ANALYSIS OF NITRIC OXIDE TRANSFER AND
RELEASE RATE FOR A CYSTEINE
MODIFIED POLYMER

By

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ANALYSIS OF NITRIC OXIDE TRANSFER AND
RELEASE RATE FOR A CYSTEINE
MODIFIED POLYMER

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<td>AA</td>
<td>Ascorbic acid</td>
</tr>
<tr>
<td>AA⁻</td>
<td>Ascorbic acid anion</td>
</tr>
<tr>
<td>AA²⁻</td>
<td>Ascorbic acid dianion</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AlbSH</td>
<td>Albumin</td>
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<tr>
<td>AlbSNO</td>
<td>S-nitrosoalbumin</td>
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<tr>
<td>APTES</td>
<td>3-Aminopropyltriethoxysilane</td>
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<td>Ar, Ar'</td>
<td>Aromatic amine</td>
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<tr>
<td>ARA</td>
<td>Arachidonate acid</td>
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<td>b</td>
<td>Half-thickness of the flow slit</td>
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<td>Boc-Cys(Trt)-OH</td>
<td>N-(tert-butoxycarbonyl)-S-trityl-L-cysteine</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSA⁻</td>
<td>Bovine serum albumin anion</td>
</tr>
<tr>
<td>BSANO</td>
<td>Nitrosated bovine serum albumin</td>
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<td>C</td>
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<td>C₀</td>
<td>Initial cysteine surface concentration</td>
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<tr>
<td>Cₐ</td>
<td>Concentration of component A</td>
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<tr>
<td>Cₐ₀</td>
<td>Initial concentration of component A</td>
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<td>Ca-CAM</td>
<td>Calcium- calmodulin complex</td>
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cAMP  Cyclic adenosine 3’,5’-monophosphate

$C_B$  Concentration of component B

$C_C$  Concentration of component C

cGMP  Cyclic guanosine 3’,5’-monophosphate

$C_{H+}$  Concentration of hydrogen ion

cm  Centimeter

$^{51}$Cr  Chromium-51

$C_T$  Total concentration of ion impurities

COOH  Carboxyl group

CyS⁻  Cysteine anion

CyS-CyS  Disulfide cysteine

CySH/Cys  Cysteine

CySH(E)  Cysteine ethylester

Cys-PET  Cysteine modified PET

CySNO  S-nitrosocysteine

$D_A$  Molecular diffusivity of component A

DAG  Diacylglycerol

DCCI  N,n’-dicyclohexylcarbodiimide

DHA  Dehydroascorbic acid

DI  Deionized

DNA  Deoxyribonucleic acid

DTNB  5,5-Dithiobis (2-nitrosbenzoic acid) (Ellman’s reagent)

DTPA  Diethylene triamine pentaacetic acid
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<th>Abbreviation</th>
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<tr>
<td>ED</td>
<td>Ethylenediamine</td>
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<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelium-derived relaxing factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>EMP</td>
<td>4-ethylmorpholine</td>
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<td>$f_B$</td>
<td>Fraction of component B</td>
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<tr>
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<td>Femtomole</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
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<tr>
<td>GA</td>
<td>Glutaraldehyde</td>
</tr>
<tr>
<td>GlyCySNO</td>
<td>S-nitrosocysteinylglycine</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSNO</td>
<td>S-nitrosoglutathione</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>H</td>
<td>Height of tube</td>
</tr>
<tr>
<td>HI</td>
<td>Hydriodic acid</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
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<td>HSANO</td>
<td>S-nitroso human serum albumin</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HMW</td>
<td>High molecular weight</td>
</tr>
<tr>
<td>HNO₂</td>
<td>Nitrous acid</td>
</tr>
<tr>
<td>HOBOt</td>
<td>1-hydroxybenzotriazole</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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$hv$  Photon’s energy

$IP_3$  Inositol 1,4,5-triphosphate

$k$  Actual rate constant

$k_1, k_2$  Actual rate constant for AA oxidation by copper

$K_1, K_2, K_3, K_4, K_5$  Equilibrium constants of reactions

$k_{A,S}$  Surface consumption rate constant of HSANO on CyS-PET

$k_{Da}$  Kilodalton

$K$  Transnitrosation equilibrium constant

$K_{a,C}$  Acid dissociation equilibrium constant for cysteine

$k_{da}$  CySNO decomposition (by ascorbic acid) rate constant

$k_{db}$  BSANO decomposition rate constant

$k_{dc}$  CySNO decomposition (by Cu$^+$) rate constant

$k_f$  Forward reaction rate constant of transnitrosation

$KI$  Potassium iodide

$k_{oc}$  Cysteine oxidation (by Cu$^{2+}$) rate constant

$k_{oa}$  Ascorbic acid oxidation (by Cu$^{2+}$) rate constant

$k_{oo}$  Cysteine autoxidation rate constant

$k_r$  Reverse reaction rate constant of transnitrosation

$k_{obs}$  Observed rate constant

$\kappa$  Nondimensionalization coefficient

$L$  Length of tube

LMW  Low molecular weight

$m$  Meter
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mol</td>
<td>Mole</td>
</tr>
<tr>
<td>mV</td>
<td>Millivolt</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>N$_2$O$_3$</td>
<td>Dinitrogen trioxide</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NEM</td>
<td>N-Ethylmaleimide</td>
</tr>
<tr>
<td>NH$_2$</td>
<td>Primary amine group</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>nmol</td>
<td>Nanomole</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NO$_x$</td>
<td>Nitrogen oxide donors</td>
</tr>
<tr>
<td>NO$^+$</td>
<td>Nitrosonium</td>
</tr>
<tr>
<td>NO$^-$</td>
<td>Nitroxyl</td>
</tr>
<tr>
<td>NO$_2^-$</td>
<td>Nitrite</td>
</tr>
<tr>
<td>NOA</td>
<td>Nitric oxide analyzer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NOS</td>
<td>NO synthase</td>
</tr>
<tr>
<td>$O_2^-$</td>
<td>Superoxide</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>$OD_0$</td>
<td>Initial optical density</td>
</tr>
<tr>
<td>ODE</td>
<td>Ordinary differential equation</td>
</tr>
<tr>
<td>ONOOH</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>PA$_2$</td>
<td>Phospholipase A$_2$</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDE</td>
<td>Partial differential equation</td>
</tr>
<tr>
<td>PET</td>
<td>Poly(ethylene terephthalate)</td>
</tr>
<tr>
<td>PET-C(a)-CySH</td>
<td>Cysteine modified carboxylated PET (amide bond)</td>
</tr>
<tr>
<td>PET-C(a)-CySH(E)</td>
<td>Cysteine ethylester modified carboxylated PET (amide bond)</td>
</tr>
<tr>
<td>PET-C(a)-Gly</td>
<td>Glycine modified carboxylated PET (amide bond)</td>
</tr>
<tr>
<td>PET-COOH</td>
<td>Carboxylated PET</td>
</tr>
<tr>
<td>PET-GA</td>
<td>Glutaraldehyde modified PET</td>
</tr>
<tr>
<td>PET-N(a)-CySH</td>
<td>Cysteine modified aminolyzed PET (amide bond, DCCI method)</td>
</tr>
<tr>
<td>PET-N(a)-Gly</td>
<td>Glycine modified aminolyzed PET (amide bond, DCCI method)</td>
</tr>
<tr>
<td>PET-N(a,e)-CySH</td>
<td>Cysteine modified aminolyzed PET (amide bond, EDC method)</td>
</tr>
<tr>
<td>PET-NH$_2$</td>
<td>Aminolyzed PET</td>
</tr>
<tr>
<td>PET-N(i)-CySH</td>
<td>Cysteine modified aminolyzed PET (imine bond)</td>
</tr>
<tr>
<td>pM</td>
<td>Picomolar</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
</tr>
<tr>
<td>PU</td>
<td>Polyurethane</td>
</tr>
</tbody>
</table>
\( \rho \)  Density of fluid

\( Q \)  Volumetric flow rate

\( \varphi \)  Nondimensionalized concentration variable

\( r \)  Independent variable in radial direction

\( r_A \)  Reaction rate of component A in bulk liquid

\( \gamma \)  Nondimensionalized variable in radial direction

\( R \)  Radius of tube

\( \Gamma \)  Wall shear rate

\( R_A \)  Surface consumption rate of HSANO in bulk liquid

\( R_{A,S} \)  Surface consumption rate of HSANO on Cys-PET

\( \text{RBC} \)  Red blood cell

\( \text{rpm} \)  Revolutions per minute

\( \text{RS}^\bullet \)  Thiol radical

\( \text{RSH, R'SH} \)  Thiol

\( \text{RSNO, R’SNO} \)  S-nitrosothiol

\( \text{RSSR} \)  Disulfide

\( s \)  Second

\( S \)  Surface area of modified polymer

\( \text{SEM} \)  Scanning electron microscopy

\( \text{sGC} \)  Soluble guanylate cyclase

\( \text{SH} \)  Sulphhydryl group

\( \text{SNAP} \)  S-nitroso-N-acetylpenicillamine

\( t \)  Time independent variable
Tris  Tris[hydroxymethyl]aminomethane
TXA₂  Thromboxane A₂
θ  Angular independent variable
µL  Microliter
µM  Micromolar
UV-Vis  Ultraviolet-visible
V  Volume of reaction system
W  Slit width
wt%  Weight percentage
z  Independent variable in axial direction
ζ  Nondimensionalized independent variable in axial direction
CHAPTER 1

INTRODUCTION

Biomaterials have been used in the medical field for approximately one hundred years [Bhat 2002]. Although there is controversy over the term “biomaterial”, a widely accepted definition for biomaterials is: "A material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body.” [Williams 1999]. It is important to note that living natural materials, such as tissues, should be called “biological materials” and should not be included in the definition of biomaterials [Shi 2005].

In recent years, there has been an increased interest in biomaterial applications in the medical field. Some common examples are artificial joints, heart valves, and contact lenses. Table 1.1 shows the major applications of biomaterials, with a total U.S. market of $9 billion in 2000 [Ratner et al. 2004]. Despite some biomaterial successes during the past few years, one of the most constant problems is the “biocompatibility” of the biomaterial. Biocompatibility is “the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinically relevant performance of that therapy” [Williams 2008]. Thus, two key Table 1.1 Numbers of Medical Devices Used Each Year in U.S. (Global numbers are typically 2-3 times the U.S. numbers) [Ratner et al. 2004].

1
<table>
<thead>
<tr>
<th>Medical Devices</th>
<th>Numbers of Usage/yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catheter</td>
<td>200,000,000</td>
</tr>
<tr>
<td>Blood Bags</td>
<td>40,000,000</td>
</tr>
<tr>
<td>Contact lens (2000)</td>
<td>30,000,000</td>
</tr>
<tr>
<td>Intraocular lens (2003)</td>
<td>2,500,000</td>
</tr>
<tr>
<td>Coronary stents</td>
<td>1,500,000</td>
</tr>
<tr>
<td>Dental implant (2000)</td>
<td>910,000</td>
</tr>
<tr>
<td>Hip and knee prostheses (2002)</td>
<td>500,000</td>
</tr>
<tr>
<td>Pacemaker</td>
<td>400,000</td>
</tr>
<tr>
<td>Renal dialyzer (2001)</td>
<td>320,000</td>
</tr>
<tr>
<td>Vascular graft</td>
<td>300,000</td>
</tr>
<tr>
<td>Heart-Lung (Oxygenators)</td>
<td>300,000</td>
</tr>
<tr>
<td>Breast implant</td>
<td>250,000</td>
</tr>
<tr>
<td>Heart valve</td>
<td>100,000</td>
</tr>
</tbody>
</table>
aspects of biomaterials need to be evaluated: performance of desired functions and assessment of interference with the biological system.

Research of biocompatible materials has drawn more and more attention since first introduced in the literature in 1967. Publication data shows that it has become an almost exponentially growing topic in research within the past twenty years as shown in Figure 1.1. Due to the complexity and diversity of the circumstances in which biomaterials are being used, biocompatibility issues are very complicated. Although, many creative works have been performed to improve the biocompatibility of materials, some key drawbacks still remain unsolved, especially in long-term implantable medical devices [Williams 2008]. One major long-term problem of current blood-contacting biomaterials is haemocompatibility, which includes platelet adhesion, activation, and aggregation, followed by thrombus formation and detaching (shedding emboli) [Hayward and Chapman 1984; Courtney et al. 1994; Williams 2008]. These adverse host responses can further lead to the severe health problems, including severe bleeding resulting from platelet depletion, life-threatening stroke due to emboli transported to the brain and ischemia (lack of oxygen), and death of downstream tissue due to thrombosis formation.

The prevalent strategy for enhancing the haemocompatibility of biomedical polymers is surface modification. Modifications include grafting anticoagulants such as heparin [Marconi et al. 1996; Thorslund et al. 2005] and hirudin [Lahann et al. 2001; Lin and Tseng 2001], increasing the surface hydrophilicity via poly(ethyleneoxide) [Amiji and Park 1993], and passivation by coupling albumin to the surface [Amiji and Park 1993; De Queiroz et al. 1997]. However, some problems still exist with such methods.
Figure 1.1 Annual numbers of articles with the word "biocompatibility" in topic (data obtained from ISI Web of Knowledge)
The immobilized active proteins, such as albumin and hirudin, are susceptible to conformational changes and enzymatic deterioration \textit{in vivo} and are often only suitable for short-term applications [Bamford and Al-Lamee 1992; Muntean 1999; Williams 2008].

Recently, nitric oxide (NO), a well-known anti-platelet factor which is endogenously generated by endothelia cells, has been used to improve the haemocompatibility of biomaterials [Bohl and West 2000; Frost \textit{et al.} 2005]. However, these biomaterials still have their limitations, including limited lifetime release of NO or a non-constant NO release rate. Recently, a polymer which uses endogenous NO to minimize platelet adhesion has been designed [Duan and Lewis 2002] and the minimum NO flux for complete platelet inhibition on the polymer surface has been determined to be 0.3-0.6 fmol/(cm$^2$·s) [Ramamurthi and Lewis 2000]. The polymer exploited endogenous NO (S-nitrosothiols, RSNO) to generate an NO flux from the polymer surface. Therefore, the polymer did not involve incorporating limited amount of NO donors into the polymer.

The design concept was based on the following theory. First, there is abundant S-nitrosothiols (mostly S-nitroso-albumin with concentrations around the $\mu$M level [MacArthur \textit{et al.} 2007]) in the blood providing an unlimited NO resource for the modified polymer [Stamler and Loscalzo 1992]. Second, NO can be transferred between different thiol compounds. The transfer rate is fast and preferentially transferred from S-nitroso-albumin to cysteine [Meyer \textit{et al.} 1994; Wang \textit{et al.} 2001]. It also has been shown that NO can be transferred \textit{in vivo} from S-nitroso bovine serum albumin (BSANO) to L-cysteine [Scharfstein 1994]. Third, S-nitrosocysteine (the compound
formed once NO is transferred to cysteine) is unstable with trace concentrations of some metal ions catalysis, such that NO is released to inhibit platelet adhesion [Askew et al. 1995; Swift and Williams 1997; Stamler and Toone 2002]. Thus, the above properties of thiols gave rise to the design of attaching cysteine to the surface of a polymer so that NO can be transferred from S-nitroso-albumin to the cysteine on the polymer and then released to inhibit platelet adhesion and aggregation.

Previous experimental results showed significant inhibition of platelet adhesion to the modified polymer surface. However, complete inhibition still has not been achieved despite several optimization attempts of this polymer [Duan 2001; Fahlenkamp 2003]. Thus, this work focused on the kinetics of the reactions between a cysteine-modified biomaterial and the S-nitrosothiols to predict the NO release rate of this modified biomaterial surface for comparison with the release rate required for complete inhibition of platelet adhesion in physiological conditions. Specifically, this work explored and developed the kinetic model of the multiple reactions between a cysteine-modified polyethylene terephthalate polymer (Cys-PET) and nitroso bovine serum albumin (BSANO) in various in vitro conditions and predicted the NO release rate from the polymer surface. Meanwhile, various potential deactivating factors of the polymer were studied so that an optimized storage condition could be determined for the polymer and the effective life time of the polymer could be estimated in the physiological condition.

Although transnitrosation kinetics in free solution have been studied earlier [Meyer et al. 1994; Hogg 1999; Wang et al. 2001; Hu and Chou 2006], this study is unique since there is no reported kinetic study for the transnitrosation and release of NO involving immobilized components applied to the minimization of platelet adhesion. In
addition, reported kinetic data for the transnitrosation between S-nitroso proteins and low molecular weight thiols are not consistent and complete [Meyer et al. 1994; Zhang and Means 1996; Hogg 1999; Wang et al. 2001], thus this study can help to fill this gap. The kinetic model will also be helpful to reveal the possible reasons that cause the incomplete inhibition of platelet adhesion. Thus, more pertinent optimizations of the polymer can be made. The work was accomplished through five objectives.

1.1 Objective #1: S-nitrosocysteine (CySNO) Decomposition in Polymer-free Solution.

The hypothesis was that various factors can affect CySNO decomposition such as light, pH and metal ion. The decomposition rate is one of the dominant parameters that can affect NO release rate from the polymer surface. Thus, by studying the CySNO decomposition in the polymer-free solution condition, behaviors of the CySNO in the immobilized form can be predicted. Because NO is extremely reactive due to its free radical essence [Wang et al. 2002], it is hard to measure the NO release rate directly from the polymer. Thus an indirect method was used through monitoring the CySNO disappearance rate which can be quantified by spectrophotometric analysis. In this study, CySNO decomposition in various buffers with different purity grades at different pH was studied and a chelate effect was also studied. In addition, light effects from the spectrophotometer were also evaluated and results from two spectrophotometers were compared. Finally, a possible mechanism for CySNO decomposition, involving metal ion concentration and pH, was presented.
1.2 **Objective #2: S-nitroso-bovine-serum-albumin (BSANO) Transnitrosation with Free Cysteine (CySH).**

In order to better understand the transnitrosation between BSANO and immobilized cysteine (CySH), transnitrosation with free CySH (not bound to polymer) was studied first. As NO transfer is hard to monitor directly as mentioned earlier, the kinetics were studied through monitoring BSANO and CySNO concentration changes with time in batch conditions. Molecular weight filtration was used to separate BSANO and CySNO and a chemiluminescence method was used to measuring their concentrations. A mathematic model was developed to fit the experiment data and key kinetic parameters were calculated using data analysis software. Ascorbic acid effects on the transnitrosation were also studied due to its well known ability of promoting the NO release from nitrosothiols [Holmes and Williams 2000; Smith and Dasgupta 2000].

1.3 **Objective #3: Immobilization of CySH on PET Surface.**

The hypothesis was that the imine bond which is formed during previous developed immobilization techniques might be unstable in neutral pH solution and the peptide bond can be a good candidate for immobilization, also CySH itself can be oxidized by various factors. Previous studies showed that cysteine can be detached from PET in acidic condition completely within 1 hour [Duan and Lewis 2002; Gappa-Fahlenkamp *et al.* 2004] probably due to imine bond hydrolysis [Vazquez *et al.* 1990; Migneault *et al.* 2004]. In order assess this hypothesis, Cys-PET made by the previous method was incubated in buffer solutions and the CySH concentration in the solution and on the polymer was monitored through Ellman’s method and chemiluminescence.
method, respectively. Afterwards, the kinetics of CySH detaching from the PET surface were studied. To overcome the potential unstable bond, several alternative methods were developed to attach cysteine or its derivatives on the PET surface via a peptide bond. The stability of the new CySH modified polymer in aqueous solution was evaluated. The immobilized CySH oxidation due to different environment conditions was studied by monitoring CySH surface concentration during various treatments, such as light, air and temperature. The light and air effect was studied only for dry conditions, while for the aqueous condition, only temperature effect was studied as the modified polymer is intended to be used in an oxygenated environment without light (in vivo). A detailed kinetic model was developed based on the experimental data and an optimized storage condition was determined.

1.4 Objective #4: BSANO Transnitrosation Kinetics with Immobilized CySH and Prediction of NO Flux from Cys-PET in a Flow System.

The hypothesis was that the partial platelet inhibition previously observed was due to insufficient NO release from the modified polymer surface. In order to assess the hypothesis, Cys-PET and BSANO transnitrosation followed by CySNO decomposition in batch conditions were monitored by measuring BSANO and cysteine surface concentration changes using a chemiluminescence based assay which was previously established. A mathematical model was developed similarly as in object #2 and key kinetic parameters were determined. The kinetic model and parameters were then applied to a tubular flow reactor (with laminar flow) and the NO flux from the polymer surface at steady state was predicted using a mathematical model. Various factors, including flow rate, BSANO concentration, cysteine surface concentration and CySNO
decomposition rate constant and transnitrosation reaction equilibrium constant, were evaluated as to their effect on the NO release rate. The predicted NO was compared with the minimum NO flux required to inhibit platelet adhesion as mentioned earlier.
CHAPTER 2

REVIEW OF LITERATURE

Platelets hold an important role in the haemostatic response. Platelets prevent the loss of blood following a wound by adhering to the site of vascular damage and then forming a thrombus that can stop bleeding. A more detailed illustration of the haemostasis sequence is shown in Figure 2.1. Briefly, platelets adhere to the vascular damage site in a few seconds after injury. Then platelet aggregation starts and it forms a loose and permeable haemostatic plug which can close up the rupture. As more platelets are attracted to the site, the plug becomes impermeable and then the coagulation cascade starts, forming fibrin fibrils which can fortify the primary thrombus and finally forming a stronger thrombus to stop bleeding [Roderique and Wynands 1967; Blockmans et al. 1995]. However, such positive mechanisms can cause significant adverse sequences when foreign materials are in contact with blood [Authi et al. 1993]. Platelet adhesion to materials, when applied to medical devices, can adversely affect the material performance.

In order to develop a haemocompatible biomaterial, the mechanism of the interaction between the foreign material and blood must be fully understood. This chapter will first focus on the mechanism of haemostatic response in the biological
Figure 2.1 Haemostasis sequence involving the platelets at different levels. Figure modified from [Roderique and Wynands 1967].
system, which includes platelet activation and inhibition. Afterwards, the biochemistry of nitric oxide (NO) and its interaction with thiols in blood, to form S-nitrosothiols, will be explored, leading to the rationale of using cysteine-modified polymers to inhibit platelet adhesion. Finally, a brief review of current haemocompatible biomaterials including the previous work done by our group will be given.

2.1 Platelet Activation and Inhibition Mechanism

2.1.1 Platelet Activation

Platelet activation can be observed when platelets change their shape from disk-like to sphere and form long extensions called pseudopodia. Although the detailed pathways of platelet activation are very complicated, the pathways all involve interactions between agonists and the corresponding receptors in the platelet membrane [Ashby et al. 1990; Blockmans et al. 1995]. These responses can be categorized into three different patterns based on the agonists that initiate the responses [Authi et al. 1993].

Direct Response. Platelets are induced by thrombin and Platelet-Activating Factor (PAF), the latter is a potent phospholipid activator. These agonist-receptor interactions can cause platelet aggregation, thromboxane A$_2$ (TXA$_2$) synthesis and secretion from all three storage granules in the platelet. ADP then interacts with TXA$_2$ to enhance platelet aggregation.

Adhesion-dependent Response. This response is initiated when platelets interact with collagen fibrils and then spread on the fibrils. This causes the secretion of TXA$_2$ synthesis from all three storage granules and an increase in the phosphatidylinerine
content of the outer leaflet of the plasma membrane. This enhanced extracellular exposure of phosphatidylserine triggers the formation of factor X and prothrombin activation complexes on the activated platelets and forms localized thrombin in the surrounding area of the site where platelets are exposed to collagen. This response only occurs in an area of vascular damage where collagen is exposed. If the collagen concentration is low, collagen alone won’t initialize this response and usually the response is additionally dependent upon the interaction with released products such as ADP and TXA2. Thus, this response can be remarkably reduced if ADP or TXA2 is removed.

Aggregation-dependent Response. If the initial aggregation caused by agonist-receptor interactions is not strong enough, TXA2 synthesis may be triggered and TXA2 will be secreted from the storage granules. This response is induced by agonists such as ADP, adrenaline and 5-hydroxytrytamine (5HT). However, this response won’t lead to lysosomal secretion or enhanced phosphatidylserine which is unlike the direct response mentioned earlier. Figure 2.2 shows different platelet agonists and responses.

Once the receptors in the platelet membrane binds the corresponding agonists, the cascade of intracellular second messengers, including inositol 1,4,5-triphosphate (IP3), and diacylglycerol (DAG), is triggered. IP3 causes Ca\(^{2+}\) secretion from the platelet dense tubular system, which leads to the arachidonate acid (ARA) release by phospholipase A2 (PA2) [Brass 1991; Haynes 1993; Puri and Colman 1997]. ARA is the precursor of TXA2 which can permeate out of cell and interact with receptors on the platelet surface and leads to further activation. Meanwhile, DAG activates protein kinase C, initiating
granule secretion and fibrinogen receptor exposure [Savi and Herbert 1996]. Another important player during the platelet activation is the G protein family, which mediates

Figure 2.2 Platelet agonists and responses. The “+” symbol indicates an excitatory agonist and the “-” symbol represents an inhibitory agonist. [Authi et al. 1993].
various agonist-receptor interactions [Brass et al. 1993]. G proteins were believed to manipulate phosphoinositide hydrolysis and 3’ , 5’-monophosphate (cAMP) formation. They are also involved in PA2 activation, which leads to the secretion of TXA2 precursor formation as mentioned earlier [Haynes 1993].

2.1.2 Platelet Inhibition

Regulation of platelet activation is critical for the homeostasis of the vascular wall because excessive platelet activation can cause a thrombo-embolic phenomena, especially in the arterial vessels [Blockmans et al. 1995]. The activation of human platelets is inhibited by various chemicals, including thrombin inhibitor (e.g. hirudin) [Glusa 1991], ADP scavengers (e.g. apyrase) [Walsh et al. 1977], TXA2 generation inhibitors (e.g. aspirin) [Vane 1994] and physiological and pharmacological agents which can elevate intraplatelet cyclic nucleotides [Haynes 1993]. In physiological conditions, vascular endothelial cells synthesize and secrete various platelet inhibitors. The most important two inhibitors are prostacyclin and the endothelium-derived relaxing factor (EDRF), which boost levels of the cAMP and cGMP, respectively, in human platelets [Haynes 1993; Vane 1994].

EDRF was first discovered by Furchgott and Zawadzki in 1980 when it was recognized as an endothelium-derived labile humeral substance which is responsible for mediating the vascular relaxations induced by acetylcholine [Furchgott and Zawadzki 1980]. Later, EDRF was found to be a potent inhibitor of platelet aggregation [Azuma et al. 1986; Radomski et al. 1992] and that it elevates cGMP levels in platelets or other target cells [Furchgott and Zawadzki 1980; Radomski et al. 1992]. This behavior of EDRF was then shown to be identical to that of nitric oxide (NO) by different research
groups [Furchgott 1988; Ignarro et al. 1988]. Additional results confirmed this analysis and it is now widely accepted that NO is EDRF [Radomski and Moncada 1993].

2.2 Biochemistry of Nitric Oxide and S-nitrosothiol

NO is a diffusible and extremely reactive molecular messenger with diverse biological functions throughout the body including the vascular, immune, and nervous systems due to its free radical essence [Moncada et al. 1991; Butler and Williams 1993; Ånggård 1994; Knowles 1997; Murayama and Nomura 1998; Louis 1999; Gladwin et al. 2003]. In the central nervous system, NO adjusts synaptic plasticity and affects brain development, memory formation and behaviors. In the peripheral nervous system, NO acts as a neurotransmitter to regulate viscera motility, local blood flow and neuroendocrine function [Christopherson and Bredt 1997; Michel and Feron 1997]. NO is also responsible for controlling infection in which macrophages express an inducible NO synthase in response to immunological or inflammatory stimulation [Nathan 1997]. Also, the continuous generation of NO by the vascular endothelia cells is critical for the regulation of blood pressure and blood flow [Michel and Feron 1997; Loscalzo 2001]. Finally, the endothelium-derived NO has a critical role in preventing premature platelet adhesion and aggregation [Moncada et al. 1991; Loscalzo 2001].

2.2.1 NO Biosynthesis

NO is synthesized by the oxidation of the guanidinium nitrogen of amino acid L-arginine, resulting in L-citrulline as the coproduct [Knowles and Moncada 1994; Williams 2003]. The mechanism is a two-stage stereo-specific reaction involving molecular oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH)
as coenzyme, with the aid of cofactors such as calcium ions and tetrahydrobiopterin (Figure 2.3) [Williams 2003]. This formation of NO from L-arginine is catalyzed by NO synthase (NOS) [Knowles and Moncada 1994; Mayer et al. 1998]. There are three different NOS isoforms that have been discovered so far:

- nNOS (neuronal NO synthase): a calcium and calmodulin (Ca-CAM)-dependent constitutive enzyme present in neurons and skeletal muscle, which generates NO as a neurotransmitter [Schmidt and Walter 1994; Christopherson and Bredt 1997; Mayer et al. 1998].
- iNOS (inducible NO synthase): a calcium-independent isoform isolated from macrophages, vascular smooth muscle cells, and hepatocytes which is induced by specific cytokines such as bacterial and viral infections [Nathan 1997; Williams 2003].
- eNOS (endothelial NOS): a Ca-CAM regulated constitutive form found in vascular endothelial cells, which produces NO and releases it into blood stream [Michel and Feron 1997].

nNOS and eNOS are also called constitutive NOS (cNOS) because they are dependent on calcium ion. The distribution of these three isoforms overlaps in many tissues and cell types and their subcellular locations are variable. Meanwhile, the same isoform in different cells may have different biological functions, and the eNOS and nNOS are also inducible [Michel and Feron 1997].

2.2.2 Platelet Inhibition by Nitric Oxide

One of the most distinguished properties of NO is its unspecified signaling due to its lipophilic and free radical properties. This is quite different from the usual signaling
Figure 2.3 NO biosynthesis is through oxidation of the guanidinium nitrogen of L-arginine. This reaction catalyzed by nitric oxide synthase (NOS) with coenzyme NADPH. Oxygen is the electron acceptor [Williams 2003].
process in which signaling messengers interact with particular receptors on target cells to induce a specific response. NO can diffuse to virtually anywhere to interact with many targets including metal- and thiol-containing molecules and soluble guanylate cyclase [Moncada et al. 1991; Radomski and Moncada 1993; Williams 2003]. Although this process may seem to be random, these unique properties of NO are critical to mediate many of its biological functions.

Guanylate cyclase exists in both cytosolic and particulate fractions of cells [Brass 1991; Ivanova et al. 1994]. Particulate guanylate cyclase is found in the plasma membrane of many cells and so far five isoforms have been recognized. The soluble isoform of guanylate cyclase (sGC) plays a essential role in the NO-cGMP signal transduction pathway [Ivanova et al. 1994]. sGC is a Mn²⁺-dependent enzyme found in the cytosolic fraction of most mammalian cells and is very abundant in platelets [Guthmann et al. 1992]. A key characteristic of sGC is the presence of heme as a prosthetic group. NO interacts with sGC through this heme group to evoke a conformational change that stimulates the conversion of GTP to cGMP [Radomski and Moncada 1993; Ånggård 1994].

It has been well documented that the key mechanism by which NO inhibits platelet activation is through activation of platelet sGC to increase levels of cGMP [Radomski and Moncada 1993; Williams 2003]. Besides the sGC pathway, NO actions that are independent of cGMP production have also been described [Gordge et al. 1996]. These include NO-mediated ADP ribosylation, inhibition of glyceraldehyde-3-phosphate dehydrogenase and inhibition of intracellular calcium mobilization [Dimmeler and Brüne...
1992; Feldman 1993; Brune B. 1998]. However, the relative importance of such non-sGC/cGMP independent actions of NO has not yet been fully understood.

The effects of NO on platelet differ substantially from those of many other antiplatelet agents. Compared to cyclooxygenase inhibitor acetylsalicylic acid (aspirin), which irreversibly inhibits platelet activation by preventing the formation of the pro-aggregatory TXA2 [Vane 1994], NO inhibits platelet activation at an earlier stage and its effects are rapid and reversible [Radomski and Moncada 1993; Williams 2003]. Thus, in addition to inhibiting platelet adhesion to the vessel wall, NO also interferes with the initial thrombus formation by inhibiting aggregation and autocrine stimulations. This interference prevents platelet recruitment to the vicinity [Freedman et al. 1995; Freedman et al. 1997; Williams 2003].

2.2.3 Nitric Oxide and S-nitrosothiol

As a messenger molecule involving many physiological functions, NO needs to be transferred to various target tissues. However, due to its high reactivity with molecular oxygen, superoxide anion and heme/non-heme iron, its half life is less than 5 seconds in biological systems [Nathan 1992; Archer 1993]. In order to overcome this problem, biological systems develop an effective method to suppress the activity of NO, namely, binding NO to thiol groups. Stamler et al. observed that NO can react with cellular thiols to form S-nitrosothiols (RSNO), thus extending the half-life and spatial impact of NO [Stamler et al. 1992b]. Stamler also suggested that RSNOs act as nitrosonium (NO⁺) and nitroxyl (NO⁻) donors with chemical reactivities different from those of NO, which donors are capable of forming and modifying proteins and are present in mammalian tissue [Stamler et al. 1992b]. S-nitrosothiols have also been shown to have similar
biological actions as NO and play an important role in many processes, including platelet inhibition, signal transduction, DNA repair, host defense, blood pressure control, ion channel regulation and neurotransmission [Radomski et al. 1992; Lander et al. 1996; Gow et al. 1997; Brune B. 1998; Persichini et al. 1998]. In order to understand the S-nitrosothiol/NO functions in the physiological conditions, the basic biochemistry of S-nitrosothiol/NO need to be elaborated. Figure 2.4 shows a summary of the major NO derivatives with different redox status. A detailed explanation of the reactions involved is discussed below.

*Synthesis.* S-nitrosothiol can be synthesized *in vitro* by mixing thiol (RSH) and acidic nitrite via the following reactions in aqueous solution.

\[
HNO_2 + H^+ \rightarrow NO^+ + H_2O \quad (2.1)
\]

\[
RSH + NO^+ \rightarrow RSNO + H^+ \quad (2.2)
\]

The S-nitrosation (or S-nitrosylation) reactions can be quite rapid (ranging from less than half minute to a few minutes) under certain circumstances [Stamler et al. 1992a; Williams 1996; Zhang et al. 1996]. Although this method was later shown to be unspecific for S-nitrosation due to its ability to nitrosate amine and hydroxyl group, it is still widely used for preparing S-nitrosothiols because the thiol groups have a much higher affinity to nitrosation agents, thus getting nitrosated very fast [Aldred et al. 1982; Zhang et al. 1996].

Nitrosation of thiols can also take place with gaseous NO in aqueous solution at neutral pH when oxygen exists. The mechanism involves oxygen converting NO into N₂O₃, which is a known strong nitrosation agent [Wink et al. 1993; Lewis and Deen 1994]:

---

22
Figure 2.4 Summary of the chemistry of the redox-interrelated forms of NO of potential biological significance. The primary reactions of NO involve oxygen, superoxide, and redox metals (M). NO is reactive with metals and with thiols (RSH). Spontaneous dimerization of NO results in the formation of N₂O. Nitrosation reactions involve RSH and addition to bases (B), including peroxide [Stamler and Loscalzo 1992].
These reaction mechanisms may serve as a pathway for \textit{in vivo} S-nitrosation formation, although no direct evidence is provided. Recently, it has been reported that even in the absence of oxygen, thiol can still be nitrosated by NO with the mediation of an hemoglobin-iron complex [Palmerini 2002]. On the other hand, it was also reported that peroxynitrite (ONOOH) may nitrosate thiols such as glutathione [Wu \textit{et al.} 1994; van der Vliet \textit{et al.} 1998] according to:

\begin{equation}
22 \text{GSH} + \text{ONOOH} \rightarrow \text{GSNO} + \text{H}_2\text{O}_2
\end{equation}

Gow \textit{et al.} proposed a mechanism for the formation of RSNOs \textit{in vivo} (under physiological conditions) [Gow, 1997]. In this mechanism, NO reacts directly with reduced thiol to produce a radical intermediate, RSNOH. This intermediate then reduces an available electron acceptor to produce RSNO. Under aerobic conditions, O$_2$ acts as the electron acceptor and is reduced to produce superoxide (O$_2^-$).

\begin{align*}
\text{RSH} + \text{NO} & \iff \text{RSNOH} \\
\text{RSNOH} + \text{O}_2 & \rightarrow \text{RSNO} + \text{H}^+ + \text{O}_2^- \\
\text{O}_2^- + \text{NO} & \rightarrow \text{ONOO}^-
\end{align*}

The above reaction mechanisms clarify that RSNO can also be synthesized under a wide variety of physiological conditions.

S-nitrosothiols have relatively high extinction coefficients in the range of 330-350 nm. This property can be used to directly measure the concentration of S-nitrosothiol at
Sub-mM levels [Stubauer et al. 1999; Vanin et al. 2004]. Table 2.1 lists the extinction coefficients of some common S-nitrosothiols at 334 nm [Gordge et al. 1996].

RSNO has also been reported to be formed in vivo from many thiol compounds [Butler et al. 1995; Lander 1997]. S-nitrosoalbumin (AlbSNO), S-nitrosoglutathione (GSNO), or S-nitrosocysteine (CySNO) have been detected and quantified in vivo and their physiological functions have been shown to be similar to NO itself [Kluge et al. 1997].

The single free cysteine of serum albumin, Cys-34, has been shown to be particularly reactive toward NO under physiological conditions, primarily because that the pKₐ of its sulphydryl group (8.5) is close to that of cysteine itself (8.0) [Tong 2003]. Human plasma contains micromolar or sub-micromolar level RSNOs, of which 96% are S-nitrosoproteins, 82% of which is accounted for by AlbSNO. However, plasma level of free nitric oxide is only in the 3 nM range [Stamler et al. 1992b; Giustarini et al. 2007]. Thus, it has been hypothesized that AlbSNO serves as a reservoir with which plasma levels of highly reactive, short-lived free NO can be regulated. The chemical half-life of released NO is very short and is inactivated within ~0.2 s [Kelm and Schrader 1990]. On the other hand, RSNOs are usually very stable compounds, especially the AlbSNO which has a half-life of 40 min [Stamler et al. 1992a].

Decomposition. The stability of most the S-nitrosothiol is much better compared to NO. The thioester bond is a slightly polar covalent bond and it is not susceptible to homolysis to form radicals unless irradiated with strong light [Hogg 2002]. However this bond is very unstable due to trace metal ions contamination in solution [John McAninly 1993; Williams 1996; Stamler and Toone 2002]. The general form of this reaction is as follows:
Table 2.1 Extinction Coefficients for Several S-nitrosothiols at 334 nm [Gordge et al. 1996].

<table>
<thead>
<tr>
<th>RSNO</th>
<th>Extinction coefficients (mM$^{-1}$cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-nitrosocysteine</td>
<td>0.74</td>
</tr>
<tr>
<td>S-nitrosohomocysteine</td>
<td>0.73</td>
</tr>
<tr>
<td>S-Nitroso-N-acetylpenicillamine</td>
<td>1.00</td>
</tr>
<tr>
<td>S-nitroso-N-acetyl-cysteine</td>
<td>0.87</td>
</tr>
<tr>
<td>S-nitrosoglutathione</td>
<td>0.85</td>
</tr>
<tr>
<td>S-nitrosoalbumin</td>
<td>0.87</td>
</tr>
</tbody>
</table>
This instability is directly related to the metal ion concentration and it leads to sporadic decomposition rates due to the various contamination level of metal ions in solution [Hogg 2002; Stamler and Toone 2002]. For example, S-nitrosocysteine (CySNO) half-life was reported to be 30 seconds by Meyer et al, while Arnelle and Stamler measured it as 60 min [Meyer et al. 1994; Arnelle and Stamler 1995]. Studies have shown that even at µM level, copper ions can effectively catalyze this decomposition [Williams 1996]. Initially, Cu$^{2+}$ was believed to be the active form that is responsible for the decomposition, but additional studies confirmed that Cu$^{+}$ is the real species. A detailed mechanism has been established as follows [Askew et al. 1995; Dicks et al. 1996; Stubauer et al. 1999]:

\[
RSO \rightarrow NO + \frac{1}{2} RSSR \quad (2.10)
\]

\[
RS^{-} + Cu^{2+} \rightarrow RS' + Cu^{+} \quad (2.11)
\]

\[
RSNO + Cu^{+} \rightarrow RS^{-} + Cu^{2+} + NO \quad (2.12)
\]

\[
2RS^{-} \rightarrow RSSR \quad (2.13)
\]

Chelating agents which can complex with Cu$^{+}$ has been shown to suppress the biological function of RSNO to some extent [Gordge et al. 1995].

On the other hand, the structure of the nitrosothiol can also affect the stability of RSNO in solution significantly, although no direct correlation has been found [Noble and Williams 2000]. For example, Nobel and Williams reported that the CySNO decomposition rate is on the order of $10^3$ faster than S-nitrosocysteinylglycine (GlyCySNO) [Noble and Williams 2000]. As for S-nitroso proteins, this stability can be even higher. Pietraforte et al. (1995) showed that S-nitroso bovine serum albumin (BSANO) is very stable in PBS buffer at 37°C even in absence of metal ion chelators.
Scorza et al. (1996) observed no NO release from BSANO within 3 hours with a chelator (DTPA) and thiol deactivator (NEM).

In biological systems, RSNO concentrations, locations and bioactivities are usually regulated by catabolic processes rather than by synthesis. Several enzymes have been shown to break down low molecular weight (LMW) RSNOs in vitro, as shown in Table 2.2 [Askew et al. 1995; Freedman et al. 1995; Hou et al. 1996; Trujillo et al. 1998]. The relative distribution of these enzymes in different tissues could dramatically alter RSNO bioactivities. In the lung, for example, $\gamma$-glutamyl transpeptidase, which converts GSNO to S-nitrosocysteinylglycine, may give a greater membrane permeability for submucosal/smooth muscle bioactivity [Hogg et al. 1997]. In addition, glutathione peroxidase may produce NO with the net effect of inactivating GSNO and eliminating NOx through NO expiration and/or reaction of NO with heme iron(II) [Freedman et al. 1995; Hou et al. 1996].

RSNO decomposition can also be induced by inorganic reaction. Intermediate Fe(S)NO formulation involving LMW thiol groups may result in RSNO catabolism [Vanin et al. 1997]. Inorganic reactions with copper are closely related to S-nitrosocysteine decomposition in vitro as previously discussed [Singh et al. 1996a]. Although these may seem to be less relevant in vivo, where free Cu$^+$ concentrations are low, protein bound Cu$^+$ is still capable of promoting NO release from RSNO at a comparatively lower speed [Dicks and Williams 1996]. It has been reported that Cu$^+$ chelation results in inhibition of bioactivities, which are thought to be related to GSNO catabolism in platelets and cardiac myocytes [Gordge et al. 1996; Mayer et al. 1998]. The RSNO catabolic effects of other biological transition metals are negligible compared
Table 2.2 Enzymes Systems that Break Down GSNO *In Vitro* [Askew *et al.* 1995; Freedman *et al.* 1995; Hou *et al.* 1996; Trujillo *et al.* 1998]

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Nitrogen oxide product</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Thioredoxin) Thioredoxin Reductase</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>Glutathione Peroxidase</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>γ-Glutamyl Transpeptidase</td>
<td>S-nitrosocysteinylglycine</td>
</tr>
<tr>
<td>(Xanthine) Xanthine Oxidase</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>Glutathione Dependent Formaldehyde</td>
<td>Hydroxylamine</td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td></td>
</tr>
</tbody>
</table>
with those of Cu and Fe [McAninly et al. 1993]. On the other hand, $O_2^-$ has been shown to cause CySNO and GSNO breakdown with second order reaction kinetics in RSNO and first order in $O_2^-$ [Aleryani et al. 1998; Jourd'heuil et al. 2000]. Based on the rate constant $k$ ($3-6 \times 10^8 \text{ M}^{-2}\text{s}^{-1}$), micromolar RSNO concentrations would be degraded only very slowly ($\sim \text{pM min}^{-1}$) by these reactions in the presence of nanomolar $O_2^-$ concentrations under physiological conditions.

2.2.4 Transnitrosation: Transfer of Nitric Oxide between S-Nitrosothiols

Although plasma albumin can react with NO to form AlbSNO in physiological conditions, the limited intracellular access of AlbSNO indicates that NO might be transferred from plasma AlbSNO pool to affect biological function. NO exchange among thiols has been already reported in non-physiological conditions [Bennett et al. 1986; Keaney 1993]. Scharfstein et al. have demonstrated that the transnitrosation between AlbSNO and LMW thiols (i.e., cysteine), occurs rapidly and completely in vitro under physiological conditions, as shown below [Scharfstein 1994].

$$\text{AlbSNO} + \text{CySH} \rightleftharpoons \text{AlbSH} + \text{CySNO} \quad (2.14)$$

After NO is transferred to LMW thiols, these smaller, more diffusible NO adducts can be used to transport NO more efficiently to the vascular smooth muscle cell or platelet surface.

Hemoglobin can also be nitrosated by NO through its single $\beta$-chain reduced Cys-93 under conditions of high oxygen affinity and the resulting SNO-hemoglobin leads to LMW RSNO formation through transnitrosation reactions [Jia et al. 1996; Gow et al. 1997; Giustarini et al. 2004]. The circulatory system may provide end organs with a stable supply of LMW RSNOs which can diffuse into tissues from the erythrocytes [Jia et
al. 1996]. Furthermore, NOS activation itself may form RSNOs, probably via intermediate ONOO$^-$ in the presence of thiols [Schmidt and Walter 1994; Gow et al. 1997]. Finally, other RSNO synthetic reactions may occur in vivo, such as protein nitrosation by nitrous acid (HNO$_2$) in acidic organelles, although these reactions remain to be verified.

2.3 Previous Research

The prevalent strategy for enhancing the haemocompatibility of biomedical polymers is surface modification. Modifications include grafting anticoagulants such as heparin and hirudin [Marconi et al. 1996], increasing the surface hydrophilicity via poly(ethyleneoxide) [Amiji and Park 1993], and passivation by coupling albumin to the surface [De Queiroz et al. 1997]. Also, polymers that have multiple functional molecules have also been studied in which heparin and albumin were both immobilized to increase the thromboresistance of biomedical polymers [Marconi et al. 1996]. However, some problems still exist with such methods. The immobilized active proteins are susceptible to conformational changes and enzymatic deterioration in vivo and are often suitable for only short-term applications. To date, none of these methods can generate a complete non-thrombogenic surface.

A new approach of haemocompatible polymer that is widely studied is a nitric oxide donor polymer. As discussed earlier, NO, a small messenger molecule, serves to inhibit platelet adhesion, aggregation, and activation as well as relax smooth muscles and modulate neurotransmission. Vascular endothelial cells synthesize and release NO and prostacyclin to make vessel walls completely compatible with platelet. The estimated NO flux rate from the blood vessel is 6800 fmol·cm$^{-2}$·s$^{-1}$ [Vaughn et al. 1998]. To mimic the
inner surface of the blood vessel, significant studies have been devoted to incorporate diazeniumdiolate NO donors into polymer surfaces to reduce platelet adhesion, aggregation, and the formation of thrombus on the thrombogenic surfaces [Annich G M 2000; Bohl and West 2000; Folts 2000].

Research on NO donor polymers demonstrates that localized NO release through incorporating NO donors into polymer surfaces may be an effective strategy for solving the haemocompatibility problems associated with blood-contacting medical devices. There are various NO donors incorporated in different polymers [Maalej *et al.* 1999; Annich G M 2000; Bohl and West 2000; Folts 2000]. However, some limitations of NO-releasing polymers, including non-constant release rates and limited life span and toxic concerns from donor residues, are difficult or impossible to be resolved which may essentially limit the applications of this technology.

2.3.1 Design of an Apparatus to Study NO Inhibition of Platelet on Surface

In order to overcome the above limitations of the current haemocompatible polymers, a new design of polymer was proposed. The first work in our research group was to establish valid guidelines for the local delivery of NO to reduce platelet deposition on biomaterials. This included the development of a suitable NO delivery device to study NO inhibition of platelet-biomaterial interactions *in vitro*, and the development of a mathematical model to predict aqueous NO concentration and flux profiles required to inhibit platelet attachment to biomaterials [Ramamurthi and Lewis 1997; Ramamurthi and Lewis 1998].

A thin-slit flow chamber device (shown in Figure 2.5) was designed to study the inhibition of platelet deposition on polymer surfaces under various flow conditions via
the local delivery of gaseous NO. A collagen-coated polymer was exposed to a flowing platelet suspension while NO gas permeated through the polymer and entering into the solution. The platelets were labeled with either a fluorescence dye or $^{51}$Cr in order to study the platelet attachment to the polymer surface either qualitatively or quantitatively. Perfusion rates were determined based on wall shear rate requirements. The study showed that platelet-surface interactions are highly dependent on wall shear rates [Ramamurthi and Lewis 2000]. Although physiologically relevant shear rates range between 40 and 5000 s$^{-1}$ [Turitto 1982], the shear rates tested were restricted between 125 and 500 s$^{-1}$, in order to minimize surface platelet aggregation and thrombus formation at higher shear rate. The wall shear rate ($\gamma$ in s$^{-1}$) was determined from laminar flow analysis in a slit flow chamber according to:

$$\gamma = \frac{3}{2} \frac{Q}{60Wb^2}$$  \hspace{1cm} (2.15)

where $Q$ is the volumetric flow rate (cm$^3$ min$^{-1}$), $W$ is the slit width (cm), and $b$ is the half-thickness of the flow slit (cm). Based on the geometry of the flow slit, a volumetric flow rate of 1 ml min$^{-1}$ corresponds to a shear rate of 250 s$^{-1}$ and a Reynolds number of 2.

In order to predict aqueous NO spatial concentrations and fluxes profiles within the slit flow chamber reactor, a steady-state dimensionless continuity equation for NO was developed and solved numerically [Ramamurthi and Lewis 1997]. This study showed that the minimum surface NO concentrations and fluxes required to inhibit platelet deposition are 0.1 nM and 0.3-0.6 fmol·cm$^{-2}$·s$^{-1}$, respectively. Comparing this value to the estimated NO flux from the vascular cell mentioned earlier (which is 6800 fmol·cm$^{-2}$·s$^{-1}$), it is obvious that the platelets can be fully inhibited at the physiological NO flux rate. For the modified polymer, since the concentration of RSNOs in plasma
Figure 2.5 Thin-slit flow chamber device used to investigate the inhibition of platelet deposition on a biomaterial surface under flow conditions via the local delivery of gaseous nitric oxide [Ramamurthi and Lewis 1998].
have been shown to be in the micromolar or sub-micromolar range [Giustarini et al. 2007], there is a sufficient supply of NO that can be transferred to the surface of the cysteine-modified polymer by transnitrosation in order to inhibit platelet deposition on the surface.

2.3.2 Cysteine Immobilization and Surface Characterization

The next part of the research was to develop a cysteine modified surface which was first characterized and then used in studies involving platelets and plasma in order to test the design’s ability to inhibit platelet adhesion to the surface [Duan 2001; Duan and Lewis 2002]. The basic concept for this modified polymer involves transnitrosation between the S-nitroso-albumin and the immobilized cysteine followed by S-nitroso-cysteine decomposition which releases NO to inhibit platelet adhesion to the polymer surface (Figure 2.6).

Two polymers which are widely used in blood-contacting applications, polyurethane (PU) and polyethylene terephthalate (PET), were chosen as the model polymer to attach cysteine. There are three reactive groups on cysteine that can be used for immobilization: the primary amine (-NH₂), carboxyl (-COOH) and the thiol (-SH). Since a free thiol group is required for the transnitrosation reaction with RSNOs in the blood followed by the release of NO into surroundings to prevent platelet attachment, therefore, only the amino group and the carboxyl group are available for immobilization. The amino group of cysteine was finally chosen as the reaction group for attaching cysteine on polymer surface because glutaraldehyde cross-linking through amino group is convenient and efficient and had been widely used [Jayakrishnan and Jameela 1996; Marconi et al. 1996; Seifert et al. 1997]. PU and PET have inert surfaces and have to be
Figure 2.6 Illustration of the concept for the design that exploits endogenous NO and naturally-occurring mechanisms to increase the haemocompatibility of blood-contacting surfaces. Nitric oxide (NO) is transferred from S-nitroso serum albumin (AlbSNO) to cysteine (CySH), which is attached on the surface of polyethylene terephthalate (PET) film. This forms unstable S-nitrosocysteine (CySNO) which then decomposes and releases NO to inhibit platelet adhesion on the PET surface.
chemically modified to introduce reactive groups for immobilization. Therefore, PET and PU were treated with ethylenediamine (ED) and 3-aminopropyltriethoxysilane (APTES) to form a primary amine group on the polymer surfaces. The complete reaction scheme used to attach cysteine to PET and PU is shown in Figure 2.7. Later on, this process was optimized and different thiol containing molecules (e.g., 2-iminothiolane, cysteine polypeptide) were successfully attached to PET [Fahlenkamp 2003].

The cysteine surface concentration was then quantified by a chemiluminescence-based assay. Modified polymers were first treated by acidic nitrite solution (nitrite in excess) so that the immobilized cysteine is nitrosated. This step also breaks off the cysteine molecules from the polymers due to the unstable imine bond under acidic conditions [Carey and Sundberg 2007]. After nitrosation, the nitrosated cysteine the solution and the excess nitrite can be converted to NO by the use of reducing agents and measured by a NO chemiluminescence analyzer. The reducing solutions are made up of acidic acid with potassium iodide and can be adjusted for measuring different nitrite product by adding different agents. For assaying the total nitrite (NO\textsubscript{2}\textsuperscript{−} + CySNO), an excess of free iodine is added in order to convert the CySNO and the excess nitrite into NO. On the other hand, adding excess of cysteine to the reducing solution can suppress 90% conversion of CySNO to NO, while exerts no effect on nitrite detection. Therefore, the concentration of the nitrosated cysteine can be determined by calculating the difference between the measurements from the two reducing solutions [Duan 2001]. The detection limit for nitrite using this method was as low as 0.02 nmol per injection. The intra-assay variability was less than 2% and the inter-assay variability was less than 5%. The calculated cysteine surface concentration on PET and PU surfaces were 8.1±1.1
Figure 2.7 Reaction schemes of immobilizing L-cysteine onto the surface of polyethylene terephthalate (PET) and polyurethane (PU) by first adding an amine group by the use of ethylenediamine and 3-aminopropyltriethoxysilane (APTES), respectively and then using glutaraldehyde as a crosslinker to attach cysteine on the polymer surface [Duan and Lewis 2002].
nmol/cm² (n=5) and 5.2 ± 0.9 nmol/cm² (n=3), respectively. Later, a modified reducing solution containing copper chloride and cysteine in phosphate buffer (pH 7.4) was developed with better accuracy by Fahlenkamp [Gappa-Fahlenkamp et al. 2004].

2.3.3 Testing the Platelet Inhibition on Modified Polymer Surface in vitro

A preliminary experiment showed that the both cysteine modified PET and PU cause significant BSANO concentration decrease during incubation in PBS buffer, indicating that the modified polymers are capable of transferring NO from BSANO in. After this, the two modified PET and PU polymers were tested in both batch and flow conditions in order to verify their haemocompatibility with platelets. The platelets were radio-labeled with isotope ⁵¹Cr so that the amount of platelets could be quantified by measuring the radioactivity of the labeled platelets. Control samples for this study were glycine-modified PET and PU samples under the same experimental conditions. The batch test showed that the cysteine modified PET and PU samples does not have noticeable antiplatelet effect when no plasma presenting in the platelet suspension. However, when 50% plasma was present in the platelet suspension, the number of platelets attached to the modified polymer surfaces was reduced by ~60% and ~50%, respectively, as compared to the control. In the flow test, similar to the batch test, the presence of plasma significantly reduced the number of platelets attached to the modified polymer to ~55% and ~25%, respectively, as compared to the control [Duan 2001]. The results of this study indicate that in order for the cysteine modified polymers to inhibit platelet adhesion on the surfaces, they must be exposed to a source of RSNOs, which are found in plasma, so that NO can be transferred to the surface of the material and then released from the surface.
In order to further improve the haemocompatibility of this cysteine modified polymer so that it can completely inhibit platelet adhesion, the NO release rate from the modified polymer under physiological condition needs to be quantified and compared to the minimum NO flux required for platelet inhibition which was mentioned earlier. Assessment of the release rate will help determine whether the incomplete inhibition results from an insufficient NO flux from the polymer surface.

To determine the release rate, the kinetics of the transnitrosation between BSANO and the cysteine on the polymer, followed by CySNO decomposition, need to be investigated since there is no available kinetic data for the transnitrosation involving immobilized cysteine. Chapters 3 and 4 focus on the transnitrosation between CySNO and BSA in free solution. Stability issues of the cysteine on the polymer are addressed in Chapters 5. Finally, the kinetics of transnitrosation reactions between the modified polymer and BSANO are discussed and a mathematical model for predicting NO flux from the modified polymer surface in a tubular reactor is established in Chapter 6. In addition, various environmental factors are examined for their influences on the NO release rate from the polymer surface.
CHAPTER 3

S-NITROSOCYSTEINE DECOMPOSITION IN FREE SOLUTION

3.1 Introduction

S-nitrosothiols (RSNOs) play a significant role in many biological processes. As the main reservoir of nitric oxide (NO), RSNOs are involved in several processes including platelet deactivation, smooth muscle relaxation, immunosupression, neurotransmission, and host defense [Richardson and Benjamin 2002]. Although the mechanisms of RSNO interactions \textit{in vivo} are complex, the biological actions of RSNOs are often associated with the transfer (transnitrosation) and/or release of NO [Kelm 1999].

In order to analyze the kinetics of the NO flux from the cysteine modified polymer, the key kinetic parameter that directly controls the release rate, which is the S-nitrosocysteine decomposition rate as mentioned in the previous chapter, must be fully studied first. Although the decomposition rate for immobilized S-nitrosocysteine might be different from that of free S-nitrosocysteine in solution (due to the conformation change after the immobilization) it has been previously shown that immobilized S-nitrosocysteine decomposition is very susceptible to metal ions, similar to free S-nitrosocysteine decomposition [Duan 2001]. Thus, it is possible that both the immobilized and free S-nitrosocysteine exert similar behaviors in response to various
environmental conditions such as temperature, pH, metals and light [Williams 1999; Stamler and Toone 2002]. Therefore, it is beneficial to study S-nitrosocysteine decomposition in free solution before assessing decomposition on an immobilized polymer.

In this chapter, studies were focused on assessing and characterizing the free CySNO decomposition rate in solution over a broad pH range with various buffer purities for further application in the polymer modeling. Previous studies have shown that CySNO is stable in acidic (pH<5) and alkaline (pH>9) pH, while in the pH range of 6-8 CySNO decomposition occurs very rapidly [Vanin et al. 1997]. However, information on how the magnitude of the decomposition rate changes over the pH range of 5 to 9, particularly near physiological pH, is not given and such information would be beneficial to understand the control in which pH has on CySNO decomposition. In addition to pH effects, several studies have shown that metal ions including copper [Askew et al. 1995], iron [McAninly et al. 1993], and mercury [Swift and Williams 1997] are responsible for RSNO decomposition, although several of the studies were performed at or near pH 7.4 rather than over a wide pH range. Analysis over a wide pH range would be beneficial for determining if pH effects on CySNO decomposition were similar among various buffers containing metal ion contaminants.

3.2 Materials and Methods

3.2.1 Materials

L-cysteine, sodium nitrite, desferal (defereroxamine mesylate salt), citric acid and Tris-base were obtained from Sigma-Aldrich. Low-purity sodium phosphate dibasic
(Certified ACS, ≥99.0%, Cat. # S374-500), low-purity sodium phosphate monobasic (Certified ACS, ≥98.0%, Cat # S369-500), 12 M hydrochloric acid, ferrous sulfate, sodium hydroxide and pH 4.0/7.0/10.0 standard buffers were purchased from Fisher Scientific. High- purity sodium phosphate dibasic (Trace Select, ≥99.99%, Cat. # 71629) and sodium phosphate monobasic (Trace Select, ≥99.99%, Cat. # 71492) were purchased from Fluka. 1M NaOH and 1 M HCl were purchased from Cole-Parmer. 10× Gibco Phosphate-Buffered Saline (PBS), pH 7.4 was purchased from Invitrogen. All solutions in this work used purified water that was prepared by passage of reverse osmosis water through a pretreatment filter to remove colloids and bacteria, then passed through two dionization columns, and finally passaged through a column to remove organics. This purified water was used for all the works in the other chapters, unless specified.

3.2.2 CySNO and Buffer Preparation

CySNO was made by acidic nitrosation as described in Chapter 2 (see section 2.2.3) [Byler et al. 1983; Kuo 2003]. Briefly, in a 15 mL centrifuge tube covered with aluminum foil, 3 mL of a 10 mM cysteine solution was mixed with 4 mL of a 10 mM sodium nitrite solution in the presence of 1 mL of 1 M HCl for 5 minutes to promote nitrosation of cysteine at acidic conditions. Following nitrosation, 1 mL of 1 M NaOH was added to neutralize the solution. A pH test paper was used to verify that the solution is neutralized. The final concentration of CySNO was approximately 3.33 mM. For studies in which the chelator desferal was used, 2 mL of 5 mM desferal was added to 8 mL of freshly-prepared CySNO. Thus, the final concentration of CySNO was approximately 2.67 mM with a desferal concentration of 1 mM.
Various buffers with different pH controlling ranges [Beynon 1996] were prepared to assess CySNO decomposition kinetics as a function of pH. The NaH$_2$PO$_4$-Na$_2$HPO$_4$ buffer, for controlling the pH between 5 and 8, was made by adding 100 mM Na$_2$HPO$_4$ slowly into 50 mL of 100 mM NaH$_2$PO$_4$ until the desired pH was obtained, with continuous mixing by micro-stirrer. The pH was continuously monitored by an Accumet semi-micro combination pH electrode (Cole-Parmer Instrument, Vernon Hills, IL) connected to an Oakton pH/CON510 Benchtop meter (Oakton Instruments, Vernon Hills, IL). The final phosphate concentration was 100 mM. This buffer was prepared using both low-purity and high-purity sources. The glycine-NaOH buffer, for controlling the pH between 7.5 and 10.5, was made similarly by slowly adding 10 M NaOH into 100 mL of 100 mM glycine until the desired pH was obtained. Since the NaOH volume added was very small (only a few droplets) compared to the glycine solution volume, the final glycine concentration after pH adjustment remained essentially at 100 mM. The glycine-HCl buffer (for pH control between 5 and 8) was similarly made by adding 12 M HCl into 100 mL of 100 mM glycine solution. For the Citric acid-Tris-NaOH buffer (for pH control between 5 and 9), 10 M NaOH was added into 100 mL of a 100 mM Citric-acid-Tris base solution. For these latter two buffers, the final concentrations were essentially 100 mM due to the small amounts of HCl or NaOH added.

### 3.2.3 CySNO Decomposition

Decomposition studies were performed by adding equal amounts of the CySNO solution with the buffer solution in either a 96-well microplate (usually 50 μL CySNO plus 50 μL buffer) for analyzing with a microplate reader or a semi-micro cuvette (usually 1 mL CySNO plus 1mL buffer was added to a 4 mL amber vial for mixing after
which a 1 ml sample was transferred into a semi-micro cuvette, as mixing the CySNO with buffer in the semi-micro cuvette directly is difficult) for analyzing with the UV-Vis spectrophotometer. Thus, for most studies, the final concentrations of buffer salts and CySNO were 50 mM and 1.67 mM, respectively. In the case where 10× Gibco PBS was used, the final total phosphate concentration of Gibco PBS was 5×, which is approximately 20 mM according to the product specification label. For the desferal studies, the final CySNO concentration was 1.34 mM. For the high purity buffer study containing added metal iron, 10 mM FeSO₄ was made freshly and diluted to a final iron concentration of 4.57 μM with high purity buffer. This final concentration of iron was made to be comparable to the maximum iron level of the low purity buffer, since 1 L of low purity buffer (pH 7.4) was made with approximately 11.50 g of Na₂HPO₄ (0.002 wt% max iron impurity) and 2.62 g of NaH₂PO₄ (0.001 wt% max iron impurity), which resulted in a potential maximum iron concentration of 4.57 μM.

Spectrophotometric absorbance changes of the mixture at 340 nm, proportional to the CySNO concentration, were monitored immediately after mixing using either a Packard® SpectraCount™ Microplate Photometer (BS10000, Packard Instrument Co., Meriden, IL) or a Varian UV-Vis Spectrophotometer (Cary 50 Bio, Varian, Inc., Palo Alto, CA). The corresponding buffer was used as blank. The spectrophotometer was used for the low-purity phosphate experiments (exemplifying faster decay) since it gives better consistency in assessing fast kinetics as compared to the plate reader (data not shown). The chemiluminescence method was not used for these experiments because the concentration of S-nitrosocysteine was high enough to be measured by a spectrophotometer directly and S-nitrosocysteine decomposition is very fast such that a
rapid and easy measurement is needed. The pH of the mixture was monitored before and after the decomposition studies using a micro pH electrode (PHR-146B, Lazarlab Research Laboratories, CA) connected to an Accumet mV/pH meter (AP62, Thermo Fisher Scientific Inc, Pittsburgh, PA) to assess any possible changes in pH after mixing the CySNO with the buffer and during the decomposition studies. Both pH meters were calibrated by 3-point standardization using pH 4.0, 7.0 and 10.0 standard buffers prior to measurement.

3.3 Results

3.3.1 CySNO Decomposition Rate Constant ($k_{obs}$)

For assessing the rate at which CySNO decomposed with time, the concentration of CySNO was measured with time at each pH and buffer condition. Since the concentration is proportional to the optical density (OD) at 340 nm, Figure 3.1 shows a plot of OD (divided by the initial OD) versus time for several of the low-purity NaH$_2$PO$_4$-Na$_2$HPO$_4$ buffer experiments. The trend shows that CySNO disappears the slowest at pH 5.85, then disappears faster as the pH increases to pH 7.33 to 7.45, then starts to slow down again after pH 7.45.

Since CySNO is known to decompose in the presence of metal ions [McAninly et al. 1993; Dicks and Williams 1996; Williams 1996; Burg et al. 2000], the CySNO decomposition was modeled as a pseudo first-order decay according to

\[
\frac{d[\text{CySNO}]}{dt} = -k_{obs}[\text{CySNO}]
\]

(3.1)

where $k_{obs}$ is the observed first-order decomposition rate constant and [CySNO] is the CySNO concentration. The metal ion concentrations contributing to the decomposition
rate are incorporated into $k_{obs}$, which is similar to the analysis used to model the decomposition of S-nitrosated glutathione (GSNO) in the presence of ascorbic acid and

![Graph](image)

Figure 3.1 CySNO decomposition profile as a function of pH in low-purity phosphate buffer. (n=2) The optical density (OD) time profile, which is proportional to the CySNO concentration, is shown as a function of pH in the presence of low-purity phosphate buffer. The solid lines are exponential fitting.
copper ions [Scorza et al. 1996; Smith and Dasgupta 2000]. The rate at which NO is generated would be represented by \( k_{\text{obs}}[\text{CySNO}] \). Since the OD is proportional to [CySNO], [CySNO] can be replaced by the OD in Equation 3.1. Integration of Equation 3.1 results in:

\[
\frac{OD}{OD_0} = e^{-k_{\text{obs}}t} \tag{3.2}
\]

Thus a plot of \(-\ln(\text{OD/OD}_0)\) versus time from the information in Figure 3.1 would yield a slope equal to \( k_{\text{obs}} \). The lines shown in Figure 3.1 represent the model (Equation 3.2) for the calculated \( k_{\text{obs}} \) values, thus confirming the correctness of the pseudo first-order decay model. In all cases, the standard error of \( k_{\text{obs}} \) obtained from the curve fit was less than 1.5%. Interestingly, if there was no buffer to control the pH (in water or 0.5 M NaCl solution), CySNO decomposition shown in Figure 3.2 did not correspond to a first-order decay model that was consistent with buffered solutions (phosphate or Gibco PBS). The lack of a first-order decay model for the non-buffered solutions was likely a result of changing pH since the pH changed 3-4 units throughout the experiment due to the formation of nitric acid from NO and oxygen [Lewis and Deen 1994].

### 3.3.2 Effect of Buffer Purity on \( k_{\text{obs}} \)

In Figure 3.3, the values of \( k_{\text{obs}} \) are plotted as a function of pH for three separate buffer solutions: a) low-purity NaH2PO4-Na2HPO4, b) high-purity NaH2PO4-Na2HPO4, and c) low-purity NaH2PO4-Na2HPO4 with the addition of the chelating agent desferal. In addition, \( k_{\text{obs}} \) is shown for one experiment in which \( \text{Fe}^{2+} \) was added to high purity NaH2PO4-Na2HPO4 buffer (final concentration of 4.57 µM) to show the effect of a metal ion contaminant on \( k_{\text{obs}} \). The error bars representing the standard errors for the curve fits
Figure 3.2 CySNO decomposition profile in non-buffer solution (water or 0.5 M NaCl) compared to buffer solution (in 50 mM low-purity phosphate buffer or Gibco 5× PBS). (n=2) The decomposition profile in non-buffer solution did not correlate with the first order decay model shown.
Figure 3.3 First-order decay constant ($k_{\text{obs}}$) for CySNO decomposition in various phosphate buffers. $k_{\text{obs}}$, as calculated from Equation 3.2 using experimental time data, is shown as a function of pH for low-purity phosphate buffer, low-purity phosphate buffer in the presence of the chelator desferal, high-purity phosphate buffer, and 4.57 μM Fe$^{2+}$ in high-purity phosphate buffer.
are not shown since they were less than 1.5% for all points. As shown in Figure 3.3, \( k_{\text{obs}} \) maximizes near physiological pH and approaches zero near pH 6 for the low-purity buffer. Thus at pH 6 and below, CySNO decomposition (or the release of NO) does not occur. There is no data for pH >8 since this buffer is only capable of controlling the pH in the 5.5 to 8 range. For each pH experiment, the pH of the solution was well controlled during the decomposition- the pH only varied within ± 0.02.

As compared to the low-purity buffer, both 1 mM desferal and high-purity buffer significantly reduced the decomposition of CySNO, essentially showing little decomposition over the entire pH range. According to product specifications (Table 3.1), the low-purity phosphate buffer contained phosphate salts that had iron contamination up to 0.002 wt% and heavy metals contamination (Ag, As, Bi, Cd, Cu, Hg, Mo, Pb, Sb, and Sn [Schmidt et al. 2005]) up to 0.001 wt%. For the desferal study, the desferal reduced the metal ion contaminants (particularly Fe\(^{3+}\) [Vanin et al. 1997]) in the low-purity phosphate buffer via chelating action. Similarly, the high-purity phosphate buffer was made of much purer phosphate salts with iron contamination less than 0.000005 wt% and heavy metals less than 0.00002 wt% (Table 3.1). With the addition of Fe\(^{2+}\) to the high-purity phosphate buffer at pH 7.44 to obtain an iron concentration similar to the maximum iron content in the low-purity phosphate salts, \( k_{\text{obs}} \) increased significantly and was close to the \( k_{\text{obs}} \) for the low-purity phosphate buffer (the small difference may be due to other metal ion contaminants). These results demonstrate the importance of metal ions in CySNO decomposition and that the metal ions for this study came from the phosphate salts. The importance of metal ions in CySNO was previously observed although the effects of pH on the process were not assessed [Burg et al. 2000; Vanin et al. 2004].
Table 3.1 Metal Contamination level for low and high purity salts (wt%)

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Low Purity Salts</th>
<th>High Purity Salts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na$_2$HPO$_4$</td>
<td>NaH$_2$PO$_4$</td>
</tr>
<tr>
<td>Fe</td>
<td>≤0.002</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>≤0.000005</td>
<td>≤0.000005</td>
</tr>
<tr>
<td>Heavy Metals</td>
<td>≤0.001 (as Pb)*</td>
<td>0.000018**</td>
</tr>
<tr>
<td></td>
<td>≤0.001 (as Pb)*</td>
<td>0.000013**</td>
</tr>
</tbody>
</table>

Note. * Fisher Scientific did not have detailed compositions regarding the heavy metal content. Usually, heavy metals concentration is determined by adding Na$_2$S into the solution and comparing the darkness of the solution with a standard Pb solution mixed with Na$_2$S (sulfide ion reacts with heavy metals to develop darkness). A common list of heavy metals includes Ag, As, Bi, Cd, Cu, Hg, Mo, Pb, Sb, and Sn [Schmidt et al. 2005]. Contaminant levels were obtained from the label.

** Sigma-Aldrich provides detailed concentration levels for each metal on the label, thus this value is calculated by adding up the corresponding heavy metal concentrations.
3.3.3 Effect of pH on $k_{obs}$

Although Figure 3.3 showed that $k_{obs}$ was negligible below pH 6 and increased to a maximum near physiological pH, further studies were performed to assess the pH effect on $k_{obs}$ over a wider pH range and in the presence of other buffers. Since the phosphate buffer is not as efficient above pH 8, a citric acid-Tris-NaOH buffer, a glycine-HCl buffer, and a glycine-NaOH buffer were also evaluated to assess the effects of CySNO decomposition over a wider pH range. Figure 3.4 shows $k_{obs}$ as a function of pH for the three buffers. Similar to the phosphate buffer studies in Figure 3.3, $k_{obs}$ maximized near physiological pH and approached zero for pH <6 and >9.5. The citric acid-Tris-NaOH buffer had higher values of $k_{obs}$ compared to the glycine buffers, which is likely a result of greater metal ion contamination in the chemicals used to prepare the buffer. The citric acid-Tris-NaOH buffer results were similar to those of the low-purity phosphate buffer, suggesting that contaminants rather than key buffer ingredients contribute to CySNO decomposition.

3.4 Discussion

The major findings from this chapter showed that the rate constant characterizing CySNO decomposition maximized near physiological pH (independent of the type of buffer), the rate constant dropped off rapidly within approximately ±1.5 pH units, and the metal ion contamination of the buffers affected the rate constant. It is interesting to note that the greatest decomposition occurred near physiological pH and that the release rate is controlled within a narrow pH band regardless of the metal ion content. Although the NO chemistry in biological systems is complex, the maximum release of NO from
Figure 3.4 First-order decay constant \((k_{\text{obs}})\) for CySNO decomposition in non-phosphate buffers. \(k_{\text{obs}}\) as calculated from Equation 3.2 using experimental time data, is shown as a function of pH for citric acid-Tris-NaOH buffer, glycine-HCl buffer, and glycine-NaOH buffer.
CySNO would thus occur near physiological pH. It would be beneficial to assess whether other RSNOs show similar trends.

The question arises as to why the decomposition rate maximizes near physiological pH. Previous studies assessing the decomposition rate of nitrosated glutathione in the presence of ascorbic acid showed that the decomposition rate was negligible below pH 5.5 but increased with increasing pH up to pH 7.7 [Smith and Dasgupta 2000]. The results showed evidence that the species promoting the greatest NO release was the ascorbic acid dianion. Equilibrium analysis that included the other two forms of ascorbic acid (ascorbic acid monoanion and ascorbic acid) showed that the dianion fraction of the total ascorbic acid increased as the pH increased, leading to a higher decomposition rate. Studies were not performed above pH 7.7 so an upper limit of the decomposition rate was not observed -- although the dianion fraction should theoretically reach unity and the decomposition rate should maintain a maximum level above a certain pH. However, these findings differ from the CySNO decomposition results where CySNO maximizes near physiological pH with metal ions and then decreases as the pH is increased or decreased. The action of Cu²⁺ on glutathione decomposition was also assessed but only at pH 7.45. Nevertheless, similar to this work that showed increasing the buffer impurity increased the decomposition rate, increasing the Cu²⁺ concentration increased the glutathione decomposition rate.

Recently, the effect of pH on CySNO decomposition in the absence of metal ions, but in the presence of excess cysteine, showed a maximum decomposition rate at approximately pH 8 with decreasing rates as the pH increased or decreased from pH 8 [Adam et al. 2005]. Thus, the results showed a similar effect of pH on the decomposition
rate as reported in this work. The mechanistic explanation for the pH effect involved an
equilibrium model of several forms of cysteine. The decomposition mechanism was
independent of metal ions and generated nitrogen gas instead of NO. In this work, excess
cysteine was not present such that the decomposition mechanism would not be
applicable. In addition, this decomposition pathway requires excessive cysteine at the
mM amounts and estimates of the cysteine concentration in human plasma is less than
100 μM [Richie et al. 1996]. Therefore, this mechanism may not be as applicable in the
physiological environment as compared to metal catalyzed decomposition—although
further experiments would need to be performed to assess the dominating pathways
leading to CySNO decomposition in a physiological environment.

Unlike the two mechanisms mentioned above involving either excess cysteine
with CySNO or ascorbic acid with nitrosated glutathione, the results of this work provide
a plausible explanation that several species of a metal ion contaminant (such as copper or
iron) exist in equilibrium and that the dominant species at physiological pH (rather than
the species at high pH in the case of ascorbic acid) is the species involved in the CySNO
decomposition. For example, various forms of a metal contaminant (ex. Cu$^{2+}$, Cu$^{+}$, and
Cu represented as A, B and C) could exist in equilibrium such that:

\[
A \rightleftharpoons B + H^+ \quad (3.3)
\]

\[
B \rightleftharpoons C + H^+ \quad (3.4)
\]

At equilibrium, \(C_A = C_B C_{H^+}/K_1\) and \(C_C = K_2 C_B C_{H^+}\) where \(K_1\) and \(K_2\) are the equilibrium
constants for the reactions in Equations 3.3 and 3.4, respectively. If only species B
contributed to the decomposition, the fraction of B \(f_B = C_B/(C_A + C_B + C_C)\) contributing to
decomposition would be pH dependent according to
In Equation 3.5, activity coefficients are considered unity. If \( k_{obs} \) was represented as \( kC_B \) = \( kf_B C_T \) where \( C_T \) is the total species of copper present in solution (which is independent of pH) and \( k \) is the true first-order decomposition rate constant (also independent of pH), then a plot of \( f_B \) versus pH would yield the same curvature as \( k_{obs} \) versus pH for a given combination of \( K_1 \) and \( K_2 \) values. For instance, with \( K_2 \gg K_1 \), at low pH the term \( K_2/C_{H^+} \) would dominate the denominator in Equation 3.5 leading to \( f_B \) (and thus \( k_{obs} \)) approaching zero. Similarly at high pH, the term \( C_{H^+}/K_1 \) would dominate the denominator such that \( f_B \) (or \( k_{obs} \)) also approaches zero. At an intermediate pH, \( f_B \) would reach a maximum.

An alternative model would be the dissolution of a single metal contaminant in solution such as was shown for the complete dissolution of cuprous oxide (represented as D below) in water to generate three forms of \( Cu^+ \) [Palmer et al. 2004] - again noting that \( Cu^+ \) is the species that has been shown to contribute to RSNO decomposition [Williams 1996; Burg et al. 2000]. In this scenario,

\[
0.5D + H^+ \rightleftharpoons A + 0.5H_2O \quad (3.6)
\]

\[
0.5D + 0.5H_2O \rightleftharpoons B \quad (3.7)
\]

\[
0.5D + 1.5H_2O \rightleftharpoons C + H^+ \quad (3.8)
\]

where A, B, and C represent the three forms of \( Cu^+ \) in solution. At equilibrium, the fraction of B in solution (\( f_B \)) is

\[
f_B = \frac{1}{(1 + C_{H^+} / K_1 + K_2 / C_{H^+})} \quad (3.9)
\]
where $K_3$, $K_4$, and $K_5$ are the equilibrium constants for Equations 3.6, 3.7 and 3.8, respectively. It is noteworthy that Equation 3.9 is of the same form as Equation 3.5. Therefore, similar to the scenario presented for Equation 3.5 above, it is feasible to have $f_B$ (or $k_{obs}$) approach zero at low and high pH and to maximize at an intermediate pH. For such a scenario to occur, $K_5/K_4$ must be much greater than $K_4/K_3$. Figure 3.5 shows $f_B$ versus pH for a variety of $K_1$ and $K_2$ values (or similarly, $K_4/K_3$ and $K_5/K_4$ values). The curvature, with values approaching zero at low and high pH, is similar to that shown in Figures 3.3 and 3.4, demonstrating the feasibility that species B in equilibrium with several other species is one plausible explanation for the effect of pH on CySNO decomposition involving metal ions. For both models presented above, species A or C participating in the CySNO decomposition would not lead to $k_{obs}$ approaching zero at both pH extremes while maximizing at an intermediate pH. For $K_2/K_1 = 10^4$, it can be seen that decreasing $K_2$ shifts the $f_B$ maximum from a lower pH to a higher pH while maintaining the same maximum value. When keeping $K_1$ constant (for example, $K_1 = 10^{-9}$), decreasing the ratio of $K_2/K_1$ increases the maximum value of $f_B$ and slightly increases the pH at which the maximum occurs. Since $k_{obs} = f_B k_{CT}$, $k_{obs}$ has a similar pH profile as $f_B$ except the height of the curve depends upon the constant factor $k_{CT}$ which is independent of pH. $k_{CT}$ is constant and independent of pH since $k$ is the true first-order decomposition rate constant and $C_T$ is the total concentration of ion impurities. Although beyond the scope of this article, further studies need to be performed to confirm the appropriate model (i.e. multiple scenarios were given above) and determine the model parameters once the metal ion contaminant(s) are determined.
The maximum decomposition occurring near physiological pH (between 7.35 and 7.45 [Cooney 1976]) indicates that blood naturally provides an optimized condition to decompose CySNO, leading to optimal release of NO. Under acidosis conditions

Figure 3.5 Fraction of species B ($f_B$) as a function of pH. Equation 3.5, representing the fraction of one form of ion species in equilibrium with other forms of the same ion species, is plotted as a function of equilibrium constants for the equilibrium reactions given in Equations 3.3 and 3.4.
(pH<7.35), the decomposition rate is relatively stable until the pH drops below 6.8. However, since the reported life-compatible pH range for blood is between 6.3 and 8.0 [Hinderling and Hartmann 2005], the CySNO decomposition rate can be reduced up to 50% at these pH extremes. Therefore, any physiological process involving CySNO decomposition in blood can potentially be affected under extreme acidosis/alkalosis conditions.

Another physiological process that can be affected by CySNO decomposition involves neurotransmission in which the localized environment in the synaptosomes is maintained at pH 7.04 ± 0.07 by bicarbonate ion [Richardson and Benjamin 2002]. According to this study, this pH indicates that CySNO decomposition (and NO release) is maximized when NO is involved in modulating the neurotransmission [Murayama and Nomura 1998]. In the event that pH control by bicarbonate is diminished such that the localized pH decreases below 7, NO release can also be diminished and potentially affect neurotransmission.

Finally, studies involving CySNO decomposition must be carefully performed and evaluated since pH and buffer purity contribute significantly to the decomposition rate. Knowledge of the decomposition rate is important for assessing the kinetics of the cysteine modified polymer and providing helpful information for temporarily preserving the CySNO solution and avoid any experimental error arising from CySNO decomposition during the measurement in the later chapters.

3.5 Conclusions

In this chapter, the CySNO decomposition rate was studied in different buffers (with varying metal ion impurities) at varying pH conditions. A chelator (deferexamine
mesylate salt) was also used to assess how scavenging metal ions affects the decomposition rate over a wide pH range. The kinetic rate constant, characterizing the rate at which CySNO decomposes with time, was measured at each condition (pH and buffer content) with results showing that the rate constant maximized near physiological pH under all buffer solutions studied. An equilibrium model of metal ions as a function of pH provides a plausible explanation for the pH dependence on the experimental decomposition rate. The results of this work validate reasons for apparent discrepancies in RSNO decomposition studies [Mathews and Kerr 1993; Meyer et al. 1994; Pietraforte 1995; Scorza et al. 1996] and demonstrate the strong correlation between physiological pH and the maximum CySNO decomposition rate.

The important findings are:

1) CySNO decomposition maximizes near physiological pH regardless of the buffer, since trace metal ion exists in all buffer salts.
2) The model presented above gives a representative curvature similar to experimental observations and provides a plausible explanation for the pH dependence on CySNO decomposition,
3) CySNO decomposition can be very sensitive to environmental conditions, especially near the physiological pH, thus an accurate spectroscopy instrument, like Cary 50 UV-Vis spectrophotometer, is recommended.
4) The buffer purity (or CT) greatly affects the decomposition rate, especially at near physiological pH.
CHAPTER 4

S-NITROSO BOVINE SERUM ALBUMIN (BSANO) TRANSNITROSATION WITH FREE CYSTEINE

4.1 Introduction

In biological systems, transnitrosation can occur with constituents containing at least one of two functioning groups, amines and thiols [Loeppky et al. 1987; Park 1988; Yanagimoto et al. 2007]. Transnitrosation reactions facilitate many signal transmissions in biological systems [Butler et al. 1994; Gaston et al. 2003; Marozkina et al. 2008]. As mentioned in Chapter 2, S-transnitrosation, which is the transfer of an NO moiety between two thiol molecules, is a key reaction involved in the mechanism of cysteine-modified polymers used to minimize platelet adhesion. The transnitrosation reaction is:

\[
RSNO + R'SH \rightleftharpoons RSH + R'SNO
\]  

(4.1)

One possible mechanism for Equation 4.1 is [Hu and Chou 2006]:

\[
R'SH \rightleftharpoons R'S^- + H^+
\]  

(4.2)

\[
R'S^- + RSNO \rightarrow RS^- + R'SNO
\]  

(4.3)

In order to conduct a kinetic analysis of transnitrosation, the reactants and the products need to be separated at a given point in the reaction and then measured. An
HPLC method has been used by several research groups to achieve this goal [Hogg 1999; Tsikas et al. 1999; Hu and Chou 2006]. The reaction is a second order reversible reaction, as determined by monitoring the concentration changes for RSNO and R'SH [Meyer et al. 1994; Hogg 1999]. Additionally, kinetic data for reactions between different thiols and S-nitrosothiols were acquired using different methods [Wang et al. 2001; Hu and Chou 2006]. However, not much kinetic analysis has been done for the transnitrosation between S-nitroso protein (such as S-nitroso bovine albumin) and low molecular weight thiols (such as cysteine).

Table 4.1 gives a recent summary of the transnitrosation kinetic data for S-nitroso albumin and low molecular weight thiols. From this list, it is evident that the current kinetic data is very limited with some inconsistencies. The kinetic data obtained was mostly acquired from spectrophotometric measurements which are not very sensitive compared to chemiluminescence methods [Samouilov and Zweier 1998; MacArthur et al. 2007]. Since this work involves transnitrosation between S-nitroso bovine albumin (BSANO) and cysteine (CySH), it would be beneficial to evaluate the kinetic constants for the transnitrosation between BSANO and free CySH before studying the transnitrosation kinetics of the cysteine modified polymer.

In this chapter, transnitrosation kinetics between BSANO and cysteine in solution, followed by S-nitrosocysteine (CySNO) decomposition, were evaluated using a molecular weight separation method coupled with a KI/I₂-based chemiluminescence analysis of RSNO (either BSANO or CySNO). Additionally, ascorbic acid (AA) effects on transnitrosation and NO release were identified since ascorbic acid has been found to
Table 4.1 Kinetic data for transnitrosation between S-nitroso albumin (RSNO) and thiols (RSH).

<table>
<thead>
<tr>
<th>RSNO</th>
<th>RSH</th>
<th>$k_f$ (μM$^{-1}$hr$^{-1}$)</th>
<th>$k_r$ (μM$^{-1}$hr$^{-1}$)</th>
<th>$K_{eq}$</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSANO</td>
<td>CySH</td>
<td>2.203×10$^{-4}$</td>
<td>0.0468</td>
<td></td>
<td>OD$_{335}$</td>
<td>[Wang et al. 2001]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.068±0.08</td>
<td>0.692±0.049</td>
<td>0.120</td>
<td>OD$_{412}$ w/ DTNB</td>
<td>[Zhang and Means 1996]</td>
</tr>
<tr>
<td></td>
<td>GSH</td>
<td>6.048×10$^{-7}$</td>
<td>0.208</td>
<td></td>
<td>OD$<em>{330-370}$ and OD$</em>{500-600}$</td>
<td>[Wang et al. 2001]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.208</td>
<td></td>
<td></td>
<td>OD$_{340}$ Griess Method and spectral deconvolution</td>
<td>[Rossi et al. 1997]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1872</td>
<td></td>
<td></td>
<td>OD$_{412}$ w/ DTNB</td>
<td>[Zhang and Means 1996]</td>
</tr>
<tr>
<td>HSANO</td>
<td>CySH</td>
<td>0.0328</td>
<td></td>
<td></td>
<td>OD$_{334}$</td>
<td>[Meyer et al. 1994]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.75</td>
<td></td>
<td></td>
<td>HPLC, OD$_{333}$</td>
<td>[Tsikas et al. 1999]</td>
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<td>GSH</td>
<td>0.01152</td>
<td></td>
<td></td>
<td>OD$_{334}$</td>
<td>[Meyer et al. 1994]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.59</td>
<td></td>
<td></td>
<td>HPLC, OD$_{333}$</td>
<td>[Tsikas et al. 1999]</td>
</tr>
</tbody>
</table>

Abbreviations: GSH, glutathione; CySH, L-cysteine; BSANO, S-nitroso-bovine serum albumin; HSANO, S-nitroso-human albumin; $k_f$, forward reaction rate constant; $k_r$, reverse reaction rate constant; $K_{eq}$, equilibrium constant. A$_{xxx}$, Spectrophotometric measurement at wave length of xxx nm; HPLC, High-performance liquid chromatography; DTNB, 5,5’-dithio-(2-nitrobenzoate).
promote the NO release from S-nitrosothiols [Holmes and Williams 2000; Smith and Dasgupta 2000; Xu et al. 2000; Aquart and Dasgupta 2004] and is contained abundantly on transnitrosation and NO release were identified since ascorbic acid has been found to promote the NO release from S-nitrosothiols [Holmes and Williams 2000; Smith and Dasgupta 2000; Xu et al. 2000; Aquart and Dasgupta 2004] and is contained abundantly in human plasma (ranging from 20 µM to 60 µM) [Khaw et al. 2001; Jamaati et al. 2006].

4.2 Materials and Methods

4.2.1. Materials

Glacial acetic acid, potassium iodide, and sodium nitrite were purchased from Fisher Scientific (Fairlawn, NJ). Low-purity sodium phosphate dibasic (Certified ACS, ≥99.0%, Cat. # S374-500), low-purity sodium phosphate monobasic (Certified ACS, ≥98.0%, Cat # S369-500), 12 M hydrochloric acid and sodium hydroxide were purchased from Fisher Scientific. 1M NaOH and 1 M HCl were purchased from Cole-Parmer. Bovine serum albumin (BSA), ammonia sulfamate, sulfanilamide, iodine, L-cysteine (CySH), N-ethylmaleimide (NEM), 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) and all the other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). NANOSEP 500 µL microcentrifuge filter tubes with a cutoff molecular weight of 10k were purchased from PALL Corporation. Kimble 4mL and 1.8 mL amber vials were purchased from VWR.

4.2.2. BSANO Preparation
The BSANO solution was made similarly as the CySNO described in Chapter 3. Briefly, 2 mM BSA was made freshly by dissolving BSANO using the HPLC water in a 15mL centrifuge tube. Then, 1760 µL of 2 mM BSA solution was mixed with 640 µL of 4mM NaNO₂. This solution can be made and stored at 4 ºC for a few months. The total moles of BSA is less than the NaNO₂ because the BSA usually contains around 0.6 thiol groups per molecule [Wang et al. 2001]. To initiate nitrosation, 800 µL 1M HCl was added to the BSA solution and the tube was set on a hand-motion shaker (Cole-Parmer, Vernon Hills, IL) shaking at 60 times/min at room temperature. After 10 minutes, the nitrosation reaction was terminated by adding 15 µL 5% ammonium sulfamate for 5 minutes to scavenge the excess nitrite. Prolonged nitrosation is not recommended as excess nitrite can react with primary amine groups in the BSA molecule to slowly form N-nitrosation products [Zhang et al. 1996] which are not desirable because N-nitroso albumin can also have transnitrosation reactions with other thiols [Yanagimoto et al. 2007]. Finally, 800 µL 1M NaOH was added to the solution to neutralize the acid and make the pH near 7. This freshly made BSANO solution was further mixed with 5985 µL HPLC water and 10 mL 100 mM NaH₂PO₄-Na₂HPO₄ (pH=7.4, low purity) buffer to bring the final volume to 20 mL containing 50mM NaH₂PO₄-Na₂HPO₄ buffer. This final mixture is referred to as the BSANO solution in the following sections. The BSANO final concentration was usually around 100 µM although, in some cases, BSANO was diluted further to around 60 µM.

4.2.3. BSANO Transnitrosation with Free Cysteine.

To initiate transnitrosation, BSANO solution (4mL) was added to a 4mL amber vial and then 20.1 µL of 10mM cysteine solution (freshly made) was added so that the
final cysteine concentration was approximately 50 μM. In the case where AA effects were studied, 10mM freshly made AA was added into corresponding vials to achieve a desired final AA concentration ranging between 20 and 500 μM. The control sample that did not contain cysteine was made by adding HPLC water with a volume equal to the cysteine solution added. In the case of AA being involved, the same amount of AA was added into the control sample. All the vials were set on a hand-motion shaker at room temperature, shaking at 60 times/min for a couple of hours. All experiments were duplicated and performed at 25 ºC.

During the reaction, at specific times (usually 5 to 6 times), 300μL sample solution was drawn using a pipette and added into a microcentrifuge filter tube with a MW cutoff of 10kDa (see Section 4.2.4) to separate CySNO from BSANO for detection. In each filter tube, 50 μL of 100 μM NEM was added and mixed by hand for 30 sec to quench the thiol so that transnitrosation stopped [Mochizuki et al. 2005]. Afterwards, 50 μL of 1N HCl was added in order to inhibit CySNO decomposition. The centrifuge filter tubes were then centrifuged at 13000 rpm for 8 min in order to obtain a retentate solution of BSANO, CySNO, and NO₂⁻ (which formed during the CySNO decomposition) and a filtrate solution of CySNO and NO₂⁻ (these two species freely flowed through the filter). The filtrate (200 μL), containing the CySNO, was moved into another tube and mixed with 50μL of 0.33% ammonium sulfamate in order to quench the residual NO₂⁻. The mixed solution (20 μL) was then measured by a KI/I₂-based chemiluminescence method (see Section 4.2.5) to determine the CySNO concentration. At the same time, 25 μL unfiltered sample was also treated with 50 μL of 100 μM NEM and 175μL 0.33% acidic ammonium sulfamate solution. The sample was then measured immediately by a KI/I₂-
based chemiluminescence method to determine the total S-nitrosothiol (BSANO + CySNO) concentration. In all cases, dilution effects were taken into account to obtain the true CySNO and BSANO concentrations during transnitrosation.

4.2.4. BSANO Separation Test

Since microcentrifuge filter tubes were used in the process to separate BSANO from CySNO following transnitrosation studies, it was important to test how well BSANO could be retained in the retentate solution. Therefore, 400 μL of a BSANO solution (without ammonium sulfamate treatment) was added into two microcentrifuge tubes. The centrifuge filter tubes were then centrifuged at 13000 rpm for 8 minutes using a Spectrafuge 16M Microcentrifuge (Labnet International, Inc, Woodbridge, NJ) in order to separate NO₂⁻ (remaining from BSANO preparation) from BSANO. Following filtration, 25 μL filtrate was collected from each filter tube and transferred into another regular centrifuge tube containing an equal volume of HPLC water. Finally, the filtrate sample was immediately measured by the Saville method (see Section 4.2.6) to measure both the NO₂⁻ concentration and total nitrite (NO₂⁻ + BSANO) concentration. It is expected that the NO₂⁻ concentration and total nitrite concentration should be the same since BSANO should not go through the filter. Meanwhile, another 20 μL un-filtered sample, which was collected directly from BSANO solution, was diluted to the same ratio as the filtrate sample and measured by the Saville method to assess the retained the NO₂⁻ concentration and total nitrite (NO₂⁻ + BSANO) concentration. The BSANO concentration was calculated by subtracting the measured NO₂⁻ concentration from the measured total nitrite concentration.
4.2.5. Chemiluminescence Method

For transnitrosation studies, it was important to measure the CySNO and BSANO concentrations with time. The chemiluminescence method has been widely used for RSNO detection for the past twenty years [Walters 1987; Conboy 1989; Samouilov and Zweier 1998; Marley R et al. 2000; Rassaf et al. 2002; MacArthur et al. 2007]. Its most distinguished advantage is high sensitivity and accuracy [Walters 1987; Conboy 1989; MacArthur et al. 2007].

The mechanism of the chemiluminescence method is based on the reaction between nitric oxide (NO) and ozone leading to the formation of an excited form of NO$_2$ (denoted NO$_2^*$), which subsequently releases electromagnetic radiation of characteristic frequency, as shown in the reaction steps below:

\[ \text{NO} + \text{O}_3 \rightarrow \text{NO}_2^* + \text{O}_2 \]  

\[ \text{NO}_2^* \rightarrow \text{NO}_2 + hv \]

Emission from NO$_2^*$ is in the red and near-infrared region of the spectrum and is detected by a thermoelectrically-cooled, red-sensitive photomultiplier tube (PMT). NO from the sample and ozone produced in the electrical discharge of the detector are mixed in the chemiluminescence reaction cell. A red cut-off filter between the reaction cell and the PMT selectively transmits the light emitted by the NO/O$_3$ reaction for detection by a recorder.

For a liquid sample containing NO$_2^-$ and RSNO, a reducing solution is required to convert NO$_2^-$ and RSNO into NO so that the NO can be detected by chemiluminescence.
One most commonly used reducing solution is a tri-iodide based reducing solution (KI/I₂) which is made by mixing 2.5 mL 0.2 M KI in saturated I₂ solution with 7.5 mL glacial acetic acid. The reaction is rapid and stoichiometric [MacArthur et al. 2007]. This reducing solution is capable of reducing both NO₂⁻ and RSNO to NO for detection. The mechanism to produce NO is [Samouilov and Zweier 1998]:

\[
I^- + I_2 \leftrightarrow I_3^{-}
\] (4.6)

\[
2NO_2^- + 2I^- + 4H^+ \rightarrow 2NO + I_2 + 2H_2O
\] (4.7)

\[
2RSNO + I_3^- \rightarrow 2NO^+ + 3I^- + RSSR
\] (4.8)

\[
2I^- + 2NO^+ \rightarrow I_2 + 2NO
\] (4.9)

Figure 4.1 shows the chemiluminescence experimental set-up that was used throughout this project. Briefly, it contains a purging vial with the reducing solution connected to a Sievers 270B chemiluminescence NO analyzer (Sievers Corporation, Boulder, CO). After the reducing solution was added to the purging vial (D × H: 20 mm × 70 mm), the solution was purged by nitrogen via a fritted disk (Pore Diameter: 25-50 μm) at the bottom of the vial. Aqueous samples from the experiments were obtaining using a gas-tight syringe (Hamilton Company, Reno, NV) and then injected into 10 ml of the reducing solution. The NO released from the sample was carried with the nitrogen purge to the detector. The volume of each sample varied between 20 and 200 μl depending on the detection limits. The output signal from the detector was sent to an HP 3396 integrator (Agilent Technologies, Inc., Santa Clara, CA) that calculated the area under each sample peak. The areas were compared to NO₂⁻ standards for quantification.
In order to measure CySNO in the filtrate following centrifugation of transnitrosation samples or BSANO + CySNO in the unfiltered sample, the sample of

![Chemiluminescence experiment setup](image)

Figure 4.1 Chemiluminescence experiment setup. Samples were injected into the purge vessel containing reducing solution. The NO released from the samples is carried by the nitrogen gas through an ice trap to remove the acetic acid which is harmful for the NOA analyzer. NOA generates ozone to react with NO which produces emission that can be detected by the photomultiplier tube. The signal was transferred to an integrator which can calculate the peak area of the signal. Figure modified from MacArthur *et al.*, 2007.
interest was treated with acidic ammonium sulfamate to scavenge NO$_2^-$ (without affecting the RSNO concentration) via the following reaction mechanism.

\[ NO_2^- + H^+ + NH_4SO_3NH_2 \rightarrow NH_4HSO_4 + N_2 + H_2O \]  

(4.10)

After all the NO$_2^-$ was consumed, the sample was then measured using the KI/I$_2$-based chemiluminescence method as previously described to give the RSNO concentration in the sample. If needed, another portion of the sample without ammonium sulfamate treatment could be measured using the same reducing solution to obtain the total nitrite (NO$_2^-$ and RSNO) concentration. The NO$_2^-$ concentration could then be calculated by subtracting the RSNO concentration from the total nitrite concentration. However, this work only reports the CySNO and BSANO concentrations during transnitrosation. In this work, the above chemiluminescence method was used in order to simplify the measurement and minimize the error propagation since the RSNO could be measured directly.

An alternative method to indirectly measure the RSNO concentration is to use two reducing solutions. In addition to the KI/I$_2$ reducing solution mentioned above, a KI/cysteine reducing solution can be made to scavenge I$_2$ and thus prevent I$_3^-$ formation and NO release from the RSNO (see Equation 4.6 and 4.8) such that [Pietraforte 1995; Robak et al. 1997]:

\[ 2CySH + I_2 \rightarrow CyS-SCy + 2HI \]  

(4.11)

Thus, this reducing solution can only liberate NO from NO$_2^-$. By using the KI/I$_2$ solution (without acidic ammonium sulfamate addition to scavenge NO$_2^-$) to measure total nitrite and the KI/cysteine solution to measure just NO$_2^-$, the RSNO concentration could be obtained by the difference [Duan 2001]. However, this method was found to be more
complicated and less accurate since the RSNO concentration is measured by a difference in two measurements.

In addition, one method to measure the RSNO directly which was used previously in the lab is using Cu\(^+\)/cysteine modified reducing solution (not acidic condition) [Fahlenkamp 2003]. Unfortunately, this method only works well with low molecular weight RSNO, such as CySNO, because when S-nitroso-protein is injected into the purge vessel, significant bubbling occurs (even using antifoam agent) due to the denaturing of the protein. However, this foaming was mostly suppressed when using acetic acid-based reducing solution probably due to the reason that the acid can break the macrostructure of the protein to reduce foaming. Therefore this Cu\(^+\)/cysteine reducing solution was not used for this work.

4.2.6 Saville Method

This test was used in some of the experiments in order to make a quick test of either free nitrite (NO\(_2\)) or RSNO concentration where a low nitrite/S-nitrosothiol detection is not required [Jocelyn 1987]. Compared to the chemiluminescence method, the Saville test is easier to set up. However, the lower detection limit for the Saville test is around 100 nM [Huang et al. 2007], while the chemiluminescence method can detect to as low as 1 nM [Jobgen et al. 2007]. The mechanism of the Saville test is described by Equations 4.12 through Eq. 4.14 [Stamler and Feelisch 1996].

\[
RSNO + Hg^{2+} + H_2O \rightarrow HONO + RS-Hg^+ + H^+ \quad (4.12)
\]

\[
HONO + ArNH_2 + H^+ \rightarrow ArN^+ \equiv N + 2H_2O \quad (4.13)
\]

\[
Ar' + ArN^+ \equiv N \rightarrow Ar-N=\overset{\equiv}{N}-Ar' \quad (4.14)
\]
Briefly, NO$_2^-$ alone or NO$_2^-$ released from S-nitrosothiols (due to decomposition catalyzed by Hg$^{2+}$) react with sulphanilamide (aromatic amine, Ar) to yield the corresponding diazonium salt which couples with N-1-naphthylethylenediamine (second aromatic amine, Ar') to form an intensely colored azo dye for colorimetric assay at OD$_{540}$.

The detailed experimental procedure is as follows. 50 µL to 100 µL samples were added into a well in a 96-well microplate, then equal volumes of Saville solution A (1% sulfanilamide in 0.5M HCl) to detect NO$_2^-$ or Saville solution B (Saville A with 0.2% HgCl$_2$) to detect total nitrite (S-nitrosothiol + NO$_2^-$) were added to the well. The plate was maintained at room temperature for 5 min in order to complete the diazonium formation (Equation 4.13). Finally, equal volumes of Saville C (0.02% N-1-naphthylethylenediamine in 0.5M HCl) were added into the well. After 5 min, the mixture was measured by a Packard® SpectraCount™ Microplate Photometer (BS10000, Packard Instrument Co., Meriden, IL) at 540 nm wavelength. The RSNO concentration was obtained by subtracting the NO$_2^-$ concentration from the total nitrite concentration. 20 µM NaNO$_2$ was made freshly for the standard. By comparing the OD$_{540}$ reading from the samples to the one from standard NaNO$_2$ solution, the total nitrite concentration or the free nitrite concentrations in the samples were calculated.

4.2.7 Cysteine Self-oxidation Assay by Ellman’s Method

Ellman’s assay [Jocelyn 1987] was used to test the cysteine self-oxidation test in the solution without transnitrosation. Briefly, 25 µM cysteine solution was freshly prepared using 50 mM low purity NaH$_2$PO$_4$-Na$_2$HPO$_4$ (pH=7.4) buffer or deionized water and then 4 mL of cysteine solution was added into a 4 mL amber vial. One group of the vials was maintained on the hand motion shaker at room temperature while the
other one was maintained at 4°C. Also, one group of vials with 25 μM cysteine was prepared using 50 mM NaH₂PO₄-Na₂HPO₄ and 50 μM AA and this one was also maintained on the hand motion shaker at room temperature. At specific time intervals, 50 μL samples were drawn from each vial and mixed with 50 μL Ellman’s reagent in a 96-well microplate for 5 minutes and then measured at 410 nm using a Packard® SpectraCount™ Microplate Photometer (BS10000, Packard Instrument Co., Meriden, IL). A 25 μM cysteine in deionized water was made freshly as the standard. By comparing the OD₄₁₀ reading from the samples to the one from standard CySH solution, the CySH concentrations in the samples were calculated.

Ellman’s reagent was made by dissolving DTNB (final concentration 5mM) in a 100 mM NaH₂PO₄-Na₂HPO₄ solution (pH 7.2) with 0.1 mM EDTA. This solution is stable for greater than 2 months if stored in the dark between 0-5°C [Sigma-Aldrich 2002].

4.3 Results

4.3.1 BSANO Nitrosation and Separation Test

Figure 4.2 shows the concentration of free nitrite (NO₂⁻), total nitrite (BSANO + NO₂⁻), and BSANO in the filtrate and the unfiltered sample following utilization of the microcentrifuge tube. As previously mentioned, the BSANO concentration was calculated according to:

\[
[BSANO] = [Total\ nitrite] - [Free\ nitrite]
\]  

(4.15)

Since the solution prior to separation was not treated by acidic ammonium sulfamate to quench NO₂⁻, and the NO₂⁻ was slightly in excess, some free NO₂⁻ was detected as
Figure 4.2 Results for microcentrifuge filter analysis. The BSANO concentration is calculated by subtracting the free nitrite data from the total nitrite data. (n=2)
expected. This NO$_2^-$ concentration remained virtually the same in both the filtrate and un-filtered samples, indicating that NO$_2^-$ is not separated but is equally distributed in both the filtrate and retentate. BSANO only existed in the un-filtered sample, indicating that BSANO cannot penetrate through the filter. The molecular weight (MW) cut-off of the filter is 10k Da which is much less than the MW of BSA/BSANO (66.43k Da).

Another important piece of information given by this result is that the measured BSANO concentration was around 93 μM. Based on this value and comparison with the maximum BSANO that could be made (176 μM BSA was nitrosated), the BSANO yield per mol of BSA was calculated as 0.53, which is consistent with the literature value of 0.6 [Zhang and Means 1996]. This incomplete nitrosation is due to the fact that 35–40% of the BSA is in the disulfide form [Carter and Ho 1994]. On the other hand, the measured total nitrite (BSANO + NO$_2^-$) was around 124 μM, which is about 97% of the moles of NaNO$_2$ added, indicating that most of the original nitrite was accounted for in either BSANO or excess nitrite (negligible N-nitrosation products formed). The 3% that was unaccounted for is probably due to the HNO$_2$ breaking down and forming NO, which quickly escapes from the solution into the headspace during nitrosation.

### 4.3.2 Transnitrosation Kinetics

The time profiles of the BSANO and CySNO concentrations during transnitrosation, with or without AA at various concentrations, are shown in Figures 4.3 through Figure 4.7. By fitting the BSANO self-decomposition (no CySH present) to a first-order kinetic model (exponential decay) according to:

$$\frac{d[BSANO]}{dt} = -k_ds[BSANO]$$ (4.16)
Figure 4.3 BSANO and CySNO concentration time profiles following the initiation of transnitrosation. (n=2) Initial conditions were 102 µM BSANO and 50 µM CySH without ascorbic acid. The solid line and the exponential equation represents first order self-decomposition model described by Equation 4.16.
the BSANO self-decomposition rate constant, $k_{db}$, can be evaluated. The associated $k_{db}$ is shown in each of the figures. In Figure 4.3, in the absence of ascorbic acid, BSANO is very stable even in the low purity phosphate buffer. There is virtually no BSANO decomposition within 24 hours. The $k_{db}$ value in the absence of AA is 0.002 hr$^{-1}$. In the presence of AA, $k_{db}$ values ranged from 0.008 to 0.011 hr$^{-1}$ (see Figures 4.4-4.7). In general, the results indicate that BSANO is relatively stable during the transnitrosation studies. Its self-decomposition rate did not appear to depend on the AA concentration, although the rate was relatively faster in the presence of AA as compared to no AA, possibly due to the fact that more reduced copper/iron is generated by AA and can catalyze BSANO decomposition [Smith and Dasgupta 2000].

When CySH was added to the BSANO solution, the BSANO concentration dropped drastically within 1 hour for all the experiments (with or without AA), indicating a very fast forward transnitrosation reaction. This was confirmed from the CySNO data which showed a rapid increase of CySNO during the first hour. In the absence of AA, the BSANO decrease was initially rapid and then essentially leveled off. In the presence of AA, the BSANO decrease was also initially rapid and then the decrease continued, with the decrease going faster with increasing AA concentration. Table 4.2 summarizes this aspect, showing the percentage of BSANO lost after 10 min and 4 hours as a function of the AA concentration. The data for 50 µM and 500 µM are not included because the starting BSANO concentration is much lower than the others due to a different dilution method mentioned earlier in Section 4.2.2.

As for the amount of NO transferred from BSANO to CysH, the studies without AA showed that approximately 30 µM NO was transferred within 15 minutes from
Table 4.2 Percentage of BSANO transnitrosated after 10 min or 4 hours with different ascorbic acid concentrations (0 to 198 µM).

<table>
<thead>
<tr>
<th>Reaction Time</th>
<th>Ascorbic Acid Conc.</th>
<th>0 µM</th>
<th>20 µM</th>
<th>148 µM</th>
<th>198 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min</td>
<td></td>
<td>33%</td>
<td>36%</td>
<td>49%*</td>
<td>46%*</td>
</tr>
<tr>
<td>4 hours</td>
<td></td>
<td>36%</td>
<td>52%</td>
<td>94%</td>
<td>98%</td>
</tr>
</tbody>
</table>

Note: *Data were acquired after 15 min.
Figure 4.4 BSANO and CySNO concentration time profiles following the initiation of transnitrosation. (n=2) Initial conditions were 88μM BSANO and 50 μM CySH with (circle) or without 20μM (triangle) ascorbic acid. The solid line and the exponential equation represents first order self-decomposition model described by Equation 4.16.
Figure 4.5 BSANO and CySNO concentration time profiles following the initiation of transnitrosation. (n=2) Initial conditions were 88 µM BSANO and 50 µM CySH with 150 µM (square) or 200 µM (circle) ascorbic acid. The solid line and the exponential equation represents first order self-decomposition model described by Equation 4.16.
Figure 4.6 BSANO and CySNO concentration time profiles following the initiation of transnitrosation. (n=2) Initial conditions were 62.27 µM BSANO and 50 µM CySH with 50 µM (circle) or 500 µM (triangle) ascorbic acid. The solid line and the exponential equation represents first order self-decomposition model described by Equation 4.16.
Figure 4.7 BSANO and CySNO concentration time profiles following the initiation of transnitrosation. (n=2) Initial conditions were 116.5 µM BSANO and 25 µM CySH with 200 µM (triangle) ascorbic acid. The solid line and the exponential equation represents first order self-decomposition model described by Equation 4.16.
BSANO to cysteine (in the form of CySNO) while the BSANO concentration decreased by approximately 30 µM (Figure 4.3 & 4.4). However, essentially no additional NO was transferred during the remainder of the experiment. Since the initial CySH was 50 µM, the amount of NO transferred was less than the initial CySH added, suggesting that CySH was not continually involved in transnitrosation. One possible explanation is that cysteine was oxidized by the oxygen in the solution with the help of metal ions [Bagiyan et al. 2003; Bagiyan et al. 2004]. Thus, there was a point where no more transnitrosation could occur since all of the CySH became oxidized.

In order to study the kinetics of cysteine autoxidation, an experiment for cysteine oxidation in buffer was conducted. The results are shown in Figure 4.8. It is evident that increasing temperature can accelerate this oxidation process and the decomposition appears to be a zero order reaction, which is consistent with literature [Ehrenberg et al. 1989; Bagiyan et al. 2004]. The zero order reaction rate constant, $k_{oo}$, was calculated by a linear fit of the data. Thus, at 25 ºC, in 50 mM low purity NaH$_2$PO$_4$-Na$_2$HPO$_4$, pH=7.4 buffer, the zero order decomposition rate constant was 0.7103 µM·hr$^{-1}$. This result also showed that 50 µM AA cannot stop this self-oxidation at all. This can be explained by the redox potential of dehydroascorbic acid/ascorbic acid (58 mV) is much higher than cystine/cysteine (-340 mV) [Fasman 1976] which means that AA cannot reduce cystine to form cysteine spontaneously.

For the transnitrosation studies with AA, more than 50 µM of NO was transferred depending on the concentration of the AA in the solution (Figures 4.4-4.6). Since the initial CySH was 50 µM, this showed that more NO was transferred than the initial amount of CySH. Thus, CySH oxidation did not completely occur following
Figure 4.8 Cysteine self-oxidation in buffers (50 mM low purity NaH$_2$PO$_4$-Na$_2$HPO$_4$, pH=7.4) and deionized water at 4 ºC and 25 ºC. (n=2) Initial cysteine concentration was 25 µM. Open diamond: in deionized water at 4 ºC. Solid square: in buffer at 4 ºC. Solid triangle: in deionized water at 25 ºC. Open square: in buffer at 25 ºC. Cross: in buffer with 50 µM ascorbic acid at 25 ºC. The solid line and the linear equation represents zero order autoxidation model reported earlier [Ehrenberg et al. 1989; Bagiyan et al. 2004].

\[ y = -0.7103x + 25 \]
transnitrosation and NO release, such that CySH could be regenerated during the transnitrosation process. Also CySH autoxidation might not be significant in this time frame because transnitrosation was completed within a few hours, whereas it takes more than 28 hours for 25 µM CySH to be fully oxidized as shown in Figure 4.8.

In order to further test the AA effect on the transnitrosation reaction, a study using less initial CySH (25 µM, compared to 50 µM previously) and 200 µM ascorbic acid was performed. In Figure 4.7, approximately 60 µM NO was transferred from BSANO, more than twice the initial amount of CySH. However, the transfer rate started to decrease after a few hours, indicating that CySH autoxidation eventually consumes most of the CySH after about 4 hours, thus no CySH is available for transnitrosation. Additionally, the CySNO concentration dropped to almost zero at approximately 4 hours, which also confirms that transnitrosation essentially stopped.

4.3.3 Transnitrosation/Decomposition Reactions and Modeling Setup

As discussed earlier (see Equations 4.2 & 4.3), the main reactions involved in transnitrosation and decomposition are:

\[
BSANO + CyS^- \xrightarrow{k_f} CySNO + BSA^- \quad (4.17)
\]

\[
BSANO + Cu^+ \xrightarrow{k_d} BSA^- + NO + Cu^{2+} \quad (4.18)
\]

\[
Cu^+ + CySNO \xrightarrow{k_c} CyS^- + Cu^{2+} + NO \quad (4.19)
\]

Since CyS^- is important in the transnitrosation reaction, it is important to understand the fate of CyS^-, which can be complicated due to various environmental conditions.
If there is no reducing agent, such as AA, Cu\textsuperscript{2+} in solution (which can initially be present or be formed according to Equation 4.19) can oxidize thiol anions to form a disulfide bond and generate Cu\textsuperscript{+} according to [Smith and Dasgupta 2000]:

\[ \text{Cu}^{2+} + \text{CyS}^- \xrightleftharpoons{k_2} \frac{1}{2} \text{CyS-CyS}^+ + \text{Cu}^+ \]  

(4.20)

This disulfide cysteine (CyS-CyS) is stable and would thus reduce the CyS\textsuperscript{-} available for transnitrosation. This is likely what happened in the studies without AA where transnitrosation stopped occurring. If a reducing agent is present, the reducing agent can suppress the available Cu\textsuperscript{2+}, keeping the thiol anion in the reduced form and minimizing the formation of disulfide bonds. For example, AA can react with Cu\textsuperscript{2+} (including Cu\textsuperscript{2+} generated from Equation 4.19) and form dehydroascorbic acid (DHA) and Cu\textsuperscript{+} [Holmes and Williams 2000; Smith and Dasgupta 2000; Aquart and Dasgupta 2004]:

\[ \text{AA} + \text{Cu}^{2+} \xrightarrow{k_2} \text{Cu}^+ + \text{DHA} \]  

(4.21)

Thus, reducing agents can allow for the continual progression of transnitrosation by inhibiting the formation of disulfide bonds. However, AA itself can react directly with CySNO, causing CySNO decomposition and the associated release of NO without the dependence on copper [Holmes and Williams 2000]. The reaction is

\[ \text{AA} + \text{CySNO} \xrightarrow{k_2} \text{CyS}^- + \text{NO} + \text{DHA} \]  

(4.22)

Finally, CySH can also be oxidized by molecular oxygen (autoxidation) in a solution containing Cu\textsuperscript{+} according to [Ehrenberg et al. 1989]:

\[ 4\text{CySH} + O_2 \xrightarrow{k_{oo}} 2\text{CyS-CyS}^+ + 2H_2O \]  

(4.23)

The value of \( k_{oo} \) measured for this work was previously shown in Section 4.3.2.
As shown in all transnitrosation results (Figure 4.3-4.7), the BSANO self-decomposition (Equation 4.18) is extremely slow and can likely be neglected compared to the transnitrosation reactions that diminish the BSANO concentration. The CySNO decomposition reactions in which NO is released (Equations 4.19 and 4.22) are the key reactions that drive the transnitrosation (Equation 4.17) towards the right hand side and keep equilibrium from being established.

Though thiol anions are the actual reactants or products in the above reactions, the rate of reaction can still be written in the form of the total thiol concentration in the solution based on a rapid equilibrium assumption where:

\[ \text{CySH} \rightleftharpoons K_{a,c} \text{CySH}^- + H^+ \] (4.24)

such that,

\[ K_{a,c} = \frac{[\text{CyS}^-][H^+]}{[\text{CySH}]} \] (4.25)

At a certain pH:

\[ [\text{CySH}] = \frac{10^{-pH} \cdot [\text{CyS}^-]}{K_{a,c}} \] (4.26)

Thus,

\[ [\text{CySH}]_T = \frac{(10^{-pH} + K_{a,c}) \cdot [\text{CyS}^-]}{K_{a,c}} = K_c \cdot [\text{CyS}^-] \] (4.27)

where \([\text{CySH}]_T\) denotes the total cysteine which includes both \([\text{CySH}]\) and \([\text{CyS}^-]\).

Assuming that Equation 4.24 is always at equilibrium (fast equilibrium), any concentration change in the CyS\(^-\) will result in a proportional change in the total CySH. Thus, all reaction rate equations involving CyS\(^-\) can be written in the form of total...
Cysteine, although the rate constant used in a reaction would include the term $K_C$ shown in Equation 4.27. A similar situation can also apply to BSA.

From the reactions given above, the mass balance for all species, assuming a well-mixed system, can be written as follows:

$$\frac{d[BSANO]}{dt} = -k_f[BSANO][CySH]_r + k_r[CySNO][BSA]_r - k_{db}[BSANO] \quad (4.28)$$

$$\frac{d[CySNO]}{dt} = -k_{dc}[CySNO] - k_{da}[AA][CySNO] - k_r[CySNO][BSA]_r + k_f[CySH]_r[BSANO] \quad (4.29)$$

$$\frac{d[BSA]}{dt} = k_f[BSANO][CySH]_r - k_r[CySNO][BSA]_r + k_{db}[BSANO] \quad (4.30)$$

$$\frac{d[CySH]}{dt} = k_r[CySNO][BSA]_r - k_f[CySH]_r[BSANO] - k_{oa} - k_{ac}[CySH]_r + k_{da}[AA][CySNO] + k_{dc}[CySNO] \quad (4.31)$$

$$\frac{d[AA]}{dt} = -k_{da}[AA][CySNO] - k_{oa}[AA] \quad (4.32)$$

The above equations assume that $Cu^{2+}$ and $Cu^+$ remain at constant values. Since the total copper concentration is very low compared to the other species in the system, and both copper species are constantly being consumed and regenerated, the change in the copper concentration of both species was assumed to be negligible. Therefore, $k_{dc}$, $k_{oa}$, and $k_{oa}$ are actually pseudo rate constants which include the $Cu^{2+}$ or $Cu^+$ concentrations.

### 4.3.4 Determination of Rate Parameters

From the above equations, there are a total of eight kinetic parameters:

- RSNO decomposition: $k_{da}$, $k_{dc}$, $k_{db}$
- Transnitrosation: $k_f$, $k_r$
CySH and ascorbic acid oxidation: $k_{oa}$, $k_{oc}$, $k_{oa}$

Rate constants, $k_{db}$ and $k_{oa}$ were measured from experimental studies shown in Section 4.3.2. The value of $k_{da}$ has previously been studied with a predicted value of 0.00094 hr$^{-1}$ [Holmes and Williams 2000]. Although $k_{dc}$ has been measured and reported in Chapter 3, different AA concentrations will affect the Cu$^+$ and Cu$^{2+}$ due to the reaction shown in Equation 4.21. Therefore, the value from Chapter 3 cannot be used. Based on the above analysis, there are five rate parameters left that needed to be determined from the experimental data.

In order to determine the above kinetic rate constants, Scientist 3.0 (Micromath, Saint Louis, MO) was initially used to fit the experiment data of Figures 4.3 to 4.7 to the ordinary differential equations (ODEs; Equations 4.28-4.32). A least square fit algorithm was used during the fitting. However, since there are five unknown parameters, some experimental results did not have enough data points for fitting (less than five) since BSANO disappeared completely within a few hours (see Figure 4.5). Thus, in order to reduce the number of kinetics parameters for fitting, a parameter sensitivity analysis was performed to find out if there were any parameters that could be considered negligible. MathCAD (PTC, Needham MA) was used to solve the ODEs numerically to simulate the BSANO concentration profile.

The base case for the parameter sensitivity analysis assumed an initial condition of 116.48 µM BSANO, 200 µM AA and 25 µM CySH. The following base-case kinetic rate constants were obtained using the previously determined values of $k_{db}$ and $k_{oa}$, the reported value of $k_{da}$ noted above, and a least square fit of the 25 µM CySH + 116.48 µM BSANO +200 µM AA study. The base-case values are:
\[ k_f = 0.2 \mu M^{-1} \cdot hr^{-1} \]
\[ k_r = 0.8 \mu M^{-1} \cdot hr^{-1} \]
\[ k_{ab} = 0.008 hr^{-1} \]
\[ k_{de} = 0.4 hr^{-1} \]
\[ k_{oo} = 0.71 \mu M \cdot hr^{-1} \]
\[ k_{oe} = 0.1 hr^{-1} \]
\[ k_{oo} = 0.2 hr^{-1} \]
\[ k_{do} = 0.005 \mu M^{-1} \cdot hr^{-1} \]

Through changing the kinetic parameters by plus or minus 10 fold and plotting the BSANO concentration profile, the model sensitivity level regarding each parameter was determined. The results are shown in Figures 4.9 through 4.13. These figures also include the base-case experimental data and the associated fit. According to the figures, a changing \( k_{oa} \) had the least effect on the BSANO concentration profile. Thus, the potential for neglecting the reaction incorporating this parameter (Equation 4.21) was considered based on the additional analysis shown below.

The detailed kinetics of ascorbic acid oxidation by copper (Equation 4.21) has been established earlier as follows [Hayakawa et al. 1973]:

\[
\frac{d[AA^+]}{dt} = k_1[Cu^{2+}]^2[AA^-] + k_2[Cu^{2+}]^2[AA^{2-}] \quad (4.33)
\]

where \( k_1 = 3.1 \times 10^4 \text{ M}^{-2} \text{s}^{-1} \) and \( k_2 = 6.1 \times 10^{10} \text{ M}^{-2} \text{s}^{-1} \). AA\(^-\) and AA\(^{2-}\) are different dissociated forms of AA according to:

\[
AA \xleftarrow{k_1} AA^- + H^+ \quad (4.34)
\]
\[
AA^- \xleftarrow{k_2} AA^{2-} + H^+ \quad (4.35)
\]

From the AA pK\(_a\) values (pK\(_1\)=4.04 and pK\(_2\)=11.34) and assuming fast equilibrium, the following results can be calculated (at pH=7.4):

92
Figure 4.9 Simulated BSANO concentration profiles using the base-case kinetic parameters along with variations in the $k_f$ parameter. The transnitrosation experimental data for the base-case scenario is also shown (solid squares).

Figure 4.10 Simulated BSANO concentration profiles using the base-case kinetic parameters along with variations in the $k_r$ parameter. The transnitrosation experimental data for the base-case scenario is also shown (solid squares).
Figure 4.11 Simulated BSANO concentration profiles using the base-case kinetic parameters along with variations in the $k_{oc}$ parameter. The transnitrosation experimental data for the base-case scenario is also shown (solid squares).

Figure 4.12 Simulated BSANO concentration profiles using the base-case kinetic parameters along with variations in the $k_{oa}$ parameter. The transnitrosation experimental data for the base-case scenario is also shown (solid squares).
Figure 4.13 Simulated BSANO concentration profiles using the base-case kinetic parameters along with variations in the $k_{dc}$ parameter. The transnitrosation experimental data for the base-case scenario is also shown (solid squares).
\[
[AA^-] = 2290[AA] \tag{4.36}
\]
\[
[AA^{2-}] = 0.2630[AA] \tag{4.37}
\]
Plugging Equations 4.36 and 4.37 into 4.33 gives:
\[
\frac{d[AA]}{dt} = k[Cu^{2+}]^2[AA] \tag{4.38}
\]
where \(k = 0.0253 \text{ \(\mu\)M}^{-2}\text{hr}^{-1}\). Comparison with Equation 4.32 in the absence of the CySNO decomposition term shows that \(k_{oa}\) is equivalent to \(k[Cu^{2+}]^2\).

For a low-purity phosphate buffer which was used in the study, the total heavy metal ion contamination in the salts is around 0.001\%wt (based on a Pb analysis) according to the product label. Although Fisher Scientific does not provide detailed compositions regarding the heavy metal content, a common list of heavy metals includes Ag, As, Bi, Cd, Cu, Hg, Mo, Pb, Sb, and Sn [Schmidt et al. 2005]. However, according to the Sigma-Aldrich product label, the total Cu is around 5.5\%wt and 7.7\%wt of the total heavy metal ion contamination for high-purity Na\(_2\)HPO\(_4\) and NaH\(_2\)PO\(_4\) salts, respectively. Thus, in 50 mM low purity buffer (which contains approximately 11.50 g of Na\(_2\)HPO\(_4\) and 2.62 g of NaH\(_2\)PO\(_4\) in 1L of 100 mM buffer), the total copper concentration is calculated to be 0.067 \(\mu\)M if the same impurity levels as shown in high-purity phosphates salts is used. If all of this was Cu\(^{2+}\), then the maximum value for \(k_{oa}\) would be \(1.14 \times 10^{-4}\) hr\(^{-1}\). It is very likely that the maximum rate would be much lower if AA is present since AA would lead to most of the copper being in the form of Cu\(^{+}\) instead of Cu\(^{2+}\).

Comparing the maximum value of \(k_{oa}\) with \(k_{da}[CySNO]\), since both terms are shown in Equation 4.32 interacting with AA, provides valuable guidance as to whether \(k_{oa}\) can be neglected in the kinetic analysis. Since the CySNO concentration was often higher than 5 \(\mu\)M during the transnitrosation studies (unless BSANO was almost
consumed and transnitrosation almost halted as observed with high AA concentration studies), $k_{da}[\text{CySNO}]$ would have a minimum value of $4.7 \times 10^{-3}$ hr$^{-1}$ based on the reported value of $k_{da}=0.000941$ µM$^{-1}$hr$^{-1}$ at the corresponding pH [Holmes and Williams 2000]. Thus, the maximum value of $k_{oa}$ appears to be more than one order of magnitude smaller than the minimum value of $k_{da}[\text{CySNO}]$. Therefore, it was concluded that kinetics involving $k_{oa}$ in Equation 4.32 could be considered negligible. This reduced the unknown kinetic parameters to four ($k_f$, $k_r$, $k_{dc}$, $k_{oc}$).

With the values of the four parameters as unknowns, all transnitrosation experimental data was fit to Equations 4.28-4.32 using Scientist 3.0. The best fit values for $k_f$ and $k_r$ are listed in Table 4.3. Figure 4.14 shows an example of the model fit for 50 µM CySH, 62.27 µMBSANO and 500 µM AA. The value of $k_f$ ranged from 0.142 to 0.995 µM$^{-1}$hr$^{-1}$ with an average of $0.332 \pm 0.272$ µM$^{-1}$hr$^{-1}$. The value of $k_r$ ranged from 0.164 to 0.822 µM$^{-1}$hr$^{-1}$ with an average of $0.504 \pm 0.226$ µM$^{-1}$hr$^{-1}$. As shown in Figure 4.15, $k_f$ and $k_r$ show no correlation with the AA concentration as expected.

Since $k_f$ and $k_r$ are supposed to be independent of the experiments, the average $k_f$ and $k_r$ were then used to again fit the experimental data to obtain values for $k_{dc}$ and $k_{oc}$. These two parameters, with fitted values for all experiments shown in Table 4.4, should be functions of AA since they both are pseudo rate constants which include the Cu$^{2+}$ or Cu$^{+}$ concentrations affected by the amount of AA. As noted in the table, both the $R^2$ and correlation values are above 0.97 for the combined fitting of BSANO and CySNO data, indicating that the model fits the data quite well. The model predictions of BSANO and CySNO are shown for all sets of data in Figure 4.16 through 4.23. In summary, the values used for the model predictions are $0.332$ µM$^{-1}$hr$^{-1}$ (average) for $k_f$, $0.504$ µM$^{-1}$hr$^{-1}$
Table 4.3 Values of $k_f$ and $k_r$ for transnitrosation experiments in low purity phosphate buffer at pH=7.4 with different ascorbic acid concentrations.

<table>
<thead>
<tr>
<th>Ascorbic Acid concentration (µM)</th>
<th>0</th>
<th>20</th>
<th>50</th>
<th>148</th>
<th>198</th>
<th>200</th>
<th>500</th>
<th>Avg.</th>
<th>Stdv.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Run #1</td>
<td>Run #2</td>
<td>Run #1</td>
<td>Run #2</td>
<td>Run #1</td>
<td>Run #2</td>
<td>Run #1</td>
<td>Run #2</td>
<td>Run #1</td>
</tr>
<tr>
<td>$k_f$ (µM$^{-1}$hr$^{-1}$)</td>
<td>0.235</td>
<td>0.229</td>
<td>0.142</td>
<td>0.995</td>
<td>0.362</td>
<td>0.484</td>
<td>0.200</td>
<td>0.160</td>
<td>0.179</td>
</tr>
<tr>
<td>$k_r$ (µM$^{-1}$hr$^{-1}$)</td>
<td>0.548</td>
<td>0.308</td>
<td>0.164</td>
<td>0.706</td>
<td>0.822</td>
<td>0.485</td>
<td>0.234</td>
<td>0.595</td>
<td>0.678</td>
</tr>
</tbody>
</table>
Figure 4.14 Scientist 3.0 regression result graphic output for the transnitrosation with 500 µM AA and 50 µM CySH with initial BSANO concentration at 62.27 µM. The lines are the fitted model and the solid and open square markers are the experiment data for BSANO and CySNO concentration respectively.
Figure 4.15 Result of data fitting for variable $k_f$ (open diamond) and $k_r$ (solid square) showed that there is no sign of correlation between the two constant and ascorbic acid concentration. Also, the range of the kinetic constant are within 0.1 to 0.7 $\mu$M$^{-1}$ hr$^{-1}$, except a few outliers, which indicating a good consistency of the data fitting.
Table 4.4 Values of $k_{dc}$ and $k_{oc}$, with fixed average $k_f$ and $k_r$ values (as shown in Table 4.3), for transnitrosation experiments in low purity phosphate buffer at pH=7.4 with different ascorbic acid concentrations.

<table>
<thead>
<tr>
<th>Ascorbic Acid concentration (µM)</th>
<th>0</th>
<th></th>
<th>50</th>
<th></th>
<th>148</th>
<th></th>
<th>198</th>
<th></th>
<th>200</th>
<th></th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Run #1</td>
<td>Run #2</td>
<td>Run #1</td>
<td>Run #2</td>
<td>Run #1</td>
<td>Run #2</td>
<td>Run #1</td>
<td>Run #2</td>
<td>Run #1</td>
<td>Run #2</td>
<td>Run #2</td>
</tr>
<tr>
<td>$k_{dc}$ (hr$^{-1}$)</td>
<td>0.225</td>
<td>0.207</td>
<td>0.423</td>
<td>3.07</td>
<td>1.729</td>
<td>2.775</td>
<td>3.031</td>
<td>0.957</td>
<td>6.596</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{oc}$ (hr$^{-1}$)</td>
<td>0.6192</td>
<td>0.4932</td>
<td>0.4868</td>
<td>0.3144</td>
<td>0.9042</td>
<td>0.319</td>
<td>0.1064</td>
<td>0.1522</td>
<td>0.0348</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.999</td>
<td>0.999</td>
<td>0.999</td>
<td>0.998</td>
<td>0.999</td>
<td>0.998</td>
<td>0.999</td>
<td>0.999</td>
<td>0.985</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation</td>
<td>0.999</td>
<td>0.998</td>
<td>0.998</td>
<td>0.990</td>
<td>0.990</td>
<td>0.990</td>
<td>0.995</td>
<td>0.999</td>
<td>0.985</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.16 Scientist 3.0 regression result graphic output for the transnitrosation with 0 µM AA and 50 µM CySH (Run #1). The lines are the fitted model and the solid and open square markers are the experiment data for BSANO and CySNO concentration respectively.
Figure 4.17 Scientist 3.0 regression result graphic output for the transnitrosation with 0 µM AA and 50 µM CySH (Run #2). The lines are the fitted model and the solid and open square markers are the experiment data for BSANO and CySNO concentration respectively.
Figure 4.18 Scientist 3.0 regression result graphic output for the transnitrosation with 50 µM AA and 50 µM CySH (Run #1). The lines are the fitted model and the solid and open square markers are the experiment data for BSANO and CySNO concentration respectively.
Figure 4.19 Scientist 3.0 regression result graphic output for the transnitrosation with 50 µM AA and 50 µM CySH (Run #2). The lines are the fitted model and the solid and open square markers are the experiment data for BSANO and CySNO concentration respectively.
Figure 4.20 Scientist 3.0 regression result graphic output for the transnitrosation with 148 µM AA and 50 µM CySH. The lines are the fitted model and the solid and open square markers are the experiment data for BSANO and CySNO concentration respectively.
Figure 4.21 Scientist 3.0 regression result graphic output for the transnitrosation with 198 µM AA and 50 µM CySH. The lines are the fitted model and the solid and open square markers are the experiment data for BSANO and CySNO concentration respectively.
Figure 4.22 Scientist 3.0 regression result graphic output for the transnitrosation with 200 µM AA and 25 µM CySH. The lines are the fitted model and the solid and open square markers are the experiment data for BSANO and CySNO concentration respectively.
Figure 4.23 Scientist 3.0 regression result graphic output for the transnitrosation with 500 µM AA and 50 µM CySH. The lines are the fitted model and the solid and open square markers are the experiment data for BSANO and CySNO concentration respectively.
(average) for $k_r$, 0.00094 hr$^{-1}$ for $k_{dra}$, 0.002 to 0.011 hr$^{-1}$ (the value determined for each experiment) for $k_{db}$, 0.7103 µM·hr$^{-1}$ for $k_{oa}$, and the fitted parameters for $k_{dc}$ and $k_{oc}$. As previously noted, the reaction involving $k_{oa}$ was assumed negligible.

Further analysis of $k_{dc}$ and $k_{oc}$ with regards to the AA concentration is shown in Figures 4.24 and 4.25, respectively. Figure 4.24 shows a general trend (with the exception of a few outliers) in which $k_{dc}$ increases as the AA concentration increases. Since $k_{dc}$ is a pseudo parameter that includes the Cu$^{+}$ concentration, this trend is consistent since AA contributes to the conversion of Cu$^{2+}$ to Cu$^{+}$. Figure 4.25 shows a general trend (with the exception of a few outliers) in which $k_{oc}$ decreases as the AA concentration increases. Since $k_{oc}$ is a pseudo parameter that includes the Cu$^{2+}$ concentration, this trend is consistent since AA contributes to the conversion of Cu$^{2+}$ to Cu$^{+}$.

4.4 Discussion

Two methods for detecting NO$_2^{-}$ or S-nitrosothiols were evaluated in this chapter, namely the Saville method and the Chemiluminescence method. Both techniques are robust as they usually give very small standard error. However, the Saville test is not as sensitive as the chemiluminescence method as described earlier and it also has the potential for interference problems with low molecular weight S-nitrosothiol detection [Tashimo et al. 2003]. Thus, it should only be used when a quick and semi-quantitive analysis is needed.

The transnitrosation results showed that transnitrosation is not as simple as previously thought, especially when there is ascorbic acid present. The key reactant of
Figure 4.24 Result of data fitting for CySNO decomposition kinetic parameter $k_{dc}$ versus ascorbic concentration (with fixed transnitrosation kinetic parameter $k_f$ and $k_r$).
Figure 4.25 Result of data fitting for CySH oxidation (by copper ion) kinetic parameter $k_{oc}$ versus ascorbic concentration (with fixed transnitrosation kinetic parameter $k_f$ and $k_r$).
transnitrosation, CySH, can be affected by various reductants or oxidants, thus changing the kinetics drastically. Oxygen is one of the key oxidants that oxidizes CySH and makes it less effective for transnitrosation. Since in this work all reactions were performed in aerobic conditions, the experimental results showed that transnitrosation was affected by CySH oxidation. In physiological conditions, it is possible that this autoxidation might be counter played by various reducing agents in the blood such as glutathione [Flagg et al. 1993; Guan et al. 2003]. On the other hand, although the kinetic study showed zero order for the CySH autoxidation, it is feasible that a lower oxygen concentration can result in different kinetics. Ehrenberg et al. found that the oxygen pressure did affect the pseudo zero order rate constant [Ehrenberg et al. 1989]. In blood vessels, the oxygen partial pressure (95 mmHg) [Martin 1999] is much lower than the atmosphere oxygen partial pressure (159.6 mmHg), thus the autoxidation rate can also be reduced and more cysteine can be kept in the reduced form. Copper is another oxidant that plays an important role during the CySNO decomposition. As Cu⁺ needs to be regenerated to cause decomposition of CySNO, two reactions (reaction 4.20 and 4.21) compete with each other to scavenge Cu²⁺ and convert it back to Cu⁺. When the ascorbic acid concentration is high, more CySH can be saved from oxidization by Cu²⁺, thus more BSANO will be transnitrosated with CySH. This effect was shown by the decrease of the \( k_{oc} \) value with increasing concentration of ascorbic acid as shown in Figure 4.25. On the other hand, as Cu²⁺ decreases, Cu⁺ increases which causes an increase in \( k_{dc} \) as shown in Figure 4.24. However, the \( k_{dc} \) value at 0 µM ascorbic acid concentration during transnitrosation (~0.2 hr⁻¹) is much lower than the result from Chapter 3 (~7.2 hr⁻¹) which measured \( k_{dc} \) in the absence of transnitrosation (no BSANO present). As noted
previously, $k_{dc}$ is a pseudo rate constant which includes the Cu$^{+}$ concentration. In the presence of BSA, it has been shown that BSA can chelate the copper ion [Dicks and Williams 1996]. Therefore, in the presence of BSA, the $k_{dc}$ value should be smaller than in the absence of BSA.

From a modeling standpoint, the high correlation value calculated from least square fitting at all ascorbic concentrations indicated that the model described the transnitrosation mechanism quite well. However, compared to the $k_f$ and $k_r$ values reported in literature for transnitrosation involving CySH (see Table 4.1), the values reported in this work are at least an order of magnitude higher due to unknown reasons. However, the calculated equilibrium transnitrosation constant $K=k_f/k_r$ obtained in this work (0.657) is similar to the literature value of HSANO and CySH transnitrosation (0.75) as shown in Table 4.1.

It should be noted that the model was based on the assumption that the Cu$^{+}$/Cu$^{2+}$ ratio remained constant during the reaction. This requires that the ascorbic acid concentration remains virtually stable during the reaction because AA can react with Cu$^{2+}$ to form Cu$^{+}$ as showed in Equation 4.21. In order assess this aspect, a simulation for the AA concentration for 25 µM CySH, 200 µM AA and 116.48 µM BSANO was performed. The result showed that the AA concentration decreased less than 5% within 15 hours (graph not shown), thus demonstrating that the AA was essentially constant for the timeframe of modeling. However, it would be better to experimentally measure the oxidized ascorbic acid (DHA) concentration to further prove this assumption.
4.5 Conclusions

In this chapter, transnitrosation between BSANO and free CySH (not immobilized CySH) was studied and a mathematic model was successfully developed to model the experimental data. The major achievements are:

1) An improved nitrite/S-nitrosothiol detection method based on ammonium sulfamate treatment and KI/I$_2$ based chemiluminescence method was established, which gives less standard error and more data consistency.

2) Transnitrosation between BSANO and free CySH was found to be sensitive to various reductants and oxidants. Oxygen and Cu$^{2+}$ both can oxidize CySH, while ascorbic acid can react with Cu$^{2+}$ so that less CySH will be oxidized. The NO transferred from BSANO increases significantly when the ascorbic acid concentration increases.

3) A mathematic model which includes transnitrosation between BSANO and CySH, CySNO decomposition and CySH oxidation was developed to model the experimental data. The results showed that the model described the transnitrosation reactions very well.

4) Experimental results showed that ascorbic acid increases the CySNO decomposition rate constant $k_{dc}$ value and reduces the CySH oxidation rate constant $k_{oc}$, which is consistent with the reaction mechanisms.

5) Model sensitivity analysis showed that the transnitrosation rate constants $k_f$ and $k_r$; and the CySNO decomposition rate constant $k_{dc}$ have the most impact on the BSANO concentration time profile, while the ascorbic acid oxidation rate constant $k_{oa}$ has less influence.
CHAPTER 5

IMMOBILIZATION OF L-CYSTEINE ON PET SURFACE

5.1 Introduction

In order to utilize endogenous NO to improve the haemocompatibility of polymer surfaces as proposed in Chapter 2, L-cysteine (CySH) needs to be attached to the polymer surface. Currently, polyethylene terephthalate (PET) has been widely used in the biomedical field, especially blood contacting applications such as vascular grafts, heart valves and surgical mesh [Vinard et al. 1988; Tweden et al. 1997] because of its relatively good haemocompatibility, good mechanical properties and good biochemical stability in vivo [Bui et al. 1993; Greenwald et al. 1994]. Thus, it is ideal to use PET as the model polymer for surface modifications as an example. Previously, researchers in this group have established a protocol for covalently attaching CySH or related thiol molecules onto the PET surface [Duan and Lewis 2002; Gappa-Fahlenkamp and Lewis 2005].

Surface modifications have been widely used to immobilize diverse molecules such as antibodies, peptide and proteins, and even cells onto different material surface in order to change the surface properties, such as improving blood compatibility, altering cell adhesion and growth, changing the hydrophilicity or lubricity and enhancing
corrosion resistance all while retaining the key bulk properties of the material [Ratner and Castner 1997].

Immobilization can be mainly divided into two categories based on the interaction between the immobilized molecules/cells and the supporting material: non-covalent and covalent. Non-covalent immobilization is basically physical absorption of a chemical on the material surface which is usually for short term applications. It, however, does have the advantage of a high degree of order and uniformity [Whitesides et al. 1991; Knobler 2007]. Covalent immobilization usually involves chemical bond formation. This can be achieved by either physical methods (such as plasma or UV radiation treatment) or chemical treatments [Jiao and Cui 2007]. For polymer surface modifications, chemical treatments are usually considered first as the process is relatively easy to control (less side reactions) and less expensive (no special equipments needed).

As an inert polymer, PET needs to be activated first to form reactive groups, so that CySH can form covalent bonds with the surface. As introduced earlier in Chapter 2, PET films were treated with ethylenediamine to introduce primary amine groups on the surface. The formation of a primary amine occurs via a polymer chain breakage as illustrated in Figure 5.1 [Avny and Ludwig Rebenfeld 1986]. The amine group was then used to react with glutaraldehyde (GA) to introduce the aldehyde group via imine bond (C=N-) formation. Finally, another unreacted aldehyde group of the GA was used to react with the amine group of CySH [Duan and Lewis 2002; Fahlenkamp 2003]. However, the imine formation reaction is reversible in aqueous solution and can be catalyzed by acid [Carey and Sundberg 2007]. The imine involved in the modified polymer has already been shown to be susceptible to acid as CySH gets detached from the polymer completely.
Figure 5.1 Reaction scheme for PET aminolysis by ethylenediamine. The reaction leads to chain breakage in the ester bond and the formation of a primary amine group.
within one hour in acidic nitrite solution [Duan 2001]. Therefore, the stability of the imine bond in neutral solution can be questionable, as water itself can serve as a weak acid. Although glutaraldehyde cross-linking has been widely used for immobilizing functional groups on different biomaterials for a long time [Bùi et al. 1993; Marconi et al. 1996; Seifert et al. 1997; Liu et al. 2008], the stability of the imine bond has rarely been addressed in these applications. However, a study done by Vazquez et al. reveals that the structure of the imine compound can significantly improve the stability of the imine bond due to the lack of ionic groups or the existence of bulky hydrophobic substituent groups near the imine bond [Vazquez et al. 1990]. Thus, for the imine bonds between CySH and GA or the imine bond between GA and ethylenediamine on the polymer, the bonds either lack one or both of the above two stabilizing factors which makes it highly possible that CySH can be detached from the polymer easily even at neutral pH. Another drawback of this modified polymer is the polymer bulk property changes after ethylenediamine treatment. As mentioned earlier, ethylenediamine causes polymer chain scission when inserting itself into the polymer, thus this treatment weakens the mechanical properties of the polymer [Avny and Ludwig Rebenfeld 1986].

In order to overcome the above problems, a new surface modification carboxylation treatment is introduced in this chapter and amide bond (-C-NH-) formation is used to attaching CySH to the polymer surface. For attaching via the amide bond, there are two functional groups on CySH that can be used: the carboxyl group and the amine group. Thus, PET needs to be activated to acquire amine or carboxyl groups on the surface, respectively, in order for attachment to occur. The amine group can be introduced by the aminolysis method as discussed above using ethylenediamine although
the polymer can be weakened. In contrast, the carboxylation treatment can add a

Figure 5.2) [Löfås and Johnsson 1990; Massia and Hubbell 1990]. Peptide bond formation is

also a widely used method for immobilization [Hermanson 1996]. Although peptide

bonds can also be hydrolyzed in neutral pH and the reaction is thermodynamically

favored (equilibrium constant is around $10^3$-$10^4$), the hydrolysis is kinetically unfavored

under physiological conditions with a reaction half life of years. Thus, peptide bonds are

still considered to be stable in physiological time scale [Aslam and Dent 1998]. In this

chapter, two different surface modification methods (imine bond and amide bond) were

evaluated and their potential stability problems were also studied in detail. In addition,

polymer surface changes were briefly characterized after different modifications.

In addition to the bond stability of modified PET, the stability of CySH itself,

particularly the thiol (-SH) group, is critical for the long term performance of a CySH-

modified polymer in biomedical applications. Thiol groups have been reported to be

sensitive to light, oxygen and ozone in either dry conditions or aqueous conditions

[Gerdes et al. 1999; Ferris and Rowlen 2000]. Although the applications of modified

PET are mostly in aqueous conditions and without light (in vivo), studying the behavior

of CySH on PET without exposure to aqueous solution is beneficial for determining an

optimized storage condition for CySH-modified PET. Therefore, this work also

evaluated the stability of CySH following attachment to PET.
5.2 Materials and Methods

5.2.1 Materials

Polyethylene terephthalate (PET) was generously provided by DuPont (Hopewell, VA). Glacial acetic acid, potassium iodide, tetrahydrofuran (THF) acetone, formaldehyde and sodium nitrite were purchased from Fisher Scientific (Fairlawn, NJ). 10× Gibco Phosphate-Buffered Saline (PBS), pH 7.4 was purchased from Invitrogen. 1M NaOH and 1 M HCl were purchased from Cole-Parmer (Vernon Hills, IL). Ethylenediamine (ED), ammonia sulfamate, sulfanilamide, iodine, L-cysteine (CySH), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), glutaraldehyde, bromoacetic acid, N,N'-dicyclohexylcarbodiimide (DCCI), N-(tert-butoxycarbonyl)-S-trityl-L-cysteine [Boc-Cys(Trt)-OH], 1-hydroxybenzotriazole (HOBt), 4-Ethylmorpholine, 2-(N-morpholino)ethanesulfonic acid (MES), 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) and all the other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Kimble 4 mL and 1.8 mL amber vials were purchased from VWR (West Chester, PA).

5.2.2 PET Modification

PET film was cut into 1×5 cm size and soaked in acetone for 24 hours followed by drying in petri dishes for 12 hours before any surface modification. The acetone treatment is for removing possible surface contaminants.

Two surface modifications: aminolysis and carboxylation were used to add either amine or carboxyl groups on the PET surface, respectively.
**Aminolysis:** Acetone treated PET was placed in an 8 mL glass culture tube and then 5 mL 40% or 60% ED solution was added into the tube to cover the entire polymer. Then, the tube was placed in a 40 °C water bath and shaken at 130 times/min for 24 hours [Gappa-Fahlenkamp and Lewis 2005]. The polymer was then washed by DI water in culture tubes for 3 cycles, each cycle consisting of three quick rinses (using 5 mL DI water each time) followed by one soaking for 5 min (in 5 mL DI water). Finally, the aminolyzed PET (PET-NH₂) was placed in a nitrogen glove box for 12 hours to let it dry.

**Carboxylation:** PET was carboxylated using the protocol described by Yang et al. [Yang et al. 2000]. Briefly acetone treated PET was placed in the culture dish and 5 mL 18.5% formaldehyde solution in 1 M acetic acid was added to cover the entire polymer. The tubes were then set on the hand motion shaker, shaking for 4 hours followed by DI water wash as previously described. This process hydroxylates the PET surface by adding a methanol residue on the benzene ring [Massia and Hubbell 1990] (Figure 5.2). After drying the hydroxylated PET (PET-OH) for 12 hours in the nitrogen glove box, PET-OH was then treated with 5 mL 1 M bromoacetic acid solution in 2 M sodium hydroxide in a glass culture tube for 16 hours followed by DI water rinsing and drying in a nitrogen glove box for 12 hours. This finally introduced the carboxyl group to the PET surface (PET-COOH) [Löfås and Johnsson 1990].

### 5.2.3 CySH Immobilization

After PET surface activation, cysteine can then be attaching using different methods. Three different approaches were attempted as follows.

**Glutaraldehyde cross-linking** (imine bond): This method was based on the previous works done by Fahlenkamp [Fahlenkamp 2003]. PET-NH₂ was put into a
Figure 5.2 Reaction scheme for PET carboxylation. The reaction adds a carboxyl group on the benzene ring without causing chain breakage.
glass tube filled with 5 mL 0.02% GA in Gibco 1×PBS (pH 7.4) buffer. Then, the tube was set on the hand motion shaker, shaking at 1 time/sec while blowing nitrogen into the tubes slowly for 15 min followed by DI water rinsing. This formed the glutaraldehyde modified polymer (PET-GA). Then PET-GA was placed in a new glass tube filled with 5 mL 0.2% CySH in Gibco 1×PBS (pH 7.4) buffer. Finally, the tube was set on the hand motion shaker, shaking at 1 time/sec for 12 hours, followed by DI water rinsing. This modified polymer with CySH on the aminolyzed PET via the imine bond is denoted as PET-N(i)-CySH.

**DCCI coupling** (amide bond): This method was modified from M. Bodanszky and A. Bodanszky [Bodanszky and Bodanszky 1994]. Briefly, PET-NH₂ was put into a glass culture tube and then 5 mL 4°C THF mixture (including 20 mM of HOBt, EMP, Boc-Cys(Trt)-OH and 22 mM DCCI) was added into the tube, followed by shaking on a hand motion shaker for 1 hour at 4°C and 1 hour at room temperature. The DCCI was slightly in excess because it is the coupling reagent. The CySH had a protecting group on both its amine and thiol groups, thus preventing CySH self-polymerization and also keeping the thiol from reacting with DCCI [Hermanson 1996]. Another experiment using higher concentration reactant (200 mM) was also made accordingly. During the reaction, white precipitate was observed which was due to the formation of dicyclohexylurea [Barany and Merrifield 1979]. After coupling, the modified polymer was rinsed by THF for 3 cycles similarly to DI water rinsing. Then, the thiol of the cysteine was de-protected by incubating the modified polymer in 80 mM I₂ dissolved in methanol for 1 hour at room temperature. Finally, the modified polymer was incubated in 1M Na₂S₂O₃ solution to remove the excess iodine on the polymer surface (The color of the polymer turn from
brown to white), followed by 3 cycles of DI water rinsing. A brief summary of these steps is illustrated in Figure 5.3. This modified polymer with CySH on the aminolyzed PET via the amide bond is denoted as PET-N(a)-CySH. A Boc-glycine modified PET was also made similarly as the blank sample and it is denoted as PET-N(a)-Gly.

EDC coupling (amide bond): This method was modified from Wissink et al. Briefly, PET-COOH was incubated with 0.25 M EDC and 0.25 M CySH in 0.1 M phosphate buffer (pH 6.8) in a culture tube which was shaken on a hand motion shaker for 16 h at 4 °C, followed by 1 cycle of 1 M NaCl solution rinsing and 3 cycles of DI water rinsing at 4°C [Wissink et al. 2001]. The CySH used here was unprotected since the thiol protected CySH used in the DCCI method was not water soluble and EDC is preferred to be used in aqueous solution [Hermanson 1996]. The only water soluble thiol protected cysteine commercially available is extremely expensive and currently there is no known method to deprotect it. However, a carboxyl group protected CySH — cysteine ethyl ester (CySH(E)) was also immobilized using the similar method in order to study the self-polymerization effect on immobilization. These two modified PET had an amide bond between the carboxyl group on the PET-COOH and the amine group on cysteine and they are denoted as PET-C(a)-CySH and PET-C(a)-CySH(E), respectively. In addition, a set of experiments with lower reactant concentrations (0.1 M EDC and 0.1M CySH) were performed in order to study the concentration effect on immobilization. A modified polymer in which glycine, rather than cysteine, was attached served as a control. This polymer is denoted as PET-C(a)-Gly.
EDC coupling was also used for ethylenediamine treated PET for attaching CySH. The immobilization is similar to the above protocol, except that 100 mM MES buffer (pH

Figure 5.3 CySH immobilization on the aminolyzed PET using DCCI coupling. The amide bond forms between the carboxyl group of CySH and the amine group on PET-NH$_2$. The thiol group was de-protected after coupling.
5.5) was used instead of 100 mM Na₂HPO₄-NaH₂PO₄ buffer (pH 6.8). This polymer is denoted as PET-N(a,e)-CySH.

5.2.4 Cysteine Surface Concentration Analysis

Immobilization of cysteine on PET-N(i)-CySH was verified using a protocol modified from Duan and Lewis [Duan and Lewis 2002]. Briefly, the polymer was cut into a 1×1 cm piece and put in a 1.8mL amber vial. Then, 250 µL 1 N HCl and 750 µL 100 µM NaNO₂ was added into the vial followed by hand motion shaking for 1 hour at room temperature. After that, 500 µL 200 µM ammonium sulfamate was added into the vial with continued shaking for 5 min to quench the excess nitrite. In this process, cysteine detaches and is nitrosated to form S-nitrosocysteine. S-nitrosocysteine was then measured by a Sievers NO chemiluminescence analyzer NOA 270B (Sievers Medical Instruments, Inc., Boulder, CO) using a potassium iodide/iodine (KI/I₂) reducing solution as mentioned in Chapter 4, Section 4.2.5. Since the excess nitrite is removed, only CySNO was measured. The surface cysteine concentration was calculated as follows:

\[
[\text{CySH}]_{\text{surface}} = [\text{CySNO}]_{\text{solution}} \cdot V / A
\]  

(5.1)

where \([\text{CySNO}]_{\text{solution}}\) is the CySNO concentration in the solution measured via chemiluminescence, \(V\) is the volume of the solution, and \(A\) is the surface area of the polymer film.

For the cysteine modified polymer via the amide bond (PET-N(a)-CySH and PET-C(a)-CySH) the amide bond is stable in the acidic condition and the above method cannot detect any S-nitrosocysteine in the solution. Therefore, an indirect method was used. Briefly, the modified polymer was incubated with 500 µL 100 µM NaNO₂ and 500 µL 1 N HCl for 30 min followed by 500 µL 1N NaOH neutralization. This reaction
reduces the nitrite concentration in the solution since the thiols on the surface are nitrosated. Meanwhile, a similar acidic nitrite solution was prepared, without exposure to a polymer, to be used as a blank since during the nitrosation some of the nitric acid can decompose to form NO and be released into the headspace of the vial which can also cause a decrease of total nitrite in the solution. Then, 20 μL samples were taken from both the polymer-exposed solution and the blank solution and the nitrite concentration was measured by the KI/I$_2$-based chemiluminescence method as previously described. The surface cysteine concentration was estimated by the following equation:

$$\left[\text{CySH}\right]_{\text{surface}} = \left(\left[\text{NO}_2^-\right]_{\text{blank}} - \left[\text{NO}_2^-\right]_{\text{sample}}\right) \cdot \frac{V}{A}$$

(5.2)

where $\left[\text{NO}_2^-\right]_{\text{blank}}$ is the nitrite concentration of the blank solution, $\left[\text{NO}_2^-\right]_{\text{sample}}$ is the nitrite concentration of the solution containing the modified PET, V is the volume of the solution and A is the area of the polymer film.

In addition to measuring the surface cysteine concentration, cysteine concentration in solution was measured during some studies using Ellman’s method [Ellman 1959; Rootwel 1967] as mentioned in the Chapter 4, Section 4.2.7. Briefly, a 100 μL sample solution was mixed with an equal volume of Ellman’s reagent (5 mM DTNB in 100 mM pH 7.2 phosphate buffer and 0.1 mM EDTA) in a 96-well microplate (Falcon 353228, BD, Franklin Lakes, NJ). After 10 min, the absorbance of the solution was measured at 410 nm using a Packard® SpectraCount™ Microplate Photometer BS10000 (Packard Instrument Co., Meriden, IL).

5.2.5 Imine Bond and Amide Bond Stability Test

All three polymers, PET-N(i)-CySH, PET-N(a)-CySH and PET-C(a)-CySH, were cut into a smaller size (usually 1×1 cm) and placed in an 4 mL amber vial which was
filled with 1.5 mL 50 mM low purity NaH$_2$PO$_4$-Na$_2$HPO$_4$ buffer (pH=7.4). All the vials were set on the hand motion shaker, shaking at 30 times/min at either room temperature or in a 37 °C incubator. At specific times, sample polymers were taken out of the vials, wiped by tissue paper and then measured by the chemiluminescence method mentioned earlier. The liquid samples were measured by Ellman’s method as previously mentioned.

5.2.6 Cysteine Modified Polymer Surface Property Analysis

The modified polymer surface was briefly characterized by scanning electron microscope (SEM) using a Jeol JSM 6360 scanning electron microscope (Jeol USA, Inc., Peabody, MA). Images of the surface were taken for unmodified polymer, aminolyzed PET (treated by different concentration of ethylenediamine) and carboxylated PET. Also, the polymer thickness change after aminolysis was analyzed by micrometer calipers.

5.2.7 Light and Air Effect on CySH Modified Polymer and Long Term Storage Test

CySH-modified PET was made by ethylenediamine treated PET followed by glutaraldehyde cross-linking immobilization as previously described. The reason for using this unstable polymer for this oxidation test was because 1) the oxidation test was under dry conditions which does not have the potential problem arising from the imine bond breakage; 2) This method was the easiest and fastest one to make the CySH modified PET; 3) This method can introduce more cysteine on the PET surface than the other methods, which will make it easier to detect the change in the surface concentration.

The PET-N(i)-CySH was cut into 1×1 cm pieces and divided into three petri dishes. One of the petri dishes was placed in the nitrogen glove box with a 60 W
incandescent light bulb turning on constantly at the distance of 20 cm. This experiment will show only the light effect on the modified PET. The other two petri dishes were exposed to air but with one of them covered with aluminum foil and one placed under a constant 60 W incandescent light bulb at a distance of 20 cm. Therefore, these two petri dishes were used for studying CySH modified PET oxidation in air only and in light + air, respectively. At specific times, one piece of the modified polymer was removed from each petri dish and treated with acidic nitrite, followed by KI/I₂ based chemiluminescence analysis as previously mentioned.

In order to study the possibility of long term storage of the modified polymer under dry conditions, one piece of a 1×5 cm polymer was placed in a 4 mL amber vial and the vial was placed in the -20 °C freezer for 62 days. Then, the polymer was cut into 1×1 cm pieces and measured by the same method which was used to analyze the PET-N(i)-CySH polymer as above.

The CySH surface concentration for oxidation in air only, light only and air + light were plotted versus time. Since the kinetic mechanism of the oxidation is unknown for the modified polymer, TableCurve2D v5.01 (Systat Software, Inc, Chicago, IL) was used to fit the graph with various embedded kinetic models in the software and then a best fit model was determined.

5.3 Results

5.3.1 Cysteine Surface Concentration Measurement

The chemiluminescence analysis for all three modified polymer showed that cysteine was attached. The surface concentrations are shown in Figure 5.4. However, the
surface concentrations are not consistent among different modification methods. The surface concentrations ranged from around 2 nmol/cm² to 20 nmol/cm², with PET-N(i)-CySH having the highest concentration and the PET-C(a)-CySH showing the lowest value. This is probably due to surface area changes or cross-linking effect, which will be addressed later. On the other hand, it is evident that increasing the reactant concentration during the carbodiimide coupling (DCCI method and EDC method) leads to a higher surface concentration of CySH (PET-N(a)-CySH (20 mM) versus PET-N(a)-CySH (200 mM) and PET-C(a)-CySH (0.1 M) versus PET-C(a)-CySH (0.25 M)). However, for DCCI coupling, since the protected CySH is relative expensive, only a 200 mM reactant concentration was tried.

For EDC coupling, two different treated PET were assessed: aminolyzed PET and carboxylated PET. The resulting surface concentrations are much lower (below 5 nmol/cm²) compared to PET-N(i)-CySH and PET-N(a)-CySH, with the modified PET coupled with cysteine ethyl ester (CySH(E)) having a relatively higher surface concentration. Also, it was also observed that a higher (around 0.5 M) concentration of CySH during the coupling is not recommended because when large amount of CySH is oxidized during the reaction, it forms a substantial amount of white precipitation (disulfide CySH) which can stick on the polymer surface and significantly interfere with coupling reaction (data not shown).

As expected, two blank polymer using glycine as the reactant: PET-N(a)-Gly and PET-C(a)-Gly showed virtually zero CySH surface concentration (data not shown).

5.3.2 Stability of Imine Bond and Amide Bond

Although the polymer using the ethylenediamine treatment and glutaraldehyde
Figure 5.4 CySH surface concentrations on PET modified by different methods. PET-N(i)-CySH: PET treated by ethylenediamine, followed by glutaraldehyde cross-linking with CySH. PET-N(a)-CySH: PET treated by ethylenediamine, followed by DCCI coupling. PET-C(a)-CySH: PET treated by carboxylation, followed by EDC coupling. PET-C(a)-CySH(E) same as PET-C(a)-CySH, but cysteine ethyl ester was used instead of cysteine. Concentrations in the parenthesis are the reactant concentrations during the coupling reaction.
cross-linking yielded the highest CySH surface concentration, the imine bond was not stable even at neutral pH. Figure 5.5 shows that cysteine detached from the polymer surface and went into the solution (The total cysteine loss is more than 70% at 37 °C and less than 50% at 25 °C). Notice that the y axis of Figure 5.5 is the total moles of cysteine on the polymer (or in the solution) instead of the CySH surface concentration (nmol/cm²) as shown in Figure 5.4. Thus, it is easy to find show that CySH loss from the polymer and the CySH increase in the solution are virtually comparable if considering the factor that some of the CySH (either on polymer or in solution) is oxidized due to the reasons which were discussed in the previous chapter. Figure 5.5 also showed that the hydrolysis is initially fast (within 5 hours) and then slows down. Increasing the temperature only increased the initial imine bond hydrolysis and the free CySH oxidation in the solution but exerted no noticeable effect on CySH detachment after 5 hours. This is evident since two curves showed the same trend of CySH surface concentration loss which is probably due to oxidation and not imine bond hydrolysis.

For the CySH modified polymer made by aminolysis followed by DCCI coupling through an amide bond (PET-N(a)-CySH), the polymer showed good stability in acidic condition as mentioned earlier. However, at neutral pH, there was still significant CySH detachment from the polymer as shown in Figure 5.6. The detaching trend was similar to the modified polymer using an imine bond (PET-N(i)-CySH). Ellman’s method again detected that there was CySH existing in the solution and the amount was comparable to the CySH amount lost from the polymer surface.

For the polymer made by carboxyl modification followed by EDC coupling (amide bond), PET-C(a)-CySH also showed significant CySH surface concentration loss
Figure 5.5 CySH surface amount and amount in the solution when the modified polymer (PET-N(i)-CySH) was incubated in low purity phosphate buffer at 25 °C and 37 °C. (n=2) The cysteine is slowly detached from the polymer (compared to acidic condition). An increase in temperature can accelerate this imine bond hydrolysis within the initial 5 hours.
as shown in Figure 5.7 (more than 80% of the CySH detached from the polymer surface). Again, CySH was detected in the solution. However, the total CySH amount in the solution was more than the amount on the polymer (almost 3 times). This inconsistency of the data will be discussed in detail later. As shown in Figure 5.8, the carboxyl group protected CySH showed instability similar to the unprotected polymer.

For the polymer made by ethylenediamine modification followed by EDC coupling (amide bond), PET-N(a,e)-CySH showed that the bond between CySH and the polymer had much better stability as shown in Figure 5.9. The CySH surface concentration decrease was probably due to oxidation, which is consistent with previous results (virtually zero order oxidation as shown in Figure 5.5 after 5 hours). Ellman’s method could not detect any CySH in the solution, thus confirming that there was no CySH detached from the polymer.

5.3.3 Polymer Surface Change after Modification

SEM images (Figure 5.10) showed that the original PET surface is quite smooth with only minor defects (some dots and lines). The carboxylated polymer also showed no polymer surface deterioration. However, for the ethylenediamine (ED) treated PET, there were many cracks formed and the size of the cracks increased with increasing ED concentration (The entire dark part of Figure 5.10(d) is one crack with a width around 5 µm while in Figure 5.10(c), the width of the cracks are less than 1 µm). The polymer thickness for the ED treated polymer also changed significantly after modification, reducing from 0.0855±0.0053 mm to 0.0525±0.0039 mm with about 60% loss after 60% ethylenediamine treatment.
Figure 5.6 CySH surface amount and amount in the solution when the modified polymer (PET-N(a)-CySH) was incubated in low purity phosphate buffer at 25 °C. (n=2) The cysteine was detached from the polymer similar to PET-N(i)-CySH, indicating that the bond was unstable.
Figure 5.7 CySH surface amount and amount in the solution when the modified polymer (PET-C(a)-CySH) was incubated in low purity phosphate buffer at 25 °C. (n=2) The cysteine was detached from the polymer similarly to PET-N(i)-CySH, indicating that the bond was still unstable. However, the CySH lost from the polymer was not consistent with the CySH observed in solution.
Figure 5.8 CySH surface concentration when the modified polymer (PET-C(a)-CySH(E)) was incubated in low purity phosphate buffer at 25 °C. (n=2) The cysteine was detached from the polymer similarly to PET-N(i)-CySH, indicating that the bond was still unstable. The solution CySH was not measured in this case.
Figure 5.9 CySH surface concentration when the modified polymer (PET-N(a,e)-CySH) was incubated in low purity phosphate buffer at 25 °C. (n=2) CySH surface concentration was reduced mostly by oxidation because there was no detectable CySH in the solution (data not shown).
Figure 5.10 SEM image of PET surface change after different treatment. (a) untreated PET, (b) carboxylated PET, (c) 40% ethylenediamine treatment, (d) 60% ethylenediamine treatment. The carboxylated PET showed similar surface as the original one while the ethylenediamine treated PET showed many cracks and the size of the cracks increased with higher ethylenediamine concentrations.
5.3.4 Light and Air Effect on CySH Modified Polymer and Long Term Storage Test.

The results shown in Figure 5.11 with only light + air treatment showed significant CySH oxidation (40% loss within 110 hours) while light or air alone virtually did not show any CySH oxidation (<10% within 110 hours). A 3rd-order simple decay model (Equation 5.3) fit the data the best with $R^2$ of 0.999:

$$C = \frac{C_0}{\sqrt{1 + 2 \cdot C_0^2 \cdot k \cdot t}}$$  \hspace{1cm} (5.3)

where $C_0$ is the initial CySH surface concentration, $k$ is the 3rd-order decay rate constant. The fitting gives the value of $C_0=26.07$ nmol/cm$^2$ and $k=(3.32\pm0.1)\times10^{-5}$ nmol$^{-2}\cdot$cm$^4\cdot$hr$^{-1}$. Long term freezer storage (Figure 5.12) showed that the CySH surface concentration remained stable at -20 °C freezer for 62 days.

5.4 Discussion

The theoretical maximum cysteine surface concentration can be estimated by assuming a tightly packed monolayer of spherical cysteine molecules on the polymer surface. The following equation can be used to estimate the radius of cysteine molecule [Fournier 1999]:

$$R = \left( \frac{3M}{4\pi\rho N_A} \right)^{1/3}$$  \hspace{1cm} (5.4)

where, M is the molecular weight of the molecule (121.16 g/mol for cysteine) and $\rho$ is the density of the molecule (1.523 g/cm$^3$). Thus, the calculated cysteine molecule radius is 0.32 nm and the maximum surface concentration is 0.52 nmol/cm$^2$. The ethylenediamine treated PET followed by glutaraldehyde cross-linking or DCCI coupling showed a much
Figure 5.11 PET-N(i)-CySH oxidation under different storage condition. (n=2) The light or air treatment virtually did not cause oxidation, only light combined with air caused CySH oxidation. The solid line is a 3\textsuperscript{rd}-order decay model (Equation 5.3) for fitting the light + air scenario.
Figure 5.12 PET-N(i)-CySH long term storage test. (n=3) CySH surface concentration remained virtually the same after 62 days in a -20 °C freezer.
higher value than the theoretical limit. There are two possible reasons for this phenomenon. First, the cracks formed can significantly increase the active surface area for immobilization; second, glutaraldehyde can self polymerize in aqueous solution to form long chains with multiple aldehyde groups [Migneault et al. 2004]. On the other hand, aminolyzed PET using the EDC method for coupling did not show a high cysteine surface concentration which was probably due to the fact that EDC was mostly consumed by CySH self-polymerization (because the unprotect CySH can react with each other to form a complex compound, therefore less CySH can be attached to aminolyzed PET).

The instability of the imine bond was verified and the possible reason was discussed above. However, there is a method to improve the imine stability by reducing the imine bond (-C=\text{N}-) to an amine bond (-C-\text{NH}-) by a strong reducing agent (e.g. NaCNBH$_3$) [Hermanson 1996]. However, this work was not pursued.

The amide bond was supposed to be stable according to many published applications [Hermanson 1996; Aslam and Dent 1998]. However, among the various modified PET that were made in this study using the amide bond, most of them (e.g. PET-N(a)-CySH, PET-C(a)-CySH and PET-C(a)-CySH(E)) appeared to be stable only in acidic conditions, only one (PET-N(a,e)-CySH) was stable at both neutral and acidic pH. One possible reason is the thiol group on the CySH. In EDC coupling, the –SH group can react with the COOH group during the EDC coupling to form a thiol ester bond [Hermanson 1996]. This bond is stable in acidic solution according to Saburo [Saburo 1999], but it is not as stable as the amide bond and can be hydrolyzed in neutral pH. Also, the activated –COOH will preferably react with a NH$_2$ group, even there is a SH group at the same concentration level [Hermanson 1996]. Therefore, during coupling, since the CySH
concentration is quite high, CySH will first react with each other to form a chain of CySH instead of reacting with the activated –COOH on the PET as shown in Figure 5.13(a). Thus, for this molecule (Figure 5.13(a)), it is easier to react with the activated –COOH on the PET via the –SH rather than the –NH₂ group because the –SH is now dominant (see Figure 5.13(b)). Therefore, when this thiol ester polymer is put into the solution, the thiol ester bond will be broken and all the cysteine will be quickly lost. However, in the acidic nitrite condition, the –SH can be nitrosated and the bond is stable. Therefore, –SH can be detected.

On another note, it has been documented that phosphate can also be activated to react with amine to form a phosphoramidate bond [Hermanson 1996], although the stability of this bond is not clear and there is no literature regarding whether activated phosphate can react with the thiol group or not. Similar reactions can still occur even if the carboxyl group of CySH is protected which is in the case of EDC coupling with CySH ethyl ester, because CySH ethyl ester might be hydrolyzed to form unprotected CySH during the long coupling process (16 hours). For EDC coupling using aminolyzed PET and CySH, since there is no thiol ester bond formation between PET and CySH (no carboxyl group on PET), all the CySH are coupled to PET via an amide bond to form a stable CySH-modified PET. This can also be used to explain the reason why in the case of PET-C(a)-CySH, a higher CySH was detected in the solution than the loss of CySH on the polymer, because the CySH chain might form a complex 3-D formation on the PET surface to prevent the thiols from reacting with acidic nitrite. Therefore, when the thiol ester bond is hydrolyzed, the chain is detached from the polymer and all of the thiol groups are exposed to Ellman’s reagent. This leads to more CySH detected by Ellman’s method as
Figure 5.13 Possible CySH chain formation (a) and thiol ester bond formation between the thiol group on the CySH chain and carboxyl group on the PET (b).
compared to the chemiluminescence method.

However, the above mechanism cannot explain why in the DCCI coupling, even when the CySH had both its thiol group and amine group protected, the resulting modified polymer was still unstable. The only explanation could be that the ethylenediamine treated polymer became more unstable after the DCCI coupling which involved a lot of different organic solvent treatments.

Although one CySH modified polymer with a stable bond (PET-N(a,e)-CySH) was finally synthesized, there were still potential problems with this polymer. The aminolyzed PET showed significant surface deterioration as shown by SEM analysis due to the reason that ethylenediamine can diffuse into the polymer fiber to cause further damage of the polymer microstructure [Avny and Ludwig Rebenfeld 1986]. Meanwhile the mechanical properties were significantly weakened as the polymer thickness reduced significantly after 60% ethylenediamine treatment. Thus, it is ideal to find a method to introduce an amine group on the PET surface without breaking the polymer chain or find a water soluble thiol protected CySH which can be deprotected easily.

The CySH surface concentration time profiles (Figure 5.5-5.8) for the unstable polymers (PET-N(i)-CySH, PET-N(a)-CySH, PET-C(a)-CySH and PET-C(a)-CySH(E)) were almost similar, with a fast initial decreasing step within a few hours and then slowing down. The first stage was likely due to bond breakage while the second stage was likely due to cysteine oxidation. By fitting the second stage of Figure 5.5 with zero order kinetics, the decay constant is 0.280 nmol/(cm²·hr). However, by fitting the data in Figure 5.9, the decay constant is 0.0433 nmol/(cm²·hr). The inconsistencies between these two constants indicate that different binding can affect the CySH oxidation decay
constant. However, the decay constant might not be affected by the temperature as shown in Figure 5.5, which is different from the free cysteine oxidation in solution mentioned in the Chapter 4.

The light and air influence study showed that except for the bond stability issue, CySH on the surface can also be oxidized in the dry condition when light and oxygen are both present, but under light or air alone, no significant oxidation occurs. A possible explanation for this phenomenon is that light serves as the energy to activate the electron in the thiol group and oxygen serves as the electron acceptor. Thiol photooxidation in dry conditions or in non-aqueous solutions has previously been studied. There is a postulated intermediate for this oxidation in both gas or liquid phase, which involves the thylperoxyl radical [Robert-Banchereau et al. 1997]:

\[
\text{R-S}^\cdot + O_2 \rightarrow \text{R-S-O-O}^\cdot \rightarrow \text{Oxidation products}
\] (5.5)

where the thiol radical might be generated under light activation. However, this work showed a 3\textsuperscript{rd}-order decay reaction, which is different from photooxidation occurring in aqueous conditions (first or zero order reaction) [Robert-Banchereau et al. 1997; Ferris and Rowlen 2000; Bagiyan et al. 2003]. Therefore, further studies need to be performed to test whether this is a unique reaction mechanism for CySH photooxidation of a modified polymer.

Cysteine-modified PET long term storage tests showed that when kept at low temperature (-20 °C), no CySH oxidation was observed for about two month. This finding is beneficial for future experiments since large amounts of CySH-modified polymer can be made in one batch and kept in the freezer for a long time. This could
potentially reduce possible inter-experimental errors for CySH surface concentration studies.

5.5 Conclusions

Cysteine immobilization using different methods was evaluated and the stability of the bond in buffer as well as cysteine oxidation in both aqueous and dry conditions were studied. The important findings are:

1) The previous protocol for making PET-N(i)-CySH lead to an unstable polymer due to imine bond breakage between either CySH and glutaraldehyde or glutaraldehyde and the amine on PET.

2) Amide bond formation using DCCI or EDC coupling increased the stability of the modified polymer in acidic condition. However at neutral pH, some of the modified polymer still showed bond breakage and only one of the newly developed polymers (ethylenediamine treatment followed by EDC coupling) showed good bond stability in both acidic and neutral pH.

3) Cysteine on all the polymers can also be oxidized slowly just as the free cysteine oxidation which followed the zero order kinetics in solution. Different immobilization methods appear to have a significant effect on the oxidation rate constant.

4) Ethylenediamine treatment can cause substantial amounts of cracks on PET surfaces and the higher concentration of ethylenediamine used, the bigger the cracks. Also, the PET thickness is reduced significantly after ethylenediamine
treatment which potentially affects the mechanical properties or the stability of the modified polymer.

5) Immobilized CySH on PET-N(i)-CySH polymer is susceptible to light and air under dry conditions. However light or air alone does not cause CySH oxidation. This oxidation was found to be represented by a 3rd-order decay reaction with regard to the CySH surface concentration, but a detailed mechanism remains unsolved. CySH modified PET can be stored in an amber vial at -20 °C for about 2 months without noticeable oxidation.

As noted in this chapter, a modified polymer that had both structural integrity and stable bonds was not achieved. In order to make a polymer with both good mechanical integrity and bond stability, two approaches can be attempted in the future: a) introduce amine group on the PET without destroying the polymer and then do the EDC coupling; b) find a water soluble thiol protected CySH (which can later be deprotected) and attach it to carboxylated PET using the EDC method.
CHAPTER 6

BSANO TRANSNITROSATION WITH CYSH MODIFIED PET AND MODELING IN FLOW SYSTEM

6.1 Introduction

As described in Chapter 1, the ultimate goal of this work is to develop a kinetic model to predict the NO flux from a CySH-modified polymer surface exposed to BSANO. A model will aid in evaluating the performance of a CySH-modified polymer under various physiological conditions. In addition, by comparing the predicted NO flux to the minimum NO flux for platelet inhibition reported by Ramamurthi and Lewis [Ramamurthi and Lewis 2000], the reason for previously incomplete platelet inhibition might be explained.

Although transnitrosation reaction kinetics in free solution were studied in Chapter 4, it is unknown whether the rate constants will change after immobilization as many enzyme activities have been found to be significantly altered after immobilization [Ollis et al. 1976; Jerzy et al. 1981; Bickerstaff 1997; Mateo et al. 2007]. Thus, in this work, the kinetics of transnitrosation between the immobilized CySH and BSANO in the solution was studied and the key parameters of the transnitrosation reaction, namely $k_f$ and $k_r$, and $k_{dc}$, were calculated based on the experimental data.
One potential difficulty of evaluating the kinetics for most CySH-modified polymers addressed in Chapter 5 (e.g. PET-N(i)-CySH) is that the bond stability was not very good. Thus, kinetic parameters could be masked by continual loss of CySH from the polymer. As noted in Chapter 5, only the modified polymer made via aminolysis followed by EDC coupling method (PET-N(a,e)-CySH) had good bond stability (although not good mechanical strength). However, this modified polymer had a very low CySH (~1 nmol/cm²) surface concentration compared to the modified PET made by aminolysis followed by glutaraldehyde cross-linking (PET-N(i)-CySH) or DCCI coupling (PET-N(a)-CySH) (~20 nmol/cm²). The low cysteine surface concentration would be difficult for kinetic studies as the BSANO concentration change due to transnitrosation might be too small to be measured accurately. The modified PET made via aminolysis followed by glutaraldehyde cross-linking (PET-N(i)-CySH) showed only a 10% loss of CySH due to imine bond hydrolysis within the first hour at 25 °C (Figure 5.5). Therefore, this CySH-modified polymer developed via aminolysis could still provide valuable information regarding the values of $k_f$ and $k_r$ for an immobilized system since these parameters could be estimated from initial transnitrosation data within the first hour. Although these parameters could vary between different types of immobilized CySH polymers, having an estimate of the parameters for one CySH-modified polymer could still provide insights regarding transnitrosation with a modified polymer. Therefore, in this chapter, transnitrosation kinetic studies between immobilized CySH (prepared via aminolysis followed by glutaraldehyde cross-linking) and BSANO were performed.

$k_f$ and $k_r$ can be estimated from experimental data according to [Duan 2001]:

\[ \text{[Duan 2001]} \]
where the left side of Equation 6.1 is the initial slope of the BSANO concentration time profile, \([BSANO]_0\) and \([CySH]_0\) are the initial concentration of BSANO and CySH, respectively, \(S\) is the total area of the modified polymer and \(V\) is the total volume of the solution. Knowledge of the initial conditions and the value of \(k_{db}\) (obtained from BSANO decomposition studies in the absence of polymer—see Chapter 4, Section 4.3.2) enables the calculation of \(k_f\) from Equation 6.1. If the equilibrium constant does not change with immobilization, \(k_r\) can be calculated by using the free solution transnitrosation equilibrium constant calculated in Chapter 4.

Once the transnitrosation studies were performed to evaluate the \(k_f\) and \(k_r\) constants following immobilization, a dimensionless mathematical model for predicting transnitrosation and the resulting NO flux was developed to mimic a CySH-modified biomaterial that could be used in a tubular system (e.g. catheters and vascular graft). A laminar flow pattern was used for the model since most of the blood flow in the human body is laminar, except in the aorta which is turbulent flow [Brant 2001]. The model was simplified according to a few assumptions as noted below. Using the model, an assessment of the BSANO concentration, CySH surface concentration and other factors was performed to determine the resulting influence on the NO flux. The predicted NO flux was compared to the minimum NO flux required to minimize platelet adhesion.

### 6.2 Materials and Methods

#### 6.2.1 Materials
Polyethylene terephthalate (PET) was provided by DuPont (Hopewell, VA). Glacial acetic acid, potassium iodide, acetone and sodium nitrite were purchased from Fisher Scientific (Faiflawn, NJ). 1M NaOH and 1 M HCl were purchased from Cole-Parmer (Vernon Hills, IL). Ethylenediamine (ED), ammonia sulfamate, iodine, L-cysteine (CySH), glutaraldehyde and all the other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Kimble 4mL and 1.8mL amber vials were purchased from VWR (West Chester, PA).

6.2.2 Transnitrosation Reaction Between PET-N(i)-CySH and BSANO Solution

PET-N(i)-CySH was prepared using the protocol described in Chapter 5. The BSANO solution was prepared using the protocol described in Chapter 4. CySH-modified PET was cut into 1×1 cm pieces and each piece was placed in a 4 mL amber vial filled with 2 mL BSANO to cover the entire polymer. Several vials were prepared. The vials were then set on a hand-motion shaker, shaking at 30 times/min at 25 °C. At specific time interval, two vials (duplicate samples) were removed from the hand motion shaker and the polymer samples were taken out of the vials to stop transnitrosation.

The polymer was wiped by tissue paper and treated with acidic nitrite to nitrosate the CySH on the polymer to form CySNO. During acidic treatment, CySNO is also detached from the polymer. The resulting CySNO was then measured using KI/I2-based chemiluminescence analysis, as previously described in Chapter 5, Section 5.2.4, to obtain the CySH concentration on the surface. In addition to un-nitrosated CySH on the polymer, there would also be CySNO on the polymer as a result of the BSANO transnitrosation. However, preliminary experiments showed that after pulling a polymer out of solution, CySNO was not detectable via chemiluminescence when the polymer
was exposed to an acidic solution (although not acidic nitrite such that any CySH was not nitrosated). Therefore, it appears that once the polymer is pulled out of solution, CySNO on the polymer perhaps undergoes decomposition and is no longer detectable but CySH can still be detected once placed back in solution and nitrosated. Therefore, the method used in this study appears to measure the CySH, but not the CySNO, on the polymer surface.

Meanwhile, 20 µL of the sample BSANO solution was withdrawn from the vials and treated by ammonium sulfamate to scavenge any NO₂⁻. The sample was analyzed for BSANO using the KI/I₂-based chemiluminescence method described in Chapter 4, Section 4.2.5. It should be noted that in the solution sample, an unknown concentration of CySNO could be present due to a small amount of CySNO release as a result of bond instability (although less than 10% in first hour). However, this CySNO concentration likely would have a negligible contribution to the BSANO analysis since CySNO decomposes very fast with half life of only 6 min at pH 7.4 (as shown in Chapter 3, Figure 3.1) and not all of the detached thiol will be CySNO (some will likely be CySH). This assumption was implied for the experimental results in Section 6.3.1.

6.2.3 Data Analysis and Modeling

The total RSNO concentration in solution (BSANO and CySNO—although most should be BSANO) and the total CySH surface concentration were plotted against time. The $k_f$ value was estimated using the first two data points of RSNO versus time according to Equation 6.1. Since the transnitrosation reaction equilibrium constant is related to the Gibbs free energy which is independent of immobilization, the free solution transnitrosation equilibrium constant calculated in Chapter 4 was used to predict $k_r$ from
the $k_f$ value. Additionally, $k_{dc}$, which represents NO release from CySNO, was also evaluated.

The modeling of transnitrosation and NO release in a tubular system through which a fluid continually flows is derived as follows. First, if CySH is maintained in a fully reduced state (i.e. never oxidized due to either CySNO decomposition or autoxidation), the CySNO surface concentration will reach a steady state value since the rate at which CySNO is generated would be a constant as a result of continuously replenished S-nitroso human serum albumin (HSANO) in a flowing system. This assumption was validated after one hour (see Figure 6.1) for CySNO in a solution using the solution transnitrosation model described in Chapter 4. It is very possible for CySH to remain in the reduced state since blood contains abundant ascorbic acid [Khaw et al. 2001; Jamaati et al. 2006] and glutathione [Svardal et al. 1990; Lim et al. 1995; Abukhalaf et al. 2002; Elena Sbrana 2004]. Ascorbic acid can keep CySH in the reduced form after CySNO decomposition according to Chapter 4 and glutathione can reduce the cystine back to cysteine [Conrad 2009]. Based on these assumptions, the following model was developed.

Similar to section 4.3.3, the transnitrosation reaction between HSANO and immobilized CySH, followed by CySNO decomposition can be illustrated as follows:

$$\text{HSANO} + \text{CyS}^- \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \text{HSA}^- + \text{CySNO} \quad (6.2)$$

$$\text{CySNO} \rightarrow_{k_d} \text{CyS}^- + \text{NO} \uparrow \quad (6.3)$$

$$\text{CySNO} + \text{AA} \rightarrow_{k_d} \text{CyS}^- + \text{DHA} + \text{NO} \uparrow \quad (6.4)$$

The major difference between these reactions and the reactions in Chapter 4 is that both CyS$^-$ and CySNO are immobilized on the polymer rather than in solution. Additionally,
the oxidation reactions (Equations 4.20 and 4.23) of CyS\(^-\) were ignored since, as mentioned above, CyS\(^-\) oxidation is not as likely in a blood system due to reducing agents.

Figure 6.1 Simulation of CySNO surface concentration time profile when PET-CySH contacts a constant concentration of HSANO. A continuously reduced CySH was assumed.
According to the above reactions, the rate of [CySNO] change is:

$$\frac{d[CySNO]}{dt} = k_f [HSANO][CySH]_T - k_r [CySNO][HSA]_T - k_{dc}[CySNO] - k_{da}[CySNO][AA]$$

(6.5)

where \([CySH]_T\) and \([HSA]_T\) are the total thiol concentrations composed of both the thiol and thiol anions that are in equilibrium (see Chapter 4, section 4.3.3). It should be noted that \([HSANO]\) and \([HSA]\) are volumetric concentrations while \([CySH]\) and \([CySNO]\) are surface concentrations. Based on a \(k_{da}\) value of 0.00094 µM\(^{-1}\)hr\(^{-1}\) [Holmes and Williams 2000] and a maximum AA physiological concentration of 60 µM [Khaw et al. 2001; Jamaati et al. 2006], \(k_{da}[AA]_{max}\) is 0.0564 hr\(^{-1}\). For \(k_{dc}\) of 2.4 hr\(^{-1}\) in the presence of 50 µM AA (which is the blood concentration), \(k_{dc} \gg k_{da}[AA]_{max}\). Thus, the \(k_{da}\) term is negligible and Equation 6.5 can be simplified to:

$$\frac{d[CySNO]}{dt} = k_f [HSANO][CySH]_T - k_r [CySNO][HSA]_T - k_{dc}[CySNO]$$

(6.6)

When CySNO is at steady state as discussed above, Equation 6.6 becomes:

$$k_f [HSANO][CySH]_T - k_r [CySNO][HSA]_T - k_{dc}[CySNO] = 0$$

(6.7)

Because the mass balance for cysteine is:

$$[CySH]_T = [CySH]_0 - [CySNO]$$

(6.8)

where \([CySH]_0\) is the initial CySH concentration, Equation 6.8 can be substituted into Equation 6.7 to give:
\[ k_f[HSANO] ([CySH]_0 - [CySNO]) - k_r[CySNO][HSA] - k_{de}[CySNO] = 0 \quad (6.9) \]

Rearrangement provides an estimate for the steady state CySNO concentration as a function of the original CySH loading on the polymer ([CySH]₀) and the amount of S-nitroso albumin (HSANO) or albumin (HSA) in blood according to:

\[ [CySNO] = \frac{[CySH]_0}{1 + \frac{k_{de}}{k_r [HSANO]} + \frac{k_{de}}{k_f [HSANO]}} \quad (6.10) \]

To further simplify Equation 6.10, the total albumin concentration (HSANO + HAS) in blood is approximately 44g/L [Rodriguez-Segade et al. 2005] and [HSANO] is below 0.2 μM [MacArthur et al. 2007]. Accordingly, [HSA] is approximately 667 μM (based on HSA MW of 66 kDa [Zunszain et al. 2003]). As shown in Chapter 4, \( k_f \) is of the same order as \( k_r \), thus \( \frac{k_r [HSA]}{k_f [HSANO]} \gg 1 \). Therefore, Equation 6.10 can be simplified to:

\[ [CySNO] = \frac{[CySH]_0}{[HSA] + \frac{k_{de}}{k_f [HSANO]}} \quad (6.11) \]

where \( K \) is the equilibrium constant, equal to \( k_f/k_r \), for the reversible transnitrosation.

Once CySNO can be approximated, the approximation can be using in the HSANO balance which is:

\[ \frac{d[HSANO]}{dt} = -k_f [HSANO][CySH]_r \cdot \frac{S}{V} + k_r [CySNO][HSA]_r \cdot \frac{S}{V} - k_{de}[HSANO] \quad (6.12) \]
where $S$ denotes the surface area and $V$ denotes the volume of the liquid. Substituting Equation 6.7 into Equation 6.12 followed by substituting Equation 6.11 into the resulting equation gives:

$$\frac{d[\text{HSANO}]}{dt} = -\frac{[\text{CySH}]_0}{[\text{HSA}]} \cdot \frac{S}{V} \cdot [\text{HSANO}] - k_{db}[\text{HSANO}] \quad (6.13)$$

Since $[\text{HSA}]>>[\text{HSANO}]$, HSA will likely remain at the concentration in blood due to rapid equilibrium between HSANO and HSA during transnitrosation. Thus, Equation 6.13 can be simplified to

$$\frac{d[\text{HSANO}]}{dt} \cdot \frac{V}{S} = k_{A,S}[\text{HSANO}] = R_{A,S} \quad (6.14)$$

where $k_{A,S} = \frac{[\text{CySH}]_0}{[\text{HSA}]} \cdot \frac{1}{K \cdot k_{dc}} + \frac{k_{db} \cdot V}{k_f \cdot S} \cdot S$.

The first term of $k_{A,S}$ is the polymer surface consumption rate of HSANO and the second term is the self-decomposition consumption rate of HSANO in the bulk solution. The total consumption rate is $R_{A,S}$ (in mol/(area·time)). As noted, $R_{A,S}$ can be approximated as a first-order irreversible reaction for HSANO under physiological conditions. As shown below for the model conditions, the second term of $k_{A,S}$ is negligible such that $k_{A,S}$ represents only the consumption at the surface.

For modeling transnitrosation and NO release in a tube through which a solution continuously flows, the model is described by a tube which has CySH coated on the inner wall. A mixture of HSANO and HSA passes through the tube (Figure 6.2). The reactions (Equations 6.2 through 6.4) only occur on the surface. Thus, the consumption of HSANO
(Equation 6.14) appears only in the boundary condition, not in the mass balance equation of the bulk liquid.

Thus, for HSANO in the bulk solution, the continuity equation is [Bird et al. 2006]:

$$\rho \left( \frac{\partial C_A}{\partial t} + v_r \frac{\partial C_A}{\partial r} + \frac{v_\theta}{r} \frac{\partial C_A}{\partial \theta} + v_z \frac{\partial C_A}{\partial z} \right) = \rho D_A \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial C_A}{\partial r} \right) + \frac{1}{r^2} \frac{\partial^2 C_A}{\partial \theta^2} + \frac{\partial^2 C_A}{\partial z^2} \right] + r_A$$

(6.15)

where \( C_A \) denotes the HSANO concentration, \( \rho \) is the density of the fluid and \( D_A \) is molecular diffusivity of HSANO. The following assumptions were made to simplify Equation 6.15:

1) Steady State for \( C_A \): \( \frac{\partial C_A}{\partial t} = 0 \).

2) No convective transport of reactant toward the wall: \( v_r = 0 \).

3) Reactant concentration profile is angularly symmetric: \( \frac{\partial C_A}{\partial \theta} = 0 \).

4) Axial diffusion can be neglected compared to axial convection: \( \frac{\partial^2 C_A}{\partial z^2} = 0 \).

5) No reaction in the bulk liquid: \( r_A = 0 \). Convective transport vanishes on the stationary surface. Thus, the mass transfer of A towards the inner wall of the tube equals the consumption rate of A on the surface.

6) Constant \( \rho \) and \( D_A \).

Based on the above assumptions, the five terms which are underscored can be neglected, resulting in the following partial differential equation (PDE):
For laminar flow, the velocity profile is a function of the radius location in the tubes [Polyanin et al. 2001]:

\[ v_z(r) = 2\overline{v}\left[1 - \left(\frac{r}{R}\right)^2\right] \]  \hspace{1cm} (6.17)

where \( v_z \) is the axial velocity, \( R \) is the inner radius of the tube, and \( \overline{v} \) is the average velocity within the tube. Substituting Equation 6.17 into Equation 6.16 gives:

Figure 6.2 Modeling geometry. A round tube with CySH coated on the inner wall of the tube was assumed with blood flowing inside the tube with a laminar flow pattern of velocity \( v \). A cylindrical coordinate system with \( z \) for the axial axis and \( r \) for the radius axis is used.
\[ 2\pi \left[ 1 - \left( \frac{r}{R} \right)^2 \right] \frac{\partial C_A}{\partial z} = D_A \left( \frac{1}{r} \frac{\partial C_A}{\partial r} + \frac{\partial^2 C_A}{\partial r^2} \right) \]  

(6.18)

Rearrangement gives:

\[ D_A \frac{\partial^2 C_A}{\partial r^2} = \frac{2\pi}{R^2} \left[ R^2 - r^2 \right] \frac{\partial C_A}{\partial z} - \frac{D_A}{r} \frac{\partial C_A}{\partial r} \]

(6.19)

The boundary equations to solve Equation 6.19 are:

At \( z=0 \) and \( r>0 \), \( C_A(r) = C_{A0} = 2 \times 10^{-4} \text{ mol/m}^3 \) (the concentration of HSANO in blood)

At \( r=R \) and \( z>0 \),

\[ k_{A,s} C_A = -D_A \frac{\partial C_A}{\partial r} \]

At \( r=0 \) and \( z>0 \), \( C_A(z) = C_{A0} = 0.2 \mu \text{mol/m}^3 \). This latter boundary condition was initially assumed and then validated with the modeling results. Generally, this assumption implies that very little HSANO is consumed in the bulk solution.

When solving a model, it is best to use a non-dimensional form of the model.

With \( r=\gamma R \), \( C_A=\phi C_{A0} \) and \( z=\zeta L \), Equation 6.19 becomes:

\[ \frac{\partial^2 \phi}{\partial \gamma^2} = \kappa \left( 1 - \gamma^2 \right) \frac{\partial \phi}{\partial \zeta} - \frac{1}{\gamma} \frac{\partial \phi}{\partial \gamma} \]  

(6.20)

where, \( \kappa = \frac{2\pi R^2}{D_A L} \)

The associated boundary conditions become:

at \( \zeta=0 \) and \( \gamma>0 \), \( \phi(\gamma)=\phi_0 = 1 \)

at \( \gamma=1 \) and \( \zeta>0 \), \( k_{A,s} \phi = -\frac{D_A}{R} \frac{\partial \phi}{\partial \gamma} \)

Since \( \gamma \) cannot be 0 in the second term on the right hand side of Equation 6.20, a very small value of \( \gamma \) was chosen as the boundary condition for \( \gamma=0 \):

at \( \gamma=0.000001 \) and \( \zeta>0 \), \( \phi(\zeta) = 1 \).
By solving the above PDE numerically in MathCAD (PTC, Needham, MA), the $C_A$ concentration profile within the tube can be predicted as a function of $\gamma$ and $\zeta$. The surface concentration of $C_A$ can then be used to calculate the CySNO surface concentration by Equation 6.11. Finally, the estimated NO flux can be calculated according to:

$$[\text{NO}]_{\text{flux}} = k_{dc}[\text{CySNO}]$$  \hspace{1cm} (6.21)

The following parameters were used for the model. Since BSA has a diffusivity of $5 \times 10^{-7}$ cm$^2$/s [Cha and Beissinger 1996] and BSA has a similar molecular structure as HSANO, the diffusivity of HSANO was assumed to be the same. The radius was chosen to be similar to an artery with a radius of 0.2 cm. For this size of an artery, the average velocity is around 10 cm/s [Stoner et al. 2004]. The length of the polymer tube was chosen as 1 m. Although this length is extremely long for polymer applications, this length would give a good idea as to how the HSANO concentration and NO flux change through the polymer tube. Based on the above information, $\kappa$ in Equation 6.20 is 16,000.

To estimate $k_{A,S}$ (Equation 6.14) used in the boundary condition, the constant cysteine surface concentration, $[\text{CySNO}]_0$, was chosen as 4 nmol/cm$^2$, which was a typical value shown in Chapter 5 and was also a typical surface concentration used in previous platelet inhibition studies in which incomplete inhibition was observed [Duan and Lewis 2002; Gappa-Fahlenkamp and Lewis 2005]. [HSA] was assumed to be the typical value in blood which is 667 $\mu$M. K was assumed to be 0.66 as measured from the free solution transnitrosation studies in Chapter 4. The value of $k_{dc}$ was assumed to be 2.4 hr$^{-1}$, which was the average value for the free transnitrosation studies in Chapter 4 involving 50 $\mu$M AA (which is the typical concentration in blood). The validity of this
assumption is shown in Section 6.3.1. The $k_{db}$ value is 0.001 hr$^{-1}$ from experimental conditions as shown in section 6.3.1. Finally, $k_f = 0.33 \mu$M$^{-1}$hr$^{-1}$ from the free transnitrosation studies of Chapter 4 was used (see discussion section to validate this reasoning). Based on these values, $k_{A,S}$ is approximately 0.0096 cm/hr of which the $k_{db}$ term of Equation 6.14 only contributed to 1% of the value. Thus, the $k_{A,S}$ term is representative of just the consumption of HSANO at the polymer surface, which was assumed for the boundary condition. As an additional point, the $k_f$ contribution was negligible such that $k_{A,S}$ is only strongly dependent upon $K$, [HSA], and $k_{dc}$.

6.3 Results

6.3.1 Transnitrosation between BSANO and Immobilized CySH

Figure 6.3 shows the BSANO concentration profile when BSANO was incubated with CySH-modified PET. Also, the BSANO concentration time profile (without CySH modified PET) is also shown in the graph for comparison. The BSANO profile without the polymer showed a first order decay constant, $k_{db}$, of 0.001 hr$^{-1}$, which is consistent with previous results in Chapter 4 (0.002 hr$^{-1}$). However, the BSANO concentration decreased significantly when CySH-modified PET existed in the system, indicating that transnitrosation followed by CySNO decomposition (on surface or in solution) played an important role.

As shown in Figure 6.4, the CySH surface concentration during transnitrosation dropped very fast within the first 6 hours (about 60% loss) and then began to stabilize. According to Figure 5.5 where no transnitrosation occurred, the CySH loss from bond instability appeared to be only 30%. Therefore, the “apparent” increase in CySH loss...
during transnitrosation may be a result of some of the CySH on the polymer being converted to CySNO and this CySNO is likely not detected by the chemiluminescence method as mentioned in section 6.2.2.

When the CySH surface concentration remained approximately constant (between 7 and 60 hours), the BSANO concentration showed a decrease with a constant slope of 0.446 μM/hr. This constant slope indicates a pseudo-steady state CySNO (and CySH) concentration which is similar to the simulation in Figure 6.1. It should be noted that although bond instability was observed at early times in Chapter 5, bond instability was not as appreciable between 6 and 30 hours, leading to the possibility of CySNO reaching pseudo-steady state value. At this point, all of the change in BSANO would be equivalent to the NO release rate, such that

\[
\frac{d[BSANO]}{dt} = -k_{dc}[CySNO] \frac{S}{V}
\]  

(6.22)

The NO release rate was obtained from Equation 6.22. The left side of Equation 6.22 is the constant slope of -0.446 μM/hr noted above. S is 2 cm² and V is 2 mL. To approximate [CySNO], the [CySH] value of 7 nmol/cm² (see Figure 6.4) was used. Since the polymer initially had 24 nmol/cm², and approximately 60% is lost after 30 hours due to hydrolysis (see Figure 5.5), then that should leave 9.6 nmol/cm² on the surface. Therefore, [CySNO] should be approximately 2.6 nmol/cm². Based on this, \( k_{dc} \) is calculated to be 0.17 hr⁻¹. This is very close to the value of 0.22 hr⁻¹ observed in Chapter 4 for free transnitrosation in the absence of AA (see Table 4.4). Thus, it appears that immobilization doesn’t significantly change the constant. Therefore, the value of \( k_{dc} \) used in the model, based on 50 μM AA, was assumed to be the same as observed in Chapter 4 for free transnitrosation.
Figure 6.3 BSANO concentration change during transnitrosation with immobilized cysteine (using PET-N(i)-CySH). (n=2) Solid diamond: BSANO without PET-N(i)-CySH, Solid square: BSANO with PET-N(i)-CySH, dashed line is the first order decay model for the data based on Equation 4.16.
Figure 6.4 CySH surface concentration (PET-N(i)-CySH) change during transnitrosation with BSANO. (n=2) The CySH surface concentration dropped very fast within the first 6 hours and then slowed down. CySH loss within 6 hours (~60%) is more than that of Figure 5.5 (~30%).
From this study, the initial surface concentration of CySH was measured as 24 nmol/cm². Based on Equation 6.1 and the initial slope of the BSANO concentration profile, together with the initial BSANO concentration, $k_f$ was calculated to be 0.0037 $\mu$M⁻¹hr⁻¹. This value is much lower than the value for free solution transnitrosation as calculated in Chapter 4 (0.33 $\mu$M⁻¹hr⁻¹) but is within the wide range reported in Table 4.1. The significantly lower value suggests that immobilization reduced the effectiveness of transnitrosation. As noted in the discussion section, the reduced effectiveness may have been due to mass transfer limitations.

6.3.2 Transnitrosation Modeling in Flow System

The results for the model simulation are shown in Figure 6.5. The dimensionless HSANO concentration is also indicated by the darkness of the grey color (the deeper color for higher concentration). It shows that concentration drops slightly near the wall and the outlet of the tube has a greater concentration gradient than the inlet of the tube. In order to get a more detailed concentration profile near the wall, a contour graph of the dimensionless concentration focusing on the dimensionless radius range from 0.9 to 1 (Figure 6.6) shows the concentration drop between the bulk solution and the surface. The maximum concentration drop is around 5%, which occurs at the outlet of the tube. As noted, the concentration in the bulk solution remains the same as the inlet, thus validating the boundary condition at r=0.

Based on the dimensionless HSANO concentration profile near the wall, the NO flux was calculated by Equation 6.11 and 6.21. Figure 6.7 shows the NO flux based on the simulated HSANO concentration profile near the inner wall of the tube. The NO flux is virtually constant throughout the entire tube, a little higher than $5.2 \times 10^{-12}$ mol/(m²·s),
Figure 6.5 Dimensionless simulation of Human Serum albumin (HSANO) concentration profile within a tube with inner surface covered with Cysteine (CySH). The graph shows only the concentration near the wall (0.8 ≤ γ ≤ 1), since the concentration is essentially constant throughout the tube except near the wall. The depth of grayscale indicates the dimensionless concentration (the darker for the lower concentration).
while the minimum NO flux to inhibit platelet adhesion is around $3-6 \times 10^{-12}$ mol/(m²·s) [Ramamurthi and Lewis 2000]. Therefore, the polymer used in previous experiments may have been sufficient to minimize platelet adhesion. However, uncertainties in physiological conditions, such as the HSANO concentration (as shown in Table 6.1), exert significant impact on the NO flux, leading to possible insufficient NO flux as discussed in detail later.

### 6.4 Discussion

6.4.1 Transnitrosation Reaction between BSANO and Immobilized CySH

Although the CySH modified polymer used for this analysis is an unstable polymer with CySH detaching from the polymer surface with time, it still showed that the modified polymer is capable of continuously transferring NO. For instance, according to Figure 6.3, 90 nmol of BSANO was transferred in the 2 ml solution compared to the 48 nmol of initial CySH on the polymer. Thus, even without ascorbic acid, CySH on the polymer performs better than free CySH in solution since CySH in solution only could transfer less NO than the amount of CySH. This finding is probably due to the reason that immobilized CySH, even if it forms a thiol radical (which is the oxidation product of thiol anion by either Cu²⁺ [Dicks et al. 1996] or O₂ [Ehrenberg et al. 1989]), may not be easy to react with each other as a result of steric hindrance to form a disulfide bond (CyS-CyS). Instead, the unreacted thiol may be reduced from other molecules (e.g. free thiol group on BSA) to form reduced cysteine.

As noted above, the transnitrosation kinetics data was different between the free solution study and the immobilized study. The value of $k_f$ for the immobilized study was
Figure 6.6 2-D contour (with concentration gradient) graph for dimensionless simulation of HSANO concentration profile within a tube with inner surface covered with CySH. The depth of the darkness indicates the concentration (lighter for higher concentration and darker for lower concentration). The graph shows part of the tube which is near the wall (dimensionless radius from 0.9 to 1). The number on the contour line is the dimensionless concentration.
Figure 6.7 NO flux profile along the length of the tube at the inner wall surface ($\gamma = 1$) of the tube calculated from the dimensionless simulation of Human Serum albumin (HSANO) concentration profile within a tube with the inner surface covered with Cysteine (CySH).
Table 6.1 S-nitroso human serum albumin (HSANO) concentration in plasma.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Method</th>
<th>Reference</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>181±150 nM</td>
<td>GC-MS + HgCl₂</td>
<td>[Tsikas et al. 1999]</td>
<td></td>
</tr>
<tr>
<td>4.2±1 μM</td>
<td>Fluorometric Assay</td>
<td>[Tyurin et al. 2001]</td>
<td>non-pregnancy women</td>
</tr>
<tr>
<td>156±64 nM</td>
<td>GC-MS + HgCl₂</td>
<td>[Tsikas et al. 2002]</td>
<td></td>
</tr>
<tr>
<td>205±96 nM</td>
<td>GC-MS + cysteine/CuSO₄</td>
<td>[Tsikas et al. 2002]</td>
<td></td>
</tr>
<tr>
<td>62±24 nM</td>
<td>HPLC + Saville</td>
<td>[Jourd'heuil et al. 2000]</td>
<td></td>
</tr>
<tr>
<td>7.2±1.1 nM</td>
<td>Chemiluminescence</td>
<td>[Rassaf et al. 2003]</td>
<td></td>
</tr>
<tr>
<td>0.59±0.31 nM</td>
<td>Chemiluminescence</td>
<td>[Wang et al. 2004]</td>
<td></td>
</tr>
</tbody>
</table>
approximately two orders of magnitude smaller than the free solution value. One possibility of the lower value, compared to free transnitrosation, is that mass transfer is controlling the rate rather than kinetics. Since BSANO is a large molecule and the nitrosated cysteine residue (Cys-34) is located in the center of the BSA molecule [Gryboś et al. 2005], the immobilized CySH within the cracks of the polymer (ethylenediamine forms crack on PET) might not be able to easily come in contact with BSANO.

According to Equation 6.1 and the measured initial values and slope, the far right side of the equation (self decomposition) is negligible. Thus, Equation 6.1 can be rewritten as:

\[
\left[ \frac{d[BSANO]}{dt} \right]_0 = -k_f [BSANO]_0 [CySH]_0 = -k [BSANO]_0
\] (6.23)

where \(k = k_f [CySH]_0\). It is interesting to note that Equation 6.23 is also consistent with a model for mass transfer limitations where \(k\) is the volumetric mass transfer coefficient for BSANO. In other words, mass transfer limitations could result in an “apparent” \(k_f\) that is lower than a “true” \(k_f\) and the apparent \(k_f\) would be related to the slower mass transfer rate. This appears to be a likely explanation for the lower \(k_f\) and further work would need to be performed to test this hypothesis. From a modeling standpoint, the model parameters noted at the end of Section 6.2 assumed a \(k_f\) of 0.33 \(\mu\)M\(^{-1}\)hr\(^{-1}\) (from free transnitrosation) rather than the apparent value shown above. The reason is that the model must include the “true” value of \(k_f\) to accurately represent the reaction at the surface as the diffusivity of the HSANO has already been included in the model.

Unlike the transnitrosation kinetics, the NO release (i.e., CySNO decomposition) rate constant was similar to the free CySNO decomposition rate constant. This seems
feasible since the rate is primarily a function of the copper ion which should easily be able to come into contact with the polymer due to its small size.

6.4.2 Factors that influence NO flux from the CySH modified polymer

There are numerous environmental factors that can affect the NO flux from the polymer. The HSANO concentration might be the most important one according to Equations 6.11 and 6.21 which shows that the predicted HSANO concentration will significantly affect the NO flux. So far there are still significant inconsistencies regarding the HSANO concentration in blood [Rassaf et al. 2003; Giustarini et al. 2007]. Table 6.1 shows recent results with the range from sub-nM to \( \mu \text{M} \). Thus, if the HSANO concentration is lower than the simulation value (200 nM), the NO flux will decrease significantly. For example, if assuming the HSANO concentration is one-tenth of 200 nM (20 nM), the NO flux would be reduced to around \( 0.52 \times 10^{-12} \text{ mol/(m}^2\text{·s)} \), although the NO flux will still remain almost constant throughout the tube (figure not shown here). This NO flux is one order of magnitude less than the minimum required NO flux to inhibit platelet inhibition. According to Table 6.1, most of the reported HSANO concentrations are below 200 nM, with an average concentration of 102 nM (not including the 4.2 \( \mu \text{M} \) value, because of the potential artifact reported for this fluorimetric analysis [Giustarini et al. 2007]). Therefore, with this average value, the predicted NO flux is estimated to be \( 2.6 \times 10^{-12} \text{ mol/(m}^2\text{·s)} \), which will likely still cause incomplete platelet inhibition. Thus, for the previous incomplete inhibition, the possible reason could be the low HSANO concentration in the blood. However, this assumption needs to be verified in the future.
Another important parameter in the model is the initial surface CySH concentration. The 4 nmol/cm² used in the simulation is the ethylenediamine treated polymer used by Duan and Fahlenkamp [Duan and Lewis 2002; Fahlenkamp 2003], which had a lot of cracks to increase the cysteine surface concentration. The theoretical CySH concentration on the polymer should be around 1 nmol/cm², unless a branch structure can be formed on the polymer by attaching a molecule with multiple thiol groups. Under the condition of 1 nmol/cm² CySH initial concentration, the NO flux would be around $1.3 \times 10^{-12}$ mol/(m²·s), which is much less than the minimum required flux. Therefore, in order to make a polymer with complete platelet inhibition and good mechanical and chemical stability, attaching a compound with multiple thiol groups or forming branches with multiple activation sites on the polymer surface might be the direction for future research.

To test the sensitivity of the decomposition rate constant $k_{dc}$ effect on NO flux, the value of $k_{dc}$ was set to 6.6 hr⁻¹ which was the highest value measured previously in Chapter 4, Table 4.4., the NO flux will be around $13 \times 10^{-12}$ mol/(m²·s) throughout the tube. Compared to the NO flux prediction ($k_{dc}=5.2 \times 10^{-12}$ mol/(m²·s)) at the base value (2.4 hr⁻¹), the model is also sensitive to $k_{dc}$ which is also a strong function of the copper ion concentration. Under physiological conditions, metal ions are usually in bound form. For example, the majority of the copper is bound to ceruloplasmin while the rest is bound to serum albumin or amino acid [Twomey et al. 2005]. Although the bound copper can still catalyze NO release from CySNO, the catalyzing rate is significantly reduced [Dicks and Williams 1996]. Therefore, the $k_{dc}$ could be much lower in vivo compared to the
value measured in chapter 4 which could be a potential problem for the modified polymer.

For the flow rate and tube diameter, these parameters only affected the HSANO concentration profile (figure not shown here) within the tube but not on the surface of the wall (which is reasonable because reaction takes place on the wall).

6.5 Conclusions

In this chapter, BSANO transnitrosation with immobilized CySH was studied in a batch system using the polymer (PET-N(i)-CySH). A mathematical model for predicting the NO flux from the modified polymer surface in a tubular flow system was established and various factors which can influence the NO flux were evaluated. The key findings are:

1) Cysteine on the polymer performs better (transferring more NO from BSANO) than cysteine in the solution even without the help of ascorbic acid.

2) The immobilized transnitrosation reaction appeared to be mass transfer limiting, likely due to the cracks on the polymer, as the $k_f$ value was two orders of magnitude smaller than the free solution transnitrosation value. This may be a result of BSANO being a large molecule and cannot easily diffuse into the cracks. However, these cracks do no effect the surface CySNO decomposition since $k_{dc}$ remained almost the same compared to the free transnitrosation studies.

3) Modeling showed that HSANO and CySH have the most significant effect on the NO flux. Increasing either of them will cause a significant NO flux increase. Also the kinetic constant of $k_{dc}$ could also affect the NO flux rate. However, the
flow rate and tube diameter does no effect the NO flux, they only change the bulk HSANO concentration profile.

4) The modeling results indicate that previous incomplete platelet inhibition by a CySH modified polymer (PET-N(i)-CySH) may likely be due to the low HSANO concentration in blood since the reported HSANO level in the plasma has a wide range (about two orders of magnitude). As the HSANO concentration decreases, the NO flux becomes insufficient if the actual HSANO concentration is about half of the 200 nM value. However, it is still possible to make a successful platelet-inhibiting polymer with good chemical and mechanic stability by attaching a compound with multiple thiol groups or introducing branch structures with multiple activation sites on the polymer for attaching thiol molecules. Such action could increase the NO flux significantly.
CHAPTER 7

CONCLUSIONS AND FUTURE WORK

The haemocompatibility of biomaterials has always been a major issue which interferes with the performance of a medical device and causes severe health threat for patients, especially for the long term applications, as a result of platelet adhesion and aggregation on the material surface. Current treatment of this problem includes systematic administration of heparin and material surface modification with functional groups. Both of these two methods have their own limitations or side effects. For example, heparin treatment can cause side effects, such as platelet depletion, which can lead to thrombocytopenia and hemorrhage [Annich et al. 2000]. On the other hand, surface modifications with functional groups always suffer from incomplete platelet inhibition or short term performance [Bamford and Al-Lamee 1992]. Nitric oxide (NO), a small signal messenger, has been found to be a key player for platelet inhibition. NO is naturally secreted by endothelia cells to prevent platelet adhesion to the blood vessel [Feldman 1993; Vaughn et al. 1998]. Thus, in order to mimic this unique property of blood vessels, a L-cysteine (CySH)-modified polymer was previously developed which can utilize endogenous NO to inhibit platelet adhesion through transnitrosation followed by fast S-nitrosocysteine (CySNO) decomposition. However, the experimental results
showed only 60% inhibition, rather than complete inhibition [Duan and Lewis 2002; Gappa-Fahlenkamp and Lewis 2005].

In this work, in order to reveal the reason for the incomplete platelet inhibition, the mechanism of transnitrosation followed by decomposition, as well as the stability of the polymer, was studied in detail. Several alternative immobilization methods were also evaluated and compared to the previous method. Finally, a kinetic model for predicting the NO flux from the polymer surface was established and various environmental effects on the NO flux were evaluated and compared to the previously identified minimum NO flux required for platelet inhibition [Ramamurthi and Lewis 2000].

7.1 Major Accomplishments of the Study

The ultimate goal of this work was to study the transnitrosation kinetics between BSANO in solution and immobilized CySH, as well as the NO release from immobilized CySNO. Following the experimental studies, a mathematical model was developed to predict the NO flux from the CySH-modified polymer under various conditions. By comparing the predicted NO flux with the minimum required NO flux established earlier, the direction for future improvement of the polymer could be established. The significant findings from this work are listed as follows.

- CySNO decomposition (which includes NO release) maximizes near physiological pH regardless of the buffer due to trace metal ions existing in all buffer salts. The buffer purity and chelating agent greatly affects the decomposition rate, especially at near physiological pH. A model involving metal ions and the associated equilibrium between different forms of the metal
ions agreed well with experimental observations. The model provided a plausible explanation for the pH dependence on CySNO decomposition.

- An improved nitrite/S-nitrosothiol detection method based on ammonium sulfamate pre-treatment and KI/I$_2$-based chemiluminescence method was established, which gave less standard error and more data consistency.

- Transnitrosation between BSANO and free CySH was found to be sensitive to various reductants and oxidants. Oxygen and Cu$^{2+}$ both can oxidize CySH, while ascorbic acid can react with Cu$^{2+}$ so that less CySH will be oxidized. The NO transferred from BSANO increases significantly when the ascorbic acid concentration increases. A mathematic model which included transnitrosation between BSANO and CySH, CySNO decomposition and CySH oxidation was developed to model the experimental data. The results showed that the model described the transnitrosation reactions very well. Experimental results showed that ascorbic acid increases the CySNO decomposition rate constant and reduces the CySH oxidation rate constant, which is consistent with the reaction mechanisms. Model sensitivity analysis showed that the transnitrosation rate constants $k_f$ and $k_r$, and the CySNO decomposition rate constant $k_{dc}$, have the most impact on the BSANO concentration time profile. However, the ascorbic acid oxidation rate constant $k_{oa}$ has less influence.

- The previous protocol for making immobilized CySH on PET (PET-N(i)-CySH) lead to an unstable polymer due to imine bond breakage between either CySH and glutaraldehyde or glutaraldehyde and the amine on PET.
Amide bond formation using DCCI or EDC coupling increased the stability of the modified polymer in acidic condition. However, at neutral pH, some of the modified polymer still showed bond breakage and only one of the newly developed polymers (ethylenediamine treatment followed by EDC coupling) showed good bond stability in both acidic and neutral pH. In addition to bond stability issues, cysteine on all the polymers can also be oxidized slowly just as the free cysteine oxidation which followed zero order kinetics in solution. Different immobilization methods appear to have a significant effect on the oxidation rate constant.

- Ethylenediamine treatment can cause substantial amounts of cracks on PET surfaces and the higher concentration of ethylenediamine used, the bigger the cracks. Also, the PET thickness is reduced significantly after ethylenediamine treatment which potentially affects the mechanical properties or the stability of the modified polymer. Immobilized CySH on PET-N(i)-CySH polymer is susceptible to light and air under dry conditions. However, light or air alone does not cause CySH oxidation. This oxidation was found to be represented by a 3\textsuperscript{rd}-order decay reaction with regard to the CySH surface concentration, but a detailed mechanism remains unsolved. It was also discovered that CySH-modified PET can be stored in an amber vial at -20 °C for about 2 months without noticeable oxidation.

- The transnitrosation reaction between the CySH modified polymer (PET-N(i)-CySH) and BSANO showed that cysteine on the polymer performs better
(transferring more NO from BSANO) than cysteine in the solution even without the help of ascorbic acid.

- The transnitrosation reaction could be affected by mass transfer limitations as the $k_f$ value was two orders of magnitude smaller than the free solution transnitrosation value. This may be a result of BSANO being a large molecule and cannot easily diffuse into the cracks. However, these cracks do not affect the surface CySNO decomposition since $k_{dc}$ remained almost the same compared to the free transnitrosation studies.

- Modeling of CySH modified polymer contacting blood in a tubular flow system showed that the S-nitroso-human albumin (HSANO) concentration and CySH surface concentration have the most significant effect on the NO flux prediction. Also the kinetic constant of $k_{dc}$ could also affect the NO flux rate. However, the flow rate and tube diameter primarily only change the bulk HSANO concentration profile. The model also predicts that using the CySH modified polymer made by previous researchers in this group (PET-N(i)-CySH with about 4 nmol/cm2 CySH surface concentration) may not achieve complete platelet inhibition mainly because of the low HSANO concentration in the blood. The HSANO level has been reported to range from 0.6 nM to 200 nM, based on different analysis methods. The NO flux will be insufficient if the actual concentration is about one-half of the 200 nM value. However, this assumption needs to be verified by accurately measuring the HSANO concentration in blood, which is very challenging so far.
7.3 Future Work

In order to improve the performance of the CySH-modified polymer in physiological conditions for long terms, there are still many issues that need to be addressed.

- A modified polymer is needed that has both structural integrity and stable bonds. In order to make a polymer with both good mechanical integrity and bond stability, two approaches can be attempted in the future: a) introduce amine groups on the PET without destroying the polymer and then do the EDC coupling or b) find a water soluble thiol protected CySH (which can later be deprotected) and attach it to carboxylated PET using the EDC method.

- Transnitrosation kinetics between the modified polymer and CySNO decomposition at 37 °C need to be studied to predict the NO flux. The current studies were only at 25 °C and the kinetics of transnitrosation and CySNO decomposition reaction rate constants are likely to be different due to a change in the temperature.

- Glutathione effects on cysteine oxidation need to be studied since glutathione is a much stronger reducing agent compared to ascorbic acid. Glutathione is present in the blood [Svardal et al. 1990; Flagg et al. 1993; Lim et al. 1995] and has been used to reduce various thiol compounds. The transnitrosation under both ascorbic acid and glutathione also needs to be studied so that a more detailed and realistic kinetic model can be established.

- An unsteady state model for the flow system needs to be developed to predict the temporal profile of the NO flux. The model can further be improved by including
a laminar pulse flow pattern that is more consistent with blood flow as compared to the constant flow pattern considered in this work. This change would give a more realistic prediction of the NO flux under physiological conditions.

- Despite the possible low HSANO in vivo, which could bring a real challenge for a successful platelet-inhibiting CySH modified polymer, the performance of the CySH-modified polymer can be improved by either modifying the polymer surface with multiple carboxyl groups such as polymethymethacrylate followed by CySH attachment or by immobilizing compounds with multiple thiol groups on the polymer surface. Increasing CySH can significantly increase the NO flux, therefore compensating for the negative effect from the low HSANO concentration.
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Pages in Study: 200               Candidate for the Degree of Doctor of Philosophy

Major Field: Chemical Engineering

Scope and Method of Study: In order to improve the haemocompatibility of biomaterials so that platelet adhesion on the surface can be minimized, a cysteine (CySH) modified polymer was developed previously and preliminary blood contacting test has shown that the modified polymer can inhibit 60% platelet adhesion. The mechanism of this modified polymer is that the immobilized cysteine on the polymer can seize nitric oxide (NO) from S-nitroso-albumin (AlbSNO) in blood through transnitrosation reaction, and then the unstable S-nitroso-cysteine (CySNO) decomposes and release NO, which is a well known platelet inhibitor. However, the reason for incomplete platelet inhibition is unclear since measuring the NO flux from the modified polymer is difficult. Therefore, a detailed kinetic model is required to predict this NO flux. In this study, the kinetics of the associated reactions is investigated and stability of the modified polymer is evaluated and finally, NO flux is estimated by modeling in a tubular flow system.

Findings and Conclusions: CySNO decomposition is sensitive to various environment conditions such as metal ion contamination level, pH and ascorbic acid concentration. Transnitrosation in free solution revealed that CySH is oxidized by metal ion or oxygen in solution during the reaction while ascorbic acid can prevent the oxidation caused metal ion, however, oxidation due to oxygen still exists. On the other hand, previous technique for immobilizing CySH led to an unstable bond which is prone to hydrolyzation in neutral pH. Increased temperature was found to accelerate this hydrolysis significantly. An improved modification method was developed and a stable bond was formed. Out of the aqueous solution, immobilized CySH can also be oxidized when both light and air exists, while at -20°C, CySH is very stable on the polymer surface for about 2 months. Immobilized transnitrosation showed that CySH on the polymer performs better than CySH in the free solution as it transferred more than three times of its own amount of BSANO before getting totally oxidized even without ascorbic acid. The calculated kinetic rate constants showed that transnitrosation has mass transfer limiting behavior which is likely due to the cracks on the polymer formed during modification while the CySNO decomposition rate constant is not affected by the cracks. The predicted NO flux by a model is sufficient for the platelet inhibition for previous in vitro blood test, however this flux is highly sensitive to AlbSNO concentration which can be much lower than the value used in the model setup.

ADVISER’S APPROVAL:    Randy S. Lewis