Controlled Delivery of Nitric Oxide for Cytotoxicity Studies

by

Chen Wang

Submitted to the Department of Chemical Engineering
on May 9, 2003 in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy in Chemical Engineering

Abstract

Endogenous synthesis of nitric oxide (NO) is essential for many physiological functions, including the immune defense. However the sustained, high production of NO by immune cells (macrophages) that accompanies chronic inflammation may be cytotoxic, mutagenic and carcinogenic. To better understand the roles of NO and its various reactive derivatives (e.g.,N_2O_3 and $ONOO^-$) in cancer, it is important to know the NO concentration and the total NO dose that tissue cells are exposed to during inflammation. To obtain this quantitative information, methods are needed for exposing cells to physiological levels of NO and its derivatives over relatively long periods of time. This research has therefore focused on developing *in vitro* release of NO.

To permit continuous NO exposures over lengthly periods, an apparatus was fabricated which uses gas-permeable polydimethylsiloxane tubing to supply both NO and O_2 to a stirred, cylindrical vessel. Mass transfer in this system was characterized by measuring the delivery rates of NO or O_2 alone, and of NO to air-saturated solutions. It was found that the total flux of nitrogen species into the liquid was 40-90% greater in the presence of O_2, depending on the NO partial pressure in the gas. Also the simultaneously measured masss transfer coefficients for NO and O_2 differed greatly from the corresponding unreactive values. An analysis of the data using diffusion-reaction models showed that NO oxidation in the aqueous boundary layer contributed very little to the nitrogen flux increase or to variations in the mass transfer coefficients. However the unusually strong dependence of the delivery rates on chemical reactions could be explained by postulating that partial oxidation of NO to N O_2 occurred within the membrane, with a rate constant of 4.4×10^5 $M^{-2}s^{-1}$ at 23° C. Using the measured mass transfer coefficients, the aqueous NO concentrations could be accurately predicted for the case of simultaneous NO and O_2 delivery.

The NO delivery system was used first to study the kinetics of plasmid DNA base deamination under pathologically relevant conditions. Three nucleobase products, 2'-deoxyxanthosine (dX), 2'-deoxyinosine (dI), and 2'-deoxyuridine (dU), were formed from 2'-deoxyguanosine (dG), 2'-deoxyadenosine (dA), and 2'-deoxycytosine (dC), respectively, at constant rates under steady state concentrations of 1.2 μM NO and 190 μM O_2. Morpholine nitrosation rates were measured under similar exposure conditions. Using a kinetic model, along with the known morpholine deamination kinetics, the three rate constants for base deamination were found to be nearly identical ($k = 1.2 \times 10^5$ $M^{-2}s^{-1}$).

The second application of the new NO delivery system was to study NO-mediated cyto- and genotoxicity in two human lymphoblastoid cell lines, TK6 (wild-type p53) and NH32 (p53-null but isogenic to TK6). The TK6 and NH32 cells were each exposed to several steady-state NO concentrations for varying lengths of time, so that the total dose (area under the concentration-time curve) covered a wide range. Endpoint assays, including lethality, apoptosis, mitochondrial damage, and mutation rate in the thymidine kinase (*TK1*) gene locus, were performed at different post-treatment times. Compared to Ar-treated control cells, significant cell death, apoptosis, mitochondrial membrane depolarization were observed in NO-treated TK6 cells, and the *TK1* gene mutation rate was elevated. Of particular importance, toxic effects were observed only when the NO concentration and dose were greater than threshold values of ~0.5 μM and of ~150 μM·min, respectively, and under such conditions, the total cell survival and the fraction of non-apoptotic cells both decreased exponentially with increasing NO dose. In general, the NH32 cells were much more resistant to NO-induced damage and death than TK6 cells, demonstrating that p53 status is an important determinant of NO-induced cytotoxicity.

Also investigated was a scheme for $ONOO^-$ delivery based on its continuous synthesis from NO and O_2^- *in situ*. The idea was to combine the NO delivery system with O_2^- generation from xanthine-oxidase-catalyzed hypoxanthine oxidation. Both hypoxanthine and xanthine oxidations by this enzyme were examined by measuring the concentration time courses of hypoxanthine, xanthine, uric acid, and O_2^- . A previously proposed model based on a ping-pong mechanism and competitive inhibition of xanthine oxidase by uric acid was used to correlate the resuls, and the kinetic parameters were evaluated by nonlinear regression. A distinction was made between the fractions of hypoxanthine and xanthine participating in the univalent reduction of O_2 to O_2^-, and those univalent fractions evaluated. The kinetic model provided an excellent description of the reactant and product concentrations over the full time course of the reactions, and thus provides an effective way to predict rates of d O_2^- generation. Uricase was used to remove uric acid, a scavenger of peroxynitrite, and the kinetics of this enzymatic reaction was also investigated. To quantify $ONOO^-$ formation in the delivery system, the rates of $ONOO^-$ -mediated tyrosine nitration and dimerization were studied. Both kinetic modeling and experiments were performed to determine the yields of the two primary products, 3, 3'-dityrosine and 3-nitrotyrosine. Simulation of a complex network that included all known reactions in the test system suggested that dityrosine is the major product. The predicted yield depended strongly on the relative rates of NO and O_2^- delivery, with the maximum occurring when they were about equal. Experiments performed under these conditions, using an HPLC method to measure dityrosine and nitrotyrosine, showed much lower levels of dityrosine than were predicted, and no detectable nitrotyrosine. The very low yields of tyrosine products are explained in part by partial inactivation of both xanthine oxidase and uricase by $ONOO^-$ -derived NO_2 and CO_3^- radicals. Overall the complexity and uncontrollability of this system makes it impractical as an $ONOO^-$ delivery tool for biological studies. Research in the future should focus on developing alternative strategies to achieve this goal.

Thesis Supervisor: William M. Deen
Title: Carbon P. Dubbs Professor of Chemical Engineering, MIT.