

The multitude of reactive species resulting from NO generation in vivo adds layers of complexity to its multifaceted role as a free radical in human physiology [12,56]. In particular, nitrosative stress expected at sites of NO overproduction (e.g. inflamed tissues) may affect a host of cellular components, among which nucleic acids are crucial for both maintaining proper genetic information and controlling gene expression. While nitrosative deamination of nucleobases has been studied using plasmid DNA and oligonucleotides as model substrates,
**Figure 3.9.** Time course for the formation of purine deamination products in RNA and DNA exposed to NO and O₂.

Kinetic profile of rI, rX, rO formation in total RNA (5 μg/mL) and dI, dX, dO formation in genomic DNA (20 μg/mL) during exposures to 1.7 μM NO and 210 μM O₂ (data represent mean ± S.D. for n = 3). Data were subjected to linear regression analysis and resulting fitted slopes (solid lines for product formation in RNA and dashed lines in DNA) were used for rate constant calculations ($k_{12}$, $k_{13}$, $k_{14}$ for deamination occurring in RNA and $k_{15}$, $k_{16}$, $k_{17}$ in DNA).
Table 3.3. Deamination rate constants derived from the reaction between N₂O₃ and nucleosides in RNA and DNA at 37 °C. Data represent mean ± S.D. for n = 3.

<table>
<thead>
<tr>
<th>rate constant</th>
<th>value (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td></td>
</tr>
<tr>
<td>( k_{12} (rI) )</td>
<td>( 3.3 (± 1.0) \times 10^4 )</td>
</tr>
<tr>
<td>( k_{13} (rX) )</td>
<td>( 1.0 (± 0.2) \times 10^6 )</td>
</tr>
<tr>
<td>( k_{14} (rO) )</td>
<td>( 4.8 (± 1.2) \times 10^5 )</td>
</tr>
<tr>
<td>DNA</td>
<td></td>
</tr>
<tr>
<td>( k_{15} (dI) )</td>
<td>( 3.0 (± 0.7) \times 10^4 )</td>
</tr>
<tr>
<td>( k_{16} (dX) )</td>
<td>( 1.2 (± 0.3) \times 10^6 )</td>
</tr>
<tr>
<td>( k_{17} (dO) )</td>
<td>( 5.9 (± 1.4) \times 10^7 )</td>
</tr>
</tbody>
</table>
there has been no evidence so far as to whether this type of chemistry affects RNA differently from DNA. Here we used a system for controlled delivery of NO (described extensively elsewhere [37,47]) to closely simulate the microenvironment believed to occur at sites of inflammation mainly due to activated macrophages [4] and maintain the steady conditions necessary to study the kinetics of deamination of nucleobases in polymeric nucleic acids and their corresponding monomers. In parallel, optimized experimental conditions permitted the rigorous quantification of an elusive nucleobase, oxanine, thus expanding the spectrum of observable deamination products in nucleic acids.

3.4.1 Formation of oxanine

Oxanine was initially detected as a secondary deamination product forming concurrently with xanthine when dG and DNA were incubated with nitrous acid (pH ~4) or when buffer solutions of dG were exposed to NO gas [14,17]. That evidence prompted a series of focused investigations in NO-treated human cells [20] and tissues from a mouse model of inflammation [21] aiming to measure levels of deamination products accumulated in cellular DNA due to nitrosative stress experienced in both instances. The experimental methods employed during those studies however were unable to detect any dO in the genome (as opposed to dX which was quantified to reach a level of ~5 lesion per $10^6$ nucleobases), leading the authors to the conclusion that the double-stranded structure of DNA in neutral pH may have prevented the formation of oxanine, a hypothesis supported by theoretical studies of guanine deamination involving a proposed ring-opened intermediate that closes to form either of the two products [57]. The previously noted absence of oxanine stands in direct contrast with results in the current work that demonstrate simultaneous formation of xanthine and oxanine in solutions of free nucleosides, RNA and DNA in neutral pH buffer when exposed to controlled levels of NO.
(Figure 3.7, Figure 3.8, and Figure 3.9). In retrospect, this disagreement can be explained by the fact that previous studies in cells and tissues used certain amines (spermine, spermidine in ref [20]; deferoxamine in ref [21]) throughout their enzymatic hydrolysis protocols as antioxidants and chelating agents. It is evident from data presented here (Figure 3.1) that millimolar levels of deferoxamine can significantly degrade dO within a fraction of the total incubation time. Hence it is possible that the aforementioned antioxidants had a similar effect on dO nucleosides present in samples derived from hydrolyzed DNA. It is possible that this specific type of degradation affected the examined samples and led to reduced amounts of intact dO, well below the limit of detection of the quantification methodologies employed. We argue that the published protocols employing amine-based antioxidants mentioned above, and elsewhere (e.g. [46]), may have inadvertently and selectively hindered the accurate determination of dO in samples of NO-treated nucleic acids (stability of dI, rI, dX and rX unaffected by deferoxamine; data not shown) whereas the present study avoided this issue altogether by omitting deferoxamine (or any type of primary amine antioxidant) from the hydrolysis procedure. Consequently, improved protocols need to be applied in future iterations of experiments that will re-examine the possibility of oxanine formation as a product of in vivo NO-associated deamination. This possibility is strongly supported by our results indicating accumulation of rO in RNA extracted from NO-treated human cells discussed in a later chapter.

3.4.2 Conversion of oxanine to xanthine

The current findings showing oxanine rearranging to xanthine in the presence of deferoxamine may complicate the interpretation of results related to previous studies of guanine deamination. As mentioned previously, nitrosation of guanine by N₂O₃ is proposed to yield a ring-opened cyanoimino-oxomethylene-imidazole intermediate [57] that closes again and gives
rise to either xanthine and oxanine as stable end products. The electrophilic C-6 position of the oxanine nucleobase however was found to be sensitive to a nucleophilic attack by primary amines (e.g., by addition of glycine in previous studies [54] and deferoxamine examined above), which leads to a different proposed ring-opened diamide derivative (Figure 3.3). This clearly suggests that oxanine is a labile nucleobase under certain conditions or environments and it presents significant challenges to its quantification, which might explain the contradictory evidence from several recent studies aiming to detect its presence in nucleic acids. The stability of the resulting diamide derivative is not yet fully explored, which raises the important question of whether the given conditions can reversibly lead back to ring closure. Previous studies claimed that when NH$_3$ is added in the presence of dO (i.e. R = H in Figure 3.3) the ring-opening reaction is expected to be irreversible [58], probably due to the inert nature of the resulting proximal amide moieties. Conversely, our data suggest that when a larger amine (in this case deferoxamine) reacts with dO, a ring closure reaction may occur. Due to the movement of an oversized R-group (shown in Figure 3.10) the bond between the carbonyl and the nitrogen atom adjacent to R is forced to rotate extensively, freeing the nitrogen’s delocalized electrons and consequently allowing them to react with the electrophilic carbon on the opposite amide. In general, large groups connected to the amide nitrogen have been previously shown to affect the availability of its electron pair [59]; altered amide chemistry is also observed through steric distortion on amide linkages [60]. Hence, we deduce that the attached deferoxamine is responsible for enhancing the reactivity of the amide nitrogen which in turn accelerates the rate of collisions that eventually produce the observed amounts of dX. No dO formation is predicted by this scheme, which is consistent with our findings.

The reactions of oxanine decomposition in the presence of amines and simultaneous
Figure 3.10. Proposed ring closing mechanism that leads to xanthine formation.
formation of xanthine can take place even when oxanine is the only base present in solution, as suggested from our data displayed in Figure 3.5. The exocyclic amine attached to the C-3 position of the oxanine nucleobase is believed to attack the C-6 position of a neighboring oxanine molecule analogously to the amine deferoxamine, although a higher temperature is required to compensate for the apparent lower efficiency of the oxanine exocyclic amine as a nucleophile. Besides the obvious implications of exposing samples with oxanine nucleosides at room temperature – or higher – in solutions that contain primary amines (e.g. at millimolar levels), this observation also points to a potential issue with purified stock solutions of dO and rO used as standards for rigorous MS quantitation, among other uses. In the latter case, the composition of the standards may be affected by partial decomposition of oxanine and gradual formation of xanthine nucleosides if the solutions maintain ambient temperature for extended periods of time (or long term storage outside a freezer). Amine-associated degradation could theoretically affect the oxanine content of nucleic acids even during enzymatic hydrolysis without deferoxamine, especially since individual samples can contain up to ~2 mM of primary amines in a mixture of native nucleosides (e.g., calculated from ~50 μg fully hydrolyzed DNA in 100 μL). However, our studies did not show any significant degradation at 37 °C, consistent with what was observed in experiments containing solely dO, which indicates the relatively unreactive nature of primary amines in nucleobases at that temperature. Putting what was discussed above in perspective, there is a possibility that the challenge in detecting oxanine in vivo may also be partly due to the local environment (e.g. the nucleophilic primary amines of histone proteins in proximity to DNA nucleobases [61]) in addition to the analytical steps following the extraction of nucleic acids from cells. It would be interesting to examine, for example, whether accumulated dO in genomic DNA could be susceptible to degradation
catalyzed by the amine-rich nuclear microenvironment and if part of what previous studies measured as total dX was in fact derived from the dO-to-dX conversion that we discussed above.

3.4.3 Comparison of deamination rates in nucleosides and nucleic acids

The current work is the first comprehensive study that provides insight on the kinetics of N₂O₃ reacting with adenine and guanine, not only as nucleobases in RNA and DNA polymers but also in the form of the corresponding monomeric nucleosides. The biologically relevant conditions of the NO-delivery system (exploited in numerous recent investigations [20,38,47,62,63]) provide steady micromolar NO levels – similar to those believed to be generated by activated macrophages in inflamed tissues [4,29] – which in turn give rise to femtomolar concentrations of N₂O₃ in oxygenated aqueous solutions, as validated by measured rates of morpholine nitrosation observed here as well as elsewhere [28]. The controlled environment of constant temperature and near-neutral pH achieved in the delivery reactors throughout exposures, along with the conditions maintained during enzymatic hydrolysis of RNA and DNA (37 °C, pH 7.4, no addition of primary amine as antioxidant), is expected to limit depurination of xanthine nucleosides (t₁/₂ > 2 years; [64]) and amine-associated degradation of oxanine nucleosides to a minimum. Since the sources of artifacts currently known to skew the quantitation of deamination products are eliminated to the best of our knowledge, a graphical comparison of all 2nd order deamination rate constants listed in Tables 3.2 and 3.3, shown in Figure 3.11, is expected to more accurately depict differences in reactivity among all the species examined and thus lead to a number of new interesting observations. Firstly, the rate at which N₂O₃ reacts with the parent purines seems only marginally affected by the type of sugar attached to the nucleobases: isolating the case of free nucleosides, comparable rate constants were derived
Figure 3.11. Calculated rate constants for the formation of deamination products in free nucleosides, RNA and DNA.

Rate constant values are reproduced from Tables 3.2 and 3.3. Data represent mean ± S.D. for n = 3.
when both 2-deoxyribo- and ribonucleosides of adenine and guanine were used as substrates during experiments. Under common conditions, the reactivity of purine nucleobases in DNA proved to be virtually indistinguishable from that observed when RNA was exposed instead.

The current data seem to suggest that such differences in the ribose structure (i.e. presence or absence of a hydroxyl in the 2’ position) have a minor effect on the electron distribution of the adjacent nucleobase moiety and thus do not significantly alter the reaction rate between N$_2$O$_3$ and the exocyclic amino group of the native nucleosides. This is consistent with our findings described above in which the kinetics of the nucleophilic attack of deferoxamine on oxanine are only modestly affected by the nature of the ribose (Figure 3.2).

Another observation stemming from the rate constant values juxtaposed in Figure 3.11 is the difference in susceptibility to deamination between free nucleosides and nucleobases in nucleic acids. Our results indicate that, upon exposure to N$_2$O$_3$, deamination products are formed up to an order of magnitude faster when adenosine and guanosine are not constrained by the secondary structure of RNA or DNA. Although, it is conceivable that base pairing and stacking interactions in both types of macromolecules – to a different degree for each biopolymer – may protect the nucleobases from exposure to the surrounding solvent and reactive species, another factor appears to have the potential to contribute to the observed difference in reactivity: the anionic phosphate backbone. It was established in previous studies that phosphate anions cause a ‘scavenging’ effect for N$_2$O$_3$ [10], in essence competing with all other nucleophiles in solution that can react with the nitrosating agent, and lead to the rapid hydrolysis depicted in Equation 4 of the Methods section. It is possible that the extensive presence of negatively charged phosphodiester linkages throughout the entire length of the RNA and DNA molecules and in immediate proximity to nucleobases may be partially inhibiting deamination (i.e., through
intramolecular competition for \( \text{N}_2\text{O}_3 \) thus leading to the lower reaction rates relative to free nucleosides – which lack the protective effect of neighboring phosphate groups – as displayed in Figure 3.11. Our observations stand in contrast with an older study by Caulfield et al. that instead showed free 2-deoxynucleosides to have a ~5-fold lower reactivity compared to single-stranded oligonucleotides (yet a ~2-fold greater reactivity compared to double-stranded oligonucleotides) when exposed to NO [9]. Those results, however, were derived from experiments involving a) delivery systems with a headspace of air that might have affected the chemistry (allowing formation of \( \text{N}_2\text{O}_4 \) in the gas phase [65]), b) acidic hydrolysis protocols and c) chemical derivatization for detection via GC/MS. The much more controlled exposures and sensitive quantification strategies employed here eliminate the aforementioned possible sources of bias and establish more confidence in the interpretation of the current findings in that, under nitrosative stress, nucleosides are indeed more reactive than nucleic acids.

Seen from a different perspective, the comparable reactivities determined above for nucleobases in RNA and DNA also suggest that the combination of solvent exposure and base-pairing may be less significant than initially expected in terms of affecting nitrosative deamination in nucleic acids. Experiments performed by Caulfield et al. yielded a ~10-fold difference in calculated rate constants between single- and double-stranded oligonucleotides, with single-stranded oligos appearing more reactive, whereas only a ~2-fold difference was noted when comparing the much more solvent exposed 2-deoxynucleosides with double-stranded oligos [9]. Those conclusions, whose discrepancies can also be attributed to the biases already listed above, contradict the findings of the current research showing that deamination proceeds in purified double-stranded genomic DNA and single-stranded RNA (mostly comprised by ribosomal RNA) at comparable rates despite differing degrees of base-pairing (Figure 3.11). It is
not known to what extent the combination of tertiary structure, base stacking and non Watson-Crick interactions in purified single-stranded RNA promotes the formation of additional hydrogen bonds or minimizes exposure to the surrounding solvent, and thus how its nucleobase accessibility compares to that in DNA. Even in that case however, it is reasonable to anticipate uninhibited diffusion of the electrophilic N$_2$O$_3$ to the entire macromolecular structure (steric exclusion would be unlikely due to its small size) and its interaction with unpaired electrons in exocyclic amine groups e.g. of adenine and guanine; the primary amines of purines engaged in Watson-Crick base-pairing donate their hydrogens as shown in Figure 3.12 but the electron pair in the nitrogen of those moieties is not occupied by any bond, is still exposed to the solvent and may be prone to attack from electrophiles. In other words, the results presented here suggest that deamination by N$_2$O$_3$ will occur independently of pre-existing hydrogen bonding and consequently that nucleobases in both single- and double-stranded configurations may be equally susceptible to that particular electrophile. In particular, guanine’s exocyclic amine can be nitrosated even while in a base pair formation with a neighboring cytidine, presumably give rise to the opened ring intermediate proposed elsewhere [57] which can close back to yield both xanthine and oxanine in the confined environment of genomic DNA (or RNA), a possible mechanism that is consistent with our experimental observations.

As already noted, the well defined conditions maintained during our in vitro investigations allow extrapolation of recently gained insight on deamination chemistry directly to biological systems. Based on what has been discussed above, it is reasonable to anticipate accumulation of comparable levels of the two guanine deamination products in nucleic acids extracted from NO-treated cells – evidence for which will be shown in a later chapter – or even tissues from animal models of chronic inflammatory stress. From a biomarker perspective, it is
Figure 3.12. Hydrogen bond formation for guanine and adenine in double stranded DNA.
interesting to determine whether endogenous damage caused by deamination can be more readily detected in DNA or alternatively in RNA. Since our data suggest that the reactivity of purine nucleobases appears to be nearly equal in both types of nucleic acids (independently of their degree of base-pairing), we deduce that additional parameters such as accessibility and repair may be of critical importance when searching for the most efficient molecular surrogate. More specifically, RNA is broadly distributed within the cytosol while there are no known mechanisms for maintaining its fidelity (unlike DNA protected in the nucleus) which motivated recent studies to quantify damaged nucleobases in RNA, including lesions expected from deamination, when examining tissues extracted from mouse models of nitrosative stress [40]. Extending a similar rationale to free nucleosides, the most reactive substrates in vitro according to Figure 3.11, it is interesting to also survey the potential of nucleoside and nucleotide pools as indicators of imbalances forming in the cytosol e.g. due to deviating enzymatic pathways [27] or chemical challenges [66]; however efforts to quantify deamination products in free nucleosides so far have been hampered by technical difficulties.

Lastly, an examination of deamination rate constants derived from the current studies suggests that guanine is more reactive toward N_2O_3 than adenine. The difference in reactivity is noted in all types of nucleic acids and nucleosides considered, indicating that neither the degree of solvent exposure (associated with base pairing) nor the presence of secondary structure (inherent to phosphodiester linkages) appear to alter it. Since the type of the sugar attached (i.e., ribose vs. 2-deoxyribose) is also unlikely to play a major role in the rate of reaction (as was the case with the kinetic profiles discussed above), the observed pattern points toward the base moiety as the source of this dichotomy. Although a direct comparison of the structures for the two purines does not lead to an obvious contrast, the literature reveals that guanosine is
characterized by a proton dissociation equilibrium at the N-1 position under basic conditions (pKa ~9) [67] whereas such a process is absent in adenosine. Hence, in the steady and constant aqueous environment of the exposures presented here (pH = 7.4), a small but significant fraction of guanine nucleobases remain negatively charged (0.02 -0.2% as mentioned in ref. [68]). We hypothesize that the charge forming in those guanosine nucleobases may be responsible for influencing the reactivity of the unpaired electrons on the exocyclic amino group (by countering its delocalization in the ring) or alternatively for contributing to an electrostatic attraction between the negative ion and the attacking electrophile. The overall effect is the increased susceptibility of guanosine to N₂O₃ – compared with adenosine – which is consistent with results from older studies demonstrating either a higher relative yield for products of guanine deamination or an increased mutation frequency in G-C basepairs when nucleic acids or cells were exposed to NO through a battery of delivery strategies [8,15,19,69]. The apparent vulnerability of guanine under nitrosative stress combined with its propensity towards oxidative damage [70] may have severe implications for its integrity within nucleic acids of cells subjected to overwhelming levels of chemical insult at inflamed tissues; the apparent sensitivity of guanine could be another reason why polymerases have evolved to follow the “A-rule” during replication. Our findings stand in contrast with a recent kinetic analysis where deamination was calculated to proceed at identical rates (k = 1.2 × 10⁵ M⁻¹s⁻¹) for guanosine and adenosine in purified plasmid DNA [28] where the use of a less sensitive quantitation method (LC-MS as opposed to LC-MS/MS employed in the current studies) may have hindered the researchers from measuring the existing difference in reactivity.

In summary, we have demonstrated a strategy that makes use of a highly reliable and widely applicable NO delivery system to determine the susceptibility of purine nucleobases in
nucleosides, RNA and DNA to deamination. The well defined chemistry of the system provides constant and predictable concentrations of NO (~1 μM), O₂ (~200 μM) and N₂O₃ (10-100 fM) – biologically relevant levels encountered at sites of inflammation – and enables the study of nitrosative damage on a wide variety of biomolecules simulating reactions expected to occur in vivo. (a) The examination of a previously unrecognized oxanine-to-xanthine rearrangement pathway, (b) the observation that base-pairing and solvent exposure may not significantly protect nucleobases against nitrosative agents, (c) the evidence that oxanine forms as a result of RNA and DNA exposure to NO, (d) the conclusion that guanine reacts faster with N₂O₃ than adenine and (e) that nucleic acids are not as susceptible to deamination as free nucleosides, constitute new information derived from results in the current work.
3.5 References


Chapter 4
Formation of abasic sites in RNA linked to nitrosative stress

4.1 Introduction

As outlined in previous chapters, reactive species originating from activated macrophages in inflamed tissues are thought to cause damage to neighboring cells by generating reactive oxygen and nitrogen species. In particular, enzyme-dependent production of NO by activated macrophages is likely to give rise to several other oxidants and agents of nitrosation and halogenation [1,2], which in turn may create pathological lesions in a variety of cellular components (nucleic acids, proteins, lipids, etc). Nitrous anhydride (N₂O₃) is one of the most reactive and short-lived molecules associated with NO generation in oxygenated aqueous environments, and it is known to react with nucleophilic amino groups in nucleobases of nucleic acids [3,4] as well as other electron-rich compounds [5,6]. Nitrosation of exocyclic amino groups in nucleobases by N₂O₃ leads to the formation of several deamination products [1], extensively discussed in Chapter 3, and guanosine intrastrand cross-links [7], which can alter the sequence – and possibly constrain the secondary structure – of nucleic acids. In addition to deamination, N₂O₃ also causes formation of abasic (i.e., apurinic/apyridinic or AP) sites by nitrosation of the N⁷ in guanosine and N⁷ or N³ in adenosine, which weakens the glycosidic bond in the resulting cationic bases [8,9] (proposed mechanism for 2-deoxyguanosine nucleotide shown in Figure 4.1, adapted from ref. [9]). This reaction introduces a parallel pathway by which N₂O₃ can modify information preserved in nucleic acids.
Figure 4.1. Depurination results from nitrosation of the N-7 position in 2'-deoxyguanosine.

Nucleobase nitrosation leads to weakening of the glycosidic bond and formation of an abasic site, shown in equilibrium between its open and closed form.
Besides nitrosation, other conditions are known to cause formation of abasic sites mainly from purines [10], including acidic, oxidative and alkylating environments [11-13], with rates greater than those estimated to occur spontaneously \textit{in vivo} ($\sim 10^4$ per genome per day in mammalian cells [14]). Abasic sites are also formed from modified or damaged nucleobases as a result of the action of DNA $N$-glycosylases as intermediate products of the base excision repair pathway [15-18]. If they remain unrepaired in DNA, abasic sites are hydrolytically unstable and eventually lead to strand scissions ($t_{1/2} = \sim 8$ d; [19]). Due to their potential for DNA strand breaks and interference with genetic information, abasic lesions are highly mutagenic [20,21] and cytotoxic [22,23], representing a major threat to the integrity and survival of the cell, which would explain why organisms have evolved appropriate responses to eliminate them by employing AP-endonucleases or AP-lyases [24-26].

While DNA has been the major focus of investigation in abasic site formation, it is reasonable to assume that they can also form in RNA. The high concentrations of coding and non-coding RNA species in a cell, their wider cellular distribution, and the greater solvent exposure of RNA nucleobases suggest that such lesions could form in RNA as well as DNA under conditions of nitrosative or oxidative stress. This possibility implies that coding information may be lost due to depurination occurring not only in critical genes but also in various elements of the translational and transcriptional processes. The biological impact of abasic sites forming in RNA alone is apparent if one considers that changes in the sequence of coding and non-coding RNA species have consequences for gene expression at the level of translation, potentially affecting the function of the translational machinery [27,28], interfering with post-transcriptional RNA editing [29], or disrupting regulatory elements strongly correlated with the progression of different types of cancer [30-32].
To investigate how nitrosative conditions arising in the microenvironment of inflamed tissues may contribute to the formation of abasic sites, a reliable method that generates steady and predictable N\textsubscript{2}O\textsubscript{3} levels in a controlled laboratory setting is indispensable. Perhaps the best characterized and most broadly applicable system that mimics the nitrosative environment at sites of inflammation was developed by Deen and coworkers using NO- and O\textsubscript{2}-permeable polydimethylsiloxane (Silastic) tubing to deliver the gases at constant and predictable levels [33,34]. An accompanying mass transfer model was developed to predict the concentrations of NO and O\textsubscript{2} in the system given the values of certain operational parameters [33,35], and a subsequent kinetic analysis estimated the levels of N\textsubscript{2}O\textsubscript{3} formed in the solution [34], making the tubing-based delivery apparatus ideal for kinetic studies of nitrosative deamination (e.g. those described in Chapter 3 and elsewhere [34]). Experimentally measured cellular consumption rates for NO and O\textsubscript{2} [35] as well as recent findings demonstrating the negligible effect of cell culture media on device performance – delineated in Chapter 2 and reported in ref. [36] – extend the system’s application to cases that involve exposure of mammalian cells to well defined levels of NO and N\textsubscript{2}O\textsubscript{3}. The ease of use and the fact that delivery is unaffected by the unknown (yet potentially complicating) effects of the secondary chemical species generated upon NO release from NO-donors [37] are additional reasons for which the system has been employed in numerous previous studies [38-41], including the current work.

Besides well defined \textit{in vitro} exposure conditions, sensitive analytical methods are also crucial for gaining insight on the mechanisms of AP site formation in nucleic acids. While several strategies have been employed in the past to detect strand breaks and putative AP sites in DNA such as the \textsuperscript{32}P-post labeling assay [42], the comet assay [43] and the plasmid nicking assay [44], direct quantitation methods via aldehyde-reactive probes can be more informative
and specific. The conception and design of those probes was based on the notion that lesions with aldehyde (or ketone) groups react with conjugated oxyamines to form stable oxime derivatives that can be purified and measured in a variety of ways. One of the early methods exploiting this chemistry used a $^{14}$C-labeled methoxyamine [45] – with subsequent versions coupling quantification of oximation products with accelerator mass spectrometry [46] or antibodies [47] – whereas other types of strategies employed biotin conjugated probes, with detection made possible by ELISA [48,49], or variations of fluorescent aldehyde reactive probes [50-52]. Among several reactive probes, N-aminooxyacetil-$N'$-biotinyl hydrazine has been employed in several studies of oxidation-induced AP lesions in DNA [48,49,53,54], as well as more recently in RNA [55,56]. The major problems limiting use of these probes is the lack of chemical specificity, limited dynamic range of the detection system and the potential for artifacts arising from incomplete removal of unreacted probe. To overcome these problems, we can turn to analytical platforms involving chromatography-coupled tandem mass spectrometry, which have emerged as attractive approaches to sensitive quantitation of AP sites generated in damaged DNA [57]. Here we have developed a novel mass spectrometry-based approach for RNA abasic sites that takes advantage of the predictable and stable products that arise in reactions of reactive carbonyl species with the biotin-conjugated aldehyde reactive probe mentioned above (mechanism depicted in Figure 4.2).

Motivated by the paucity of knowledge about RNA abasic sites as products of inflammation, the goal of this work was to estimate the rate at which AP sites accumulate when RNA is exposed to well-controlled steady levels of $\text{N}_2\text{O}_3$, similar to those believed to occur in close proximity to inflamed tissues. The kinetic parameters describing the anticipated
Figure 4.2. Reaction between abasic sites and biotinylated aldehyde reactive probe (ARP).

A stable oxime product results from the reaction between the open form of an abasic site and the $N$-aminooxyacetyl-$N'$-biotinyl hydrazine aldehyde reactive probe.
degradation of glycosidic bonds will be compared to similar information previously derived from DNA under comparable levels of nitrosative stress. The methodology employed to measure AP sites in RNA was developed in collaboration with Dr. Wan Chan and complements recent sensitive and chemically specific mass spectrometry-based protocols [58] aiming to accurately measure the same lesion in DNA as an index of exposure to endogenous sources of nitrosative or oxidative stress.

4.2 Materials and Methods

4.2.1 Chemicals

Na₂HPO₄ and NaH₂PO₄ were obtained from American Bioanalytical, LC-MS grade acetonitrile from EMD, ethanol from Koptec. Glacial acetic acid and morpholine (Mor) were from Sigma (98% or higher purity). N-aminooxyacetyl-N'-biotinyl hydrazine was from Dojindo Molecular Technologies, Inc. Water purified through a Milli-Q system (Millipore) was used throughout the study.

4.2.2 Human cell cultures for total RNA extraction

TK6 human lymphoblastoid cells obtained from ATCC were grown in large cultures for the purpose of harvesting purified RNA. Cells were maintained in exponentially growing suspension cultures at 37°C in a humidified atmosphere of 95% air and 5% CO₂ in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated donor horse serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. Stock cell cultures were routinely passaged to maintain an optimal growth density (~1.0 × 10⁶ cells/mL) in 150 mm dishes. Cell pellets harvested by centrifugation were washed with phosphate buffer saline (PBS) and processed using Qiagen’s RNeasy Maxi Kits following the manufacturer’s instructions.
Extracted total RNA concentrations (2-4 mg per pellet containing ~600 × 10^6 cells) were determined by UV spectroscopy and stored at -80 °C until further use. RPMI-1640, L-glutamine, streptomycin /penicillin and PBS were obtained from Lonza, donor horse serum from Atlanta Biologicals, and trypan blue (4% in saline) for cell counting from Sigma.

4.2.3 NO/N₂O₃ delivery system

A delivery system described in full detail in Chapter 2 [33,36] was employed to expose purified RNA to accurately controlled constant levels of NO and O₂ (and N₂O₃). Briefly, two distinct gas streams (one for NO the other for O₂) were fed to reactors (modified 120 mL PFA screw-top jars from Cole Parmer) containing solutions of total RNA, with NO and O₂ diffusing separately into the liquid through gas permeable Silastic tubing loops (6-7 cm long, 1.96 mm o.d., 1.47 mm i.d. from Dow Corning). The flow rates were 100 standard cubic centimeters per minute (sccm) for the NO-feeding stream and 200 sccm for the O₂- feeding stream, for every reactor used. Gas flow rates were regulated via mass flow controllers obtained from Porter Instruments, Parker-Hannifin. The contents of each reactor were kept stirred using a magnetic stirrer plate from IKA (stirring speed at 100 rpm; stir-bar placed in every reactor) and maintained at 37 °C within a water bath. Mixtures of 10% NO in argon, 50% O₂ in N₂ (and 5% CO₂) and pure argon (for controls) were obtained from Airgas.

4.2.4 Exposing RNA and morpholine to NO

The nitrosative conditions achieved in the delivery system described above were exploited to induce generation of abasic sites in RNA. Stock solutions of total RNA, derived as described above, were washed twice with purified water using spin-filters (NanoSep 10K Omega, Pall) before being treated in the delivery system. Solutions of washed total RNA (9.5
μg/mL) in 50 mM sodium phosphate (Na-PO₄) buffer pH 7.4 (prepared from Na₂HPO₄, NaH₂PO₄ as described in ref. [59]) were exposed in reactors (~115 mL solution per reactor). A mass transfer model that simulates the system’s performance [33,36] was used to determine lengths of tubing loops necessary to achieve constant physiological levels of the dissolved gases in each reactor: steady-state NO concentration of ~1.7 μM was reached by passing 10% NO in argon through a 7.0 cm Silastic tubing loop and 50% O₂ in N₂ (with 5% CO₂) through a 6.3 cm loop led to constant oxygen levels (~210 μM) throughout the exposures. At various time points after initiating gas flows, 2.5 mL aliquots were withdrawn from each reactor and replaced with an identical volume of fresh solution. All buffers and reactors were autoclaved before exposures took place to completely neutralize the activity of remaining RNases; samples were properly handled to avert artifactual RNA degradation.

The rate of NO-induced formation of abasic sites in RNA was compared to rates of morpholine (Mor) nitrosation for the purpose of accurately calculating the corresponding rate constant, as shown below. The experimental procedure involving exposure of 2 mM Mor solutions (in 50 mM Na-PO₄ buffer pH 7.4) to 1.7 μM NO and 210 μM O₂ in the delivery reactor was previously described in full detail in section 3.2.5 of Chapter 3.

4.2.5 Derivatization of RNA with aldehyde reactive probe

The biotinylated aldehyde reactive probe (ARP) was employed to derivatize the abasic sites present in RNA samples, based on a modification of a recent protocol [56] and the probe manufacturer’s instructions. RNA aliquots collected during NO exposures (9.5 μg/mL in 50 mM Na-PO₄ pH 7.4) were concentrated ~3-fold under reduced pressure before adding N-aminooxyacetyl-N'-biotinyl hydrazine to a final concentration of 2 mM. Samples were incubated
at 37 °C for 1 h and the derivatized RNA solutions were filtered through NanoSep 10K spin filters, with the RNA (retentate residing on the filter membrane) redissolved in 60 μL water; resuspended RNA samples were subsequently quantified by UV spectroscopy before hydrolysis to nucleosides.

4.2.6 Enzymatic hydrolysis of RNA

Derivatized RNA samples (~20 μg) were hydrolyzed to nucleosides employing a variation of a previously developed protocol [60]. Briefly, a combination of nuclease P1 (Sigma), phosphodiesterase I (USB) and alkaline phosphatase (Sigma) were added in the presence of antioxidants deferoxamine and butylated hydroxytoluene (Sigma) and deaminase inhibitors coformycin (DTP/NCI) and tetrahydrouridine (Calbiochem), as described previously [61] (no 15N-labeled internal standards added). Enzymes were removed at the end of the hydrolytic digestion process by NanoSep 10K spin filters and filtrates were concentrated under reduced pressure to a final volume of 50 μL before analysis by LC-MS/MS.

4.2.7 LC-MS/MS quantification of derivatized abasic sites in hydrolyzed RNA (in collaboration with Dr. Wan Chan)

Levels of ARP-derivatized AP sites in hydrolyzed RNA samples were measured using an Agilent 1100 series HPLC system interfaced with an AB Sciex API 3000 tandem quadrupole MS with a turbo ion spray source. Resolution of derivatized AP sites was achieved on a Thermo Fisher Hypersil Gold aQ C18 reverse-phase column (150 x 2.1 mm, 3 μm particle size) using 0.1% acetic acid (solvent A) with a stepwise increasing gradient of pure acetonitrile (solvent B), at a 0.2 mL/min flow rate; details of the chromatographic method are given in Table C.1 (Appendix C). The MS was operated in positive ion mode with the first and third quadrupoles
fixed to unit resolution. Multiple reaction monitoring (MRM) mode was used for sample
detection with dwell times set to 200 ms. Parameters for the mass spectrometer were optimized
for maximal sensitivity and are displayed in Table C.2 (Appendix C). High mass accuracy
experiments were performed on a AB Sciex QSTAR XL quadrupole TOF mass spectrometer
with a standard electrospray ionization interface.

A linear external calibration curve ($r^2 > 0.99$) was generated using a synthesized standard
for the ribose-ARP conjugate (Figure 4.3). The standard was generated by reacting 1mM ribose
and 10 mM ARP in 50 mM Na-PO$_4$ (pH 7.4) at room temperature for ~8 hours, subsequently
purified by LC (using the reverse-phase column mentioned above), based on a variation of a
previously used protocol [56].

### 4.2.8 Detection of morpholine nitrosation

In morpholine (Mor) nitrosation experiments, the formed product $N$-nitroso-morpholine
(NMor) was quantified by UV absorbance as described in section 3.2.9 of Chapter 3.

### 4.2.9 Reaction scheme for NO-induced nitrosation and formation of abasic sites

The reaction scheme is identical to the one defined by Equations 1-5 in section 3.2.11 of
Chapter 3. Briefly, the reactions of interest include generation of N$_2$O$_3$ from NO and O$_2$ and its
consumption by inorganic phosphate in the buffer and morpholine (when present in the solution).
Alternatively, when RNA is exposed to N$_2$O$_3$ in the absence of Mor, the following reaction is
considered where nitrosated purine nucleosides in RNA give rise to apurinic (AP) sites (based on
the mechanism presented in Figure 4.1):
Figure 4.3. (A) Biotinylated ARP-ribose conjugate ($m_{total} = 463$) with annotated fragments ($m_1 = 332$, $m_2 = 227$) and (B) linear calibration curve for the ARP-ribose standard.

Limit of quantification $\sim 2.4$ fmol and limit of detection $\sim 0.8$ fmol derived from the calibration curve (data represent mean $\pm$ S.D. for $n = 3$).
Adenosine and guanosine in RNA are lumped into the term purines of reaction A while abasic site formation resulting from nitrosation of pyridines is assumed insignificant compared to purines [10]. Reaction A occurs in parallel to the deamination reactions analyzed in detail in Chapter 3, however AP site formation is much slower than deamination, as will be discussed below.

Rate constants for reactions 1-5 are listed in Table 3.1 in section 3.2.12 of Chapter 3 whereas $k_{AP}$ was derived by comparing observed rates for NMor and AP formation under the same nitrosative conditions.

4.2.10 Kinetics of nitrosation and AP formation – estimation of concentrations and rate constant

Following the same analysis as the one delineated in section 3.2.12 of Chapter 3 and applying the rate law to reaction A defined above (formation of AP lesions from purines in RNA in lieu of nucleobase deamination products) yields the following equation:

$$k_{AP} = \alpha k_5 \frac{[\text{Mor}^0]}{([rA]+[rG])} \frac{d[\text{AP}]}{dt} \frac{d[\text{NMor}]}{dt} \quad (B)$$

Equation B is used to calculate the rate constant for AP accumulation ($k_{AP}$), as long as slopes of AP and NMor formation are experimentally available, and the nitrosation products do not significantly affect the initial concentration of the reactants (which was the case in the...
present experiments). Parameter $\alpha$, i.e. the ratio of the bulk steady-state $\text{N}_2\text{O}_3$ concentration in Mor experiments to that estimated in RNA experiments, was also previously defined in section 3.2.12 of Chapter 3.

4.3 Results

4.3.1 Development of LC-MS/MS method to quantify RNA abasic sites (by Dr. Wan Chan)

The major fragment ions observed when the synthesized standard for the ribose-ARP conjugate (depicted in Figure 4.3) was analyzed by collision-induced-dissociation are shown in Figure 4.4, a typical product ion scan of the molecular ion (m/z 464) in positive ionization mode. The predominant ion peak in Figure 4.4 (m/z 227) is likely derived from cleavage at one of the amide bonds of the biotinylated probe (m/z 332), illustrated in Figure 4.3. Also shown in Figure 4.4 is the high-accuracy MS analysis of the ribose-ARP standard which agrees with a previously measured m/z value (464.2) of the same conjugate [56].

Positive ionization mode with the multiple-reaction-monitoring (MRM) transition at m/z 464→227 was used to measure AP sites in hydrolyzed RNA samples; the MS/MS parameters were optimized by flow injection analysis (values shown in Table C.2 of Appendix C). Figure 4.5 shows representative ion peaks resulting from the LC-MS/MS analysis of the ribose-ARP conjugate standard and samples from NO- and Argon-treated hydrolyzed RNA. A linear external calibration curve was generated using the ribose-ARP standard (Figure 4.3) and the method’s quantification limit was found to be ~2.4 fmol or ~4 lesions per $10^8$ nucleotides (based on 20 $\mu$g RNA).
Figure 4.4. (A) MS/MS and (B) high accuracy MS spectrum for the biotinylated ARP-ribose conjugate.
Figure 4.5. Extracted ion chromatograms of the MRM transition at m/z 464→227 derived from representative samples.

(A) Biotinylated ARP-ribose conjugate, (B) hydrolyzed NO-treated RNA and (C) hydrolyzed argon-treated RNA. Chromatographic conditions described in the Methods section.
4.3.2 Determination of $N_2O_3$ concentration in the NO-delivery system

The conditions in the delivery device (buffer composition, temperature, steady-state NO and $O_2$ concentrations, etc) established during experiments aiming to investigate the NO-induced formation of abasic sites in RNA are identical to those specified in Chapter 3 regarding the deamination of nucleic acids and nitrosation of Mor. Hence, the NMor formation data shown in Figure 3.6 of Chapter 3 and the calculations described in section 3.3.2 of Chapter 3 can be re-applied to calculate the $N_2O_3$ concentration in the reactor (i.e., $[N_2O_3] = 39$ fM in the bulk liquid region with $[N_2O_3] = 59$ fM an effective increase in the aforementioned value due to the presence of the boundary layer) as well as the values for some of the parameters required in Equation B, namely $\alpha = 0.76$, $d[NMor]/dt = 0.105 \mu M/\text{min}$ and $[Mor^o] = 274 \mu M$.

4.3.3 Kinetics of abasic site formation in RNA exposed to nitrosative conditions

Based on evidence from previous studies indicating that a nitrosative environment leads to depurination of DNA [8,9,34], the accumulation of abasic sites was measured in RNA exposed to well defined $N_2O_3$ concentrations. Employing the predictable conditions maintained in the NO-delivery reactor, solutions of purified RNA (9.5 $\mu g/mL$) in Na-PO$_4$ buffer were exposed to steady femtomolar levels of $N_2O_3$ for several hours while 2.5 mL aliquots were withdrawn from the reaction vessels at regular intervals. Collected RNA samples were first treated with a biotinylated probe (Figure 4.2) that reacts with the aldehyde groups expected to arise at sites along the macromolecule where nucleobases are lost due to nitrosation. The derivatized samples were subsequently hydrolyzed to nucleosides with a combination of enzymes discussed in the Methods section. Levels of probe-derivatized ribose were then quantified using a sensitive LC-MS/MS approach and results revealed a time-dependent increase
of N₂O₃-induced apurinic lesions. The time course of AP sites accumulating in RNA throughout the exposure is shown in Figure 4.6.

A linear least squares fit on the data set presented in Figure 4.6, which indicate a negligible consumption of parent RNA, allowed calculation of the corresponding rate constant \(k_{\text{AP}}\). The fitted slope \((d[\text{AP}]/dt)\) was determined to be \(1.6 \pm 0.3 \times 10^{-8} \text{μM/min}\) while \(r_A\) and \(r_G\) remained nearly constant at approximately 7 μM each, based on the total amount of RNA in solution. Incorporating the values of parameters discussed above \((\alpha, d[N\text{Mor}]/dt, [\text{Mor}^\circ])\) as well as the rate constant for Mor nitrosation \(k_5 = 6.5 \times 10^7 \text{M}^{-1}\text{s}^{-1}\) at 37 °C; listed in Table 3.1 in section 3.2.12 of Chapter 3), equation B yields: \(k_{\text{AP}} = 1.5 \pm 0.3 \times 10^2 \text{M}^{-1}\text{s}^{-1}\).

To our knowledge, this is the first time AP site formation is observed in RNA exposed to precisely controlled levels of NO \textit{in vitro} and it is the first quantitative estimate of the rate constant describing cleavage of glycosidic bonds in RNA due to nitrosative stress.

4.4 Discussion

Overproduction of NO in the microenvironment of inflamed tissues is believed to lead to a battery of reactive chemical species [1,2,62] that can potentially attack many elements controlling function and structure in host cells. Among those reactants, the nitrosating agent N₂O₃ has been shown \textit{in vitro} and \textit{in vivo} to damage nucleic acids [4,34,38,61,63], which raises questions about its role in interfering with storing genetic information and regulation of cell division and growth. In particular, nitrosation of nucleobases in DNA predominantly gives rise to deamination products (discussed in detail in Chapter 3). However, there is evidence that the same type of chemistry results in cleaved glycosidic bonds in purines and the formation of basic sites [9,34]. Supporting the concept that RNA is susceptible to the same types of damage as DNA, we
**Figure 4.6.** Time course for the formation of abasic sites in RNA exposed to NO and O$_2$.

Abasic site formation in total RNA (9.5 μg/mL) during exposure to 1.7 μM NO and 210 μM O$_2$ (data represent mean ± S.D. for n = 3). Data were subjected to a linear regression analysis and the resulting fitted slopes were used for the rate constant calculation.
investigated the rate at which apurinic lesions accumulate in total RNA exposed to well-defined levels of N$_2$O$_3$. To achieve this, we used a system for controlled delivery of NO (described extensively in Chapter 2 [33,36]) to simulate the nitrosative conditions believed to occur in proximity to sites of inflammation mainly because of activated macrophages [62] and maintain the steady conditions necessary to study the kinetics of AP site formation in RNA.

One of the crucial components to measuring levels of AP sites was the selection of an aldehyde-reactive probe that could efficiently capture the presence of base-free sugars along the nitrosatively damaged RNA macromolecules. We chose $N$-aminooxyacetyl-$N'$-biotinyl hydrazine since this particular probe was previously used to detect AP sites in DNA [48] and, although several modified probes have since been developed exploiting the reactivity of the aminooxy group towards aldehyde-containing open-ringed deoxyribose [64], because its reactivity with abasic sites in RNA has been recently investigated [55,56]. Employing the biotinylated aldehyde-reactive probe in DNA blot analyses, Tanaka et al. established that RNA abasic sites were readily generated in vitro. This raised the possibility of glycosidic bonds being cleaved in RNA when it is exposed to various sources and levels of oxidative stress in vivo, similarly to what has been observed with DNA. The current work used the same probe to show that formation of abasic sites can also be detected in RNA exposed to nitrosating agents, consistent with experimental findings suggesting that nitrosated purines in DNA lead to the formation of base-free deoxyribose [8,9,34]. These results (in parallel with what was presented in Chapter 3) reinforce the notion that, despite major differences in structure and distribution in the cellular milieu, RNA and DNA are susceptible to the same type of damaging chemistries and these chemistries give rise to the same types of lesions or adducts (e.g. 8-OH-guanine, deamination products, apurinic sites). Secondly, exposure to NO seems to be associated with the
formation of abasic sites in both types of nucleic acids, adding nitrosation to the list of chemical stresses that likely lead to depurination in vivo [2]. This raises the question of whether a synergistic phenomenon resulting in considerably higher levels of the particular damage could take place in inflamed tissues, where overproduction of NO coincides with the abundance of oxidants.

Formation of apurinic sites is considered to be a biologically important DNA damage product [10]. It is believed that oxidative modifications on nucleobases weaken the glycosidic bond, leading to the generation of base-free sugars that contain an aldehyde group at the 1’ position of the open ring form of 2-deoxyribose. Alternatively, base-free 2-deoxyribose is formed by DNA glycosylases that cleave the glycosidic bond connecting the sugar to a damaged base [15-18]. These abasic sites are eventually removed from the macromolecule by abasic site endonucleases as part of the cellular base excision repair system [24-26]. However, abasic lesions formed in RNA – as a result of oxidative [56] or nitrosative stress – are presumably not subjected to the same protective mechanisms of repair and could potentially be more persistent in the cellular environment due to their greater chemical stability, compared to those in DNA [65]. If created and maintained in the cellular environment, abasic lesions may have a significant effect on the translation process in vivo as suggested by the impact of enzymatic mRNA and rRNA depurination on translational activity [66,67]. The implications of damage in the form of base-free ribose in RNA extend to inaccuracies concerning the information stored in coding and non-coding transcripts, interference with RNA editing processes [29], the response of the translational machinery under cellular stresses [27,28], and imbalances over regulating elements linked to the progression of cancer [30-32].
We have developed a sensitive LC-MS/MS approach to quantifying RNA abasic sites. The limit of quantification of this method is ~2 fmol or ~4 lesions per \(10^8\) nucleotides in 20 µg of RNA (Figure 4.3), which is lower than the chemically non-specific blot quantification method of Tanaka and coworkers, which has a limit of detection of 10 fmol (1 lesion per \(10^5\) nucleotides in 100 µg of RNA) reported in ref. [56]. This sensitivity enabled us to quantify low levels of apurinic sites in hydrolyzed RNA damaged by controlled levels of N\(_2\)O\(_3\). The data presented above are the first indicating that RNA exposed to nitrosative conditions can generate abasic sites, which is consistent with previous exposures of plasmid DNA in a similar experimental setting [34]. To gain a quantitative measure of the rate at which these lesions form under the predicted steady N\(_2\)O\(_3\) levels, we employed a kinetic analysis to determine a rate constant for the accumulation of AP sites (\(k_{\text{AP}} = 1.5 \times 10^2\) M\(^{-1}\)s\(^{-1}\)). Performing the same analysis on the data reported by Dong et al., we calculated the corresponding kinetic parameter to be approximately 50-fold greater in DNA (8 \(\times\) \(10^3\) M\(^{-1}\)s\(^{-1}\); assuming a typical activation energy of 20 kJ/mol to extrapolate from 23 °C to 37 °C), which is consistent with the observation that AP sites form spontaneously in RNA more slowly than in DNA [12,68]. The well defined conditions maintained during the \textit{in vitro} investigations undertaken in the current work allow extrapolation of recently gained insight on apurinic site formation to biological systems. Hence, assuming that nitrosation and formation of apurinic sites are similar \textit{in vitro} and in cells, it is reasonable to anticipate at least an order of magnitude greater accumulation of abasic sites in DNA compared to RNA extracted from e.g. NO-treated cells or tissues from animal models of chronic inflammatory stress. From a biomarker perspective, it is not directly apparent whether AP sites can be more readily detected in DNA or RNA. Our current data suggest that there is a difference in how fast nitrosated nucleobases are lost from the two types of nucleic acids. However,
parameters such as accessibility and repair may be of critical importance when deciding on the
most efficient molecular surrogate. At any rate, depurination induced by nitrosation turns out to
be orders of magnitude slower than nitrosative deamination (rate constants determined in
Chapter 3 range between \( \sim 10^4 \) and \( \sim 10^7 \) M\(^{-1}\)s\(^{-1}\)), at least as measured under our experimental
settings and methods, making apurinic sites less promising as biomarkers compared to
deamination products. On the other hand, it is possible that the methodologies using aldehyde-
reactive probes underestimate the total number of base-free sugars accumulating in nucleic acids
since the majority of abasic sites exist at all times in a hemiacetal (ring-closed) form [11,69,70]
rather than the aldehyde (ring-opened), with only the latter being able to react with the
aforementioned probes.

Lastly, the protocol presented in this work can increase our understanding of the
generation and fate of abasic sites in the intracellular environment. As mentioned already, the
majority of the studies investigating formation of apurinic sites maintain their focus on DNA as a
substrate while only a subset of those have the necessary sensitivity and chemical specificity to
unequivocally quantify biologically relevant levels of the particular lesion [57,58]. An equally
effective method measuring abasic sites in RNA not only addresses the recent need to monitor
RNA species as targets of the same nitrosative or oxidative chemistries that damage the genome
[71] but also can reveal potential protective effects associated with the cytosol. If experimentally
measured levels of AP sites in DNA and RNA extracted from challenged cells reflect the
difference in reactivity established \textit{in vitro}, then the cellular milieu can be assumed to be
spatially homogeneous in terms of dealing with the imposed insult. On the other hand, in the
event that formation of AP sites in RNA is determined to be faster compared to DNA, a number
of questions can be raised regarding a) the compartmentalization of the cell and how it affects
concentrations of antioxidants, b) the possibility of nuclear proteins limiting the exposure of DNA to damaging agents, c) a possible synergistic effect originating from mitochondria and amplifying the observed damage, and d) differential rates of repair linked to different types of nucleic acids. In any case, the progress made in measuring AP sites in other species besides DNA can assist in finding more accurate markers of exposure to endogenous sources of nitrosative or oxidative stress.

In summary, we have developed a platform to quantify the rate at which purine nucleobases in RNA give rise to abasic sites in a controlled nitrosative environment. The delivery system generates constant and predicable concentrations of NO (~1 μM), O₂ (~200 μM) and N₂O₃ (~100 fM) – comparable to levels expected to form at sites of inflammation – that lead to the accumulation of several lesions along the macromolecule, including the base-free ribose presumed to result from the cleavage of nitrosated purines. Subsequently, a specialized derivatizing agent that reacts with the aldehyde moiety of the open form of an apurinic site in combination with a sensitive LC-MS/MS method enables the detection of the lesion in hand. The kinetic profile of abasic site formation in RNA revealed a slow dose-response increase, indicating that nitrosation of RNA does indeed lead to depurination, and the calculated relevant kinetic parameter, \( k_{\text{AP}} = 1.5 \pm 0.3 \times 10^2 \text{M}^{-1}\text{s}^{-1} \), suggests that genesis of base-free sugars from nitrosated purines is a slower process in RNA compared to DNA.
4.5 References


Chapter 5

Estimation of cytosolic levels of \( \text{N}_2\text{O}_3 \) in human cells under nitrosative stress

5.1 Introduction

Nitric oxide (NO) is a free radical with diverse physiological roles in mammals, including regulation of vasculature, signal transmission between neurons, and pathogen killing through the innate immune system [1-3]. In particular, micromolar NO concentrations generated at sites of inflammation [4-7] are believed to give rise to a battery of reactive nitrogen species [8] that not only attack foreign microorganisms but also cause damage to surrounding host cells.

Peroxynitrite (\( \text{ONOO}^- \)) is formed in a reaction of NO with superoxide (\( \text{O}_2^- \)), whereas the nitrogen dioxide radical (NO\(_2\)) and nitrous anhydride (\( \text{N}_2\text{O}_3 \)) are produced from NO in the presence of molecular oxygen [9]. These reactive chemical mediators of inflammation can damage all types of cellular components, including lipids, nucleic acids and proteins [10], while elevated levels of these species sustained over prolonged periods of time are implicated in the initiation and progression of diseases linked to chronic inflammation, such as cancer [11-14]. In contrast to the oxidizing properties of \( \text{ONOO}^- \), \( \text{N}_2\text{O}_3 \) is a potent nitrosating agent (Table 1 in ref. [8]) that reacts with amino groups in heterocyclic bases of free nucleosides and nucleic acids [15,16] – among other nucleophilic targets such as inorganic anions [17] and thiols [18] – and gives rise to nucleobase deamination products, abasic sites and DNA crosslinks [10,19].

Interestingly, nucleobase deamination in DNA and RNA can also arise by several nitrosation-independent mechanisms, such as simple hydrolysis [20,21], reactions with bisulfite [22], and enzymatic mechanisms [23,24], as well as a result of altered purine nucleotide metabolism [25].
While hydrolysis and bisulfite reactions do not present problems for nucleobase deamination artifacts, we can control adventitious deamination by deaminase contamination arising during cell processing and from commercial enzyme preparations using deaminase inhibitors [26,27].

The mechanism of nucleobase deamination involves reaction of electrophilic N₂O₃ which arises in both weakly acidic solutions of nitrite and in oxygenated NO solutions at physiological pH [15,28-31] with nucleophilic nitrogens in nucleobases to form an N-nitroso species that rearranges to an aryl diazonium intermediate, with the diazonium ion undergoing nucleophilic substitution by water to replace the original amino group by an oxygen [32]. Nitrosative deamination leads to the formation of modified nucleobases such as xanthine (X) and oxanine (O) derived from guanine (G), hypoxanthine (I) from adenine (A), and uracil (U) from cytosine [10,19], as illustrated in Figure 5.1. Although nitrosation leads to other lesions such as 2-deoxyguanosine (dG) intra-strand cross-links [33] and abasic sites [34,35], the accumulation of nucleobase deamination products seems to be the major pathway as revealed in previous studies [26,36] and in the kinetic analyses described in Chapters 3 and 4. Although the majority of studies have focused on investigating the potential of DNA deamination products as indicators of exposure to NO *in vitro* [15,26,37] and *in vivo* [27,38,39], modifications in nucleic acids other than genomic DNA may be more efficient markers for nitrosative stress. In particular, RNA may be more susceptible to nucleobase damage since it is more widely distributed in the cytosol, is associated to a lesser degree with proteins and undergoes less repair compared to nuclear DNA [40]. This is consistent with recent findings reporting higher levels of deamination products detected in RNA versus DNA extracted from inflamed murine tissues [39]. A similar differential reactivity in nucleic acids, albeit from the perspective of propensity to oxidative damage, has also been established in bacteria, human cells and mice [41-43]. Assessing levels of
Figure 5.1. Products of DNA nucleobase deamination.

The same deamination products arise in free ribonucleosides or ribonucleosides as constituents of polymeric RNA (adapted from ref. [19]).
modifications in RNA nucleosides becomes even more significant if one considers that changes in the sequence of coding and non-coding RNA species have consequences for gene expression by affecting the function of the translational machinery [44,45], interfering with post-transcriptional RNA editing [46,47], or disrupting regulatory elements strongly correlated with the progression of different types of cancer [48-50].

Determining the extent of nitrosative damage in nucleic acids during exposure to inflammatory conditions in vivo is critical for gauging the health risks that may be associated with chronic NO overproduction. However, measuring concentrations of reactive nitrogen species formed at inflamed tissues is unfeasible both due to limited technology and the short half-life of derivatives of NO such as ONOO\(^-\), NO\(_2\) and N\(_2\)O\(_3\). To circumvent the impracticality of their direct detection in vivo, levels of reactive nitrogen species can be estimated using kinetic models that include rates of relevant reactions established in controlled studies. The accuracy of the model-derived concentration predictions can subsequently be validated by measuring stable end products (i.e., biomarkers) that are quantifiable in treated cells or in the extracellular space surrounding the cells. Based on this principle, micromolar NO levels measured in activated macrophage cultures [5] led to the development of a series of models describing concentration profiles of reactive species in simulated cell cultures [51,52]. Incorporating elements of cellular synthesis and consumption in the formulation of kinetic models [53] allowed for a more precise representation of the local microenvironment at sites of inflammation [54]. To gain further insight into the cellular fates of NO during inflammation, detailed models of intracellular chemistry have been developed to calculate the kinetics of NO-derived species in exposed cells [55] and to predict steady-state levels of reactive intermediates expected to arise in cells under biologically relevant nitrosative conditions [56]. In the latter case, Deen and coworkers provided
the first estimates for cytosolic steady-state concentrations of NO₂ (~1 pM) and N₂O₃ (1-50 fM) arising at an inflammation-relevant NO concentration of 1 μM and predicted that glutathione (GSH) acts as the main scavenger for both NO derivatives and that a 10-fold decrease in cellular GSH levels can lead to a ~10-fold higher N₂O₃ abundance in the cell. These values were calculated using kinetic data derived from *in vitro* studies and assuming that the effect of the cytosolic milieu on the experimentally determined rates is negligible. As described in this chapter, this is not the case since protein thiols may represent a more significant and relatively constant scavenging factor for N₂O₃ levels.

Toward the goal of defining levels of N₂O₃ at sites of inflammation in humans, we have undertaken studies to estimate N₂O₃ levels in human cells exposed to biologically relevant levels of NO using RNA deamination products as a surrogate for the short-lived N₂O₃. A critical feature of the studies involves controlled delivery of predictable and biologically relevant steady-state levels of NO to cells. NO can be introduced into cell cultures by a variety of strategies. A popular but problematic technique involves the use of “NONOates” that release NO with various kinetics to provide transient, non-uniform levels of NO that must be averaged over time to define the exposure level [57]. The difficulty of controlling the steady-state concentration of NO with NONOates over longer periods of exposure [57,58] is coupled with the fact that interpretation of data obtained from NONOates is complicated by unknown effects of non-NO free radical species generated along with NO, generation of alternative reactive nitrogen species in addition to NO [57], and the need for several different NO donor compounds to span the range of physiologically relevant NO concentrations [59]. Perhaps the best characterized and most broadly applicable system that mimics the nitrosative environment at sites of inflammation was developed by Deen and coworkers using NO- and O₂-permeable polydimethylsiloxane (Silastic)
tubing to deliver the gases to cell cultures at constant and predictable levels [27,60-63]. A mass transfer model was developed to predict the concentrations of NO and O₂ in the system given the values of certain operational parameters [64], and a subsequent kinetic analysis estimated the levels of N₂O₃ formed in the solution [26], making the tubing-based delivery apparatus ideal for kinetic studies of nitrosative deamination.

The other critical feature of these studies was the choice of a surrogate for the short-lived N₂O₃. Nitrosative damage products in RNA were chosen as the exposure indices given the widespread distribution and relatively high concentration of RNA in cells, the availability of methods for rigorous quantification of RNA deamination products, and the foundation of kinetic data for RNA deamination from the studies of Chapter 3. By comparing the time course for the formation of rI, rX and rO in RNA extracted from cells under NO-induced stress with in vitro kinetic analyses involving exposure of purified RNA to similar conditions (discussed in Chapter 3), we were able to estimate the apparent intracellular levels of N₂O₃ (0.2-40 fM) that led to the observed generation of deamination products in vivo. Repeating the experiments using an inhibitor of cellular GSH synthesis revealed the modest effect of cytosolic GSH levels on mitigating the N₂O₃-associated damage, suggesting that additional antioxidants or nucleophiles may react with the nitrosating agent in the cytoplasm. Our experimentally-derived calculations agree with estimates from recent mathematical models that predict steady-state N₂O₃ concentrations in cells of inflamed tissues [56].

5.2 Materials and Methods

5.2.1 Chemicals
Na$_2$HPO$_4$, NaH$_2$PO$_4$, and ammonium acetate were obtained from American Bioanalytical, LC-MS grade acetonitrile from EMD, ethanol from Koptec, glacial acetic acid, inosine (rI), and xanthosine (rX) were from Sigma (98% or higher purity) whereas oxanosine (rO) was synthesized by bubbling pure NO gas (Airgas) through saturated guanosine (Sigma) solutions in purified water at room temperature and subsequently isolating the product via HPLC. The product was identified by HPLC coelution, MS and UV spectroscopy relative to published data [65]. Water purified through a Milli-Q system (Millipore) was used throughout the study.

5.2.2 Human cell culture

TK6 human lymphoblastoid cells (ATCC) were maintained in exponentially growing suspension cultures at 37°C in a humidified atmosphere of 95% air and 5% CO$_2$ in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated donor horse serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Stock cell cultures were routinely passaged to maintain an optimal growth density (~1.0 × 10$^6$ cells/mL) in 150 mm dishes. To obtain glutathione-depleted cells, appropriate amounts of filter-sterilized solutions of buthionine sulfoximine (BSO) in water were added to cultures at a final concentration of 125 μM 24 h prior to NO exposure and subsequently incubated in the humidified atmosphere described above; optimal BSO concentration for GSH depletion was selected based on previous studies which also demonstrated negligible toxicity of BSO towards TK6 cells [66]. BSO-treated cultures were resuspended in fresh supplemented RPMI-1640 medium immediately before the NO exposures took place. RPMI-1640, L-glutamine, streptomycin /penicillin and PBS were obtained from Lonza; donor horse serum from Atlanta Biologicals; buthionine sulfoximine from Sigma.

5.2.3 Synthesis of isotopically labeled internal nucleoside standards
Uniformly $^{15}$N-labeled internal standards of the RNA deamination products rI, rX and rO, were synthesized as described previously [39,65] from uniformly $^{15}$N-labeled ribonucleosides (rA, rG) as starting materials (Cambridge Isotope Laboratories). Standards were purified by HPLC, characterized by high-resolution mass spectrometry, and quantified by UV absorbance using published extinction coefficients [65].

5.2.4 NO delivery system

A delivery system described in full detail in Chapter 2 [61] was employed to expose cells to precisely controlled levels of NO and O$_2$. Briefly, two distinct gas streams – one for NO the other for O$_2$ – were fed to reactors (modified 120 mL PFA screw-top jars from Cole Parmer) containing the cultures with NO and O$_2$ diffusing separately into the liquid through gas permeable Silastic tubing loops (3.5-9.5 cm long, 1.96 mm o.d., 1.47 mm i.d. from Dow Corning). The gas flow rates were 100 standard cubic centimeters per minute (sccm) for the NO-feeding stream and 200 sccm for the O$_2$-feeding stream in every reactor. Flow rates were regulated via mass flow controllers obtained from Porter Instruments, Parker-Hannifin. The contents of each reactor were kept stirred using a magnetic stirrer plate from IKA (stirring speed at 100 rpm; stir-bar placed in every reactor) and maintained at 37 °C within a water bath. Gas tanks of pure NO, 50% O$_2$ in N$_2$ (and 5% CO$_2$) and pure argon (for controls) were obtained from Airgas.

5.2.5 Exposure of cells to NO

Immediately before exposures, TK6 cells were transferred in sterilized reactors at a density of $\sim 8.5 \times 10^5$ cells/mL in $\sim 115$ mL of fresh supplemented RPMI-1640 medium. A mass transfer model that simulates the delivery system performance (discussed extensively in Chapter
2 [61,64]) was used to determine the exact lengths of tubing loops needed to reach specific NO and O\textsubscript{2} concentrations in the reactors; the reactors with tubing loops fully attached were autoclaved before each exposure. The model incorporated cellular gas consumption rates discussed elsewhere [60] and assumed negligible consumption of NO from the organics in the cell culture media as shown in recent studies [61]. According to the model, a steady-state NO concentration of \(~5\ \mu\text{M}~\) – with oxygen levels maintained constant at \(~200\ \mu\text{M}~\) – was achieved by passing NO through a 3.5 cm Silastic tubing loop and 50% O\textsubscript{2} in N\textsubscript{2} (with 5% CO\textsubscript{2}) through a 9.5 cm loop. For control experiments, pure argon (instead of NO) was supplied through a 3.5 cm Silastic tubing loop and 50% O\textsubscript{2} in N\textsubscript{2} (with 5% CO\textsubscript{2}) through a 7.0 cm loop; oxygen concentration was also kept constant at \(~200\ \mu\text{M}~\) throughout the control exposures. Shorter tubing loops were required for O\textsubscript{2} delivery in control reactors, to compensate for the reduced rate of O\textsubscript{2} consumption in the absence of NO. The time for the concentrations to reach their steady-state values in every reactor was \(~10\ \text{mins}~\) and after that point aliquots (6-7 mL) were withdrawn at regular intervals and replaced with an identical volume of fresh supplemented medium. From every aliquot, 0.1 mL was used for determining cell survival, 1.5 mL for measuring cellular GSH levels and the residual volume for quantifying deamination products in RNA, as described below.

5.2.6 Cell viability analysis

Live cells were identified by trypan blue exclusion (Sigma Chemical; 4% solution in saline), and the relative viability was calculated as the ratio of the live (unstained) cell number in NO experiments to that in argon controls (expressed as a percentage). In a separate set of exposures, aliquots of NO- and argon-treated cultures were withdrawn, centrifuged to remove the supernatant, resuspended in an equal volume of fresh supplemented medium and incubated for
48 h in the humidified atmosphere described above; cell viability was determined after the 48 h recovery period following the exposures as specified above. Analysis with trypan blue produces results comparable to plating efficiency and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assays, as demonstrated in previous work [67,68].

5.2.7 Glutathione measurement

Reduced (GSH) and oxidized glutathione (GSSG) levels were measured in NO- and argon-exposed cells using a commercial kit (Trevigen) based on the principle of glutathione reductase recycling assay [69,70]. Aliquots of cell cultures withdrawn during exposures (~0.6-1.4 × 10^6 cells) were centrifuged to remove the supernatant, washed with phosphate-buffered saline (PBS; Lonza), centrifuged to remove the supernatant again and the resulting cell pellet was lysed in 100 µL ice-cold 2.5% (w/v) sulfosalicylic acid (Sigma) followed by rigorous pipetting and freeze-thaw cycles. Cell debris were precipitated by centrifugation at 4 °C and the clarified lysates were analyzed using a microplate reader (BioTek), following the manufacturer’s instructions. Lysates were analyzed for total GSH content without any further processing however they were incubated (after dilution) at room temperature with 2% 4-vinylpyridine and 2% triethanolamine (v/v) before GSSG analysis. Total GSH and GSSG content in every sample was calculated based on standard curves.

5.2.8 Enzymatic hydrolysis of cellular RNA

Total RNA extracted from NO- and argon-treated cells was hydrolyzed to ribonucleosides for subsequent quantification of the levels of deamination products. Aliquots of cells withdrawn from the exposure reactors (2.5-4.5 × 10^6 cells) were centrifuged to remove the supernatant, washed with PBS, centrifuged once more to remove the supernatant and the
resulting cell pellets were processed using Qiagen’s RNeasy Mini Kits following the manufacturer’s instructions. Extracted total RNA from each sample (30-50 µg) was inspected for its integrity using a Bioanalyzer RNA Nano 6000 on-chip electrophoresis system (Agilent) and quantified by UV spectroscopy. RNA samples were subsequently hydrolyzed using a combination of nuclease P1 (Sigma), phosphodiesterase I (USB) and alkaline phosphatase (Sigma) in the presence of deaminase inhibitors (coformycin from DTP/NCI, tetrahydouridine from Calbiochem) and the antioxidant butylated hydroxytoluene (Sigma), as described previously [39,65]. Antioxidant and chelating agent deferoxamine was omitted from the protocol due to its effect on oxanine stability demonstrated in Chapter 3. The following average amounts of internal standards were added to the digestion mixture per sample: 1.2 pmol $^{15}$N-rI, 0.7 pmol $^{15}$N-rX and 0.5 pmol $^{15}$N-rO. Enzymes were removed at the end of hydrolytic digestion process by spin filters (NanoSep 10K Omega, Pall) and filtrates were concentrated under vacuum.

### 5.2.9 HPLC prepurification of RNA deamination products

Samples of ribonucleosides were dissolved in water to a total volume of 100 µL and individual nucleosides were resolved and collected using an Agilent 1100 series HPLC system equipped with a Phenomenex Synergi C18 reversed-phase column (250 x 4.6 mm, 4 µm particle size, 80 Å pore size) with elutions performed at a 0.5-1 mL/min flow rate of 8 mM ammonium acetate solvent (A) with a stepwise increasing gradient of pure acetonitrile (B); details are given in Table B.1 of Appendix B. Empirically determined retention times, which were defined in previous studies [39,65], for the native and target nucleosides are shown in Figure B.1 of Appendix B, whereas the output from a typical hydrolyzed RNA sample is depicted Figure B.3 of Appendix B. Fractions containing the target nucleosides (rI, rX, rO) were dried under vacuum, redissolved in 50 µL of water and analyzed by LC-MS/MS.
5.2.10 LC-MS/MS quantification of deamination products in RNA

Levels of deamination products in RNA extracted from NO- and argon-treated cell cultures that have been subsequently hydrolyzed were analyzed using an Agilent 1100 series HPLC system interfaced with an AB Sciex API 3000 tandem quadrupole MS with a turbo ion spray source. Resolution of target nucleosides was achieved on a Thermo Fisher Hypersil Gold aQ C18 reverse-phase column (150 x 2.1 mm, 3 µm particle size) using isocratic conditions of 0.1% acetic acid (A) and 0.7-3% v/v acetonitrile (B), depending on the target nucleoside, at a 0.2 mL/min flow rate; elution times, content of solvent B and column temperature for each of the target nucleosides are listed in Table B.2 of Appendix B. The MS was operated in positive ion mode with the first and third quadrupoles fixed to unit resolution. Multiple reaction monitoring (MRM) mode was used for sample detection with dwell times set to 200 ms. Mass spectrometric parameters were optimized for maximal sensitivity and are displayed in Table B.3 of Appendix B. Linear calibration curves were generated (r² > 0.98) for the isotope-labeled and unlabeled forms of each of the deamination products.

5.2.11 Expression of N₂O₃ concentration as a function of cellular species

To model the nitrosative chemistry arising in cells exposed to NO and better understand in quantitative terms the damage inflicted by N₂O₃, the intracellular environment is approximated as an aqueous solution at physiological pH where NO reacts with oxygen, as illustrated in the reactions below.

\[ \text{2NO} + \text{O}_2 \xrightarrow{k_1} \text{2NO}_2 \]  \hspace{1cm} (1)
When NO is the only nitrogen oxide added to the system, intermediates NO$_2$ and N$_2$O$_3$ are present only in trace quantities [31], which justifies pseudo-steady state approximations for their concentrations, and N$_2$O$_3$ is the principal nitrosating agent in the solution [17]. The majority of N$_2$O$_3$ forming in the aqueous environment of neutral pH is directly hydrolyzed to the stable end product nitrite (NO$_2^-$) as shown in reaction 3. However hydrolysis is effectively accelerated in the presence of certain anions that act as ‘catalysts’ (including phosphate, chloride and bicarbonate [16,71]), decreasing the N$_2$O$_3$ available to react with other targets in the solution. Reaction 4 represents an example of the parallel hydrolysis pathways attributed to those anions, in particular inorganic phosphate (P$_i$):

\[
\text{N}_2\text{O}_3 + P_i + H_2O \xrightarrow{k_p} 2\text{NO}_2 + P_i + 2H^+ \quad (4)
\]

Similar reactions occur with chloride (Cl$^-$) and bicarbonate (HCO$_3^-$) with the corresponding rate constants defined as $k_C$ and $k_B$. In addition to anions, the cytosol contains certain antioxidants that are known to react with N$_2$O$_3$, such as ascorbate (AH$^-$) and glutathione (GSH), and the kinetics of those reactions are explored elsewhere [18,72] – with assigned rate constants $k_A$ and $k_G$, respectively, in this scheme. The same compounds also act as ‘scavengers’ for other radicals in the cytosol including NO$_2$ [73,74] thus influencing the N$_2$O$_3$ concentration in more than one way (N$_2$O$_3$ is produced from NO$_2$ according to reaction 2). The residual N$_2$O$_3$ that
is not consumed by the aforementioned species can attack other biomolecules in the cytosol, including RNA nucleosides that are of great interest to the current study. The reactions between $\text{N}_2\text{O}_3$ and native purines in RNA are depicted as follows.

$$
\begin{align*}
\text{k}_1 & \quad \text{N}_2\text{O}_3 + \text{rA} \rightarrow \text{rI} + \text{NO}_2^- + \text{H}^+ \\
\text{k}_x & \quad \text{N}_2\text{O}_3 + \text{rG} \rightarrow \text{rX} + \text{NO}_2^- + \text{H}^+ \\
\text{k}_o & \quad \text{N}_2\text{O}_3 + \text{rG} \rightarrow \text{rO} + \text{NO}_2^- + \text{H}^+
\end{align*}
$$

(5) (6) (7)

Deamination products $\text{rX}$ and $\text{rO}$ are expected to form simultaneously from $\text{rG}$, as indicated by in vitro studies discussed in Chapter 3, assuming the same chemistry takes place in the cells. The current scheme is limited only to purines since the reaction between $\text{N}_2\text{O}_3$ and cytidine yields uridine, a naturally abundant nucleoside in RNA, making detection of small amounts generated by deamination alone very challenging. As it will be discussed shortly, by measuring the rate at which $\text{rI}$, $\text{rX}$ and $\text{rO}$ accumulate in the RNA of NO-exposed cells the apparent steady-state intracellular $\text{N}_2\text{O}_3$ concentration that forms in the cytosol can be indirectly estimated.

To deduce an expression for $\text{N}_2\text{O}_3$ levels in cells exposed in the NO-delivery device while minimizing the calculations, spatial variations of the species concentrations governing the cell environment were ignored. That simplification was in accordance with previous findings that intracellular concentrations of reactive nitrogen intermediates remain approximately the same no matter the distance from the center of the cell [52]. Besides having a uniform value throughout
the entire cytosol, the concentrations involved in the reactions shown above were also approximated as time-independent. Namely, the major dissolved gases NO and O_2 were maintained at constant levels in the delivery system and, as shown in previous work [52], their concentrations in the bulk culture medium could safely be equated with those in the cytosol. The ions and antioxidants in cells were assumed to vary little during each exposure, with a duration equal to 50% of the cell doubling time (~24 h; ref. [67]), whereas their concentrations were specified based on values from the literature. Finally, the reactive intermediates formed in trace levels are expected to have a slow permeation across the cell membrane relative to intracellular reactions since the concentration driving force for diffusion is very small, hence during exposures the various chemical species in the cytosol will be at a steady state with rates of formation and consumption being in continuous balance within the closed system that each cell represents. Although there is evidence that NO oxidation is accelerated in hydrophobic media [75] the current analysis examines only aqueous chemistry, assuming that intramembrane reactions have a negligible effect on the concentrations of interest. Simplifying the model formulation by not including a lipid phase is also supported by the results of a recent study that investigated concentrations of reactive nitrogen species employing similar approximations to those stated above [56].

Applying the steady state approximation on N_2O_3 and including all terms of formation vs. consumption involving the species discussed earlier and solving for the N_2O_3 concentration yields:

\[
[N_2O_3] = \frac{k_2[NO][NO_2]}{k_{-2} + k_3 + k_P[P_1] + k_C[Cl^-] + k_B[HCO_3^-] + k_A[AH^-] + k_G[GSH] + k_I[A^+] + (k_X + k_O)[rG]} \]

(8)
Some terms in the denominator of equation 8 may be less significant than others, and thus ignored, whereas new sinks of N$_2$O$_3$ can be added to more accurately represent the nitrosative chemistry affecting the cellular environment. Rate constants for the reactions mentioned above are listed in Table 5.1 whereas estimates for the species concentrations are shown in Table 5.2.

### 5.2.12 Intracellular N$_2$O$_3$ concentration from *in vivo* deamination kinetics

On the basis of recent studies that determined rate constants for *in vitro* RNA deamination occurring in well-defined systems, the goal here is to employ the established kinetic parameters and conversely calculate levels of N$_2$O$_3$ to which RNA is exposed in the cytosol of NO-treated cells. As there is no direct way to measure trace levels of N$_2$O$_3$ forming in cells (in contrast to NO which can be detected *in vivo* [76,77]) a surrogate for its effects on components of the cellular environment is crucial for a quantitative analysis. The nitrosative damage associated with N$_2$O$_3$ is likely broadly distributed and affects multiple nucleophiles in the cytosol. However, this work focuses on nucleoside deamination lesions resulting from the generation of N$_2$O$_3$ and its immediate interaction with the cellular RNA species (primarily ribosomal RNA). NO and O$_2$ freely diffuse through the cell membrane – their levels equaling those in the extracellular medium – and react in the cytosol to produce N$_2$O$_3$, among other species, leading to the reactions discussed above. The constant levels of N$_2$O$_3$, as a trace steady-state intermediate, give rise to accumulating amounts of deamination products (rI, rX, rO) that can be measured, depending on the sensitivity of the analytical approach employed. In that case, expressing the rate law for reactions 5, 6 and 7 yields:

\[
\frac{d[rI]}{dt} = k_1[rA][N_2O_3]_{cyt} \quad (9)
\]
Table 5.1. Reaction rate constant values.

<table>
<thead>
<tr>
<th>rate constant</th>
<th>value (37 °C)</th>
<th>units</th>
<th>ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1$</td>
<td>$2.4 \times 10^6$</td>
<td>M$^{-2}$ s$^{-1}$</td>
<td>[31]</td>
</tr>
<tr>
<td>$k_2$</td>
<td>$1.1 \times 10^9$</td>
<td>M$^{-1}$ s$^{-1}$</td>
<td>[78]</td>
</tr>
<tr>
<td>$k_{-2}$</td>
<td>$8.0 \times 10^4$</td>
<td>s$^{-1}$</td>
<td>[78]</td>
</tr>
<tr>
<td>$k_3$</td>
<td>$2.1 \times 10^3$</td>
<td>s$^{-1}$</td>
<td>[72]</td>
</tr>
<tr>
<td>$k_P$</td>
<td>$1.1 \times 10^6$</td>
<td>M$^{-1}$ s$^{-1}$</td>
<td>[17]</td>
</tr>
<tr>
<td>$k_C$</td>
<td>$2.2 \times 10^5$</td>
<td>M$^{-1}$ s$^{-1}$</td>
<td>[17]</td>
</tr>
<tr>
<td>$k_B$</td>
<td>$1.8 \times 10^6$</td>
<td>M$^{-1}$ s$^{-1}$</td>
<td>[71]</td>
</tr>
<tr>
<td>$k_A$</td>
<td>$1.2 \times 10^9$</td>
<td>M$^{-1}$ s$^{-1}$</td>
<td>[72]</td>
</tr>
<tr>
<td>$k_G$</td>
<td>$6.6 \times 10^7$</td>
<td>M$^{-1}$ s$^{-1}$</td>
<td>[18]</td>
</tr>
<tr>
<td>$k_1$</td>
<td>$2.9 \times 10^4$</td>
<td>M$^{-1}$ s$^{-1}$</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>$k_X$</td>
<td>$1.3 \times 10^6$</td>
<td>M$^{-1}$ s$^{-1}$</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>$k_O$</td>
<td>$7.7 \times 10^5$</td>
<td>M$^{-1}$ s$^{-1}$</td>
<td>Chapter 3</td>
</tr>
</tbody>
</table>

Values for $k_2$, $k_{-2}$ reported in 20 °C; $k_3$ estimation at 37 °C based on activation energy also reported in ref. [72]; $k_P$, $k_C$ calculated based on $k_3$ values as indicated in ref. [17]; $k_B$ calculated assuming a typical activation energy of ~15 kJ/mol.
**Table 5.2.** Concentrations of NO and cellular scavengers of N₂O₃ in the cytosol.

<table>
<thead>
<tr>
<th>species</th>
<th>concentration</th>
<th>ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td>5 µM</td>
<td></td>
</tr>
<tr>
<td>P_i</td>
<td>80 mM</td>
<td>[79]</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>5 mM</td>
<td>[79]</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>10 mM</td>
<td></td>
</tr>
<tr>
<td>AH</td>
<td>0.5 mM</td>
<td>[80]</td>
</tr>
<tr>
<td>GSH</td>
<td>0.5-10 mM</td>
<td>[81]</td>
</tr>
</tbody>
</table>

NO levels were imposed by the delivery device; HCO₃⁻ levels were calculated based on a 5% CO₂ atmosphere at neutral pH; pKₐ = ~4 for ascorbic acid [82] and pKₐ = ~9 [83] hence at pH = 7 ascorbic acid will be mainly anions (A⁻) and glutathione will be in its neutral form (GSH).
\[
\frac{d[rX]}{dt} = k_X[rG][N_2O_3]_{\text{cyt}} \quad (10)
\]
\[
\frac{d[rO]}{dt} = k_O[rG][N_2O_3]_{\text{cyt}} \quad (11)
\]

A time-independent value for the cytosolic N\textsubscript{2}O\textsubscript{3} concentration, [N\textsubscript{2}O\textsubscript{3}]_{\text{cyt}}, combined with unchanged levels of native RNA nucleosides (assuming the RNA content of each cell varies very little during the entirety of the NO-exposure) result in a proportional linear increase of deamination products in RNA, as our experimental findings show below. Rate constants for deamination of RNA nucleosides at the examined temperature (37 °C) were determined in Chapter 3.

5.3 Results

5.3.1 Accumulation of deamination products in RNA of NO-exposed cells

To estimate N\textsubscript{2}O\textsubscript{3} levels at sites of inflammation, we used a model system of controlled exposure of human cells to NO and quantification of RNA deamination products as a surrogate for the short-lived N\textsubscript{2}O\textsubscript{3}. Cultures of TK6 cells were exposed to NO at a steady concentration of ~5 μM which is the same order of magnitude as the concentration measured in media surrounding activated macrophages [5], while O\textsubscript{2} was kept constant at ~200 μM. The levels of dissolved gases in the delivery device were determined with accuracy using a mass transfer model that simulates the reaction between the diffusing gases in the liquid while also incorporating rates of cellular consumption [60]; the organics in cell media were recently found not to affect the function of the delivery device thus reinforcing the predictive power of the model [61]. During exposures, cell aliquots were withdrawn from the reactors and total RNA
was immediately extracted from lysed cells and subsequently hydrolyzed to nucleosides minimizing the effect of artifactual generation or loss of deaminated nucleobases (coformycin, tetrahydrouridine used as deaminase inhibitors; deferoxamine was omitted due to its reactivity with oxanosine discussed extensively in Chapter 3). The target nucleosides (rI, rX, rO) were resolved from significantly more abundant native ones in a HPLC purification step, enriched fractions were collected and subsequently analyzed via isotope-dilution LC/MS/MS. As shown in Figure 5.2, a dose response increase was observed for the formation of all three deamination lesions examined relative to levels detected in control experiments (i.e. exposure to argon instead of NO).

Using this system, we observed that controlled exposure of cells to NO and O₂ for up to 12 hours led to time-dependent increases in RNA deamination products – approximately a 2-fold increase determined for rI, 5-fold for rX and 10-fold for rO – departing from baseline levels of 20 (±6) rI per 10⁶ nt, 2 (±0.5) rX per 10⁶ nt and 4 (±2) rO per 10⁷ nt.

To investigate the effect of GSH levels on N₂O₃-induced deamination, we incubated TK6 cells with 125 μM BSO for 24 h immediately prior to NO exposure to cause a 99% reduction in GSH (see below) and observed that this reduction did not significantly affect the time course for the formation of nucleobase deamination products (Figure 5.3). Only marginally higher amounts of rI, rX and rO were measured at almost every time point in BSO-treated cells compared to cells that remained untreated before being challenged by the same exposure conditions (Figure 5.3). These results indicate that such a significant decrease in cellular GSH does not seem to expose the intracellular environment to greater damage associated with nitrosative chemistry, at least as quantified by the designated surrogate of deamination lesions in RNA.
Figure 5.2. Fold-increase in nucleoside deamination products measured in RNA of NO-challenged TK6 cells compared to argon controls.

Results presented as relative fold-increase of rI, rX, rO levels in NO-treated cells over those in argon controls. The NO dose was calculated by multiplying the steady state NO concentration (~5 μM) with exposure time (final time point at 12 h). Data represent mean ± S.D. for n = 2-3.
Figure 5.3. Formation of nucleobase deamination products rI, rX and rO in RNA extracted from and BSO-treated and untreated TK6 cells.

Cells were exposed to constant levels of NO (5 μM) in the delivery device. Data for untreated cells represent mean ± S.D. for n = 2-3.
5.3.2 Effects of NO exposure on levels of GSH and GSSG

To further assess the significance of GSH as a scavenger of N\textsubscript{2}O\textsubscript{3} in the intracellular milieu, reduced and oxidized levels of glutathione (GSH, GSSG) were quantified during the time course of NO exposures. Aliquots of cells were withdrawn during treatment with NO (and argon) as described earlier, and the resulting cell pellets were lysed and the concentrations of GSH and GSSG measured using a GSH reductase recycling assay summarized in Figure 5.4. Results depicted in Figure 5.5 show a substantial difference in cytosolic GSH levels in cells challenged with NO compared to argon controls, which suggests that exposure to 5 μM steady-state levels of NO causes a net reduction in GSH levels. However, even at the highest dose during the exposure, GSH levels in the NO exposed cells did not fall below ~5 mM. GSSG measurements, on the other hand, showed a significant increase at 900 μM min compared to argon controls, but were otherwise unchanged by NO exposure and represented only 0.1-2% of the total GSH content in all cases (Figure 5.6). The latter is consistent with earlier reports [84,85].

To further gauge the role of GSH in mitigating nitrosative chemistry, GSH levels were reduced by treatment of cells with BSO, a γ-glutamylcysteine synthetase inhibitor that efficiently blocks the pathway of de novo GSH synthesis [86] (depicted in Figure 5.7). In cells treated with 125 μM BSO for 24 h before NO exposure, GSH levels decreased to 1% of the level in untreated cells and did not change further when cells were challenged with NO (Figure 5.8). GSSG was undetectable in lysates of BSO-treated cells. Previous studies showed that incubation of TK6 cultures with 125 μM BSO does not affect cell viability [66,84,85].

5.3.3 Effect of NO exposure on cell viability
Figure 5.4. Schematic of the glutathione reductase recycling assay.

GSH reacts with DTNB to form TNB and the GS-TNB conjugate. GS-TNB is continuously reduced (recycled) back to GSH by glutathione reductase in the presence of NADPH, releasing TNB once again. The rate of TNB formation – proportional to GSH concentration in the sample – can be quantified at 412 nm. Pre-existing GSSG is also reduced to GSH in the reaction mixture thus adding to the total TNB generation. To isolate the contribution of GSSG (usually significantly less abundant than GSH) the lysate is first derivatized with 4-vinylpyridine which reacts with GSH, but not GSSG, before analysis (adapted from ref. [70]).
Figure 5.5. Levels of reduced glutathione monitored during exposure of TK6 cells to NO and argon.

Intracellular concentrations were calculated assuming an average cell volume of 500 fL (based on ref. [87]). Time point at $t = 12$ h corresponds to a total NO dose of 3600 μM min. Data represent mean ± S.D. for n = 3-4.
Figure 5.6. Levels of oxidized glutathione (GSSG) in cells exposed to NO.

Intracellular concentrations were calculated assuming an average cell volume of 500 fL (ref. [87]). Time point at $t = 12$ h corresponds to a total NO dose of 3600 μM min. Data represent mean ± S.D. for $n = 2-3$. 
**Figure 5.7.** Inhibitory mechanism of buthionine sulfoximine (BSO).

The enzyme \( \gamma \)-glutamylcysteine synthetase (or \( \gamma \)-glutamate cysteine ligase) is the rate-limiting enzyme in glutathione (GSH) synthesis. Adapted from ref. [86].
Figure 5.8. Levels of reduced glutathione (GSH) in BSO-treated cells exposed to NO and argon.

Intracellular concentrations were calculated assuming an average cell volume of 500 fL (ref. [87]). Time point at $t = 12$ h corresponds to a total NO dose of 3600 μM min. Data represent mean ± deviation about the mean for $n = 2$. 
The cytotoxic effects of NO exposure were assessed by trypan blue uptake in aliquots of cells withdrawn from the delivery device during NO exposures. Figure 5.9 shows the expected dose-dependent decrease in cell viability at 2 days post-exposure to NO, with little apparent cytotoxicity immediately after exposure. For argon-exposed controls, the delivery reactor was previously reported to have little or no effect on cell proliferation [60], so the observed effects can be attributed to the presence of NO alone. The results presented here agree with previous studies examining NO effects on TK6 viability [27]. Although cell integrity appears to be maintained for most cells during exposure, the substantial difference in viability measured immediately and following a recovery period (48 h) indicates the cytotoxic effect of NO leading to cycle arrest and apoptosis, as shown in earlier studies [88].

5.3.4 Estimation of intracellular N₂O₃ concentration

Using the data for NO-induced deamination of RNA nucleobases, we proceed to estimate the cytosolic N₂O₃ concentration using a mathematical model. The steady-state hypothesis entails N₂O₃ formation in constant (trace) amounts in the intracellular milieu during NO/O₂ exposure, and the apparent linear increase of rI, rX and rO in extracted RNA over time (Figure 5.2) supports that assumption. In other words, equations 9, 10 and 11 sufficiently describe the kinetics of nucleobase deamination occurring in the cytosol and can be solved to determine the unknown term [N₂O₃]cyt. A volume of ~500 fL is calculated for a mammalian cell with a mean diameter of ~10 μm [87] and UV measurements indicate that ~10 pg of total RNA is extracted per cell. Combining this information leads to a calculated intracellular RNA concentration of ~60 mM; [rA] and [rG] are each expected to be a quarter of that value. Formation rates for rI, rX and rO can be calculated from the corresponding slopes in graphs presenting the accumulation of deamination lesions as a function of time (using information shown in Figure 5.3). The slopes
Figure 5.9. Cell viability determined by the trypan blue exclusion assay at various doses of NO.

Relative cell viability (expressed as a percentage) calculated as the ratio of cell number in NO exposures to those in argon controls immediately after exposure (blue circles). Relative cell viability was also determined after a 48 h recovery period following exposure to NO (red squares). Data represent mean ± S.D. for n = 2-3.
were determined to be 2.2, 0.37 and 0.1 lesions/10⁶ RNA nt/h for rI, rX and rO, respectively. The formation rates – required input in equations 9, 10 and 11 – are derived from the experimental slopes by multiplying with the cytosolic RNA concentration addressed above. Finally, the rate constants describing the deamination chemistry of RNA nucleobases were determined from in vitro exposures of purified RNA in the delivery device discussed in Chapter 3, and their values are listed in Table 5.1. Using the values mentioned above, equations 9, 10 and 11 each give an estimate for the apparent [N₂O₃]ₙₘ₂ calculated to be 73, 0.4 and 0.2 fM, respectively. The concentration range suggested by these calculations is in reasonable agreement with estimates from kinetic models predicting intracellular concentrations for reactive nitrogen species in conditions simulating the environment of inflamed tissues [56].

To assess the possibility that other nucleophilic biomolecules can lower the steady-state levels of N₂O₃ in the cytosol, [N₂O₃]ₙₘ₂ was expressed in terms of concentrations of cellular constituents known to react with the nitrosating agent (equation 8). As mentioned above, calculations based on experimental data from the current study (i.e., the experimentally derived [N₂O₃]ₙₘ₂ = 0.2 fM) indicate that the NO-associated nitrosating species may be more extensively depleted within cells than previously estimated. Recent mathematical models predicted GSH to be the most important cytosolic sink for N₂O₃ with its levels predicted to increase by an order of magnitude – along with the resulting nitrosative damage – from a ~10-fold reduction in GSH concentration [56]. However, our results demonstrate that BSO-treated TK6 cells (with only ~1% of the GSH content of untreated cells) sustained virtually the same levels of deamination products under nitrosative stress compared to cells with normal GSH levels. Equation 8, as defined above, requires input only for the NO₂ concentration, since all other parameters have been specified in the literature, listed in Table 5.1 and Table 5.2, or alternatively derived from
measurements in this work ([GSH] = 5-8 mM from Figure 5.5; [RNA] = 60 mM). With the calculations of Lim et al. providing the only available estimate of NO₂ concentration in cells under nitrosative stress, with a value of ~1 pM in a cellular environment with GSH levels higher than 5 mM [56], equation 8 yields [N₂O₃] = 4-5 fM. Similar values are obtained even when scavenging from DNA is added to the denominator of equation 8; DNA nucleobases were assumed to follow the same deamination kinetics as those in RNA (as discussed in Chapter 3) and a 1:1 ratio of DNA to RNA per cell was assumed as an upper limit [89,90]. Neglecting the GSH concentration altogether in equation 8 – which simulates the cellular environment in BSO-treated cells – led to only a 30-50% increase in N₂O₃ levels, indicating the abundance of other scavenger considered and diminishing the role of GSH in depleting N₂O₃, consistent with our experimental findings. Those observations combined with the sub-femtomolar levels of N₂O₃ forming inside cells calculated based on in vivo kinetics may be pointing toward the possibility of other major scavenger(s) in the cytosol, such as protein thiols or other reducing species.

5.4 Discussion

To shed light on how chemical stresses implicated in the immune response affect the intracellular environment of cells in proximity to inflamed tissues, previous studies subjected biomolecules and cells to controlled experimental environments that closely mimic those expected to arise at sites of inflammation [27,60,85,88]. Based on that principle, we used a system for precise and steady delivery of NO (described extensively in Chapter 2 [60,61]) to simulate the nitrosative conditions resulting from activated macrophages [5] and investigate the formation of nucleobase deamination products in RNA extracted from stressed cells. In vitro studies presented in Chapter 3 established the kinetic parameters of nitrosative deamination in purified RNA using the same delivery device. The current work describes a novel approach to
estimate intracellular levels of the nitrosative agent \( \text{N}_2\text{O}_3 \) by quantifying the rate at which \( rI \), \( rX \) and \( rO \) accumulate in the RNA of NO-challenged cells while incorporating the previously defined \textit{in vitro} rate constants. An algebraic expression was also developed to reassess the degree to which cellular GSH levels affect the damage caused by \( \text{N}_2\text{O}_3 \) formed in the cytoplasm.

### 5.4.1 Formation of inosine, xanthosine and oxanosine in cellular RNA

Exposure of TK6 cells to steady levels of nitrosative stress in the delivery device led to quantifiable increases in all three purine deamination products. The dose-dependent rise of these modified nucleosides in total RNA extracted from intact cells (Figure 5.2) reinforces the observation that nitrosative deamination linked to relatively high NO levels indeed damages a variety of nucleophiles in the cytosol, including nucleosides in RNA and DNA [27]. In particular, measuring \( rO \) for the first time in the RNA of NO-exposed cells supports the assumption that the chemistry occurring in the cellular milieu does not differ from the nitrosative mechanism observed in buffered solutions (Chapter 3), yielding the same set of modified nucleosides resulting from the reaction with locally generated \( \text{N}_2\text{O}_3 \). The particular lesion – albeit as a 2-deoxynucleoside – was initially found to form concurrently with 2-deoxyxanthosine when free 2-deoxyguanosine was exposed to NO [29]. This prompted subsequent investigations to re-examine the spectrum of deamination products in genomic DNA extracted from NO-treated human cells [27] and tissues from a mouse model of inflammation [38], however the experimental techniques employed in those investigations were unable to detect the presence of 2-deoxyoxanosine. Those earlier observations stand in direct contrast with the results presented here as well as those from Chapter 3 that show simultaneous formation of xanthine and oxanine in solutions of free nucleosides, RNA and DNA exposed to NO. The disagreement can be explained taking into account that previous studies in cells and tissues used primary amines
(spermine, spermidine in ref [27]; deferoxamine in ref [38]) as antioxidants or chelating agents during enzymatic hydrolysis and that millimolar levels of deferoxamine can significantly degrade (deoxy)oxanosine within a fraction of the total incubation time as discussed in Chapter 3. We propose that the aforementioned antioxidants may have exerted a similar effect in samples derived from hydrolyzed DNA thus hindering the detection of 2-deoxyoxanosine in previous studies. It is possible that this amine-induced loss led to greatly reduced amounts of 2-deoxyoxanosine, well below the limit of detection of the employed method. We avoided this problem here by omitting all types of amine-containing antioxidants during RNA hydrolysis, and employing sensitive LC-MS/MS techniques to measure the minute amounts of rO forming during NO exposures. Considering our results and the similarity of the deamination kinetics between DNA and RNA (Chapter 3), one would expect similar oxanine levels to accumulate in genomic DNA under the nitrosative conditions described above as long as a) improved protocols are applied in future experiments that will re-assess the possibility of oxanine formation in nucleic acids as a result of NO-induced deamination in vivo and b) the nucleophilic primary amines of histone proteins in the nucleus do not significantly affect the fate of this particular lesion.

5.4.2 Determination of intracellular N₂O₃ concentration using in vivo deamination kinetics

The results presented in the current studies represent the first attempt to use a kinetic approach to determine cytosolic levels of the potent nitrosating agent N₂O₃ in cells challenged by NO exposure. The controlled environment of the delivery system (previously used in numerous investigations [27,60,61,85,88]) provides constant micromolar levels of dissolved NO to which cells are exposed over several hours, simulating the environment of tissue cells in proximity to
activated macrophages [5,54]. Earlier characterization of the NO delivery device revealed the existence of a ~1 μm thick boundary layer adjacent to the NO-feed tubing loop where the concentrations of reactive nitrogen species greatly exceed those in the well-stirred bulk liquid [64]. TK6 cells, however, are almost fully excluded due to their larger size (~10 μm) and remain virtually unaffected by the presence of the layer. This allows us to predict with confidence the concentration of dissolved NO constantly surrounding the cells using the mass transfer model already discussed in detail in Chapter 2 [61,64]. Due to its permeability in the lipid membrane [52], the extracellular concentration of NO is expected to roughly equal that in the cytosol. Thus, the delivery device results in predictable and constant levels of NO and O₂ in the cytosol, with the expectation of constant N₂O₃ intracellular concentrations, according to the kinetic scheme of NO consumption defined in the Methods section. Since trace amounts of N₂O₃ are impossible to measure directly in the cytosol, we used the well-characterized nucleobase damage cause by N₂O₃ as a surrogate to gauge its abundance in the cytoplasm. A linear increase of RNA nucleoside deamination products detected in intact cells implies that constant levels of N₂O₃ are generated in the cellular milieu and validates the steady-state assumption. As mentioned above, the data corresponding to the increase in rI (Figure 5.3) led to a calculated intracellular N₂O₃ concentration of ~70 fM whereas using the information from the observed increases in rX and rO yields a mean concentration of ~0.3 fM. Cytosolic N₂O₃ levels in the femtomolar range (1-60 fM) were first predicted by a kinetic model developed by Lim et al., which involved ~65 reactions with reactive nitrogen and oxygen species and cellular components such as antioxidants, amino acids and proteins [56]. However, our results are the first experimentally-derived calculations that support the aforementioned order-of-magnitude predictions and provide evidence derived from biological systems that confirm the accuracy of mathematical models in
determining the fate of reactive nitrogen intermediates in vivo. The same kinetic approach can be readily employed to determine trace levels of other mediators of inflammation (i.e. NO₂) in the cell, as long as reliable delivery methods (e.g., the system described in ref. [91]) and sensitive detection protocols [92] are available. Experiments aiming to measure NO₂ in vivo would complement the current work by testing the validity of some of our assumptions (i.e., intracellular NO₂ levels expected in the range of ~1 pM) and thus reinforcing our confidence in predicting N₂O₃ concentrations.

5.4.3 Effect of GSH in scavenging N₂O₃-induced related damage in the cytosol

To assess the role of GSH, a potent scavenger for reactive nitrogen species, in mitigating the nitrosative stress imposed by N₂O₃ in vivo, we studied the formation of deamination products in cells significantly depleted of GSH. By incorporating the established kinetics of the reaction between GSH and N₂O₃ [18,93] in their recent model, Lim et al. concluded that GSH is the most significant scavenger for the nitrosative agent in the cytosol. In particular, a roughly order-of-magnitude increase in N₂O₃ levels was expected to occur if the cellular GSH concentration dropped from 5 to 1 mM [56]. To examine whether that prediction accurately represents the cytosolic environment, BSO-treated and untreated cells were challenged with the same NO doses in the delivery device. The anticipated effect of the inhibitor blocking de novo GSH synthesis was confirmed by the 100-fold reduction in GSH content of BSO-treated cells, yet little change in RNA deamination was detected (Figure 5.3). The observation that nitrosative damage accumulating in the cytoplasm appears to be insensitive to levels of one of the most abundant antioxidant agrees with previous studies showing negligible changes in levels of DNA deamination products when BSO was employed to alter the cytosolic GSH content [66,85].

These results indicate that GSH may not be the single most important sink for N₂O₃ in the cell,
as proposed by Lim et al., most likely because the authors included only the catalytic effects of bicarbonate in the N\textsubscript{2}O\textsubscript{3} hydrolysis pathway in addition to the reaction with GSH ignoring other sinks such as those appearing in the denominator of equation 8. On the other hand, the current work suggests that there is some other reducing species in the intracellular environment that scavenges N\textsubscript{2}O\textsubscript{3} more effectively than GSH (Tables 5.1 and 5.2). A wider array of N\textsubscript{2}O\textsubscript{3}-consuming biomolecules is probably the main reason why only modest changes in the N\textsubscript{2}O\textsubscript{3} levels are calculated by eliminating the term associated with GSH in equation 8. Such a dynamic seems to be more representative of the experimental trend presented in this work.

The results shown in this work suggest that the list of cytosolic sinks for N\textsubscript{2}O\textsubscript{3} is incomplete, which raises the question of the identity of other N\textsubscript{2}O\textsubscript{3} scavengers. The calculations described above yielded a range of experimentally-derived [N\textsubscript{2}O\textsubscript{3}]\textsubscript{cyt} values, depending on which increase in deamination product was employed. In particular, rI is a naturally occurring nucleoside in RNA [94] and measuring modest increases over a pre-existing high background level may lead to large errors when determining the corresponding formation rate. There is also a possibility that cellular processes regulating rA editing in RNA [46] could become erratic while cells are challenged by high doses of NO, hence a cytosolic N\textsubscript{2}O\textsubscript{3} estimate based on measured increases of rI may be less accurate compared to those calculated relying on the rX and rO accumulation rates. In addition, rX and rO are not physiological nucleosides and their formation in nucleic acids likely reflects the nitrosative damage occurring in the intracellular environment. Choosing the more reliable estimate for the intracellular N\textsubscript{2}O\textsubscript{3} concentration (~0.3 fM) returns a value ~10-fold lower than that calculated from equation 8 (4-5 fM), suggesting that additional terms need to be incorporated into the model to better represent the experimental findings. Cellular protein is an ideal candidate among abundant species that can react with N\textsubscript{2}O\textsubscript{3} in the
cytosol. Reported levels of 100 pg of protein per cell [95] with a mean molecular weight of 53 kDa [96] yield an intracellular concentration of ~4 mM (alternatively estimated at ~15 mM according to ref. [97]). Assuming that each protein molecule has at least 5-6 nucleophilic residues that can be nitrosated by N₂O₃ (i.e., glutamine, proline, cysteine, tryptophan and the N-terminus among others), an effective concentration for the protein reactive sites can be defined. Multiplying the protein concentration value determined above by the number of nucleophilic residues gives rise to the cytosolic concentration for protein reactive sites ([Prot]), estimated in the range of ~20-25 mM. Coupling this concentration with a rate constant value (kₚ) based on published kinetics of amino acid N-nitrosation by N₂O₃ (10⁷-10⁸ M⁻¹ s⁻¹ [98,99]) yields the term kₚ[Prot] which when added to the denominator of equation 8 leads to a lower N₂O₃ estimate (~1 fM), increasing the total number of N₂O₃ scavengers by one while simultaneously diminishing the relative importance of the GSH-associated sink. If the concentration for protein reactive sites is adjusted to include an additional contribution from the free amino acid pool (~35 mM; [100]), the value represented by [Prot] increases to ~60 mM which subsequently leads to a sub-femtomolar [N₂O₃] estimate derived from equation 8. The hypothetical class of amino acid /protein scavengers may also include species such as thioredoxin [101] and metallothionein [102] already known to be affected by nitrosative stress. It is interesting to consider that products of the reaction between proteins and N₂O₃ could potentially be involved in the regulation of cell function through posttranslational protein modification, however the significance of the formation of N-nitrosated proteins in vivo still remains unclear and information on their nitrosation kinetics is scarce.

In summary, we have demonstrated a strategy that employs a precisely controlled NO delivery system and a sensitive LC-MS/MS analytical method to determine the degree to which
nitrosative deamination modifies RNA nucleosides in NO-treated cells. The well defined conditions of the system provide constant and predictable concentrations of NO and O₂ to which cell cultures can be exposed. Measuring rates of formation for rI, rX and rO in RNA extracted from stressed cells and applying the kinetic information to basic expressions of the rate law led to the calculation of intracellular N₂O₃ concentration estimates (0.2-70 fM). Our results also revealed that drug-induced depletion of GSH levels in cells does not significantly alter the accumulation of deamination products in RNA, suggesting that additional scavengers are likely mitigating the damaging effects of N₂O₃ in the cytosol.
5.5 References


Chapter 6
Conclusions and Future Directions

6.1 Conclusions

The overarching goal of this thesis was to estimate the concentration of nitrosating agent N$_2$O$_3$ in the cytosol of mammalian cells exposed to doses of NO similar to those encountered in proximity to inflamed tissues. To determine levels of this reactive and short-lived mediator of inflammation – previously estimated only via mathematical modeling – nucleobase deamination products in RNA were chosen as surrogate markers for the damage it inflicts in the intracellular milieu. Sensitive analytical methods were developed to quantify the corresponding lesions and the kinetics of NO-induced deamination monitored both in purified RNA in solution and RNA extracted from intact cells under nitrosative stress were then compared.

First we investigated the function of a delivery system that simulates the nitrosative environment at sites of inflammation and assessed its reliability under conditions approximating exposure of cell cultures to NO [1]. To challenge biomolecules or cell suspensions with precise doses of NO we chose to employ the Silastic tubing-based reactor developed by Deen and coworkers [2] which had been characterized while containing buffer solutions, but not in the case of cell culture media. Hence, we exposed different commercial culture media in the device, measuring the dissolved NO and O$_2$ concentrations with appropriate polarographic probes and compared our data with predictions from an accompanying mass transfer model that describes the gases profile generated in the system. Our findings indicated that the complicated constitution of media – glutathione, salts, amino acids, sugars and vitamins among others – does
not interfere with diffusion of NO and O\textsubscript{2} and the reaction between them while no extraneous consumption of the radical gas was observed. In particular, the micromolar levels of riboflavin, whose photoactivation produces superoxide – a molecular “sink” for NO in the solution – under certain wavelengths, did not affect the system’s performance whether experiments were performed in light or darkness. We concluded that the device will operate as expected during treatments of cell cultures and the levels of NO can be predicted with confidence, a crucial parameter in calculating the amount of N\textsubscript{2}O\textsubscript{3} generated in the cytosol of stressed cells.

To establish the rate at which RNA nucleobases are damaged by N\textsubscript{2}O\textsubscript{3}, in the absence of other cellular components that may mitigate its effect, we quantified the increase of deamination products resulting in total RNA from exposure to a well defined regime of nitrosative stress. Measurements of RNA treated to precise levels of NO – and a steady N\textsubscript{2}O\textsubscript{3} concentration – in the delivery reactor revealed differential kinetics for the formation of rI, rX and rO (calculated first order rate constants 2.9 × 10\textsuperscript{4}, 1.3 × 10\textsuperscript{6} and 7.7 × 10\textsuperscript{5} M\textsuperscript{-1}s\textsuperscript{-1}, respectively) indicating a considerably greater susceptibility of rG to deamination compared to rA. We noticed similar kinetics for the aforementioned deamination lesions – as well as the disparity between adenine and guanine – when we exposed purified genomic DNA to the same controlled environment and we observed even higher reactivities when free (deoxy)ribonucleosides were used as substrates, suggesting that whereas a protective effect is inherent to macromolecular structure there is no detectable difference between double- and single-stranded nucleic acids. In every case, the generation of oxanine reinforces the conclusion that deamination of guanine leads to comparable amounts of produced xanthine and oxanine, illustrating the complexity of the specific chemistry and expanding the spectrum of products previously thought to occur in cells under nitrosative stress. Specific to the fate of oxanine, our results underlined the importance of rigorously
controlling the conditions for the analytical methods employed as millimolar levels of amine-containing antioxidants such as deferoxamine can affect the stability of the oxanine heterocyclic ring (via nucleophilic attack) during sample processing and contribute to artifactual formation of xanthine (via a previously undocumented oxanine-to-xanthine rearrangement) as shown by our data.

Combining the recently gained confidence in defining conditions in the NO-delivery device during cell culture exposures and kinetic information on RNA nucleobase damage caused by N₂O₃ \textit{in vitro}, we examined the protective effect against nitrosative stress that the cytosol of NO-challenged cells confers. Employing our sensitive LC/MS/MS analytical platforms, small but measurable increases in deamination products were detected in total RNA isolated from TK6 cells treated in the delivery reactor (maintained at predictable and constant concentrations of NO and O₂ comparable to those at sites of inflammation). The observed formation rates for rI, rX and rO – together with the \textit{in vitro} rate constants mentioned above – were applied to fundamental expressions of the rate law and thus provided estimates for the intracellular N₂O₃ concentrations ranging between 0.2 and 33 fM. These experimentally derived estimates compare favorably with predictions stemming from mathematical models, supporting the accuracy of the incorporated kinetic information and illustrating their significance in determining the fate of reactive nitrogen species in inflamed tissues. Further experiments revealed that a roughly 100-fold drug-induced depletion of GSH levels in cells prior to being challenged in the reactor did not alter the accumulation rate of rI, rX and rO, which contradicts previous kinetic analyses that projected marked increases in N₂O₃-related damage with a diminishing GSH cellular content [3]. Our calculations – involving the steady-state hypothesis while including previously reported biomolecules that consume N₂O₃ with known rate constants – suggested that additional sinks are
likely to play an important role in mitigating the damaging effects of N$_2$O$_3$ in the cytosol and that would also explain the negligible contribution of GSH in protecting against nitrosative stress, as indicated by our experiments.

Aside from examining the extent to which nitrosative deamination modifies the parent nucleobases, we also investigated whether depurination can be the result of exposing RNA to NO and N$_2$O$_3$. The study, performed in collaboration with Dr. Wan Simon Chan, focused on the formation of abasic sites in purified RNA exposed to the well controlled and predictable nitrosative environment maintained in the delivery reactor. Using a sensitive LC/MS/MS strategy and derivatization of the treated RNA with a hydroxylamine-based reactive aldehyde probe we were able to show for the first time the occurrence of such damage in RNA, albeit with very slow rates (1.5 × 10$^2$ M$^{-1}$s$^{-1}$) that proved to be orders of magnitude less than the same reaction observed in DNA or the formation of deamination products.

6.2 Future Perspective

The unexpected observation of oxanine formation in RNA due to nitrosative stress, primarily resulting from the application of rectified analytical methods (i.e. omitting amine-containing antioxidants), adds a different perspective to the argument of whether oxanine is a biological lesion encountered in cells exposed to NO. Robust increases in rO content were measured during in vitro exposures of pure RNA to NO whereas smaller rates of formation were detected in RNA extracted from NO-challenged cells; both cases present strong evidence that deamination of guanosine indeed gives rise to more than one product, as initially suggested by previous experimental findings [4]. Our in vitro studies also indicate that there is minimal difference in reactivity between double-stranded DNA and RNA based on values determined for
deamination rate constants and that the spectrum of products is the same in both types of macromolecules. Hence, since our sensitive LC/MS/MS methods enabled the detection of rO in total RNA of treated cells, it is likely that dO can also be measured in genomic DNA purified from cell aliquots (exposed to the largest NO dose during exposure in the delivery reactor and currently stored in -80 °C) by readily modifying the analytical protocols. If the nitrosative environment in the cytosol of cells treated in the reactor led to the formation of rO in ribosomal RNA then we would also expect dO to form in the genome, as long as cross-reaction with basic molecules localized in the nucleus (e.g. histones and polyamines among others [5,6]) or efficient DNA repair do not significantly decrease the levels of the particular lesion.

Based on the notion that amino acids in proteins may be competing with other targets in the cytosol for the reaction with N₂O₃, it would be interesting to investigate how aspects of the ribosomal supramolecular structure affect the deamination of nucleobases in rRNA. The RNA molecules comprising the ribosomal units are in very close proximity to proteins in the ribosome – proteins localized mainly on the surface of the structure – and it is conceivable that in a nitrosative environment those proteins may act as scavengers, the same way phosphate in solution, for example, has been shown to be for nucleobases [7]. Especially after noting that the rate constants for deamination are orders of magnitude smaller compared to those for amino acid N-nitrosation [8,9], it is possible that nucleobases close to the proteins decorating the rRNA strands are relatively more protected against N₂O₃-induced damage rather than those found in the core of the ribosomal units. That scenario comes in contrast to studies arguing that the hydrophobic parts of proteins may accelerate NO auto-oxidation [10] in which case the opposite pattern would prevail, with the protein-free core exhibiting lower levels of accumulated deamination products. To examine whether the presence of proteins noticeably affects
deamination of nucleobases in the RNA ribosomal unit, intact ribosomes can be isolated and exposed to defined levels of NO/N₂O₃ before measuring rI, rX and rO with our optimized protocols. The acquired rates of formation of deaminated nucleobases can then be compared to those observed with purified (protein-free) rRNA in an effort to detect any potential shielding effects related to the presence of protein. Furthermore, attempting to map the deamination events on the RNA macromolecule and search for consistent patterns that may arise due to nitrosative stress could shed more light on the possibility of local hotspots; the preserved sequence inherent to rRNA is expected to facilitate the detection of altered bases. The same study could potentially reveal whether solvent accessibility of the ribosomal units (i.e. strands located in the surface vs. the core) is another factor controlling which nucleobases become more susceptible to modification via N₂O₃. In addition, comparing rates of rI, rX and rO formation between intact ribosomes and protein-free rRNA may provide deamination rate constants that better represent the chemistry as it occurs in the crowded cytosol and thus assist in calculating a more accurate estimate of the intracellular N₂O₃ concentration.

Finally, given the potential for free amino acids and proteins to readily react with N₂O₃ in vitro, we propose to further investigate the possibility of N-nitrosation being a pathway parallel to nucleobase deamination that may damage elements of cellular function and structure. Exposure of thiols to a nitrosative environment has been shown to lead to products of S-nitrosation [11] that can interfere with cellular signal transduction associated with numerous pathologies linked to severe biological implications [12]. Thiols however are not the only protein residues that can be nitrosated since studies have shown at least one more amine-containing side-chain (tryptophan) to react with N₂O₃ at comparable rates with thiols [9]. This suggests that N-nitrosation could be a significant reaction in vivo (especially since amino groups are in general
more abundant than sulfhydryls) however relevant information is scarce and other nucleophilic residues besides tryptophan that may participate in similar pathways have not been characterized yet. More recent studies showed that although denitrosation of nitrosated tryptophan residues is possible in the presence of certain cellular constituents (e.g. GSH [13] or xanthine oxidase [14]) it occurs at a slow enough rate that significant amounts of nitrosated residues are hypothesized to accumulate in vivo. In that case, it is not unreasonable to expect multiple other amine-containing residues to contribute (in addition to thiols and tryptophan) towards lowering the steady-state levels of N₂O₃ forming in the cytosol of cells overwhelmed with NO. Assuming the rate of denitrosation in such cells is sufficiently small, products of N-nitrosation may remain in the intracellular milieu long enough to be detected. To that end, we propose a proteomic mapping of nitrosyl adducts in amine-containing residues of peptides isolated from the most abundant proteins in the cytosol (e.g. listed in ref. [15]) of cells challenged with NO. This strategy will shed some light on the significance of this pathway, if any, in terms of defining the complex network of dysregulated pathways that may lead NO-stressed cells to aberrant growth and will test our hypothesis that nucleophilic protein residues actively scavenge N₂O₃ allowing for a more complete representation of all the relevant molecular sinks in the cell milieu.

Altogether, the proposed studies should provide new insights into the chemical basis for endogenous nucleic acid and protein adducts that could be useful as biomarkers of exposure to NO and assist in more fully understanding the mechanisms that lead to uncontrolled cell growth at sites of inflammation.
6.3 References


Appendix A

Parts list and assembly and operation instructions for the peripheral tubing system complementing the NO-delivery apparatus

A.1 Introduction

The detailed instructions below provide sufficient information for assembling and operating the peripheral tubing system that delivers controlled gas flow to the NO-delivery device while maintaining stirring speed and temperature in safety.

A.2 Detailed assembly instructions for the peripheral tubing system

The peripheral tubing system consists of three independent gas feed lines, each for every required gas mixture, labeled ‘NO-line’, ‘Ar-line’ and ‘O₂-line’. The three gas lines are assembled independently and generally do not merge, with the only exception being a single point where the ‘NO-’ and ‘Ar-line’ connect through a valve (Points A and B in Figure 2.3, Chapter 2). Beginning with the assembly of the ‘NO-line’, the NO cylinder (1%, 10% or 99% NO; Item #8 in Table A.1), is fitted to the NO-regulator (Item #11) that is compatible with all cylinders of varying NO composition. The outlet of the regulator is fitted with 1/8” tubing (Item #15), via a 1/8” stainless nut (Item #22) housing a 1/8” back and front ferrule (Items #23 and #24) that securely anchor the tubing within the fitting. The relative order and orientation of Items #22, #23 and #24, through which Item #15 is threaded, just before the stainless nut is screwed on the outlet of the regulator, is depicted in the upper right inset of Figure 2.3 (Chapter 2); the same layout is implicitly inferred for every stainless nut (Items #22 or #25) appearing on the schematic. The 1/8” tubing connected to the regulator’s outlet –and every other piece of tubing
Table A.1. List of items required for the assembly of the peripheral tubing system.

The assembly part numbers on this list are helpful in relating each item with the schematic in Figure 2.3 (Chapter 2), a visual guide on how to connect the components of the peripheral tubing system (see also Figure A.1).

<table>
<thead>
<tr>
<th>Assembly Part Number</th>
<th>Part Description</th>
<th>Company</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gas mass flow controller</td>
<td>Porter Instrument Company</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Model 201)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Control module</td>
<td>Porter Instrument Company</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Model CM2)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Cable assembly</td>
<td>Porter Instrument Company</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(KC18043-010)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10-position magnetic stir-plate with water-bath</td>
<td>IKA</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(RO 10 power IKAMAG)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Immersion thermostat</td>
<td>IKA</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(EH 4 basic)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Argon Cylinder</td>
<td>Airgas</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(AR300)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Oxygen Mix Cylinder (50% O₂, 5% CO₂, 45% N₂)</td>
<td>Airgas</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Z03NI4532002133)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td>Manufacturer</td>
<td>Model/Part Number/Order Code</td>
</tr>
<tr>
<td>---</td>
<td>--------------------------------------------------</td>
<td>-----------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>8</td>
<td>NO Mix Cylinder</td>
<td>Airgas</td>
<td>(NCCP200 – 99% NO)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(X02AR90C20000X6 – 10% NO)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(X02AR99C15A04Z5 – 1% NO)</td>
</tr>
<tr>
<td>9</td>
<td>Argon Regulator</td>
<td>VWR</td>
<td>(55850-626)</td>
</tr>
<tr>
<td>10</td>
<td>Oxygen Mix Regulator</td>
<td>Airgas</td>
<td>(Y11-244B500)</td>
</tr>
<tr>
<td>11</td>
<td>HP 742 Stainless Steel Regulator</td>
<td>Harris Specialty Gas</td>
<td>(742-50-660-E)</td>
</tr>
<tr>
<td>12</td>
<td>Product moisture trap</td>
<td>Harris Specialty Gas</td>
<td>(4302722)</td>
</tr>
<tr>
<td>13</td>
<td>Manifolds with luer locks, 4 ports, 180° rotation</td>
<td>Cole-Parmer</td>
<td>(EW-30600-42)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(EW-30600-67)</td>
</tr>
<tr>
<td>14</td>
<td>PVC tubing with luer ends</td>
<td>Cole-Parmer</td>
<td>(EW-06407-42)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(EW-06407-44)</td>
</tr>
<tr>
<td>15</td>
<td>PTFE tubing, 1/16&quot;ID x 1/8&quot;OD</td>
<td>Cole-Parmer</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>PTFE tubing, 3/16&quot;ID x 1/4&quot;OD</td>
<td>Cole-Parmer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td>Brand</td>
<td>Quantity</td>
</tr>
<tr>
<td>---</td>
<td>------------------------------------------------------</td>
<td>----------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>17</td>
<td>Male luer with lock ring x 1/16&quot; hose barb</td>
<td>Cole-Parmer (EW-45503-00)</td>
<td>2 packs</td>
</tr>
<tr>
<td>18</td>
<td>Male luer lock plug</td>
<td>Cole-Parmer (EW-45503-56)</td>
<td>1 pack</td>
</tr>
<tr>
<td>19</td>
<td>Union Tee</td>
<td>Swagelok Fittings (SS-200-3)</td>
<td>2</td>
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<tr>
<td>20</td>
<td>Stainless Reducer</td>
<td>Swagelok Fittings (SS-200-R-4)</td>
<td>6</td>
</tr>
<tr>
<td>21</td>
<td>Ball Valve</td>
<td>Swagelok Fittings (SS-41GS2)</td>
<td>1</td>
</tr>
<tr>
<td>22</td>
<td>1/8” Stainless Nut</td>
<td>Swagelok Fittings (SS-202-1)</td>
<td>10</td>
</tr>
<tr>
<td>23</td>
<td>1/8” Stainless Back Ferrule</td>
<td>Swagelok Fittings (SS-204-1)</td>
<td>10</td>
</tr>
<tr>
<td>24</td>
<td>1/8” Stainless Front Ferrule</td>
<td>Swagelok Fittings (SS-203-1)</td>
<td>10</td>
</tr>
<tr>
<td>25</td>
<td>1/4” Stainless Nut</td>
<td>Swagelok Fittings (SS-402-1)</td>
<td>10</td>
</tr>
<tr>
<td>26</td>
<td>1/4” Stainless Back Ferrule</td>
<td>Swagelok Fittings (SS-404-1)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Item Description</td>
<td>Supplier</td>
<td>Quantity</td>
</tr>
<tr>
<td>---</td>
<td>------------------------------</td>
<td>-----------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>27</td>
<td>1/4” Stainless Front Ferrule</td>
<td>Swagelok Fittings</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(SS-403-1)</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Poppet Check Valve</td>
<td>Cambridge Valve &amp; Fitting, Inc.</td>
<td>2</td>
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<tr>
<td></td>
<td></td>
<td>(SS-4C-1/3)</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>Female connector</td>
<td>Swagelok Fittings</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(SS-400-7-4)</td>
<td></td>
</tr>
</tbody>
</table>

(Table A.1)
Table A.2. List of items required for the assembly of the delivery apparatus.

The 11/16" O-rings, 9/64" O-rings and stainless steel fitting required only for the version of the device that houses the NO/O₂ probes. Last three parts on the list required for the adherent cell version only.

<table>
<thead>
<tr>
<th>Part Description</th>
<th>Company</th>
<th>(Catalog #)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFA Wide-Mouth Sample Jar, 120 mL</td>
<td>Cole-Parmer</td>
<td>(EW-06103-40)</td>
</tr>
<tr>
<td>Magnetic stirrer bar</td>
<td>Cole-Parmer</td>
<td>(EW-08551-00)</td>
</tr>
<tr>
<td>Viton O-Ring, 3/8&quot; O.D.</td>
<td>McMaster-Carr</td>
<td>(9464K15)</td>
</tr>
<tr>
<td>Silastic tubing 1.47mm I.D. x 1.96 mm O.D.</td>
<td>Dow Corning</td>
<td>(508-006)</td>
</tr>
<tr>
<td>Female luer bulkhead x 1/16&quot; hose barb</td>
<td>Cole-Parmer</td>
<td>(EW-45500-30)</td>
</tr>
<tr>
<td>Viton O-Ring, 11/16&quot; O.D.</td>
<td>McMaster-Carr</td>
<td>(9464K21)</td>
</tr>
<tr>
<td>Viton O-Ring, 9/64&quot; O.D.</td>
<td>McMaster-Carr</td>
<td>(9464K102)</td>
</tr>
<tr>
<td>Stainless Steel Fitting</td>
<td>Swagelok</td>
<td></td>
</tr>
<tr>
<td>------------------------------</td>
<td>-------------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(SS-8-UT-1-6)</td>
<td></td>
</tr>
<tr>
<td>Impeller assembly (50 mL)</td>
<td>Bellco Biotechnology</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(1967-30050)</td>
<td></td>
</tr>
<tr>
<td>Glass impeller shaft (7mm O.D. x 155 mm)</td>
<td>Bellco Biotechnology</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(1969-70250)</td>
<td></td>
</tr>
<tr>
<td>Black PP compression fitting for 7 mm O.D. glass shafts (part 10)</td>
<td>Bellco Biotechnology</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(A523-204)</td>
<td></td>
</tr>
</tbody>
</table>

(Table A.2)
Figure A.1. Picture of the peripheral tubing system.

Picture of the peripheral tubing system with elements of temperature control and stirring assembled under a fume hood.
depicted— is arbitrarily long so the peripheral tubing system can fit the physical dimensions of the fume hood within which it will be installed. Since NO is toxic, assembling the peripheral system in a hood is imperative to protect the user(s) from exposure to the gas. The other end of the tubing extending from the regulator’s outlet is connected to the first port (out of three) of a union tee (Item #19). Starting from the second port of the same union tee, another piece of 1/8” tubing leads to the inlet of a custom-made NOx impurities trap, essentially a product moisture trap (Item #12) emptied of its initial absorbent material and refilled with soda lime which removes traces of oxidized forms of NO. The third port of the union tee (Point A in Figure 2.3, Chapter 2) will be connected later with the fully assembled ‘Ar-line’ via a valve, allowing for a fraction of the Argon flow to be redirected through the ‘NO-line’ for purging purposes. From the NOx impurities trap outlet, another piece of 1/8” tubing extends and enters a stainless reducer (Item #20). The reducer enables the increase of tubing dimensions (following the direction of the flow, from 1/8” to a 1/4” diameter) for compatibility purposes with the NO mass flow controller (Item #1). The larger end of the reducer is fitted with a 1/4” nut (Item #25) –1/4” back and front ferrules (Items #26 and #27) included— to the inlet of a poppet check valve (Item #28). The check valve is used to restrict airflow to a single direction, preventing backflow, and its outlet is connected to a short piece of 1/4” tubing (Item #16). The other end of that tubing is fitted to the inlet of the NO mass flow controller (Item #1; see Table A.3 for custom specifications), plugged to the flow control module (Item #2; not shown in Figure 2.3, Chapter 2) via its cable assembly (Item #3). The control module allows the user to manually set the NO-controller’s flow from 0% to approximately 100% of its maximum flow capacity (500 standard cubic centimeters per minute for the NO mass flow controller). The controller’s outlet is connected to another reducer (Item #20) and the smaller end of that reducer is attached to a piece of 1/8” tubing. The other end
Table A.3. Custom specifications for mass flow controllers installed in the peripheral tubing system.

Custom specifications for ordering the two mass flow controllers (Item #1 in Figure 2.3, Chapter 2 and Table A.1). The listed parameters describe characteristics of the (approximate) flow conditions for which the controllers will be used.

<table>
<thead>
<tr>
<th></th>
<th>NO mass flow controller</th>
<th>O$_2$ mass flow controller</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fluid</strong></td>
<td>90% Ar/10% NO</td>
<td>21% O$_2$/79% N$_2$</td>
</tr>
<tr>
<td><strong>Flow</strong></td>
<td>500 sccm*</td>
<td>2000 sccm*</td>
</tr>
<tr>
<td><strong>P1</strong></td>
<td>40 psig</td>
<td>40 psig</td>
</tr>
<tr>
<td><strong>P2</strong></td>
<td>0 psig</td>
<td>0 psig</td>
</tr>
<tr>
<td><strong>Temp</strong></td>
<td>70F/21.1C</td>
<td>70F/21.1C</td>
</tr>
</tbody>
</table>

* sccm: standard cubic centimeter per minute
of the 1/8" tubing is capped with a plastic barbed hose male luer adapter (Item #17) with its hose barb firmly inserted into the tubing and its locking nut screwed on the inlet of a 4-port manifold (Item #13). The outlet of the manifold is sealed with a luer plug (Item #18) allowing the entering gas flow to be divided through its 4 ports. By default, the manifold’s stopcocks knobs are all limited to a 180 degree rotation, but overriding (by breaking) the stops in each knob that obstruct free 360° rotation gives a fully functional manifold where 1, 2, 3 or 4 ports can be used simultaneously (not possible granted the manifold’s original design). As a last step in the assembly of the ‘NO-line’, four pieces of 1/8" tubing, equal in length, are capped at both ends with male barbed hose luer adapters (Item #17) and the locking nut from one end of each is screwed to one of the 4 ports on the manifold. Hence 4 equal pieces of tubing are attached to the 4 manifold ports with their unengaged ends free to fit onto 4 identical delivery devices (connection points denoted by asterisks in Figure 2.3, Chapter 2). The manifold is firmly suspended at an appropriate height above the 10-position magnetic stir-plate with water-bath (item #4) so that the 4 pieces of tubing can extend freely downwards and attach to up to 4 devices, with all devices resting in the water-bath at the preset positions on the magnetic stir-plate (allowing for proper rotation of the stirrer bars inside each device). The ‘NO-line’ connects at a single point only with the ‘Argon-line’, whose assembly is described next.

The Argon cylinder (Item #6) is connected to the Argon regulator (Item #9) and the regulator’s outlet is fitted with a stainless reducer (Item #20), securely tightened with a 1/4" nut (Item #25; 1/4" back and front ferrules Items #26 and 27 included). The reducer is subsequently attached to a stretch of 1/8" tubing (Item #15) via a 1/8" nut (Item #22) while the other end of that tubing is inserted into the first port of a union tee (Item #19). From the second port of the same union tee (Point B; Figure 2.3, Chapter 2), 1/8" tubing extends to one end of a ball valve
(Item #21) while the other end of the valve is connected to the previously unoccupied port of the tee in the ‘NO-line’ (Point A; Figure 2.3, Chapter 2) with another stretch of 1/8" tubing. This valve, uniting (when in the open position), or separating (when in the closed position), the ‘Ar-‘ and ‘NO-lines’, is labeled “purging valve” and its purpose will be discussed shortly. The third and final port of the union tee on the ‘Ar-line’ (Point B) is connected to one end of 1/8" tubing while the tubing’s other end is capped with a male barbed hose luer adapter (Item #17). That luer is fitted on the inlet of a manifold (Item #13), modified to allow independent opening of all its ports in a similar fashion to the one in the ‘NO-line’. As before, the manifold’s outlet is sealed with a luer plug (Item #18) and the 4 ports are connected with 4 equal pieces of 1/8" tubing (capped at both ends with male barbed hose luer adapters), while the manifold is suspended above the 10-position magnetic stir-plate with water-bath. The 4 pieces of tubing extending from the 4 ports can connect to 4 separate delivery devices situated in the water-bath (connection points denoted by asterisks in Figure 2.3, Chapter 2). The principal purpose of the ‘Ar-line’ is to enable negative control experiments to be performed, during which the contents of the devices are exposed to inert Argon and O₂, rather than NO and O₂. With the given configuration, up to 4 independent devices can be connected to the ‘NO-line’ while 4 others can be connected to the ‘Ar-line’ in parallel, thus reaching the maximum capacity of the peripheral system (O₂ delivered to all 8 devices by the ‘O₂-line’ described next). A second purpose for the ‘Ar-line’ involves purging the mass flow controller in the ‘NO-line’ from residual NO (residues of formed nitric acid may corrode the steel fittings of the controller): using the “purging valve” (Points A and B in Figure 2.3, Chapter 2) in its open position allows a fraction of the Argon gas flow to travel through the ‘NO-line’, flushing NO traces from the controller’s chamber. With “purging valve” in the closed position (default state during typical exposures), ‘Ar-‘ and ‘NO-lines’ are
completely separated and the two gases are delivered individually through the corresponding manifolds.

The last part of the peripheral tubing system involves assembling the ‘O2-line’: the O2 cylinder (Item #7) is fitted with the oxygen regulator (Item #10), while a female connector (Item #29) is attached to the regulator’s outlet. The female connector has the appropriate dimensions so that a stainless reducer (Item #20) can be anchored to its outlet with the help of a 1/4” nut (Item #25). The smaller end of the reducer is connected to 1/8” tubing (Item #15), firmly secured in place with a 1/8” nut (Item #22), and the other end of the tubing extends towards an arrangement of fittings mirroring the configuration of the ‘NO-line’ Briefly, the O2 mass flow controller (Item #1; see Table A.3 for custom specifications) is integrated into the ‘O2-line’ through the following sequence of items connected with each other: reducer (Item #20), poppet check valve (Item #28), 1/4” tubing (Item #16), O2 mass flow controller, reducer, 1/8” tubing. The O2 mass flow controller is plugged to the control module (Item #2; not shown in Figure 2.3, Chapter 2) via its cable assembly (Item #3). The 1/8” tubing from the flow controller’s outlet connects, via a male barbed hose luer adapter (Item #17), to the inlet of a 4-port manifold (item #13) whose outlet is connected to a second identical manifold, through a short piece of 1/8” tubing capped at both ends with male barbed hose luer adapters; the second manifold downstream is sealed at its outlet with a luer plug (Item #18). Both manifolds were modified to allow simultaneous opening of up to 4 ports each (similarly to the ‘NO-line’ and ‘Ar-lines’) and both are situated above the 10-position magnetic stir-plate with water-bath (Item #4). All 8 ports combined from both manifolds, are fitted with 8 PVC tubing with luer ends (Item #14) that extend their unoccupied locking nuts towards the water-bath. With this arrangement, up to 8 ports can be open at any given time, allowing O2 to be distributed to up to 8 separate delivery devices (connection points
denoted by asterisks in Figure 2.3, Chapter 2) which at the same time are attached to the ‘Ar-line’ and ‘NO-lines’. The devices are positioned on top of the stir-plate, within the water-bath whose water is maintained at a constant temperature by an immersion thermostat (Item #5).

A.3 Operation instructions for the peripheral tubing system

A.3.1 Flow adjustment through the gas lines of the peripheral tubing system

Both ‘NO-line’ and ‘O₂-line’ have mass flow controllers (Item #1 in Figure 2.3 in Chapter 2 and Table A.1) installed in-line, communicating through cable assemblies (Item #3) with the control module (Item #2): the NO mass flow controller is plugged to ‘Channel 1’ of the module and the controller for O₂ to ‘Channel 2’. Switching the module between ‘Channel 1’ and ‘Channel 2’ and using the corresponding dials, allows the user to manually change the flow through each controller from 0% to approximately 100% of its maximum flow capacity (500 sccm for the NO controller and 2000 sccm for the O₂ controller; Table A.3). For every delivery device planned to be supplied with NO, the digital reading on ‘Channel 1’ (representing percent of maximum flow for the NO controller) needs to be increased by 20.0 units. For every device supplied with O₂ (also taking into account negative controls), the digital reading of ‘Channel 2’ (percent of maximum flow for the O₂ controller) needs to be increased by 10.0 units. In the hypothetical scenario of a single delivery device exposed to NO and O₂, ‘Channel 1’ should have a reading of “20.0” and ‘Channel 2’ a reading of “10.0”. In the full capacity scenario of 4 delivery devices supplied with NO, O₂ and another 4 with Argon, O₂ (negative controls), the readings should be “80.0” for ‘Channel 1’ and “80.0” for ‘Channel 2’. Due to the absence of a mass flow controller for the ‘Ar-line’, the Argon regulator (Item #9) needs to be opened until minimal argon flow is achieved, either for exposure or purging purposes. Audible hissing
coming from the outlets of the ‘Ar-line’ is an indication that the current flow is excessively high and that it should be decreased via the Argon regulator to a minimum.

A.3.2 Operation procedure of peripheral tubing system connected with delivery device(s)

Once the desired number of devices are prepared for exposure (equipped with tubing loop pairs of specific lengths, subsequently autoclaved if used for cell culture exposure and then filled with the fluid of interest), they are attached using the designated gas-inlet luer adapters (female luers) on top of their caps to connect with the luer locks (male luers) extending from the manifolds of the ‘NO-line’, ‘Ar-line’ and ‘O₂-line’ (connection points denoted by asterisks in Figure 2.3 in Chapter 2). The connected devices are positioned on top of the operating magnetic stir-plate (rotating speed set at 100 rpm) while being immersed in the water-bath. To efficiently isolate the contents of the devices from the water filling the water-bath, and vice versa, each device is inserted in a small clear (sandwich-style) plastic bag before being immersed into the temperature-regulated water. Once the content in the devices reaches the desired temperature and after making sure the “purging valve” (Points A and B in Figure 2.3, Chapter 2) is closed, in other words, properly separating the two gas lines, the regulators on the gas cylinders are opened (digital reading of both channels on the control module should be approximately zero before any flow is engaged). Based on how many devices are connected, the manifolds are adjusted so that gases are channeled only through lines of tubing that connect with a device; flow is restricted for any line of tubing extending from the manifolds that is not engaged. The control module is adjusted so that the digital read-out of each channel is consistent with how many devices are connected (i.e., 20.0 units on ‘Channel 1’ per device supplied with NO, 10.0 units on ‘Channel 2’ per device supplied with O₂). After flow from all gas lines has been initiated, small droplets of leak detection solution are deposited on top of the gas-outlet luer adapters on each connected
delivery device to determine whether proper flow is achieved: formation of bubbles indicates unobstructed gas flow through the tubing loops attached inside the devices and thus proper operation of the delivery device. However, if no bubbles are formed after application of the leak detection solution it may be possible that the flexible loops of Silastic tubing are chocked due to a kink, twist or drop of liquid, blocking the normal flow of gas and that issue needs to be remedied immediately. Finally, when testing the flow with the leak detection solution is complete, the gas-outlet luer adapters are wiped dry to remove any residual solution that could potentially enter and plug the tubing. As already mentioned, the entire system needs to be located in a fume hood, such that none of the NO waste streams that escape the devices becomes a hazard while the exposure is taking place.

After completion of the exposure, the procedure to shut the system down begins by choking the regulators on NO and O₂ cylinders (Items #10, 11 in Figure 2.3, Chapter 2) and removing the devices from the water-bath, keeping any disengaged lines of tubing from accidentally dipping in the bath’s water. Once flow from the ‘NO-’ and ‘O₂-line’ has fully decreased, the “purging valve” (Points A and B in Figure 2.3, Chapter 2) is opened and the ‘Ar-line’ manifold is blocked, such that the entire Argon flow is being redirected through the NO line to purge the NO flow controller. After 10 minutes of purging, the regulator on the Argon cylinder is chocked and when the flow finally diminishes the “purging valve” is reset to its default closed position.

A.3.3 Safety with handling the NO gas

NO gas is toxic so all experiments should be performed in a certified fume hood and personal protective equipment should be used to minimize contact with or inhalation of the
hazardous gas. Regular tests for leaks on connections and parts of the system that are outside the fume hood (e.g. regulators, tanks) should take place during usage.
Appendix B
Parameters for the analysis of nucleobase deamination products

Table B.1. Chromatographic conditions for HPLC prepurification of hydrolyzed RNA and DNA samples.

Solvent A 8 mM ammonium acetate. Solvent B acetonitrile. Instrument Agilent 1100.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% solvent B</th>
<th>Flow (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hydrolyzed RNA</td>
<td></td>
<td></td>
</tr>
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<tr>
<td>74</td>
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<tr>
<td>76</td>
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<tr>
<td>hydrolyzed DNA</td>
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<td></td>
</tr>
<tr>
<td>0</td>
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<td>0.5</td>
</tr>
<tr>
<td>45</td>
<td>3.5</td>
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</tr>
<tr>
<td>50</td>
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<tr>
<td>55</td>
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<tr>
<td>78</td>
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</tbody>
</table>

Column temperature at 20 °C for hydrolyzed RNA and 32 °C for hydrolyzed DNA samples.
Table B.2. Isocratic chromatographic conditions employed for LC-MS/MS quantification of RNA and DNA deamination products.

Solvent A 0.1 mM acetic acid. Solvent B acetonitrile. Instrument AB Sciex API 3000. Flow rate maintained constant at 0.2 mL/min.

<table>
<thead>
<tr>
<th>nucleoside</th>
<th>% solvent B</th>
<th>elution time (min)</th>
<th>column temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rI</td>
<td>0.7</td>
<td>~7.5</td>
<td>45</td>
</tr>
<tr>
<td>rX</td>
<td>1</td>
<td>~18</td>
<td>30</td>
</tr>
<tr>
<td>rO</td>
<td>1</td>
<td>~20</td>
<td>30</td>
</tr>
<tr>
<td>dI</td>
<td>3</td>
<td>~8.5</td>
<td>25</td>
</tr>
<tr>
<td>dX</td>
<td>3</td>
<td>~10.5</td>
<td>25</td>
</tr>
<tr>
<td>dO</td>
<td>3</td>
<td>~15.5</td>
<td>25</td>
</tr>
</tbody>
</table>


**Table B.3.** Mass spectrometry parameters for LC-MS/MS quantification of deamination products in hydrolyzed RNA and DNA samples.

Instrument AB Sciex API 3000.

<table>
<thead>
<tr>
<th></th>
<th>rI</th>
<th>rX/rO</th>
<th>dI</th>
<th>dX/dO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nebulizer gas (psi)</td>
<td>10</td>
<td>14</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Curtain gas (l/min)</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Collision gas (l/min)</td>
<td>12</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Ion spray voltage (V)</td>
<td>3500</td>
<td>4250</td>
<td>5000</td>
<td>3500</td>
</tr>
<tr>
<td>Gas temperature (°C)</td>
<td>425</td>
<td>425</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>Declustering potential (V)</td>
<td>19</td>
<td>21</td>
<td>15</td>
<td>22</td>
</tr>
<tr>
<td>Focusing potential (V)</td>
<td>120</td>
<td>130</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>Entrance potential (V)</td>
<td>9</td>
<td>9</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Collision energy (V)</td>
<td>13</td>
<td>13</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>Collision cell exit potential (V)</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
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<tr>
<td>Transition Q1→Q3 (m/z)</td>
<td>269→137</td>
<td>285→153</td>
<td>253→137</td>
<td>269→153</td>
</tr>
</tbody>
</table>
Table B.4. Chromatographic conditions employed for LC-MS/MS quantification of deamination products in ribonucleosides.

Solvent A 0.1 mM acetic acid. Solvent B acetonitrile. Instrument Agilent 6410 QQQ.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% solvent B</th>
<th>Flow (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rI</td>
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<td>0.2</td>
</tr>
<tr>
<td>19</td>
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<td>0.3</td>
</tr>
<tr>
<td>21</td>
<td>100</td>
<td>0.3</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>0.3</td>
</tr>
<tr>
<td>36</td>
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<tr>
<td></td>
<td>rX/rO</td>
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</tr>
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<td>0</td>
<td>0.2</td>
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<td>0</td>
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<tr>
<td>9</td>
<td>0</td>
<td>0.3</td>
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<tr>
<td>10</td>
<td>0</td>
<td>0.3</td>
</tr>
<tr>
<td>16</td>
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<td>0.3</td>
</tr>
<tr>
<td>19</td>
<td>100</td>
<td>0.3</td>
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<tr>
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<td>0.3</td>
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<td>0.2</td>
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<tr>
<td>36</td>
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<td>0.2</td>
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</tbody>
</table>

LC flow diverted to MS only during the 9-17 min window while quantifying rI, 14-25 min window for rX/rO; column temperature maintained constant at 23 °C in every case.
Table B.5. Chromatographic conditions employed for LC-MS/MS quantification of deamination products in 2-deoxyribonucleosides.

Solvent A 0.1 mM acetic acid. Solvent B acetonitrile. Instrument Agilent 6410 QQQ.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% solvent B</th>
<th>Flow (mL/min)</th>
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</thead>
<tbody>
<tr>
<td>dI</td>
<td></td>
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<td>0</td>
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<td>100</td>
<td>0.3</td>
</tr>
<tr>
<td>24</td>
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<td>40</td>
<td>0</td>
<td>0.2</td>
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<td>dX</td>
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<tr>
<td>64</td>
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<tr>
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<td>56</td>
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</table>
Table B.6. Mass spectrometry parameters for LC-MS/MS quantification of deamination products in nucleosides.

Instrument Agilent 6410 QQQ.

<table>
<thead>
<tr>
<th></th>
<th>rI</th>
<th>rX/rO</th>
<th>dI</th>
<th>dX/dO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nebulizer gas (psi)</td>
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<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Gas flow (l/min)</td>
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<td>Gas temperature (°C)</td>
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<td>300</td>
<td>250</td>
<td>250</td>
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<td>Capillary potential (V)</td>
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<td>3900</td>
<td>3900</td>
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<td>Fragmentor potential (V)</td>
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<td>75</td>
<td>70</td>
<td>50</td>
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<tr>
<td>Collision energy (V)</td>
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<td>4</td>
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<td>0</td>
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<tr>
<td>Transition Q1→Q3 (m/z)</td>
<td>269→137</td>
<td>285→153</td>
<td>253→137</td>
<td>269→153</td>
</tr>
</tbody>
</table>
**Figure B.1.** Composite chromatograms of ribonucleosides and 2-deoxyribonucleosides.

Resolution and elution times of (A) ribonucleosides and (B) 2-deoxyribonucleosides standards during the HPLC pre-purification step. Instrument Agilent 1100. Absorbance at 260 nm.
Figure B.2. Experimental UV signature of oxanosine.
Figure B.3. HPLC resolution of nucleoside mixture derived from hydrolyzed total RNA.

Elution times of native nucleosides annotated in a typical chromatographic analysis of hydrolyzed total RNA extracted from TK6 cells. Instrument Agilent 1100. Absorbance at 260 nm.
Appendix C

Parameters for the analysis of abasic sites in RNA

Table C.1. Chromatographic conditions employed for LC-MS/MS quantification of ARP-derivatized AP sites in hydrolyzed RNA.

Solvent A 0.1 mM acetic acid. Solvent B acetonitrile. Instrument AB Sciex API 3000. Flow rate maintained constant at 0.2 mL/min.

<table>
<thead>
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<th>Time (min)</th>
<th>% solvent B</th>
</tr>
</thead>
<tbody>
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<td>0</td>
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<td>26</td>
<td>1</td>
</tr>
<tr>
<td>40</td>
<td>1</td>
</tr>
</tbody>
</table>

LC flow initially diverted to waste and switched to the ion source 8 min after sample injection; column temperature maintained constant at 23 °C in every case.
Table C.2. Mass spectrometer parameters for LC-MS/MS quantification of ARP-derivatized AP sites in hydrolyzed RNA.

Instrument AB Sciex API 3000.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nebulizer gas (psi)</td>
<td>10</td>
</tr>
<tr>
<td>Curtain gas (l/min)</td>
<td>8</td>
</tr>
<tr>
<td>Collision gas (l/min)</td>
<td>10</td>
</tr>
<tr>
<td>Ion spray voltage (V)</td>
<td>3000</td>
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<tr>
<td>Gas temperature (°C)</td>
<td>300</td>
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<tr>
<td>Declustering potential (V)</td>
<td>30</td>
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<tr>
<td>Focusing potential (V)</td>
<td>200</td>
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<tr>
<td>Entrance potential (V)</td>
<td>10</td>
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<tr>
<td>Collision energy (V)</td>
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<td>Collision cell exit potential (V)</td>
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<tr>
<td>Transitions Q1→Q3 (m/z)</td>
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</tr>
<tr>
<td></td>
<td>464→332</td>
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