2003

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CHEMICAL, BIOCHEMICAL, AND CELLULAR ASPECTS OF S-NITROSO ThiOLS

BY

Shirin Akhter

A Dissertation
Submitted to the Faculty of Graduate Studies and Research
through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements for
the Doctor of Philosophy at the
University of Windsor

Windsor, Ontario, Canada
2003
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Abstract

Nitric oxide is involved in many physiological processes including vascular control, immune responses and neurotransmission under physiological conditions. Many functions of NO are triggered by the formation of its metabolite, $S$-nitrosothiol. $S$-nitrosothiols play an important role in the delivery, storage and transport of NO. In addition, they regulate the activity of a variety of proteins and enzymes.

The first part of my study is focused on studying the regulatory role of $S$-nitrosothiol. In this study, $S$-nitrosothiols including $S$-nitrosoglutathione (GSNO) have been demonstrated to regulate the activity of a key protein in plasma, fibrinogen, without chemically modifying it. GSNO and a few other RSNO derivatives have demonstrated the ability to inhibit thrombin catalyzed fibrinogen polymerization via their effects on fibrinogen, whereas the activity of thrombin itself remained intact upon incubation with GSNO. The percentage of inhibition obtained ranged from 43% to 68%. Upon incubation with GSNO, the $\alpha$-helix content of fibrinogen increased by 15%. The GSNO fibrinogen interaction was allosteric and reversible with an estimated dissociation constant of 3-10 $\mu$M. Fibrinogen has been demonstrated to contain 2 binding sites for GSNO.

The second part of my study deals with the chemical and cellular aspects of $S$-nitrosothiols. I formed a coloured nitrite adduct of sinapinic acid (SA) which has shown the ability to $S$-nitrosate thiol-containing amino acids and proteins. In addition, this nitrite adduct has demonstrated the potential for spectrophotometric detection of NO derived species, NO$^+$ or peroxynitrite in vitro or under physiological conditions. In addition, I synthesized an S-nitroso derivative of 1-octadecane thiol, $S$-nitrosooctadecane (SNOD).
We also designed SNOD-BSA nanoparticles, which were capable of delivering large amounts of SNOD to human fibroblasts. In preliminary studies, the illumination of SNOD-BSA loaded fibroblasts induced apoptosis in 58% of the fibroblasts.

The third part of my study deals with the molecular aspects of S-nitrosothiols. Under physiological conditions, the reaction between NO and O$_2$$^•$ produces peroxynitrite. I have shown previously that upon exposure to light, an air-saturated GSNO solution can also give rise to peroxynitrite. Peroxynitrite causes inactivation of many proteins and enzymes by nitrating their tyrosine residues. We created three different tyrosine mutants of rat calmodulin namely, CaM Y$_{99A}$, CaM Y$_{138A}$, and CaM Y$_{99A}$.Y$_{138A}$ for assessing the roles of its two tyrosine residues. Mutations of the tyrosine residues apparently affected calmodulin’s stability and its Ca$^{2+}$ binding ability. S-nitrosothiols are also capable of causing DNA damage. For the purpose of decontaminating platelet-rich plasma, we synthesized two fluorophore labelled S-nitrosothiols namely N-dansyl-S-nitrosohomocysteine (Dns-HCysNO) and N-dansyl-S-nitroso-glutathione (Dns-GSNO), which could mediate the cleavage of DNA upon exposure to light. Dns-HcysNO solutions degraded R773 plasmid DNA completely upon exposure to light and the extent of degradation was a function of exposure time. In contrast, only a high concentration of Dns-HCysNO degraded plasmid DNA partially without exposure to light. Thus, Dns-HCysNO could be potentially utilized for light-induced DNA cleavage in order to decontaminate platelet rich plasma.
Dedicated to my husband, MD. Tamizur Rahman
And my parents Gulzar Begum & M.A. Jalil
And my two lovely children Sifat Rahman & Ashiqur Rahman
Acknowledgements

First of all, I would like to thank my supervisor for providing me the opportunity to carry out research under his supervision and for his trust and confidence in me throughout my graduate studies. I also wish to thank Dr. Brian Edwards for serving as my external examiner as well as my committee members; Dr. Keith E. Taylor, Dr. Lana Lee, and Dr. Andrew Hubberstey for reviewing my research progress each year. I am grateful to Dr. P. G. Wang, Wayne State University, Detroit, USA for supplying me the NONOates. My gratitude is extended to Dr. J. Kornblatt and Dr. A. English, University of Concordia, Montreal, Canada for performing the isothermal titration calorimetric studies for me. I also want to thank Dr. Thatcher, Queen’s University, Kingston for carrying out one experiment for me.

Special thanks to three really wonderful persons, Paul, Shane, and Arianna for all their help and support in the last few years. I am grateful to Dr. Katz for critical reading of part of my thesis. I would also like to thank Niro, Okey, Jiyun, Arun, Inga, Vivian, Kelly, and Dave for their help and company.

I would also like to thank Dr. S. Ananvoranich for critical reading of part of my thesis and Dr. S. Pandey for his academic advice and support. My special thanks to Fatme, for critical reading of my thesis, all scientific discussions, suggestions, and being there whenever I needed her. Fatme, your continuous help and support will always be remembered. I would also like to thank Jenny, Elita, Bakhos, Monique, Mauro, Jafar, Mollika, Umesh, Acek, and Ju for their technical suggestions and friendship. I would also like to thank all the staffs in the department for their help.

Finally, I would like to thank my husband for giving me inspiration, encouragement and support throughout the course of my studies.
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**List of Abbreviations**

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<tbody>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSA-NO</td>
<td>S-nitroso bovine serum albumin</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CaM Y\textsubscript{99A}</td>
<td>Tyrosine at position 99 in the amino acid sequence of Calmodulin substituted with alanine</td>
</tr>
<tr>
<td>CaM Y\textsubscript{138A}</td>
<td>Tyrosine at position 138 in the amino acid sequence of Calmodulin substituted with alanine</td>
</tr>
<tr>
<td>CaM Y\textsubscript{99A}Y\textsubscript{138A}</td>
<td>Tyrosine at positions 99 and 138 in the amino acid sequence of Calmodulin substituted with alanine</td>
</tr>
<tr>
<td>Captopril-SNO</td>
<td>S-nitrosocaptopril</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>Chromozym TH</td>
<td>tosylglycylprolylarginine-4-nitranilide acetate</td>
</tr>
<tr>
<td>DHR</td>
<td>Dihydrorhodamine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s minimal essential medium</td>
</tr>
<tr>
<td>DMF</td>
<td>N, N-dimethyl formamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dns-Cl</td>
<td>Dansyl Chloride</td>
</tr>
<tr>
<td>Dns-GSNO</td>
<td>N-dansyl-S-nitrosoglutathione</td>
</tr>
<tr>
<td>Dns-HCysNO</td>
<td>N-dansyl-S-nitrosohomocysteine</td>
</tr>
<tr>
<td>Dns-Octadecane</td>
<td>S-dansyloctadecane</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobis-2-nitrobenzoate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethyleneglycol bis (2-aminoethylether) N,N,N, N tetraacetic acetic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescin isothiocyanate</td>
</tr>
<tr>
<td>Fpg</td>
<td>Formamidopyrimidine-DNA glycosylase</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein Liquid Chromatography</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSNO</td>
<td>S-nitrosoglutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidized glutathione</td>
</tr>
<tr>
<td>HCA</td>
<td>Hydroxycinnamic acid</td>
</tr>
<tr>
<td>HO’</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>H₄B</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>HcysNO</td>
<td>S-nitrosohomocysteine</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropylthiogalactoside</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>LRSC</td>
<td>Lissamine rhodamine B sulfonyl chloride</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>NHF</td>
<td>Normal human fibroblast</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>'NO₂</td>
<td>Nitrogen dioxide radical</td>
</tr>
<tr>
<td>NO’</td>
<td>Nitric oxide radical</td>
</tr>
<tr>
<td>NOH arginine</td>
<td>N-hydroxy arginine</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>O-nitroso SA</td>
<td>O-nitrososinapinic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
</tbody>
</table>
PEG  Polyethylene Glycol
PMSF  Phenylmethylsulfonyl fluoride
PTIO  2-phenyl-4,4,5,5-tetramethylimidazo-1-oxyl-3-oxide
Pyrrolidium NONOate  Pyrrolidinyldiazenium dilolate
rCaM  rat Calmodulin
RNA  Ribonucleic acid
RH  Rhodamine
RITC  Rhodamine B-Isothiocyanate
RSNO  S-nitrosothiols
RSSR  Oxidized thiols
RS'  thyl radical
SA  Sinapinic acid
SAM  S-adenosylmethionine
SDS  Sodium dodecyl sulfate
SDS-PAGE  Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIN-1  3-morpholinosydnonimine N-ethyl-carbamide
SMCC  Succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate
SNAP  S-nitroso-N-acetyl penicillamine
SNOAC  S-nitroso-N-acetylcysteine
SNOD  S-nitrosooctadecane
TEMED  N, N, N', N'-tetramethylene diamine
Trp  tryptophan
Tyr  tyrosine
UV  Ultraviolet
Vis  Visible
wt-CaM  Wild type calmodulin
PART I

Regulation of fibrinogen polymerization

by S-nitrosothiols
1.0 Abstract

*S-Nitrosoglutathione (GSNO, 50 μM) inhibited the initial rate of thrombin-catalyzed human and bovine fibrinogen polymerization by ~50 % to 68 % respectively. Inhibition was also observed with other structurally varied RSNOs including sugar derivatives of *S-nitroso-N-acetylpenicillamine (SNAP). The fact that the same concentration of GSNO had no effect on thrombin-dependent hydrolysis of tosylglycylprolylarginine-4-nitroanilide acetate suggested that this inhibition was due to GSNO-induced changes in fibrinogen structure. This was confirmed by circular dichroism (CD) spectroscopy where GSNO increased the α-helical content of fibrinogen by 15 %. On the other hand, *S-carboxymethylamido derivatives of glutathione had no effect on the secondary structure of fibrinogen. The GSNO-dependent secondary structural effects were reversed upon gel filtration chromatography suggesting that these effects were allosteric.

Further evidence for fibrinogen-GSNO interactions was obtained from GSNO dependent quenching of the intrinsic fibrinogen Trp fluorescence and the reduction of the GSNO circular dichroic absorbance in the presence of fibrinogen solution. The *K_Ds of 3 to 10 μM for fibrinogen-GSNO interactions with a stoichiometry of 2:1 (GSNO: fibrinogen) were estimated from isothermal titration calorimetry and fluorescence quenching, respectively.

These results suggest that *S-nitrosothiols induce changes to fibrinogen structure by interacting at specific aromatic rich domains. Three such putative RSNO-binding domains have been identified in the unordered, aromatic residue-rich, C-termini of the α-chains of fibrinogen.
1.1 Introduction

1.1.1 Nitric oxide

Nitric oxide (NO) is a colorless free radical gas that is sparingly soluble in water (2 mM at room temperature and atmospheric pressure). The paramagnetic NO contains an odd number of electrons that renders it highly reactive. The major breakdown product of NO in aqueous solutions is nitrite. However, the kinetics of NO autooxidation in aqueous solutions is dependent on the concentration of NO (Ford et al., 1993). NO is lipophilic and possesses 6- to 8-fold higher solubility in non-polar solvents (Shaw and Vosper, 1977) and lipid membranes compared to water. NO does not react with sulfhydryl directly; however, a potent nitrosating species is formed upon reaction of NO with oxygen (Wink et al., 1994). NO$_x$ reacts about $10^5$ fold faster with GSH than with H$_2$O suggesting that intracellular GSH may act as an efficient scavenger for reactive nitric oxide species and play an important role in the detoxification of NO under aerobic conditions (Mayer et al., 1995a).

1.1.1.1 Biosynthesis of NO

Biosynthesis of nitric oxide is catalyzed by nitric oxide synthase (NOS). It involves a two-step oxidation of L-arginine to L-citrulline, along with the production of NO. The reaction consumes 1.5 mol of NADPH, and 2 mol of oxygen for the formation of each L-citrulline (Griffith and Stuehr, 1995). In the first step, hydroxylation of L-arginine leads to the formation of N-hydroxy-L-arginine, which in turn is oxidized by NADPH generating L-citrulline and nitric oxide (Abu-Soud et al., 1997, Figure 1).
1.1.1.2 *Nitric oxide synthase (NOS)*

NO synthesis is catalyzed by nitric oxide synthase (NOS) *in vivo*. Three NOS isoforms have evolved to function in animals; all of which utilize NADPH and O$_2$ as cosubstrates for the synthesis of NO (Nathan and Xie, 1994). Two of the three isoforms are constitutively expressed and they synthesize NO in response to increased Ca$^{2+}$ or in some cases are elicited by shear stress (Fleming *et al.*, 1998). These constitutive enzymes are designated neuronal NOS (nNOS) and endothelial NOS (eNOS) named after the cell types in which they were first isolated. They were also located in other cell types later.
1.1.1.2.1 Structure of NOS: Each NOS polypeptide is comprised of an amino terminal oxygenase domain and a carboxyl terminal reductase domain. A recognition sequence containing 30 amino acid residues for the Ca\(^{2+}\)-binding protein calmodulin (CaM) is located between the two domains (Masters et al., 1996; Hemmens and Mayer, 1997).

The oxygenase domain in NOS has binding sites for its substrate L-Arginine, heme and tetrahydropterin (Figure 2). The amino terminal region that is located upstream from the oxygenase domain varies in length among the NOS isoforms. This domain is involved in cellular targeting and may sometimes affect NOS structure or catalysis. The reductase domain contains a binding site for flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and NADPH (Figure 2), in close homology with cytochrome P-450 reductase. The flavins in the reductase domain facilitate the transfer of electrons from NADPH to the heme iron during NO synthesis, thus allowing it to bind and activate O\(_2\), and eventually catalyze NO synthesis (Masters et al., 1996; Hemmens and Mayer, 1997; Stuehr, 1997). CaM binding enhances the electron transfer in the reductase domain and allows heme reduction and NO synthase activity (Matsuoka et al., 1994; Abu-Soud et al., 1994). Inducible NOS (iNOS) is usually expressed at high levels only following induction by cytokines or other inflammatory mediators. The activity of iNOS does not depend on the change on Ca\(^{2+}\) concentration (Abu-Soud et al., 1993).

All the NOS isoforms are only active in their homodimeric form (Hierholzer et al., 1998; Kroncke et al., 1996). In dimeric form, two NOS subunits align in a head to head manner, with the reductase domains attached as independent extensions (Figure 2).
Figure 1.1.2: Structural organization of Nitric Oxide Synthase (Stuehr, 1997)

Newton et al. (1998) demonstrated that calmodulin binding was not required for electron transfer within the reductase domain of human iNOS (Newton et al., 1998). Later, it was demonstrated that CaM binding does not alter the reduction potential of the flavins instead, a large structural rearrangement induced by CaM controls the electron transfer in the reductase domain of nNOS (Daff et al., 2001). Recently, Rozhkova et al. (2002) have shown that the most prominent role of CaM binding to NOS is the reorientation of the two domains. With the use of a reconstituted system, they demonstrated that Ca\textsuperscript{2+}/CaM binding to the oxygenase or reductase domain decreased the
rate of NO synthesis instead of increasing it. On the other hand, the rate of NADPH oxidation and cytochrome c reduction rates were increased (Rozhkova et al., 2002).

1.1.1.2.2 Regulation of NOS: NOS activity is regulated by a large number of post-translational mechanisms. One of the most prominent modes of regulation is through the complexation with NO. Since all NOS's are heme proteins, their heme iron can readily bind NO irrespective of their oxidation state (Hurshman and Marletta, 1995; Abu-Soud et al., 1995). NOS-ferric or ferrous-NO complexes form within seconds of the initiation of NO synthesis. NO synthesis can only be resumed following the dissociation of NO from the heme in the enzyme. The dissociation rates are maintained at sufficiently low level to force the NOS to operate only at a fraction of their maximum activity during the steady state (Hurshman and Marletta, 1995; Abu-Soud et al., 1995). Formation of the iNOS-NO complex is dependent on the level of NO in solution. In contrast, nNOS-NO complex formation appears to be independent of the concentration of NO in solution. The formation and breakdown of this complex can occur without losing the activity of the enzyme in the presence of sufficient L-arginine and tetrahydropterin (H₄B).

NO can also slowly inactivate NOS, if its synthesis occurs in the presence of sub-saturating H₄B (Kerwin et al., 1995; Griffith and Stuehr, 1995). Even though the mechanism of this inhibition has not been investigated directly, a few possible pathways were suggested. A few possible factors, that might play a role in this type of regulation, are as follows: uncoupling of O₂ reduction (Klatt et al., 1994; Mayer et al., 1997), dimer dissociation into monomers (Stuehr, 1997), or NO binding to the heme and subsequent breakage of the cysteine thiolate-heme iron bond. This mode of NOS inactivation could
be important if cellular H₄B level is limiting. H₄B is a crucial cofactor for NOS due to its involvement in coupling iron reduction to NO synthesis. H₄B binding has been found to be anticooperative, binding of the first H₄B lowers the affinity for binding of the second H₄B by at least an order of magnitude.

eNOS has been reported to be myristoylated, palmitoylated, farnesylated, acetylated, and phosphorylated all within its oxygenase domain (Hemmens and Mayer, 1997). These modifications assist in localizing the enzyme to cytosol, membrane components, or organelles. These modifications do not alter the activity of the enzyme except for phosphorylation.

1.1.1.3 Physiological functions of NO

Most of the physiological actions of NO are brought about by its activation of the soluble guanylate cyclase. Binding of NO to the heme moiety of this enzyme induces a conformational change that increases the activity of the enzyme by ~ 400-fold leading to the formation of the intracellular second messenger, cyclic guanosine monophosphate (cGMP) (Ignarro, 1991). cGMP acts in turn as a second messenger, activating cGMP dependent protein kinase, which decreases cytosolic Ca²⁺ concentration and modulates ion channel function leading to relaxation of vascular smooth muscle cells (Lincoln and Cornwell, 1993, Figure 3).


**Figure: 1.1.3:** The mechanism of action of NO and NO 'donor' drugs on vascular smooth muscle (Butler et al., 1995)

In other cell types, the accumulation of cGMP leads to other physiological effects. In platelets, cGMP is formed by soluble guanylate cyclase following stimulation by NO which is derived either from the vascular endothelium or by endothelial-type NOS within the platelets themselves. The produced cGMP results in the inhibition of platelet aggregation (Radomski et al., 1987).
In addition to regulating the vascular tone, endothelium-derived NO also inhibits platelet activity, vascular smooth muscle cell growth, and adhesion of inflammatory cells to the endothelial surface (Moncada and Higgs, 1993). NO produced in macrophages (Beckman et al., 1990; Drapier, 1991) as well as in neutrophils (Salvemini et al., 1989) through inflammatory and immune reactions is involved in phagocytic and non-phagocytic removal of foreign or damaged cells. NO produced in large concentration through these reactions is cytotoxic.

1.1.2 NO donor compounds

1.1.2.1 S-nitrosothiols

S-nitrosothiols are compounds with the generic structure R-SNO. Older chemical literature refers to these compounds as thionitrites. S-nitrosothiols are endogenous metabolites of nitric oxide that have been detected in extra- and intracellular spaces. They are formed by the reaction of small molecular weight and protein thiols either with the nitrosonium ion or by NO under aerobic conditions (Wink et al., 1997). It has been suggested that the formation and the decay of low molecular weight S-nitrosothiols, such as S-nitrosoglutathione and S-nitrosohomocysteine, may represent a mechanism for the storage or transport of NO (Myers et al., 1990; Girard and Potier, 1993).

RSNO’s are not only synthesized and administered clinically, but are also produced endogenously. Stamler et al. (1992a) reported that the human plasma contains 7 μM RSNO, in contrast to the free concentration of NO, which is in the low nanomolar range (Malinski and Taha, 1992).
Stubauer et al. (1999) proposed a novel mechanism of S-nitrosothiol formation and degradation in the biological system using bovine serum albumin as a model protein. They suggested that the generation and the breakdown of S-nitrosothiols could be mediated by Cu\textsuperscript{2+} ions present \textit{in vivo}. The sulfhydryl group of Cys-34 of BSA binds Cu\textsuperscript{2+} forming a copper thiol complex, which reacts with NO to yield S-nitrosobovine serum albumin (BSA-NO). Both BSA and Cu\textsuperscript{2+} are required for the reaction with NO according to this suggested mechanism as opposed to the mechanism proposed earlier where they implicated the direct involvement of NO\textsuperscript{+} in the reaction (Kharitonov et al., 1995; Vanin et al., 1997; Boese et al., 1995).

Albumin is the most abundant, thiol-containing serum proteins. It was suggested that albumin could serve as a reservoir for low molecular weight S-nitrosothiols by forming S-nitrosoalbumin through transnitrosation reaction from low molecular weight thiols in plasma. This S-nitrosoalbumin can then deliver the NO to another thiol by transnitrosation (Heuil et al., 2000).

Nedospasov et al. (2000) later demonstrated that hydrophobic interiors of proteins could catalyze the formation of S-nitrosothiols. Hydrophobic pockets of protein interior concentrate hydrophobic O\textsubscript{2} and NO thereby promoting N\textsubscript{2}O\textsubscript{3} formation and preventing the breakdown of newly formed N\textsubscript{2}O\textsubscript{3} by sequestering it from the aqueous environment. N\textsubscript{2}O\textsubscript{3} formed in the hydrophobic interior of the protein can S-nitrosate the thiol group in the protein itself, or it can transnitrosate other low molecular weight thiols in the aqueous phase \textit{via} a series of NO\textsuperscript{+} transfer reactions (Nedospasov et al., 2000, Figure 4).
Recently, it has been demonstrated that these protein hydrophobic pockets rapidly absorb NO and slowly release it. The half-life of NO in those pockets has been reported to be 7 min. These hydrophobic pockets have been suggested to potentially control the vascular tone in human plasma. According to their proposed model, these hydrophobic pockets can absorb NO from the aqueous phase thereby causing vasoconstriction and can deliver the NO upon demand via NO$^+$ transfer to low molecular weight thiols thus causing vasodilation (Rafikova et al., 2002).

**Figure 1.1.4:** RSNO formation in protein hydrophobic pockets (Nedospasov et al., 2000)
1.1.2.2 NONOates

Although adducts of NO with nucleophiles have long been known in the chemical literature (Drago, 1962), their biological properties have not been explored until recently. NONOates can be prepared by exposing nucleophilic compounds (X') dissolved in ether or other solvents to a few atmospheres of NO gas, as shown in equation below:

\[ X' + 2\text{NO} \rightarrow X^-[\text{N(O)NO}]^- \quad (1) \]

These products are generally stable as solids, but decompose in solution at a rate that appears to depend on pH, temperature, and the identity of the carrier nucleophile. The decomposition of a NONOate generally regenerates NO in solution in a reaction, which can yield up to 2 mol NO per mole of NONOate decomposed.

\[ X^-[\text{N(O)NO}]^- \rightarrow X' + 2\text{NO} \quad (2) \]

The structures of five representative NONOates are shown in Figure 5. All NONOates contain the anionic [N(O)NO]^- functional group which is the characteristic of this class of compound. The nucleophiles that are produced when these compounds generate NO according to Equation 2 are primary amine (isopropylamine, IPA) from IPA/NO; a secondary amine (diethylamine) from DEA/NO; the biologically important polyamine, spermine, from SPER/NO; and water from the oxide adduct, OXI/NO. The SULFI/NO produced on reaction of NO with sulfite ion disproportionates to sulfate and nitrous oxide when dissolved in water rather than reverting to reactants.
Figure 1.1.5: Structures of five representative NONOates
As such, SULFI/NO can serve as a useful control for biological work with the NONOates, since it contains the same $[\text{N(O)NO}]^-$ functional group as the other compounds but produces negligible NO (Morley and Keefer, 1993).

These compounds have potent *in vitro* vasodilatory potential, and they act by activation of soluble guanylate cyclase. It has been demonstrated that DEA/NO and SPER/NO have similar vasodilatory potency without acute toxic effects in an *in vivo* animal model (Diodati *et al.*, 1993a). DEA/NO has also been shown to have potent antiplatelet activity both *in vitro* and *in vivo* (Diodati *et al.*, 1993b).

1.1.2.3 *Sugar-S-nitroso-N-acetylpenicillamines (Sugar-SNAPs)*

Due to the growing potential use of RSNO, recent interest is focused on designing and synthesizing new RSNOs with optimized pharmacokinetic properties. A series of sugar-$S$-nitrosothiols with NO releasing properties has been developed by a number of researchers in the last few years (Hou *et al.*, 1999; Ramirez *et al.*, 1996). These sugar-SNAP compounds are composed of an aglycone unit conjugated with mono or oligosaccharides. Aglycone moiety is responsible for providing the pharmacological activity, while the carbohydrate unit is responsible for the enhancement of water solubility, cell penetration, and drug receptor interaction (Hou *et al.*, 1999; Ramirez *et al.*, 1996).
1.1.3 Mechanism of action of S-nitrosothiols

RSNO decomposition can occur through both homolytic and heterolytic cleavage of S-N bond leading to the release of NO and NO$^+$ or NO$^-$ respectively (Arnelle and Stamler, 1995).

Homolytic decomposition:

$$\begin{align*}
\text{RS} - \underset{N=O}{\rightarrow} & \rightarrow \text{RS}^* + \text{NO}^* \\
& (1)
\end{align*}$$

Heterolytic decomposition:

$$\begin{align*}
\text{RS} - \underset{N=O}{\rightarrow} & \rightarrow \text{RS}^+ + \text{NO}^- \\
& (2)
\end{align*}$$

$$\begin{align*}
\text{RS} - \underset{N=O}{\rightarrow} & \rightarrow \text{RS}^- + \text{NO}^+ \\
& (3)
\end{align*}$$

**Figure 1.1.6**: Homolytic and heterolytic decomposition of RSNO (Wang et al., 2000).

Most biological effects of RSNO are elicited by the release of NO through homolytic cleavage (Bulter and Williams, 1993). The biological reactions of RSNO have focused on three main reactions: nitric oxide release, transnitrosation and S-thiolation.

1.1.3.1 *Nitric oxide release*

RSNO are susceptible to decomposition by a number of mechanisms that ultimately release NO. The prominent pathways of NO release are as follows:
1.1.3.1.1 *Metal ion catalyzed release of NO*: The presence of metal ions such as copper (Williams, 1996) and iron (Vanin et al., 1997) and reducing agents promote the release of NO from RSNOs. Since the human body contains a fairly large amount of copper (0.1 g copper per 75 kg body weight), copper-catalyzed RSNO decomposition has drawn a lot of attention. Cu$^{2+}$ can directly and catalytically decompose RSNO (Askew et al., 1995). The real reaction catalyst is Cu$^{+}$, which is formed via reduction of Cu$^{2+}$ by thiolate ion generated by hydrolysis of RSNO or free thiol. The produced Cu$^{+}$ can catalyze RSNO decomposition through the formation of a complex intermediate Y, proposed as structure (1) and (2) as shown in Figure 7.

\[
\begin{align*}
\text{RSNO} + H_2O & \quad \overset{\text{\text{RSNO + H2O}}}{{\longrightarrow}} \quad \text{RS}^- + \text{NO}_2^- + 2H^+ \\
\text{Cu}^{2+} + \text{RS}^- & \quad \overset{\text{\text{Cu}}^{2+} + \text{RS}^-}{{\longrightarrow}} \quad \text{Cu}^{+} + \text{RS}^* \\
\text{Cu}^{+} + \text{RSNO} & \quad \overset{\text{\text{Cu}^{+} + RSNO}}{{\longrightarrow}} \quad \text{Y} \quad \overset{\text{\text{Y}}}{{\longrightarrow}} \quad \text{Cu}^{2+} + \text{RS}^- + \text{NO}' \\
\text{2RS}^- & \quad \overset{\text{\text{2RS}^-}}{{\longrightarrow}} \quad \text{RSSR}
\end{align*}
\]

\[\text{Figure 1.1.7: Copper catalyzed decomposition of RSNO}\]
Both Cu$^{2+}$ and RS$^-$ are regenerated and required only in catalytic amounts. However, substoichiometric copper ions are unable to completely decay GSNO, since the newly formed GSSG is an efficient Cu$^{2+}$ chelator that will inhibit further decay of GSNO (Singh et al., 1999). Consequently, a catalytic amount of Cu$^{2+}$ will be rapidly chelated out by GSSG.

1.1.3.1.2 Photo-induced release of NO: RSNOs are photosensitive, especially to UV light. The irradiation of GSNO at absorption band either at 340 nm or at 545 nm results in the release of NO and thiyi radical (Sexton et al., 1994). The newly formed thiyi radicals will react directly with GSNO in the absence of oxygen to form disulfide GSSG and NO. However, in the presence of oxygen, GSOO$^*$ radical is first generated, which in turn reacts with another GSNO to form GSSG and NO. Thus, NO can be produced via GSNO homolysis as well as from the reactions of GSNO with GS$^*$ and GSOO$^*$ as shown in Figure 8.

\[
\begin{align*}
RSNO & \longrightarrow RS^- + NO^-
\\
RS^- + RSNO & \longrightarrow RSSR + NO^-
\\
RS^- + O_2 & \longrightarrow RSOO^*
\\
RSOO^* + RSNO & \longrightarrow RSSR + NO^- + O_2
\end{align*}
\]

Figure 1.1.8: Photoinduced release of NO (Sexton et al., 1994)
1.1.3.1.3 **Enzymatic release of NO**: Askew *et al.* (1995) demonstrated that γ-glutamyl transpeptidase can cause decomposition of GSNO. Following γ-glutamyl cleavage, an S-nitrosothiol of greatly reduced stability as compared to GSNO is formed, Figure 9, that readily releases NO in the presence of Cu\(^{+}\) ion.

![Chemical Reaction Diagram](image)

**Figure 1.1.9**: Mechanism of γ-GT-dependent GSNO decomposition (Hogg *et al.*, 1997)
In the presence of purine or pterin substrate, xanthine oxidase, a superoxide
generator, has been demonstrated to induce the decomposition of S-nitrosothiol and S-
nitrosocysteine under aerobic conditions. This decomposition has been shown to be
mediated by superoxide (Trujillo et al., 1998)

1.1.3.2 Transnitrosation

This process involves the transfer of NO from S-nitrosothiols to other thiols. If the
transition occurs from relatively stable S-nitrosothiols, such as GSNO or SNAP, to a thiol
such as cysteine, which is a relatively abundant thiol in vivo (Butler et al., 1998), a
relatively unstable S-nitrosothiol, such as S-nitrosocysteine, may be formed. This newly
formed S-nitrosothiol can decompose in the presence of Cu⁺ to release NO (Butler et al.,
1998).

1.1.3.3 S-thiolation

Even though S-thiolation reaction of RSNO was known for a long time (Park,
1988) only recently, has it drawn a lot of attention. This reaction involves the
nucleophilic attack on the sulfur of RSNO by a thiolate anion resulting in a disulfide and
nitroxyanion as products. Mixed disulfide has been suggested to be synthesized
according to this mechanism upon incubation of a thiol with an S-nitrosothiol (Oae et al.,
1977). It has been reported recently that the protein component of cells undergoes an
extensive S-thiolation upon exposure to thiols-S-nitrosothiol mixtures. In addition,
glyceraldehyde-3-phosphate dehydrogenase has also been shown to undergo S-thiolation
upon incubation with GSNO (Mohr et al., 1999). Konorev et al. (2000) demonstrated that
the degree of $S$-thiolation and $S$-nitrosation depends not only on the protein but also on the chemical nature of the $S$-nitrosothiol (Konorev et al., 2000, Figure 10). Creatine kinase has been demonstrated to be predominantly $S$-thiolated by GSNO, but $S$-nitrosated by SNAP. On the other hand, bovine serum albumin became $S$-nitrosated by both GSNO and SNAP. It has been suggested that these differences are likely due to both steric

\[ \text{Figure 1.1.10: } S\text{-nitrosation and } S\text{-thiolation of protein thiols by } S\text{-nitrosothiols (Konorev et al., 2000)} \]
considerations and pKa of the protein thiol groups involved. In general, S-thiolation is a more stable modification than S-nitrosation (Konorev et al., 2000).

1.1.4 Biological functions of S-nitrosothiols

The biological activities of S-nitrosothiols were realized before the landmark discovery that nitric oxide was an endogenously generated molecule (Ignarro et al., 1981). Several studies suggested that S-nitrosothiols formed from the NO-mediated S-nitrosation of thiol-containing peptides and proteins can serve as intermediates in the metabolism of NO in the circulation (Stamler et al., 1992b; Lander, 1997). Nitrosation of protein thiols has been implicated in the NO-dependent regulation of many enzymes, including protein kinase C (Gopalakrishna et al., 1993) and glyceraldehyde-3-phosphate dehydrogenase (Clancy et al., 1994).

It has been reported that normal human serum contains S-nitrosoalbumin (Stamler et al., 1992a). S-nitrosoalbumin has been proposed to act as an endogenous regulator of vascular tone later (Scharfstein et al., 1994). S-nitrosothiols are potent antiplatelet agents and vasodilators. Both of these functions are usually attributed to nitric oxide release. Pawloski et al. (1998) have shown that S-nitrosohemoglobin inhibits platelet aggregation, and the mechanism of inhibition is independent of cGMP (Pawloski et al., 1998). S-nitrosation of Factor XIII, a transglutaminase involved in the crosslinking of fibrin monomers during blood coagulation, has been implicated in the inhibition of blood clot formation (Catani et al., 1998). Moreover, Simon et al. (1993) showed that S-nitrosoproteins are potent antiplatelet agents, and the mechanism of their inhibition is
through the increase in cyclic-GMP, like NO and other related S-nitroso compounds (Simon et al., 1993).

1.1.5 Fibrinogen

Fibrinogen is a large (340 kDa) dimeric glycoprotein found in the blood plasma of all vertebrate animals (Hall and Slayter, 1961, Figure 11). Its most prominent function is to form fibrin clots after thrombin-mediated activation, preventing the loss of blood upon vascular injury or causing thrombosis in pathological state. Moreover, fibrinogen also plays an important role in cell adhesion, migration and proliferation during wound healing, inflammation, angiogenesis and tumorogenesis. It comprises 2-3% of plasma protein (Mosesson et al., 2001).

Figure 1.1.11: Crystal structure of modified bovine fibrinogen taken from Protein Data Bank (Structure code 1DEQ)
1.1.5.1 Amino acid sequence composition of human fibrinogen

Fibrinogen is comprised of two sets of three polypeptide chains termed Aα, Bβ and γ. Aα chain contains 610 amino acid residues with a molecular weight of 66 kDa, Bβ contains 461 amino acid residues and γ chain contains 411 amino acid residues with a molecular weight of 52 and 46 kDa respectively. The α-chain is the largest polypeptide chain of the three non-identical chains that constitute the human fibrinogen molecule. It does not contain covalently bound carbohydrates unlike β and γ chains (Gaffney, 1972; Pizzo et al., 1972).

The amino acid sequence of human fibrinogen α chain has revealed a structure that can be divided into three zones of unique amino acid composition (Doolittle et al., 1979). These regions are designated ZN, ZM and ZC and correspond roughly to the amino terminal third (ZN), the middle third (ZM) and the carboxyl terminal third (ZC) respectively. In zone ZN, the first 194 residues from the amino terminus contain a very long proline-free stretch, which is also deficient in glycine residues. This part of the α chain is linked to the β and γ chains by two sets of unique six-cysteine combinations that are called disulfide rings. There are three 111-residue segments, which are surrounded by these disulphide rings at each end. The three chains exhibit a large amount of homologous structure consistent with a set of coiled-coils in which the non-polar residues are pointing inward and the polar side chains extend out into the aqueous solvent (Doolittle et al., 1978).

The molecular weight of Aα chains vary among mammalian species while those of Bβ and γ chains remain fairly constant (Doolittle, 1973). The γ polypeptide chain of normal human fibrinogen has been shown to be heterogeneous with respect to charge
(Galanakis et al., 1978; Mosesson et al., 1972), molecular weight (Henschen and Edman, 1972) and sialic acid content (Gati and Straub, 1978). The least anionic, lowest molecular weight γ chain, denoted as the γ or γA chain, accounts for about 92% of the chains in human plasma (Mosesson et al., 1972). The highest molecular weight chain, denoted as γ or γB chain, comprises most of the remaining plasma γ chains. This form differs from the low molecular weight form in that the C terminal tetrapeptides in the γA chains are exchanged for 20- and 12- residue anionic oligopeptides in human fibrinogen (Chung and Davie, 1984; Wolfenstein-Todel and Mosesson, 1981).

Henschen and Lottspeich (1977) demonstrated 31% amino acid homology between Bβ and γ chains of fibrinogen. The middle and carboxyl terminal parts of the chains show higher homology than that of amino terminal parts. Cysteine, tryptophan and glycine were the most conserved residues; nine out of ten cysteines in the γ-chain could be perfectly aligned with cysteines in the β chain. Therefore, it was hypothesized that the peptide chains of fibrinogen, at least the γ-and Bβ chain, have evolved from a common ancestor (Henschen and Lottspeich, 1977).

1.1.5.2 Carbohydrate

Fibrinogen contains 5% carbohydrate. There are four carbohydrate clusters, each of which has a molecular weight of 2.5 kDa in fibrinogen (Marder et al., 1982). Two are located in each lateral domain in the β chain and two on each arm on the γ chain, near the central domain (Marder et al., 1982). The oligosaccharide chains are linked to each molecule of fibrinogen via N-glycosyl bonds at N-Bβ364 and N-γ52. Surprisingly, the Aα chains lack carbohydrate side chains. (Pizzo et al., 1972; Gaffney, 1972).
1.1.5.3 Domain organization of Fibrinogen

The chemical structure of fibrinogen has been established in numerous biochemical studies. Fibrinogen consists of two identical subunits, each of which is formed by three non-identical polypeptide chains, Aα, Bβ and γ (Doolittle, 1984; Henschen and McDonagh, 1986). The three polypeptide chains share many structural features which suggest a common ancestry. Both of the subunits and polypeptide chains in fibrinogen are covalently linked together by disulfide bonds, and assemble to form at least 20 distinct domains (Privalov and Medved, 1982; Medved et al., 1997). These domains are again grouped into four major regions: the central E region, 2 identical terminal D regions, and the α-C domains. The amino terminal portions of all six chains together constitute the central E region. This globular domain is also called a disulfide knot because this domain contains almost half of fibrinogen’s disulfide bonds. The carboxyl terminal portions of the Bβ and γ chains and a portion of the Aα chains form the distal D regions. This D-E-D arrangement corresponds to the three major nodules revealed by electron microscopy of intact fibrinogen (Hall and Slayter, 1961). Some fibrinogen molecules were reported to have a fourth domain (Weisel et al., 1985) that was suggested to correspond to two interacting α C domains, each consisting of the carboxyl terminal two thirds of the Aα chain. This nodule appeared to interact with the middle nodule and seemed to be attached by a thin filamentous structure with the D domain.

The αC domains of fibrinogen are easily cleaved by plasmin and other proteases. Due to their high susceptibility to proteolysis, it was suggested that they are unordered in
fibrinogen thus forming “free swimming appendages” (Henschen and McDonagh, 1986; Doolittle, 1984).

1.1.5.4 Biosynthesis of fibrinogen

Fibrinogen is synthesized in hepatic parenchymal cells (Miller et al., 1954; Straub, 1963). The plasma level of fibrinogen is very sensitive to physiological changes in vivo. They become elevated in pregnancy (Regoecezi and Hobbs, 1969), and during the body’s response to injury and stress (Koj, 1974).

1.1.5.5 Crystal structure of fibrinogen

Single molecules of fibrinogen have been visualized in electron micrographic studies only at very low resolution. A 30 kDa recombinant protein corresponding to the carboxyl terminal domain of the γ-chain of human fibrinogen was the first crystal structure ever reported (Yee et al., 1997).

Brown et al. (2000) have recently determined the crystal structure of proteolysed bovine fibrinogen to 4-Å resolution (Brown et al., 2000) using a lysine-specific protease from P. aeruginosa (Elliott and Cohen, 1986). Even though the carboxyl terminal third of the Aα chain and the first 60 residues of the Bβ –chain have been removed by proteolysis, the resulting molecule fully retains its ability to clot (Weisel et al., 1978). It has been reported that the crystals of modified bovine fibrinogen consist of end-to-end bonded molecules that form flexible filaments. The axis of the α-helical coiled-coil rod adopts a sigma shape. Fibrinogen’s overall architecture is crucially dictated by these coiled coil segments that constitute 75% of the total length of the 450 Å. The dimeric
fibrinogen molecule has an approximate two-fold axis oriented nearly perpendicular to the plane containing most of the coiled-coil axis. Near the middle of the molecule, the two Aα chain α helices of the two half molecules at the N-termini of the two coiled coils, are positioned on the same side of the coiled coil. The two Bβ chains and α-helices on the other hand, point out in opposite directions. Near the middle of the coiled-coil region the plasmin-sensitive segment is located within a hinge region that assumes a variable conformation. Owing to the flexible nature of the coiled-coil region, relatively variable relationships between the half molecules in the distal carboxyl terminal regions are obtained. The two β domains are positioned approximately on the opposite sites of the coiled-coil axis whereas the GHR binding pockets on these domains are almost facing each other in the intact molecule. The coiled coil stretch into the two γ-domains is located at the ends of the molecule (Spraggon et al., 1997).

The conformation of the coiled coil fragment between the E and D regions is the most variable segment of the coiled-coil backbone in the crystals. Crystals of fibrinogen have shown to have a conserved linkage between the ends of the 450 Å-long molecules that form filaments in the previous low-resolution studies of various microcrystals and crystals of fibrinogen (Weisel et al., 1981; Cohen et al., 1983). Recently, crystallography studies on modified fibrinogen have demonstrated that the specific end-to-end bonding arrangement between γ domains of the symmetry related molecules are similar to that observed in dimer D derived from the human fibrin clot earlier (Spraggon et al., 1997).

The crystal structure of native chicken fibrinogen has been determined at a resolution of 5.5 Å recently. Excluding the floppy αC domains, fibrinogen is sigma shape. The structure of this 460 nm long protein resembles the image obtained by
negative-staining electron microscopy and is virtually superimposable on the recently reported bovine fibrinogen structure. The coiled-coil connections are significantly bent as expected for a three-stranded coiled coil. The central domain is defined by sets of radial spokes on each side of the covalent dimer forming oblate disk like shape. When visualized along an axial projection from either coiled coil, the disulfide rings adopt a triangular aspect. The $\gamma$-, $\beta$- and $\alpha$-chains appear to diverge along the three legs of a Y. It has been implicated that the amino terminal segments of the $\alpha$- and $\beta$- chains that constitute the fibrinopeptides A and B respectively, are highly flexible and floppy because they were not visible in the electron density maps. A very weak signal was obtained for the flexible $\alpha C$ domains, which was impossible to be assigned with certainty to particular molecules. Nevertheless, it has been suggested that in the crystal the $\alpha C$ domains of adjacent fibrinogen molecules interact rather than within the same molecule (Yang et al., 2000a).

1.1.5.6 Polymerization of fibrinogen

Removal of two small peptides namely fibrinopeptide A and fibrinopeptide B from the $\alpha$ and $\beta$ chains of fibrinogen respectively by thrombin leads to the transformation of fibrinogen into fibrin. Since these fibrinopeptides were known to carry negative charges, removal of these peptides was suggested to give rise to a net electrostatic attraction that is responsible for the non-covalent association of fibrin monomers (Blomback and Yamashina, 1958). Later, Doolittle (1977) suggested two possible reasons for spontaneous polymerization of fibrinogen following the removal of the fibrinopeptides. He hypothesized that polymerization occurs either due to the
exposure of key polymerization sites that was previously masked by the fibrinopeptides or due to the reduction of electrostatic repulsion that was caused by the net negative charge of the fibrinopeptides (Doolittle, 1977).

A number of studies have supported a hypothesis that the removal of the fibrinopeptides A and B activates separate binding sites in fibrinogen (Laudano and Dolittle, 1979; Shainoff and Dardick, 1979; Blomback et al., 1978). It has been reported that these sites are not similar. Under physiological conditions, the site exposed upon removal of fibrinopeptide A is sufficient to induce fibrinogen polymerization while the site activated by the loss of fibrinopeptide B is not. Olexa and Budzynski (1980) later identified four different polymerization sites in fibrinogen. One of the binding sites ("a") has been demonstrated to be in the D domain that is available even prior to the cleavage of fibrinogen by thrombin. Another site ("A") is complementary to the first one ("a") and the site ("A") is located in the amino terminal domain that is only available upon activation by thrombin. Alignment of the D domains fragments on two fibrin monomers upon fibrinogen polymerization had been demonstrated to give rise to a third polymerization site ("b") which is not activated by thrombin. This alignment is further stabilized by the crosslinking with Factor XIIIa. The fourth polymerization site ("B") located in the amino terminal domain requires activation by thrombin. This site is complementary to the "b" site. The two polymerization sites "A" and "B" can be easily distinguished even though both of them are located in the amino terminal domain. Thus, stable fibrin clot formation from fibrinogen actually involves three sequential steps: 1) the removal of fibrinopeptides A and B from fibrin monomer; 2) lateral association of the fibrin monomers; and 3) covalent cross-linking of fibrin monomers by Factor XIIIa.
Yang et al. (2000b) proposed a stepwise model of fibrin formation from fibrinogen. In the first step, a pair of A knobs that are exposed in the central E domain upon removal of fibrinopeptide A, joins the end of the protofibrils by fitting knobs into the holes located in the γC domains. This knob-hole interaction leads to the formation of two- molecule thick oligomer with a half-molecule stagger. In the second step, these protofibrils are crosslinked by Factor XIII between the carboxyl terminal segments of the γ-chains. The γ-chains laterally associate through two different segments of the γ-chains 350-360 and 370-380 in the third step. In the fourth step, the B knob that are exposed following removal of fibrinopeptide B fill a pair of holes in the βC domains of the protofibril already linked together by the A knob hole interaction. The B knob-hole interaction promotes lateral association between the protofibrils along their β chains residues (β 330- 375). This region of the β-chain that is involved in lateral association is homologous to the region of γ-chain that is involved in linking the ends of the protofibrils together (Yang et al., 2000b). Previously it was suggested that the fibrinogen C domains participate in the lateral association of the protofibrils during polymerization (Medved et al., 1985). They were also suggested to be involved in crosslinking events (Mckee et al., 1970).

1.1.5.7 Interaction of platelet with fibrinogen

Fibrinogen is essential for the aggregation of platelets following their activation by thrombin or by other agonists. Fibrinogen has binding sites for glycoprotein receptors I Ib/IIa on the platelet membranes (Bennett, 2001).
1.1.5.8 Fibrinolysis

Fibrin clots do not remain permanently in the damaged blood vessel. The clot is dissolved by the fibrinolytic system composed of plasminogen and plasminogen activators (Mosesson et al., 2001). Tissue type plasminogen activator circulates in the blood (Collen, 1980; Levin, 1983). The presence of fibrin polymers stimulates the activation of plasminogen by tissue type plasminogen activator (Hoylaerts et al., 1982; Mosesson et al., 1998). Formation of a ternary complex among fibrin, tissue type plasminogen activator and plasmin results in the activation of plasmin (Hoylaerts et al., 1982). Fibrinogen contains binding sites for both plasmin and antiplasmin. Plasmin is responsible for clot dissolution and antiplasmin inactivates plasmin after its task has been accomplished.

1.1.5.9 Interaction with fibronectin

In addition to preventing loss of blood following vascular injury, fibrin clot also serves as matrix for cell adhesion and migration at the site of injury during subsequent tissue repair. Interactions among cell surface receptors, fibrin and fibronectin promote these processes. Fibronectin is incorporated in the fibrin clot upon fibrinogen polymerization. Fibronectin is required for the migration of fibroblasts into a plasma clot and the adhesion and spreading of fibroblasts (Knox et al., 1986; Corbett et al., 1996). Incorporation of fibronectin into fibrin also prevents the migration of macrophages mediated by fibrin (Lanir et al., 1988).

Recently, Makogonenko et al. (2002) have demonstrated that the cryptic high-affinity fibronectin binding site of fibrinogen is in the αC domain. This site is not
accessible in fibrinogen but becomes exposed in fibrin. The formation of the cross-link between fibronectin and fibrin involves the reaction of a glutaminyl residue in fibronectin with a lysyl amino group in the $\alpha$ chain of fibrin (Mosherr and Johnson, 1983).

1.1.6 Thrombin

Thrombin is a serine protease that plays a crucial role both in homeostasis and thrombosis. It is generated from its zymogen, prothrombin, through the concerted action of several factors involved in the blood coagulation pathway (Fenton, 1981; Chang, 1985). Thrombin is a vitamin K-dependent protein that is synthesized in the liver. Vitamin K is required for the post-ribosomal formation of the residues in the amino terminal lead peptide of the zymogen (Magnuson et al., 1975). These are believed to be involved in Ca$^{2+}$ binding and formation of salt bridges to phospholipids during prothrombin activation (Chang, 1985; Sheraga, 1977).

1.1.6.1 Structural organization of thrombin

Prothrombin is composed of two chains: chain A and chain B, which are connected to two homologous kringle structures (Magnuson et al., 1975). The two chains of thrombin, chain A and chain B, are covalently linked by disulfide bonds (Suttie and Jackson, 1977). The A chain runs in the back of the molecule, opposite to the front hemisphere of the B chain that contains the active site and all the functional epitopes of the enzyme. The B chain has the typical fold of serine proteases (Lesk and Fordham, 1996), with two six stranded $\beta$ barrels of similar structures that pack together
asymmetrically to accommodate at their interface the residues of catalytic triad H 57, D102, and S195.

Thrombin B chain is more closely related to chymotrypsin than any other pancreatic serine proteases (Esmon et al., 1982; Chang, 1981). There are considerable structural and functional differences between the two halves of the prothrombin (Fenton 1981; Fenton et al., 1979). The amino terminal moiety is involved in regulation of thrombin generation such as zymogen biosynthesis, circulation, and activation (Fenton, 1981), whereas the regulatory functions such as clotting of fibrinogen are carried out by the carboxyl terminal moiety.

Prothrombin forms a complex with activated Factor X, activated Factor V, Ca$^{2+}$ and phospholipid called prothombinase complex (Suttie and Jackson, 1977; Mann et al., 1981). Two sequential cleavages of the prothrombin in this complex generate the disulfide linked thrombin A and B chains (Suttie and Jackson, 1977). In addition to possessing the classical active site, thrombin contains several additional sites called exosites or subsites for the recognition of substrate and receptors. Subsites of this recognition center include anion-binding exosite 1 (also called the fibrinogen recognition site) and anion binding exosite 2 (also called heparin binding site). These exosites facilitate specific and selective binding with substrates and receptors and ensure selective catalytic cleavage of peptide bond (Stubbs and Bode, 1993; Fenton, 1995).

In addition to the native form (α-thrombin) other forms of enzymatically active thrombin exist. Human α-thrombin slowly degrades to β- and subsequently to γ-thrombin by autoproteolysis with concurrent loss of clotting activity. Trypsin converts thrombin very rapidly to α to β- and γ-thrombins (Fenton et al., 1977).
Thrombin has a binding site for Na\(^+\). The domain, which contains the Na\(^+\) binding site, controls the specificity towards fibrinogen, protease activator receptors (PAR-1) and protein C. Na\(^+\) is the major procoagulant cofactor of thrombin. The Na\(^+\) bound form of thrombin has higher catalytic activity toward fibrinogen and the PARs compared to the Na\(^+\) free form, whereas the two forms cleave the anticoagulant protein C with similar values of \(k_{cat}/K_m\). Several mutations that reduce Na\(^+\) binding to thrombin have been demonstrated to elicit an anticoagulant effect. It has been concluded that these mutations induce allosteric effects, since none of the mutated residues has been observed to bind fibrinogen or PAR-1 (Stubbs et al., 1992; Mathews et al., 1994).

1.1.6.2 Functions of thrombin

One of the many important functions of thrombin is the removal of fibrinopeptides from the soluble plasma fibrinogen that subsequently leads to its polymerization thus forming fibrin clot. Thrombin acts as a powerful agonist for a variety of cellular responses to vascular injury apart from its major role in blood clot formation. Thrombin participates in the regulation of numerous physiological and pathophysiological processes namely blood coagulation, anticoagulation, thrombus formation and fibrinolysis, regulation of vascular tone, developmental processes, and also inflammation, tissue reparative processes, atherogenesis, carcinogenesis, and Alzheimer’s disease (Strukova et al., 1998; Henrikson et al., 1999; Grand et al., 1996).

Thrombin acts as an anticoagulant, when it activates protein C. This function unfolds upon binding to thrombomodulin, a receptor on the membrane of endothelial
cells. Binding of thrombomodulin enhances the affinity of thrombin 1000 fold towards the zymogen protein C (Esmon, 1989), but suppresses simultaneously the ability of thrombin to cleave fibrinogen or PAR-1. Upon activation, protein C cleaves and inactivates Factor Va and VIIIa, two essential cofactors of coagulation Factors Xa and IXa that are required for thrombin generation. As a result, both the amplification as well as progression of the coagulation cascade is downregulated upon binding of thrombin to thrombomodulin (Davie et al., 1991). The conversion of fibrinogen into an insoluble clot upon thrombin generation is thus prevented through this scavenging of thrombin by thrombomodulin and activated protein C. In addition, thrombin is irreversibly inhibited at the active site by the serine protease inhibitor antithrombin III with the assistance of heparin.

Thrombin is also a very potent activator of platelet aggregation and release. Binding and subsequent cleavage of the transmembrane protease-activated receptors (PAR)-1 by thrombin initiates the activation of glycoprotein receptors IIb/IIIa on the platelet surface thus activating platelets (Schwartz et al., 1995). Upon activation, platelets become crosslinked by fibrinogen and other plasma proteins with specific amino acid sequence (Arg-Gly-Asp = RGD)(Bennett et al., 1988).

Thrombin binds to and activates endothelial cells via a combination of receptor dependent activation. The subsequent signals alter the cell’s cytoskeleton structure and induce secretion of von Willebrands factor, prostacyclin, endothelium-derived relaxing factor (EDRF), tissue plasminogen activator, plasminogen activator inhibitor, and platelet derived growth factor. Thrombin binds to at least three putative receptors, endothelial thrombomodulin (Esmon and Owen, 1981), cell surface heparin sulfate (Machovich,
1985), and a receptor similar to protease nexin (Levoy-Viard et al., 1989). An endothelial cell surface protein that acts as a substrate for thrombin has been identified later (Vu et al., 1991). Following cleavage, the new amino terminus of the protein may serve as a surface-signaling molecule.

A number of changes occur intracellularly upon binding of thrombin to endothelium. Phospholipase C hydrolyzes phosphatidyl inositol, 4,5-biphosphate (PIP$_2$) into 1,4,5-triphosphate (IP$_3$) leading to an increase in intracellular calcium levels (Brock and Capasso, 1988) and diacylglycerol (DAG), which activates protein kinase C. Numerous cells such as monocytes, blood T-lymphocytes, and connective tissue mast cells, involved in the inflammatory and reparative response become activated by thrombin formed at the site of vascular damage (Strukova et al., 1998; Henrikson et al., 1999). Mast cells are involved in the activation of endothelial cells, leukocyte migration, edema formation, and other processes related to tissue repair. Upon activation by immune and non-immune liberators, mast cells release inflammatory mediators and modulators (Galli, 2000).

Thrombin plays opposing roles in controlling vascular tone depending on the existing physiological conditions (Yang et al., 1994; Ogletree, 1996). It can stimulate vasodilation by releasing nitric oxide and prostacyclin (PGI) from endothelial cells. In contrast, thrombin can also cause the release of endothelin-1 from endothelial cells and thromboxane A from platelets thus promoting vasoconstriction (Yang et al., 1994; Ogletree, 1996).
1.2 Purpose of the study

RSNO mediated inhibition of blood clot formation (Catani et al., 1998) has been reported earlier. S-nitrosation of a critical cysteine residue in Factor XIII, a transglutaminase, by RSNO has been suggested to be responsible for the observed inhibition of blood clot formation. The process of blood clotting is very complex. Many enzymes and proteins are involved in this process. The purpose of our study was to examine whether RSNOs had any effect on the structure and function of fibrinogen, a key protein involved in blood clot formation. In addition, we intended to characterize the type of possible interaction and to understand the mechanism of the interaction. Figure 12 shows the structures of the RSNO’s studied.
$S$-nitroso-$N$-acetylpenicillamine (SNAP)  

Captopril-SNO

Glucose-1-SNAP

Fructose-1-SNAP

Figure 1.1.12: Structures of RSNOs employed in this study
1.3 Chemicals and instrument

1.3.1 Chemicals

Crystallography grade human thrombin, and human fibrinogen were purchased from Haematologic Technologies Inc. (East Junction, VT).

Bovine fibrinogen, fraction I, type I-S, bovine thrombin, 5,5'-dithiobis-2-nitrobenzoate, glutathione, α-tocopherol and iodoacetamide were purchased from Sigma-Aldrich (St. Louis Mo.).

Tosylglycylprolylarginine-4-nitranilide acetate (Chromozym TH) was purchased from Roche Molecular Biochemicals Canada (Montreal, PQ).

2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) was purchased from OxisResearch (Portland, OR).

Glycine, Sodium chloride, Trishydroxymethylethanolamine, Na₂HPO₄, KH₂PO₄, potassium chloride (KCl) - Sigma

1x Phosphate buffered saline (PBS)- 0.137M NaCl, 12.7Mm KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4

1.3.2 Instruments

Perkin-Elmer Fluorometer model LS 4 - Perkin Elmer (Norwalk, CT)

Aviv CD spectrometer model 62A - Aviv Associates (Lakewood, NJ)

Agilent 8453 UV-visible spectrophotometer with a Pelletier temperature controller – Agilent Technologies (Mississauga, ON)

Fast protein liquid chromatography (FPLC)- BioRad (Hercules, CA)

MicroCal VP ultrasensitive titration calorimeter –MicroCal (Northampton, MA)
1.4 Methods

1.4.1 Synthesis of GSNO: Glutathione was dissolved in ice cold 0.5M HCl. Equimolar sodium nitrite was added, and the reaction was carried out in the dark at 4°C for 40 min. The pH of the reaction mixture was adjusted to 7.4. The product was precipitated from the solution by the slow addition of cold acetone and successively washed with ice-cold acetone, water and diethyl ether.

1.4.2 Synthesis of RSNO’s and pyrrolidinyl diazenium diolate (Pyrrolidium NONOate): S-nitroso bovine serum albumin (BSA-NO) was synthesized according to the method described earlier (Hogg, 1999). Captopril-SNO, glucose-1-SNAP, fructose-1-SNAP, and pyrrolidium NONOate were synthesized in Dr. Peng G. Wong’s laboratory in Wayne State University according to previously published methods (Loscalzo et al., 1989; Ramirez et al., 1996; Hou et al., 2001; Saavedra et al., 1997).

1.4.3 Preparation of pyrrolidium NONOate solution: Pyrrolidium NONOate was dissolved in methanol in a drum vial and sealed with a septum. Pyrrolidium NONOate solution was degassed with nitrogen in order to prevent the reaction of released NO with oxygen. The pyrrolidium NONOate solutions were made fresh before use.

1.4.4 Fibrinogen polymerization: Fibrinogen was first dissolved in 50 µL of glycine-NaOH buffer (50 mM, pH 8.5). After dissolved the fibrinogen solution was diluted in phosphate buffered saline (PBS) to a final concentration of 0.38 µM. The polymerization
was initiated by the addition of 0.03 μM thrombin. The turbidity of the solution was measured at 660 nm. The initial rates were calculated from the Δ O.D. within the first 20 s subsequent to mixing of the reagents. When the polymerization was performed in the presence of GSNO, the fibrinogen sample was incubated with GSNO for 10 min in the dark.

1.4.5 Thrombin assay: The activity of thrombin was determined with the colorimetric pseudo substrate Chromzym TH. Thrombin (0.6 μM) either preincubated with 50 μM GSNO for 15 min or not, was added to a 0.2 mM solution of tosylglycylprolylarginine-4-nitranilide acetate (Chromzym TH) in PBS (25°C). The absorbance at 405 nm was measured as a function of time in the spectrometer described in section 1.3.2. The blank hydrolysis rate was subtracted from the enzymatic rate.

1.4.6 Circular dichroism measurements: Fibrinogen (0.3 μM) in PBS buffer was placed in a 1 cm quartz cuvette. The CD spectra were measured over the wavelength region of 190 nm to 260 nm (protein 2° structure) or 500 nm to 600 nm (RS-NO environment) in an AVIV CD spectrometer model 62A DS. Each spectrum is an average of 3 different scans obtained by collecting data 1 nm intervals with an integration time of 10 s, at a constant temperature of 25 °C. The secondary structure composition was determined with the aid of CDNN deconvolution software (Böhm et al., 1992).
1.4.7 *Determination of free SH content:* The free thiol content of the samples were determined colorimetrically with the aid of 5,5′-dithiobis-2-nitrobenzoate (DTNB) ($\varepsilon_{412}=13600 \text{ M}^{-1}\text{cm}^{-1}$) (Ellman, 1959).

1.4.8 *Blockage of free thiols:* GSH (5 mmol) was dissolved in tris-acetate buffer (50 mM, pH 8.5). To this solution 5 mmol iodoacetamide was added. The reaction was allowed to proceed at room temperature for 2 h. The obtained product was then diluted appropriately in PBS and used without further purification.

1.4.9 *Fluorescence measurements:* The intrinsic Trp fluorescence spectra of fibrinogen solutions in PBS were measured with the aid of a Perkin-Elmer Fluorometer. The samples were excited at 290 nm. The emission spectrum 300 nm to 400 nm was monitored as a function of [GSNO]. The absorbance of the solutions at 290 nm did not exceed 0.1. Fractional quenching at a given [GSNO] was estimated by dividing this value by the maximal quenching obtained at the largest [GSNO]. These data were then fitted to a saturation function ($\Delta F/\Delta F_{\text{tot}} = [\text{GSNO}]/(K_D+[\text{GSNO}])$) in order to estimate the $K_D$.

1.4.10 *Isothermal titration calorimetry (ITC):* Fibrinogen solution was dialyzed extensively against PBS. Then the dialyzed sample (2.6 μM) was added to the 1.4-mL calorimeter sample cell. GSNO solution (0.6 mM in PBS buffer) was injected to the sample cell in 1-5 μL aliquots at 4-8 min intervals. The heat of reaction per injection (μcal/s) was determined by integration of peak areas using Origin software (MicroCal).
The heat of dilution was determined from the baseline at the end of titration and subtracted from the observed heat of binding. Heat evolved per mole of substrate injected was plotted against the GSNO/fibrinogen molar ratio. $\Delta H^\circ$, the stoichiometry of binding $(n)$, and the dissociation constant $(K_d)$ were estimated from the best fit. The Gibbs free energy of binding $[\Delta G^\circ = RT \ln (1/K_d)]$ and the entropy of binding $(T \Delta S^\circ = \Delta H^\circ - \Delta G)$ were calculated from the experimental values.
1.5 Results

1.5.1 Fibrinogen polymerization

1.5.1.1 Effect of GSNO: Thrombin catalyzes the transformation of fibrinogen into fibrin by removing the small polar peptides, fibrinopeptides A and B, from the parent molecule. Removal of the peptides uncovers the knobs that lead to the polymerization of fibrinogen. This process can be monitored *in vitro* by measuring the increase in turbidity (660 nm) of a fibrinogen solution in the presence of catalytic amounts of thrombin. As can be seen in Figure 1 (squares) the initial rate of bovine fibrinogen polymerization (2.5x $10^{-5}$/s) decreased by $60 \pm 8\%$ (circles) and $68 \pm 10\%$ (triangles) in the presence of 20 μM and 50 μM GSNO, respectively. These experiments were repeated with human fibrinogen and thrombin where 20 μM and 50 μM GSNO resulted in $43 \pm 7\%$ and $50 \pm 8\%$ inhibition, respectively (Figure 2).

1.5.1.2 Effect of GSSG and pyrrolidium NONOate: There is a possibility that the observed inhibition of initial rate of fibrinogen polymerization is due to NO$_X$-protein reactions from thermally or photolytically released NO from the RSNOs employed. Another possibility is that GSSG, which could be formed as a result of the loss of NO from GSNO, could be affecting the polymerization. To test for these possibilities fibrinogen polymerizations were carried out in the presence of pyrrolidium NONOate, which is known to rapidly release NO in solution ($t_{1/2} = 1.8$ s) or GSSG. However these compounds had no effect on the initial rates of fibrinogen polymerization (Figure 3).
Figure 1.5.1: The effect of GSNO on thrombin catalyzed fibrinogen polymerization. The turbidity of the solution of bovine fibrinogen (0.38 μM) plus thrombin (0.03 μM) was monitored as a function of time at 660 nm in the presence of (□) buffer alone; (○) 20 μM GSNO and (Δ) 50 μM GSNO. Data points depicted under each condition are representatives of the average of five different experiments.
**Figure 1.5.2:** The effect of GSNO on human fibrinogen polymerization. The turbidity of the solution of human fibrinogen (0.38 μM) plus thrombin (0.003 μM) was monitored as a function of time at 660 nm in the presence of (□) buffer alone; (○) 20 μM GSNO and (△) 50 μM GSNO. Data points depicted under each condition are representatives of the average of five different experiments.
Figure 1.5.3: The effect of GSSG and pyrrolidium NONOate on bovine fibrinogen polymerization. The turbidity of the solution of fibrinogen (0.38 µM) plus thrombin (0.03 µM) was monitored as a function of time at 660 nm in the presence of (□) buffer alone; (○) 10µM pyrrolidium-NONOate; (△) 50 µM GSSG. Data points depicted under each condition are representatives of the average of five different experiments.
1.5.1.3 The effect of PTIO on GSNO induced inhibition of fibrinogen polymerization:
In order to further eliminate the possibility that the observed inhibition is due to NO released from the RSNOs or other NO\(_x\) species subsequently formed, the polymerizations were repeated in the presence of 50 \(\mu\)M PTIO as a NO scavenger (Akaike and Maeda, 1996). In addition these solutions contained \(\sim\) 5 \(\mu\)M \(\alpha\)-tocopherol to scavenge any NO\(_2\) that might be produced. In the presence of PTIO/\(\alpha\)-tocopherol GSNO (50 \(\mu\)M) inhibited polymerization by 60\% \(\pm\) 9\% (Figure 4). This is yet another line of evidence that NO or NO\(_x\) species are not causing the inhibition.

1.5.2 Effect of GSNO on thrombin: In order to examine whether GSNO affected thrombin or fibrinogen, thrombin was incubated with 50 \(\mu\)M GSNO for 15 min in the dark. The thrombin activity was then measured with the protease pseudo-substrate Chromozym TH. It was found that the activity of thrombin was not affected by GSNO as the initial rates of Chromozym-TH hydrolysis were indistinguishable with \((7.31\times10^{-3} \pm 5.57\times10^{-5})/\text{s}\) or without GSNO \((7.33\times10^{-3} \pm 1.8\times10^{-4})/\text{s}\) (Figure 5). It is also conceivable that the RSNOs did not inhibit thrombin but contaminating amounts of factor XIII, which has an active site thiol that can be inactivated via transnitrosation (Catani et al., 1998). This does not appear to be the case, since the fibrinogen (human and bovine) polymerization rates in the absence of thrombin were negligible \((7\times10^{-6} \pm 8 \times10^{-6} \text{ s}^{-1})\).

Other structurally diverse RSNOs also inhibited the process (Table 1) with similar IC\(_{50}\)s within experimental error, and the maximal inhibition varied from 68\% in the case of GSNO to 43\% with fructose-1-SNAP.
Figure 1.5.4: The effect PTIO on GSNO induced fibrinogen polymerization inhibition. The turbidity of the solution of human fibrinogen (0.38 μM) plus thrombin (0.003 μM) was monitored as a function of time at 660 nm in the presence of buffer containing: (□) 5 μM α-tocopherol 50 μM PTIO; and (Δ) 50 μM GSNO, 5 μM α-tocopherol and 50 μM PTIO. Data points depicted under each condition are representative of the average of five different experiments.
Figure 1.5.5: The effect GSNO on thrombin activity. The rate of Chromozyme TH hydrolysis by thrombin without (control) and with (treated) pre-incubation with GSNO was measured by monitoring the absorbance change at 405 nm as a function of time. Mean values ± SD from four different experiments are shown.
Table 1.5.1. Apparent inhibition constants between RSNO’s and fibrinogen.

<table>
<thead>
<tr>
<th>RSNO</th>
<th>Apparent IC$_{50}$ (µM)</th>
<th>Maximal inhibition (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSNO</td>
<td>4.0 ± 1.0</td>
<td>68</td>
</tr>
<tr>
<td>SNAP</td>
<td>5.0 ± 2.1</td>
<td>64</td>
</tr>
<tr>
<td>Captopril-SNO</td>
<td>6.5 ± 2.2</td>
<td>46</td>
</tr>
<tr>
<td>Glucose-1-SNAP</td>
<td>7.5 ± 2.7</td>
<td>56</td>
</tr>
<tr>
<td>Fructose-1-SNAP</td>
<td>6.0 ± 2.3</td>
<td>43</td>
</tr>
<tr>
<td>BSA-NO</td>
<td>7.0 ± 3.4</td>
<td>46</td>
</tr>
</tbody>
</table>

*An initial rate of fibrinogen polymerization catalyzed by thrombin in the presence of RSNO was determined by following the same experimental conditions described in Figure 1 (section-1.3.4). Fractional inhibition at a certain [RSNO] was estimated by dividing each single inhibition by the maximum inhibition obtained at the largest [RSNO]. Then the values were fitted to a saturation function to obtain the apparent inhibition constants (IC$_{50}$). Fibrinogen polymerization rates were monitored at RSNO concentration range of 0.1 µM to 100 µM. Values are the means ± SD from five experiments.*
1.5.3 Circular dichroism studies

1.5.3.1 Effect of GSNO on fibrinogen Secondary structure: Both bovine and human fibrinogen secondary (2°) structure were monitored by CD as a function of [GSNO] (Figures 6 and 7). GSNO increased the negative ellipticity at 207 nm and decreased the positive ellipticity at ~190 nm. This change appears to be RSNO specific as S-amidomethylGSH (Figure 8) failed to alter the CD spectrum of fibrinogen. The GSNO-dependent effect on fibrinogen 2° structure was reversible as the fibrinogen CD spectrum was very close to that of native spectrum subsequent to separation of GSNO from fibrinogen by FPLC (Figure 9).

Upon deconvolution, the GSNO-dependent change in the CD spectrum corresponded to a 15 % increase in helicity at the expense of 10 % loss in β sheet and 5 % decrease in random coil content.

1.5.4 NO detection with NO electrode: The S-NO bond in GSNO is photolabile. It is therefore conceivable that the observed conformational changes result from NO generated from photolysis of S-NO, by the monitoring beam during the CD experiment, which might react with protein functional groups. NO electrode is capable of detecting NO in nanomolar range. Therefore, the possibility of NO generation was tested by inserting a NO-specific electrode into the cuvette containing a solution of GSNO (.05 μmol) plus fibrinogen, in the sampling chamber of the CD spectrometer. There was no detectable NO produced during the CD experiment.
Figure 1.5.6: The effect of GSNO on the CD spectra of bovine fibrinogen. The far UV CD spectra of bovine fibrinogen (0.3 μM) were measured in the presence of (□) buffer alone; (○) 25 μM GSNO; (×) 30 μM GSNO; (△) 50 μM GSNO. The depicted CD spectra are representative of the average of four different experiments. In each experiment average of three different scans was recorded. SD values are within the dimension of the symbols used in the figures.
Figure 1.5.7: The effect of GSNO on the CD spectra of human fibrinogen. The far UV CD spectra of human fibrinogen (0.03 μM) were measured in the presence of (□) buffer alone; (Δ) 50 μM GSNO. The depicted CD spectra are representative of the average of four different experiments. In each experiment average of three different scans was recorded. SD values are within the dimension of the symbols used in the figures.
Figure 1.5.8: The effect of  S-amidomethylGSH on the CD spectra of bovine fibrinogen. The far UV CD spectra of fibrinogen (0.3 μM) were measured in the presence of (Δ) buffer alone, (□) 25 μM S-amidomethylGSH; (□) 30 μM S-amidomethylGSH; (X) 50 μM S-amidomethylGSH. The depicted CD spectra are representative of the average of four different experiments. In each experiment average of three different scans was recorded. SD values are within the dimension of the symbols used in the figures.
Figure 1.5.9: CD spectra of native and GSNO treated fibrinogen following FPLC. The far UV CD spectra of fibrinogen (0.3 μM) were measured in the presence of (X) buffer alone; and after exposure to GSNO for 10 min followed by chromatography on Sephadex G-25 (○). The depicted CD spectra are representative of the average of four different experiments. In each experiment average of three different scans was recorded. SD values are within the dimension of the symbols used in the figures.
1.5.5. Fluorescence studies of fibrinogen: The intrinsic Trp fluorescence spectrum was monitored as a function of [GSNO]. The fibrinogen fluorescence was quenched in a saturable manner (Figure 10) indicating that the process is not due to collisional quenching. The fit of the quenching data to a saturation function resulted in an estimated $K_d$ of 10 μM.

In an effort to estimate the stoichiometry of GSNO/fibrinogen interaction, 100 μM fibrinogen was titrated with [GSNO]. Since this is above the estimated $K_d$, the fluorescence quenching should represent stoichiometric binding. Under these conditions the quenching profile was characterized by two slopes which when extrapolated gave an approximate ratio of 2:1 (GSNO/fibrinogen) (Figure 11).

1.5.6 The effect of fibrinogen on the CD spectrum of GSNO: The CD absorption spectrum (550 nm) of RSNos is spectrally well separated from that resulting from protein secondary structure (Mohney and Walker, 1997). Low molecular weight RSNos where the -SNO is free to rotate, have a strong CD absorption in the 500 to 600 nm region. However, this signal is lost, if rotation is restricted as is the case in serum albumin-NO (Mohney and Walker, 1997). When 25 μM fibrinogen (50 μM in estimated GSNO site concentration) was added to 100 μM GSNO the magnitude of GSNO-CD spectrum decreased by ~1/2 (Figure 12). This is an indication that the rotational freedom of the SNO moiety is restricted upon interacting with fibrinogen. In addition, the CD studies confirm the 2 to 1 GSNO/fibrinogen stoichiometry determined by the fluorescence titrations (Figure 12).
Figure 1.5.10: The effect of GSNO on fluorescence emission of fibrinogen. The intrinsic Trp fluorescence spectra of fibrinogen solutions. The emission spectrum 300 nm to 400 nm was monitored as a function of [GSNO]. Fractional quenching at a given [GSNO] was estimated by dividing this value by the maximal quenching obtained at the largest [GSNO]. This data was then fitted to a saturation function ($\Delta F/\Delta F_{\text{tot}} = [\text{GSNO}]/(K_D+[\text{GSNO}])$) in order to estimate the apparent $K_D$. The absorbance of the solutions at 290 nm did not exceed 0.1.
Figure 1.5.11: Determination of Fibrinogen: GSNO stoichiometry from fibrinogen fluorescence quenching as a function of [GSNO]. The fluorescence emission at 330 nm ($\lambda_{ex}$ 290 nm) of a 100 $\mu$M solution of fibrinogen was monitored as a function of [GSNO] in a 0.2 cm path length fluorescence cuvette. Fractional quenching at a given [GSNO] was estimated by dividing this value by the maximal quenching obtained at the largest [GSNO].
Figure 1.5.12: The effect of fibrinogen on GSNO CD spectrum. The CD spectra 100 μM GSNO (♦) plus 25 μM fibrinogen (○).
1.5.7 Isothermal titration calorimetry (ITC): In an effort to characterize the GSNO-fibrinogen interactions by another independent technique, 2.6 μM fibrinogen was titrated in a calorimeter with 1 μL to 5 μL aliquots of GSNO (0.6 mM in syringe concentration). The data were best fitted with 2 binding sites/fibrinogen with a $K_D$ of $3.31 \pm 0.9$ μM (Figure 13). The process appears to be entropy driven with a $\Delta S$ of 63.7 and a $\Delta H$ of 1.12 kcal/mol.
Figure 1.5.13: Determination of dissociation constant ($K_d$) between fibrinogen and GSNO using isothermal titration calorimetry. 2.6 µM fibrinogen sample was taken into the 1.4 ml calorimeter sample cell. The sample was then titrated by injecting 1-2 µl aliquot of (0.6mM) GSNO solution at 4-8 min intervals. The heat evolved (kcal) per mole of GSNO added was plotted against [GSNO]/[Fibrinogen]. The line represents the best fit.
1.6 Discussion

It has been reported earlier that RSNOs inhibit blood clot formation (Catani et al., 1998). This inhibition had been demonstrated to be due to the inactivation of Factor XIII, a transglutaminase, involved in γ dimer formation during the fibrin clot formation. Fibrin clot is stabilized by the formation of the γ dimers between the fibrin monomers. The S-nitrosation of a thiol group in a critical cysteine residue of Factor XIII has been demonstrated to be responsible for this loss of functional inactivation (Catani et al., 1998). Several studies implicated a role of RSNO also in the inhibition of platelet aggregation (Mendelsohn et al., 1990; Loscalzo, 1992; Stamler et al., 1992; Simon et al., 1993).

Mendelsohn et al. (1990) have demonstrated that S-nitroso-N-acetylcysteine (SNOAC) causes a remarkable inhibition of fibrinogen binding to platelets. The binding interaction between fibrinogen and activated platelet is a key event that precedes platelet aggregation (Peerschke, 1985). In the presence of SNOAC, an increase in the apparent dissociation constant of platelet receptor for the fibrinogen and a reduction of the total number of bound fibrinogen to the activated platelet was also reported. In addition, there was a dose dependent increase in the platelet cGMP level that correlated well with the inhibition of fibrinogen binding to the activated platelet by SNOAC. The extent of inhibition was further increased in the presence of cGMP phosphodiesterase. It was suggested that RSNOs might exert their inhibitory effect on platelet aggregation through the stimulation of cGMP production (Mendelsohn et al., 1990). The S-nitroso derivative of tissue type plasminogen activator (S-nitroso-t-PA) had also been demonstrated to
cause vascular relaxation and to inhibit platelet activation. An increase in the cGMP level accompanied by both of these effects demonstrated by S-nitroso-t-PA (Stamler et al., 1992). Simon et al. (1993) also demonstrated the inhibition of platelet aggregation by different S-nitrosoproteins including S-nitroso-BSA. Incubation of the platelets with S-nitrosoproteins also increased the level of cGMP. In addition, the exposure of platelets to S-nitrosoproteins reduced the number of platelet bound fibrinogen molecules (Simon et al., 1993).

In the present study, GSNO was observed to inhibit thrombin-catalyzed bovine and human fibrinogen polymerization. Thrombin did not appear to be affected, since the initial rates of Chromozym TH hydrolysis were unchanged after a 15 min exposure to GSNO (Figure 5). This observation suggested that the GSNO inhibited polymerization via its action on fibrinogen. Since GSNO is photolabile, the possibility remains that the released NO or other reactive NO species subsequently generated from thermal or photolytic decomposition of GSNO is interacting with fibrinogen. Such an interaction could lead to the inhibition of polymerization. However, this did not appear to be the case, because the rate of polymerization was not apparently affected by both the NO donating compound, pyrrolidinyl diazenium diolate or oxidized glutathione (GSSG). The possibility of NO fibrinogen interaction was further eliminated by the fact that the employment of a NO scavenger, PTIO failed to prevent GSNO induced inhibition of fibrinogen polymerization. Moreover, a number of structurally different RSNO’s also exhibited similar inhibitory actions on the thrombin mediated fibrinogen polymerization like GSNO.
These observed results were intriguing since there are no free thiols in fibrinogen, all of the thiols are involved in disulfide bridges. The mechanism of platelet aggregation inhibition in all of the earlier reported cases has been attributed to either the NO-mimicking activity of RSNOs in triggering the NO/cGMP pathway or has involved the transnitrosation or S-thiolation of an essential free thiol(s) on the proteins participating in thrombosis or platelet activation. The activity of several enzymes not involved in hemostasis, namely glyceraldehyde-3-phosphate dehydrogenase (Mohr et al., 1999), creatine kinase (Konorev et al., 2000) papain (Xian et al., 2000a), protein tyrosine phosphatases (Xian et al., 2000b), human rhinovirus 3C protease (Xian et al., 2000c) have also been shown to be attenuated by RSNO-dependent transnitrosation or S-thiolation.

Using creatine kinase as a model, Konorev et al. (2000) have demonstrated that the extent of S-thiolation and S-nitrosation of a protein by RSNO depends not only upon the environment of the protein thiol, but also on the chemical nature of the S-nitrosothiol involved. GSNO had been demonstrated to mainly S-thiolate creatine kinase, whereas SNAP mainly caused S-nitrosation of the protein (Konorev et al., 2000). A number of S-nitrosothiols including SNAP, GSNO, Captopril-SNO have demonstrated to inactivate the cysteine protease, papain. However, the enzyme activity could be restored upon incubation with dithiothreitol. The functional inactivation of the cysteine protease has been reported to be due to the mixed disulfide formation between RSNO and protein thiol. A direct nucleophilic attack of the highly reactive thiolate, Cys$^{25}$, in the active site of the enzyme on the sulfur of S-nitrosothiol has been suggested to be responsible for the formation of the mixed disulfide (Xian et al., 2000a). The protein tyrosine phosphatases
namely recombinant mammalian protein tyrosine phosphatase 1B and Yersinia protein tyrosine phosphatase have also been reported to become inactivated by a series of low molecular weight S-nitrosothiols. The enzyme activity was lost in a time- and concentration-dependent manner. The inactivation of the enzymes by S-nitrosothiols had been shown to be a reversible process, since reduction by dithiothreitol could partially restore the activity of the enzyme. S-nitrosothiols induced inhibition of PTPase could be partially prevented by inorganic phosphate, a competitive inhibitor of PTPase thus suggesting interaction of RSNO with the active site of the enzyme. Both S-nitroso human serum albumin (HSA) and poly-S-nitrosated HSA resulted in the inhibition of the enzyme Yersinia PTPase. The efficiency of inactivation was reported to be higher with poly-S-nitrosated HSA than the single S-nitrosated HSA. The inactivation by S-nitrosoHSA was hindered in the presence of inorganic phosphate and the activity of the enzyme could be reverted upon incubation of the inactivated enzyme with DTT (Xian et al., 2000b). GSNO has also been reported to inactivate glyceraldehyde-3-phosphate dehydrogenase via S-thiolation of a thiol group of the protein both in vitro and in physiological conditions. The enzyme activity could be restored following incubation with DTT (Mohr et al., 1999).

Before proceeding further with my studies I ensured that the GSNO samples did not contain any free thiols (i.e. unreacted GSH). The amount of free thiol was determined by DTNB titrations to be < 1% of the [GSNO] and these were blocked by iodoacetamide. Without the S-NO moiety, S-amidomethylGSH did not perturb fibrinogen 2α structure as determined by CD. On the other hand, GSNO increased the negative ellipticity at ~ 207 nm and decreased the positive ellipticity at ~ 190 nm which
translated to 15% increase in α-helix content at 50 μM GSNO. Higher RSNO concentrations could not be utilized owing to the absorptivity of the solutions.

All the thiols in fibrinogen are involved in disulfide bond formation. Furthermore, these disulfide bridges could not be possibly reduced because we ensured that the GSNO solution was free of any contaminating GSH. Based on these facts, S-nitrosation of the protein is not possible. Therefore, S-nitrosation or S-thiolation of a thiol group in fibrinogen is not responsible for the observed structural modulation. In addition, upon removal of GSNO by gel filtration chromatography, the CD spectra of fibrinogen solutions exposed to GSNO were identical to the native protein, thus indicating that the RSNO-induced structural perturbations were reversible.

These observations point to a mechanism where GSNO alters fibrinogen structure not through a chemical reaction, but instead, it induces a conformational change in the protein by binding at specific site(s). We hypothesize that these RSNO-binding sites might be in regions of the protein that are disordered and are rich in aromatic side chains. The requirement for GSNO-binding domains being in unstructured regions of the protein is assumed, since GSNO increased the α-helical content of the protein thus suggesting that it interacts with regions with little or no structure and induces α-helix formation. The recent crystal structure (Yang et al., 2000a; Brown et al., 2000) of fibrinogen indicates that the protein consists of a ~400-Å long sigmoidal coiled coils that connect globular C-terminal domains with a central domain consisting of the N-terminal regions of the α2 β2 and γ2 subunits. Despite a large degree of structure in the protein, there are
several domains that are largely unstructured. By far the most unstructured of these is the C-terminal end of the α-subunits termed αCA and αCB. In the crystal structure of chicken fibrinogen (Yang et al., 2000a), αCA and αCB appeared disordered in the electron density map. Despite the fact that the chicken protein is shorter by 119 residues and lacks the 10 X 13 repeated sequences (Doolittle et al., 1979; Murakawa et al., 1993) that add additional floppiness to the bovine and human proteins. These αC-domains termed “free-swimming appendages” often interfere with crystal formation and had to be proteolyzed in the structure as reported earlier (Brown et al., 2000).

The second hypothesis, which states that the GSNO binding site is rich in aromatic residues, is evidenced by the intrinsic fibrinogen fluorescence quenching by GSNO. The observed fluorescence quenching is attributed to energy transfer between Trp residues (ex 290 nm em 330 nm) of the protein and the S-NO moiety of GSNO (acceptor-absorbance maxima 343 nm, 543 nm). We have previously observed that the fluorescence of N-dansylhomocysteine was quenched upon its S-nitrosation (Ramachandran et al., 1999). The quenching was attributed to energy transfer between the dansyl moiety donor (ex 340 nm em 540 nm) and RS-NO (acceptor, absorbance 543 nm) and in order for this to occur the RS-NO must “lay over” the dansyl ring. Recent calculations by Chen and his coworkers (Chen et al., 2001) indicated that the interaction between the SNO moiety and dansyl ring is energetically favourable.

The fact that, in the present study, the quenching of Trp fluorescence, was saturable and occurred at a concentration much lower than expected for collisional quenching, suggests that GSNO interacts at specific sites on fibrinogen via SNO-aromatic
interactions. This binding is thought to yield conformational changes, which inhibit thrombin-catalyzed fibrinogen polymerization.

Examination of the protein data base (PDB) files 1DEQ and 1EI3 revealed that aromatic amino acid residues are abundant in the following non-helical structures: globular αC-domains which are on or close to the central amino-terminal disulfide-knot (Veklich et al., 1993), and globular carboxyl terminal domains of Bβ (twelve Trp) and γ (ten Trp) in fibrinogen. The αC-domains are likely GSNO-binding sites both from the point of lacking structure and being Trp-rich in that there are 12 Trp between residues 291 to 610, and being much closer to the central domain. Veklich et al. (1993) have reported that the interaction between the αC fragment and αC domains inhibits fibrinogen polymerization by preventing intermolecular interaction between fibrin monomers. This is also consistent with the 2:1 stoichiometry of GSNO-fibrinogen complexation observed in our fluorescence and calorimetric studies. The reduction of CD signal of GSNO (100 μM) in the presence of 25 μM fibrinogen by 50% further supports our postulated stoichiometric ratio between GSNO and fibrinogen.

An examination of the homologous αC-domains of human and bovine fibrinogen (Figure 14) revealed three regions (in yellow) that have sparked our interest as potential GSNO-binding domains. These regions are rich in Trp and Phe as well as contain cationic Lys or Arg residues. The cationic residues in these homologous domains could be involved in additional electrostatic contacts with GSNO.
Figure 1.5.14: BLAST (nr databases): comparison of human and bovine fibrinogen α-chain.

A BLAST search of the protein database for short, nearly exact matches to the αC domains in yellow (Figure 14) has indicated the presence of these sequences in several human proteins including A20, an endothelial Zn-finger protein induced by tumour necrosis factor (Klinkenberg et al., 2001); peripheral benzodiazepine receptor-associated protein 1; melatonin receptor 1B; short form transcription factor C-MAF; nestin; protocadherin α-1; effector cell proteinase receptor 1; G-protein β-subunit like protein; general transcription Factor IIIA as well as a highly conserved phosphoenolpyruvate carboxylase (Matsumura et al., 1999) domain. In addition, these domains were present
in several prokaryotic proteins including DNA topoisomerase; Ala-tRNA synthase; and DNA polymerase (Hepatitis B virus).

This study indicates that low molecular weight RSNOs can drastically alter fibrinogen structure without chemically reacting with the protein. Both experiments and quantum chemistry calculations (Chen et al., 2001) are in agreement that the interaction between RSNO and aromatic amino acids may contribute to the observed GSNO-fibrinogen interactions. RSNO-dependent allosteric interactions are predicted to occur in regions of the αC chains that are rich in aromatic residues, thus converting them to more ordered α-helices. The functional consequence of these interactions is the in vitro inhibition of thrombin-catalyzed fibrinogen polymerization. This inhibition was also observed with serum albumin-NO. This suggests that the S-nitroso derivative of the Cys 34 of albumin, which is at the end of a ~9 Å cleft (Sugio et al., 1999), is accessible to the S-NO binding peptides of fibrinogen. However, the group of researchers who have solved the crystal structure of albumin, suggested that the backbone conformation of albumin in solution might be different than that observed in the crystal structure hence bringing the Cys 34 thiol toward the exterior of the protein (Sugio et al., 1999). In support of this idea, we have recently shown that BSA-NO was able to act as a substrate for cell surface protein-disulfide isomerase (Ramachandran et al., 2001) indicating this residue is accessible to large proteins.

Assuming a fibrinogen: serum albumin-NO $K_D$ of ~ 1-3 μM (from ITC) and a reported circulating [HSA-NO] in normal adults of ~200 nM (Pietraforte et al., 1995),
16% of fibrinogen would be bound to serum albumin-NO. This would give rise to ~10% inhibition in the clotting process. The effect on fibrinogen-dependent processes at the cell surface might be larger as [albumin-NO] on the surface may be many-fold larger since many cell types contain surface receptors for serum albumin (Pietraforte et al., 1995).

The significance of the present study is that it demonstrates that RSNOs can potentially regulate essential physiological processes via allosteric interactions, in addition to chemical interactions, with their targets.
PART II

Chemistry of $S$-nitrosothiols: potential analytical and cell biological applications
2.0 Abstract

2.0.1 Synthesis of a coloured nitrite adduct from sinapinic acid and NO$^+$ donors: potential applications

Sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid, SA) reacted with peroxynitrous acid at neutral pH with a second order rate constant of 812 M$^{-1}$ s$^{-1}$, to yield a red product ($\lambda_{\text{max}}$, 532 nm). The fact that the identical coloured product could be formed with decomposed peroxynitrous acid solutions or nitrite in acidic solutions (0.1 M HCl) at slower rates (8.32 M$^{-1}$ s$^{-1}$), 10% acetic acid, (K= 0.0004 M$^{-1}$ s$^{-1}$) suggested that $O$-nitrososinapinic acid (3,5-dimethoxy-4-nitrosooxycinnamic acid) was being formed. This compound is red with an extinction coefficient of 8,419 M$^{-1}$ cm$^{-1}$ at 510 nm in the presence of 10% acetic acid 90% acetonitrile. NO$^+$ transfer from GSNO to SA can also result in $O$-nitrososinapinic acid ($O$-nitrosoSA) formation. $O$-nitrososinapinic acid in turn can $S$-nitrosate low molecular weight thiols and protein thiols. SA was also shown to act as a peroxynitrite sink, as it effectively prevented the oxidation of dihydroorhodamine under physiological conditions. The fact that $O$-nitrososinapinic acid is stable and can be used to $S$-nitrosate thiol containing amino acids, peptides and proteins makes it a potentially useful reagent in the study of $S$-nitrosothiol biochemistry and physiology. In addition, the relatively high extinction coefficient of $O$-nitrososinapinic acid means that it could be utilized as an analyte for the spectroscopic detection of peroxynitrite or NO$^+$ donors in the sub-micromolar range.
2.0.2 Bovine serum albumin nanoparticles as carriers of NO

There is a growing evidence for the reaction between $\text{N}_2\text{O}_3$ and thiols in the hydrophobic-hydrophilic interfaces of biomembrane and hydrophobic pockets of protein leading to the formation of $S$-nitrosothiols (Nedospasov \textit{et al.}, 2000; Rafikova \textit{et al.}, 2002; Ramachandran \textit{et al.}, 2000). I synthesized an $S$-nitroso derivative of thiolipid, $S$-nitrosooctadecane (SNOD) that demonstrated NO releasing properties like other $S$-nitrosothiols upon exposure to light. In this study I demonstrated that the newly synthesized SNOD was thermally stable, as it could be stored in solution in a quartz cuvette at room temperature for up to two weeks. Laser-irradiated oxygen-saturated SNOD solution in hexane could also $S$-nitrosate aqueous small molecular weight thiol in solution, namely glutathione, as evidenced by the formation of characteristic $S$-NO absorption peak at 340 nm following the addition of GSH solution to the irradiated SNOD solution. However, inclusion of a $\text{N}_2\text{O}_3$ quencher, $\alpha$-tocopherol, in the oxygen saturated SNOD solution during irradiation inhibited this process significantly. On the other hand, nitrosation of glutathione did not occur under an oxygen-free environment. These observations indicated the involvement of $\text{N}_2\text{O}_3$ and oxygen in this nitrosation process. In addition, $S$-nitrosoglutathione was not formed, when similar experiments were carried out in the dark.

The development of methods for delivering drugs to the target site with high therapeutic index without causing toxicity to the neighbouring cells has been a hot area of research in the last decade. Nanoparticles have emerged as a popular delivery vehicle due to their non-toxic and non-antigenic properties over the last few years.
S-nitrosoglutathione (GSNO) has been demonstrated previously to kill cancer cells in culture by the release of NO upon cell illumination with visible light (Tannous et al., 1997). However, a delivery method would still be required to deliver the GSNO into the cancer cells in vivo due to the potential loss of NO to the abundant protein thiol in the plasma. I attempted to deliver to the cells a large amount of SNOD, which could trigger a large intracellular influx of NO upon illumination with light.

I prepared defatted BSA nanoparticles, which were readily taken up by normal human fibroblast cells in culture. Loading of these nanoparticles with SNOD or S-dansyloctadecane was successful as confirmed by monitoring the uptake of S-dansyloctadecane loaded rhodamine labelled nanoparticles by fluorescence microscopy. Illumination of the fibroblasts following the particle uptake resulted in 58% cell death by apoptosis. These SNOD-loaded nanoparticles could be employed to deliver NO into the fibroblasts.

Even though these particles showed the potential to kill fibroblasts in culture, the particles had to be modified in such a way to target them into the site of interest, before being utilized in vivo to deliver NO. Therefore, the particles surface was conjugated to SMCC, a heterobifunctional linker to bind to the cell surface thiols by covalent linkage. The SMCC conjugated BSA nanoparticles demonstrated the ability to bind to the surfaces of only 28% of the fibroblasts. 15% of the control cells (surface thiols blocked with iodoacetamide) could also bind the SMCC conjugated BSA nanoparticles. Thus, targeting of the nanoparticles following this method of modification was demonstrated to be successful, but not very efficient.
2.1 Introduction

2.1.1 Synthesis of a coloured nitrite adduct from sinapinic acid and NO$^+$ donors: potential applications

2.1.1.1 Nitrosonium ion (NO$^+$): Nitrosonium ion is the oxidized form of nitric oxide. It is a key species involved in the process of nitrosation, in which the NO$^+$ group is transferred (usually from a carrier compound) to a nucleophilic centre, which often is sulfur or a nitrogen lone pair electrons. NO$^+$ is rapidly hydrolyzed in aqueous solution to give nitrous acid. The equilibrium constant for this reaction is $10^{-6.5}$ at 25$^\circ$C (Williams, 1988; Ridd, 1971) and the lifetime of NO$^+$ in water is about $3\times10^{-10}$s. NO$^+$ is only found in aqueous solution at very high acidity. Nitrosonium cation (NO$^+$) is the active electrophile in the nitrosation of phenols (Williams, 1988) and anilines. It is also an effective oxidant in electron transfer from other electron-rich compounds (Olah et al., 1989).

2.1.1.2 Peroxynitrite: Peroxynitrite is a relatively long-lived toxic compound. In biological systems, peroxynitrite is formed from near the diffusion controlled ($k = 6.7\times10^9$ M$^{-1}$s$^{-1}$) reaction of nitric oxide and superoxide (Huie and Padmaja, 1993). This reaction rate is four times faster than the scavenging of superoxide with copper/zinc superoxide dismutase ($k = 6.7\times10^9$ M$^{-1}$s$^{-1}$). This reaction rate is also higher than the reaction of nitric oxide with ferrous heme compounds ($\sim10^7$ M$^{-1}$s$^{-1}$) (Pryor and Squadrito, 1995). Peroxynitrite was shown to be relatively stable at highly alkaline pH. However, at physiological pH, it becomes protonated and rapidly isomerizes to nitrate.
with a decay constant of 1.3 s\(^{-1}\) (Koppenol et al., 1992). It has been suggested that the decomposition process proceeds through a rate-limiting isomerization reaction, which yields a potent reactive intermediate capable of hydroxylating organic substrates (Van der Vliet et al., 1994a). Inflammatory cells including neutrophils (McCall et al., 1989) and macrophages (Ischiropoulos et al., 1992a) as well as endothelial cells (Kooy and Royal, 1994) can generate superoxide and/or NO simultaneously with the potential of peroxynitrite formation.

Peroxynitrite is capable of oxidizing many biological molecules such as protein and non-protein sulphhydrils, deoxyribonucleic acid, and membrane phospholipids (Radi et al., 1991; King et al., 1992). Peroxynitrite is also capable of causing significant DNA damage, including base modification, sugar oxidation, and strand breaks (Burney et al., 1999). Spectrophotometric determination of dihydrorhodamine 123 oxidation is a sensitive well known method used for the specific detection of peroxynitrite at submicromolar concentrations (Kooy and Royal, 1994).

The reaction of peroxynitrite in biological systems depends on the constituents of the biological environment. In the presence of plasma, proteins, glucose or glutathione, peroxynitrite forms intermediates that act as NO donors (Moro et al., 1994, 1995). Recent studies have shown that CO\(_2\) derived from bicarbonate reacts rapidly with peroxynitrite forming an ONOO^- adduct (Lymar and Hurst, 1995). This adduct rapidly decomposes to CO\(_2\) and nitrate due to the homolytic cleavage of the weak O-O bond (Lymar and Hurst, 1998, Figure 1).
Figure 2.1.1: Peroxynitrite formation and the reaction with CO₂ (Gu, 2002)

Although ONOOCO₂ is a less potent oxidant than peroxynitrite, it is a more powerful nitrating agent than peroxynitrite (Goldstein and Czapski, 1997; Lymar and Hurst, 1998). Homolytic decomposition that leads to the formation of carbonate radical and nitrogen dioxide promotes the nitration reaction.

Peroxynitrite is also capable of nitrating free and protein associated tyrosines and other phenolics via the metal-catalyzed formation of the nitronium ion (Ischiropoulos et al., 1992b). Peroxynitrite has been demonstrated to nitrate tyrosine residues of a variety of proteins (Beckman and Koppenol, 1996; Ye et al., 1996; Beckmann, 1996). Consequently, tyrosine nitration can lead to the inactivation of enzymes and receptors
that depend on tyrosine residues for their activity (e.g. by blocking phosphorylation of key tyrosine residues important for signal transduction etc.) (Ischiropoulos et al., 1992b; Kong et al., 1996; Martin et al., 1990). Since nitrotyrosine is very stable, it can also serve as a marker for the involvement of peroxynitrite in certain pathological conditions. Nitrotyrosine has been detected in human diseases associated with oxidative stress and has been detected in atherosclerosis plaques of human coronary vessels, in acute lung injury, sepsis, and in adult respiratory distress syndrome (Haddad et al., 1994).

Pathological conditions such as the activation of inflammatory cells, sepsis, and reperfusion of ischemic tissue may induce tissues to simultaneously produce superoxide and nitric oxide thus resulting in the formation of peroxynitrite. The presence of peroxynitrite has also been reported in activated alveolar macrophages (Ischiropoulos et al., 1992a). Peroxynitrite has been implicated in many pathophysiological conditions including stroke (Dawson et al., 1991), heart disease (Matheis et al., 1992), and atherosclerosis (Hogg et al., 1993).

Peroxynitrite reacts with glucose and other hydroxylated compounds to produce relatively stable products that can cause tissue or thiol dependent generation of NO and a prolonged relaxation of vascular tissue (Moro et al., 1995; White et al., 1997). However, the vasoactive products formed through the reaction with glucose are apparently significant only at high concentration of peroxynitrite (Dowell and Martin, 1997).

Peroxynitrite induces the depletion of low molecular weight antioxidants, the oxidation of –SH groups and the peroxidation of lipids (Van der Vliet et al., 1994b). Formation of GSNO through the reaction of peroxynitrite and glutathione has been reported earlier (Moro et al., 1994; Mayer et al., 1995b). It has been suggested previously
to be formed by one or two electron oxidations and with the intermediate formation of the thyl radical (Quijano et al., 1997). However, recently GSNO formation has been reported to occur via the direct nucleophilic attack of the thiolate ion on the nitrogen atom of ONOOH along with the removal of HOO· (Van der Vliet et al., 1998).

\[ \text{GS}^- + \text{ONOOh} \rightarrow \text{GSNO} + \text{HOO}^- \]

This mechanism is similar to the S-nitrosation mediated by nitrous acid or alkyl nitrite. S-nitrosation occurs in those cases via the removal of OH· and alkoxide ion (RO·) respectively (Butler and Rhodes, 1997).

2.1.1.3 Nitrosation

The nitrosation reaction introduces a nitroso (-NO) group (Williams, 1988) into an organic molecule leading to the formation of C-nitroso, N-nitroso, O-nitroso, or S-nitroso derivatives of the parent molecule. Commonly used nitrosating agents include HNO₂, N₂O₃, and N₂O₄ (Williams, 1988). Nitrosation is an important pathway in the metabolism of nitric oxide. NO is involved in a variety of physiological and pathophysiological processes. Oxidation of NO generates *NO₂ and N₂O₃ (Figure 2), which can, in turn, cause nitrosation (Figure 3).

\[ 2\ ^*\text{NO} + \text{O}_2 \rightarrow 2\ ^*\text{NO}_2 \]  \hspace{1cm} (1)

\[ ^*\text{NO}_2 + ^*\text{NO} \rightarrow \text{N}_2\text{O}_3 \]  \hspace{1cm} (2)

**Figure 2.1.2:** Oxidation of NO
A significant portion of biosynthesized-NO reacts with molecular oxygen to produce N$_2$O$_3$. N$_2$O$_3$ can then nitrosate biologically relevant thiols like the Cys residues of serum albumin and glutathione to form S-nitrosothiols (RSNOs).

\[ \text{NO}^+ + \text{H}_2\text{O} \rightarrow \text{HNO}_2 + \text{H}^+ \quad (1) \]

\[ \text{RSH} + \text{NO}^+ \rightarrow \text{RSNO} + \text{H}^+ \quad (2) \]

**Figure 2.1.3: Nitrosation**

Nitrosation of DNA bases, thiols, indoles and other biotargets that occurs within nitric oxide producing systems can have pathophysiological consequences in living organisms. Nitrosation of DNA bases leads to oxidative deamination that can consequently cause mutagenesis (Tamir et al., 1995; Merchant et al., 1996). Nitrosation of thiols results in the formation of S-nitrosothiols (RSNO). For example, in a strongly acidic solution, glutathione reacts with NO to form S-nitrosothiol. This reaction can also occur in solutions of lower acidity where the actual nitrosating agent is H$_2$NO$_2^+$. A S-nitrosothiol can transfer NO$^+$ to a second thiol or to another nucleophile. This process is called transnitrosation (Barnett et al., 1994). Even though free NO$^+$ cannot exist in physiological environments, S-nitrosothiols can be viewed as a biological source of NO$^+$ owing to their ability to transfer NO$^+$ (Butler et al., 1995).

RSNO's function as a reservoir for NO, acting to buffer its concentration (Butler et al., 1995). RSNO's are involved in signaling pathways, immune responses and in the actions of nitrovasodilating compounds (Myers et al., 1990; Park and Kostka, 1997;
Ignarro et al., 1981). More recently, we have directly demonstrated the involvement of cell surface protein-disulfide isomerase in the transfer of NO$^+$ from extracellular RSNOs to the cytosolic thiols (Ramachandran et al., 2001).

In acidic media, nitrous acid mediated nitrosation of phenol results in the formation of C-nitroso compounds. These compounds have been demonstrated to form via a neutral dienone intermediate from which proton loss is usually rate limiting. Sometimes, nitrocompounds are isolated as the final product owing to the rapid oxidation of the initially formed nitroso product (Challis and Higgins, 1973).

2.1.1.4 Sinapinic acid and hydroxycinnamic acid derivatives: Sinapinic acid (SA), 3,5-dimethoxy-4-hydroxycinnamic acid, is the most abundant phenolic acid in rapeseeds, including its genetically improved variety, canola. Free SA and its ester constitute 9 and 90% respectively, of the total phenolics in canola meal (Krygier et al., 1982), a by-product of canola seed oil extraction. Among the esters, the most abundant is choline ester, sinapine (SIN) that represents up to 70% of all sinapinic acid esters.

Hydroxycinnamic acid (HCA) derivatives including ferulic acid, sinapinic acid and caffeic acid have been shown to be good scavengers of nitrogen dioxide radical (NO$_2^*$) as demonstrated by a pulse radiolysis study (Zhou et al., 1998). NO$_2^*$ is a strongly oxidizing free radical and a toxic agent. It induces lipid peroxidation and is believed to cause membrane damage and cell death (Pryor and Lightsey, 1981; Pryor et al., 1982). Oxidation of cysteine and tyrosine residues in peptides by NO$_2^*$ leads to the loss of enzyme activity (Forni et al., 1986; Prutz et al., 1985). However the rate constant of oxidation of amino acids by NO$_2^*$ is much lower than the rate constant of the reaction
between NO₂• and sinapinic acid (Zhouen et al., 1998). Therefore, it has been suggested that the HCA derivatives could be potentially utilised as a good protecting agent against NO₂• mediated oxidation of cell membrane and enzymes.

The reaction of sinapinic acid with nitrogen dioxide radical forms phenoxyl radical. The rate constants for the reactions of NO₂• with hydroxycinnamic acid derivatives were calculated to be much higher than those with phenol and p-methoxyphenol (Hue and Neta, 1986). It has been hypothesized that the extended conjugation of HCA derivatives renders them more susceptible for oxidation. Therefore, HCA derivatives had been also demonstrated to be capable of repairing the oxidized hydroxyl radical adducts of deoxyguanosine monophosphate (dGMP) (Jiang et al., 1997).

Sinapinic acid was demonstrated to inhibit peroxynitrite-mediated tyrosine nitration of proteins in vitro (Niwa et al., 1999). The treatment of a protein with peroxynitrite in the presence of sinapinic acid resulted in the formation of a novel product as determined by high performance liquid chromatography. Following purification, the product was identified as a monolactone type dimer of sinapinic acid by nuclear magnetic resonance and liquid chromatography-mass spectrometry (Niwa et al., 1999). SA and a number of SA analogues employed in this study are shown in Figure 4.

2.1.1.5 3-morpholinosydnonimine N-ethyl-carbamide (SIN-1): SIN-1 is commonly used to generate peroxynitrite in situ (Uppu et al., 1996). In solution SIN-1 decomposes to release NO• and O₂•− simultaneously in a 1:1 stoichiometry in the presence of oxygen (Kelm et al., 1997). It also has been demonstrated to react with biological targets in similar way as authentic peroxynitrite (White et al., 1994).
3, 5-dimethoxy-4-hydroxycinnamic acid

3, 5-dimethoxy-4-hydroxyhydrocinnamic acid

3, 4, 5-Trimethoxycinnamic acid

4-hydroxycinnamic acid

3-(3, 4, 5-trimethoxyphenyl)propionic acid

**Figure 2.1.4:** SA and SA analogues employed in this study
2.1.2 Bovine serum albumin nanoparticles as carriers of NO

A major goal in modern drug therapy is the delivery of the drug to a specific site (Kreuter, 1991). Drugs can be targeted to the specific site using different colloidal drug carriers namely, microemulsions, liposomes, niosomes, and nanoparticles.

2.1.2.1 Liposomes: Liposomes are vesicular structures that are encapsulated by one or more phospholipid bilayers. These lipid bilayers are also called lamellae. Liposomes can differ widely in their size (0.02 to 10 μm) and in the number of lamellae depending on the procedure of their preparation (Gregoradis, 1993; Vemuri and Rhodes, 1995; Watev and Bellare, 1995). They are divided into three categories based on their size and lamellarity (number of bilayers), namely small unilamellar vesicles (SUVs) or oligolamellar (OLVs), large unilamellar vesicles (LUVs) and multilamellar vesicles (MLVs). Unilamellar liposomes possess only one phospholipid bilayer whereas multilamellar liposomes have several concentric bilayers that alternate with aqueous layers. Lipid soluble drugs can be incorporated into the lipid layers and water-soluble drugs are entrapped in the aqueous phases.

Liposomes can interact with living cells in one of four different ways: adsorption, endocytosis, lipid exchange and fusion. They have been used as potential drug carriers instead of the conventional dosage forms for several reasons. They have the ability to protect drugs from degradation, target the drug to the site of action and reduce the drug’s toxicity or side effects (Knight, 1981). However, liposomes were demonstrated to have low encapsulation efficiency and poor storage stability. In addition, water-soluble drugs
rapidly leak out of the liposomes in the presence of blood components (Soppimath et al., 2001).

2.1.2.2 Nanoparticles

Nanoparticles are solid colloidal particles, ranging in size from 10 to 1000 nm, consisting of various macromolecules (synthetic or natural). Due to their polymeric nature, nanoparticles are more stable than liposomes in biological fluids and upon storage. Kreuter and Speiser (1976) successfully prepared a polyacrylamide nanoparticles for the first time in the presence of antigens during the development of a vaccine adjuvant.

There are two types of nanoparticles namely, nanospheres (solid framework) and nanocapsules (liquid central cavity surrounded by a wall).

2.1.2.2.1 Nanocapsules: Nanocapsules are vesicular systems in which an aqueous or oily cavity is surrounded by a single polymeric membrane. Nanocapsules may, thus, be considered as a “reservoir system” (Couvreur et al., 1996).

2.1.2.2.2 Nanospheres: Different macromolecules have been used over the years to prepare nanoparticles namely poly(D,L-lactide), poly(lactic acid) PLA, poly(D,L-glycolide) PLG, poly(lactide-co-glycolide), poly(cyanoacrylate) PCA, poly(ε-caprolactone) PCL, poly(alkylcyano-acrylate), PACA, polymethylidene malonate, gelatin, chitosan, sodium alginate and bovine serum albumin (Soppimath et al., 2001). Nanoparticles can be administered through different routes. These include intravenous,
intramuscular, subcutaneous injection, peroral, ophthalmic and transdermal administration (Cappel and Kreuter, 1991).

2.1.2.2.3 Importance of nanoparticles in drug targeting and delivery: Conjugated monoclonal antibodies have previously been utilized to transport anticancer drugs to the target sites (Davis and Baldwin, 1983; Couveur, 1983). Even though drug-antibody conjugates are capable of accumulating in the tumor sites, conjugation can lead to the inactivation of the drug or the antibody. The concentration of the deposited antibody also has to be sufficient in order to achieve the required dose of the therapeutic reagent. The development of an effective drug delivery system has been the focus of many researchers for many years to circumvent these difficulties. One of the most important features of an effective drug delivery system is to ensure high selectivity of the carrier for the desired organ or tissue (Banker et al., 1983; Roll, 1983).

Nanospheres have been demonstrated to be more suitable than liposomes for drug delivery. They can be utilized as novel drug carriers to tissues throughout the body. Therapeutic drugs can be adsorbed, entrapped in the matrix or covalently attached to these particles. They can also be freeze-dried and rehydrated without modification of their size and drug content (Verdun et al., 1986). Their structure permits better retention of the drug inside the polymeric network. Nanospheres are also slowly degraded by esterase (Kante et al., 1982). If designed appropriately, nanospheres may act a drug vehicle capable of targeting tumor tissues or cells. In addition, proper designing of the particles would protect the drug from premature inactivation during its transport.
Both the polymeric composition (type, hydrophobicity, biodegradation profile) of the nanoparticles and the associated drug (molecular weight, charge, localization in the nanospheres: adsorbed or incorporated) have a great influence on the drug distribution pattern in the reticuloendothelial organs (Brigger et al., 2002). The release of drugs from the particle may occur by desorption, diffusion through the nanoparticle matrix or polymer wall or disruption of the nanoparticle following its reception by the target tissue. The rate of antibiotic release from nanospheres correlated very well with the degradation rate of the polymer by esterases. The colloidal carriers can be degraded in endosomes by lysosomal esterases subsequent to their endocytosis by phagocytic cells (Guise et al., 1987). Even though the release of the drug from nanoparticle is low in esterase-free medium it is greatly increased in the presence of carboxyesterases (Lenaerts et al., 1984).

2.1.2.2.4 Designing nanoparticles: Many factors need to be considered during the development of drug containing nanoparticles for successfully delivering them to the target. One of the most important is the carrier capacity of the nanospheres (Kreuter, 1983). Ideal drug carriers should be non-toxic, biodegradable, biocompatible and excreted rapidly from the body (Oppenheim, 1981). A lot of research has been undertaken in the last few decades to evaluate the potential application of biodegradable polymers in colloidal drug delivery system for selective and targeted delivery of various therapeutic agents (Murakami et al., 1997). However, most of these polymers are expensive and utilize toxic solvents in production. A few of these including polylactic acid, polylactic glycolic acid, and polyalkyl cyanoacrylate have been observed to produce in vitro cytotoxic effects (Almeida et al., 1996).
Different materials such as acrylic acid derivatives and poly(lactic acid) have been employed earlier to prepare nanoparticles. Monoclonal antibodies were demonstrated to be well adsorbed onto the surface of acrylic particles, but serum compounds led to a competitive displacement of the drug from the surface (Illum et al., 1983). The charged drugs with ion exchange characteristics bound to the surface of nanoparticles are displaced by the ionic buffer (Langer et al., 1997). An alternative approach that can be used to stabilize the drug binding is to link the compounds to the surface of the carriers covalently. Aqueous carbodiimide and cyanogen bromide reaction has been used previously to crosslink protein to the latex spheres (Molday et al., 1974).

2.1.2.2.5 Protein nanoparticles: To date, bovine serum albumin (BSA) and gelatin are the two different proteins that have been used to prepare protein nanoparticles.

2.1.2.2.5.1 BSA nanoparticles: Akasaka et al. (1988) were the first to prepare bovine serum albumin nanospheres. They were also able to covalently link them with rabbit anti-human IgG by the glutaraldehyde crosslinking method to render them immunospecific. A number of extensive studies have demonstrated that albumin nanoparticles are suitable for drug delivery (Lin et al., 1993; Merodio et al., 2001) since they are biodegradable (Morimoto and Fujimoto, 1985), non-toxic (Rhodes et al., 1969), and non-antigenic (Arshadi, 1990). Moreover, since albumin has a defined primary structure, and a relatively highly charged amino acid content, positive- or negatively-charged molecules could be easily adsorbed electrostatically onto the albumin based nanoparticles. In addition, the preparation of albumin nanoparticles does not require maintaining rigorous
experimental conditions. They can be prepared either by coacervation or controlled desolvation processes. In addition, a variety of different molecules can be incorporated into them (Lin et al., 1993).

The properties of serum albumin and different methods for the preparation of particles have been reviewed recently. Glutaraldehyde is the most commonly used reagent for the crosslinking of the particles. The bifunctional aldehyde reacts with the terminal amine group of lysine (Sokoloski and Royer, 1984) resulting in the crosslinking of albumin. Since the reaction is not stoichiometric, a portion of glutaraldehyde reacts only monovalently thus leaving free aldehyde functional groups on the surface of the particle. These aldehyde groups can be utilized subsequently to couple primary amines onto the surface of the particle (Sokoloski and Royer, 1984; Lubig et al., 1981).

Langer et al. (2000) have demonstrated that the surface of the BSA nanoparticles can be chemically modified following their formation. They were able to successfully introduce thiol groups onto the surface of BSA nanoparticle. The introduced surface groups can be covalently conjugated to avidin through a bifunctional linker that reacts with both avidin and thiol groups. With the use of biotinylated peptide nucleic acid as a model compound for biotinylated drugs, it has been demonstrated that these conjugated nanoparticles could be effectively coupled with the covalently attached avidin through complexation (Langer et al., 2000).

2.1.2.5.2 Gelatin nanoparticles: Gelatin nanoparticle preparation by desolvation process was first reported in 1978 (Marty et al., 1978). Gelatin is one of the basic materials that can be used to produce nanoparticles. The properties of this material vary
with its manufacturing method (acidic or alkaline), its origin (bovine or pig), the type and number of amino acids and molecular weight. Gelatin is suitable for nanoparticle preparation due to its possession of low antigenicity (Schwick and Heide, 1979).

2.1.2.2.6 Conventional nanoparticles: Conventional nanoparticles are nanoparticles lacking any surface modification. Drugs loaded in conventional nanoparticles are mainly delivered to the mononuclear phagocytes system (MPS) (liver, spleen, lungs and bone marrow). They are rapidly taken up by the reticuloendothelial system (RES) and massively cleared by the fixed macrophages of the MPS organs namely liver, spleen, lung etc. after entering the bloodstream (Grislain et al., 1983). As a consequence of this rapid removal by the RES, less than 1% of the injected dose stays in the blood circulation after 15 to 30 minutes. Both the polymeric composition (type, hydrophobicity, biodegradation profile) of the nanoparticles and the associated drug (molecular weight, charge, localization in the nanospheres: adsorbed or incorporated) have a great influence on the drug distribution pattern in the reticuloendothelial organs (Couvrer et al., 1980). The contribution of conventional nanoparticles to the enhancement of anticancer drugs efficacy is limited to targeting tumors at the level of MPS organs. Owing to their very short circulation time, targeting of nanoparticles to other tumoral tissues is not feasible. The mean half-life of conventional nanoparticles is 3-5 min after intravenous administration.

2.1.2.2.7 Surface modified nanoparticles: Coating the particles with surfactants or surface modification of nanoparticles with polyethylene glycol (PEG) can reduce the
uptake of the particles by reticuloendothelial organs. Moreover, the blood circulation
time of surface modified nanoparticles is also considerably increased. Two methods are
mainly used to modify the surface of biodegradable and long-circulating polymeric
nanoparticles: i) surface coating with hydrophilic polymers/surfactants; and ii)
development of biodegradable copolymer with hydrophilic segments. Some of the widely
used surface-coating materials are: PEG, polyethylene oxide (PEO), poloxamer,
poloxamine, polysorbate (Tween-80) and lauryl ethers (Brij-35). These coatings provide
a dynamic cloud of hydrophilic and neutral chains at the particle surface that repel plasma
proteins as modeled by a group of researchers (Jeon et al., 1991; Jeon and Andrade,

2.1.2.2.7.1 Modification with PEG: The formation of long circulating nanoparticles by
modifying the surface with hydrophilic, flexible and non-ionic polymers, such as PEG
has been reported earlier (Tobio et al., 1998; Quellec et al., 1998). Poly(lactide)-poly-
(ethylene glycol) nanoparticles can be coated with PEG. Coating with PEG prevents the
uptake of the particles by the mononuclear phagocyte system thereby increasing their
half-life in the blood. They are more efficient in controlled delivery of the
pharmacological agents to its target (Perachhia et al., 1999; Torchilin, 1998).
Nanoparticles prepared from the blends of poly(lactic-co-glycolic acid)-polyoxyethylene
and diblock copolymers of poly(lactic-co-glycolic acid)-polyoxyethylene showed a
prolonged half-life of these nanoparticles in the blood (Gref et al., 1993).
2.1.2.7.2 Polysorbate-coated nanoparticles: It has been a challenge to target drugs to the brain because of the blood brain barrier. The tight endothelial cell junctions of the capillaries within the brain form the blood brain barrier. This barrier limits the ability of many drugs to penetrate into the brain tissue in order to enter the central nervous system. However, once the anti-inflammatory drugs that act on the central nervous system (CNS) were delivered in one study through surface modified nanoparticles, the blood brain barrier could easily be passed (Schroeder and Sabel, 1996; Schroeder et al., 1998). An increased permeability of the drug through the biological membrane was also observed, when it was delivered by polysorbate-80 coated nanoparticles (Cavallaro et al., 1994).

2.1.2.8 Delivery of antisense RNA

Antisense oligonucleotides are synthetic fragments of ribo- and deoxyribonucleic acids that recognize and bind specifically to the complementary sequence of a gene or its messenger RNA. Their hybridisation is believed to interfere with the processing, transport and/or translation of the mRNA. The elicited target mRNA degradation results in the inhibition of the target gene expression. It has been demonstrated previously that these oligonucleotides could be potentially utilised as therapeutic agents to control the expression of deleterious protein associated with viral, neoplastic or other diseases (Crooke, 1999). The low efficiency of oligonucleotide-based therapies is due to their instability in biological fluids (e.g. fast degradation by ubiquitous exo- and endonucleases). Since the penetration of these short nucleotide fragments is hindered by their negative charges, their therapeutic efficacies are reduced (Juliano et al., 1998).
To circumvent these difficulties, recent research has focused on designing carriers to deliver oligonucleotides to the target site. A carrier is expected to provide protection from enzymatic hydrolysis and prevent protein binding, thus allowing targeting of the drug to specific cells or tissues and ensuring improved therapeutic index (Juliano et al., 1999). When delivered through cyanoacrylate nanoparticles, 100-fold lower concentration of the antisense nucleotide targeted against ras oncogene in HBL100 cells was required to achieve the tumor growth inhibition. The antisense nucleotides were ion-paired with a cationic hydrophobic detergent, leading to the formation of a hydrophobic complex. This complex was then adsorbed onto the hydrophobic and negatively charged surfaces of cyanoacrylate nanosphere (Schwab et al., 1994).

2.1.2.2.9 Nanoparticle as the carrier for drugs: Nanoparticles have been demonstrated to be useful in delivering drugs to the target tissue. Recently, Santhi et al. (2000) have shown that a versatile anticancer drug, methotrexate, could be loaded onto BSA nanoparticles. Approximately 40 mg of the drug could be loaded onto per 125 mg of the particles. In vitro studies showed a burst release of the drug within 15 min. Up to 50% of the total drugs was released within the first 12 hours whereas the remaining drug was released very slowly over 24 hours period. The total percentage of the methotrexate release was 90% (Santhi et al., 2000).

2.1.2.2.10 Targeting tumors: Nanoparticles have been shown to accumulate in a number of tumors. Leakiness of the tumor blood vessels, and a possible higher endocytotoxic activity of tumor associated cells in the blood vessels or in their vicinity are believed to
be responsible for promoting the accumulation of nanoparticles within the tumor (Grislain et al., 1983; Gipps, et al., 1986).

2.1.2.11 Other uses of nanoparticles: Nanoparticles can be used for qualitative or quantitative in vitro detection of tumor cells. They help in the detection process by concentrating and protecting a marker from degradation thus rendering the analysis more sensitive. For example, streptavidin-coated fluorescent polystyrene nanospheres Fluospheres (green fluorescence) and TransFluospheres (red fluorescence) were used in single colour flow cytometry to detect the epidermal growth factor receptor (EGFR) on A431 cells (human epidermoid carcinoma cells) (Bhalgat et al., 1998). The fluorescent nanospheres demonstrated 25-fold higher sensitivity compared to streptavidin-fluorescein conjugate. The encapsulation of fluorescent markers resulted in objects that were brighter and more concentrated than when simple conjugates of single dyes were used. The coating of nanoparticles by surfactants can also be used to monitor the uptake of the particles into the solid tumors (Beck et al., 1993) and into blood macrophages (Schäfer et al., 1992)

2.1.2.12 Advantages of using nanoparticles in drug targeting: The major advantage of colloidal drug carriers is the possibility of drug targeting by a modified body distribution (Kreuter, 1983) as well as the enhancement of cellular uptake of a number of substances (Schäfer et al., 1992). Undesired toxic side effects of the free drug can be avoided by using nanoparticle to deliver the drug (Narayani and Rao, 1993).
Nanoparticles were demonstrated to enable an enhanced uptake of important drugs namely anti-HIV drugs into a number of cells, especially macrophages (Schäfer et al., 1994; Bender et al., 1994). These macrophages serve as important reservoir for HIV and play a detrimental role during the progression of AIDS (Orenstein et al., 1997).

The crosslinking reaction of succinamyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), a heter bifunctional linker, used to to link to the surface of the BSA nanoparticles employed in this study is shown in figure 5.
Figure 2.1.5: The heterofunctional linkage reaction between succinimidyld 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), a primary amine (R-NH₂) and a thiol (R'-SH)
2.2 Purpose of study

2.2.1 Synthesis of a coloured nitrite adduct from sinapinic acid and NO$^+$ donors:

potential applications

Peroxynitrite is a major cytotoxic agent produced in many pathological conditions. Therefore, compounds that can selectively and rapidly react with peroxynitrite are in demand as they could potentially be used in the biological milieu to protect against peroxynitrite-induced toxicity.

The goal of this study was twofold: first to investigate whether sinapinic acid (SA) could be used for the colourimetric detection of NO$^+$ in biological systems or in vitro; and second to test whether SA can be used to prevent peroxynitrite-induced oxidation of dihydrorhodamine which is frequently used for assaying the formation of cell and tissue derived peroxynitrite.

2.2.2 Bovine serum albumin nanoparticles as carriers of NO

In previous studies in our laboratory we reported that the death of cancer cells in culture could be induced by irradiation with visible light of the $S$-nitrosoderivative of apo-metallothionein, thionein-NO (T-NO) (Tannous et al., 1997). However, T-NO is difficult to prepare owing to the fact that it is prone to polymerization through its many free thiols. In addition T-NO would readily lose its NO via transnitrosation and $S$-thiolation reactions in plasma.

We therefore decided to pursue alternative methods for packaging, carrying and delivering large amounts of RSNOs to cells for their subsequent photoactivated release.
Therefore, we intended to assess the potential of using defatted BSA nanoparticles as carriers of S-nitrosolipids, which can be easily prepared from inexpensive thiolipids like 1-octadecanethiol.

The second aim of these studies was to determine whether the photolysis of the lipid-SNO:BSA nanoparticle complexes could induce N₂O₃ mediated S-nitrosation in hydrophobic environments and whether this could induce death in cells.
2.3 Chemicals, supplies and instruments employed

2.3.1 Chemicals and supplies

2.3.1.1 Synthesis of a coloured nitrite adduct from sinapinic acid and NO⁺ donors:

potential applications

Glutathione, Sinapinic acid (SA), 4-hydroxy cinnamic acid, 3,4,5-trimethoxycinnamic acid, 3,4,5-trimethoxy hydro cinnamic acid, 3-(3,4,5-trimethoxyphenyl)propionic acid, bovine serum albumin, sodium nitrite, nitrosonium tetrafluoroborate (BF₄NO), Sodium phosphate (dibasic), tryptophan, and tyrosine -Sigma-Aldrich (St. Louis Mo).

DHR-6G was purchased from Molecular Probes (Eugene, OR)

Hydrochloric acid, acetonitrile- EM Industries Canada Inc. (Brampton, ON)

Methanol, Acetic acid- BDH Inc. (Toronto, ON)

2.3.1.2 Bovine serum albumin nanoparticles as carriers of NO

Bovine serum albumin (BSA), 25% aqueous glutaraldehyde solution, 1-octadecane thiol, Lissamine rhodamine B sulfonyl chloride (LRSC), sodium chloride, potassium sodium bicarbonate, dansyl chloride, 95% anhydrous hexane, succinimidy1-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC), Sephadex G-25, iodoacetamide, Neocuproine -Sigma-Aldrich

Activated charcoal - Norit® SX plus

Chloroform-BDH Inc. (Toronto, ON)

Dimethyl sulfoxide – Fisher Scientific (Toronto, ON)
N, N-dimethylformamide (DMF) – Caledon (Georgetown, ON)

Methanol, Ethanol- EM science Merck KgaA (Darmstadt, Germany)

Triethylamine, n-propanol – Anachemia Chemical Ltd. (Montreal, CA)

Normal human fibroblast - ATCC cultures (Manassas, VA)

Dublico’s minimal medium (DMEM), heat inactivated fetal bovine serum (FBS),
antibiotic antifungal reagent - Gibco-BRL (Burlington, ON)

Petri-dishes, culture flasks, sterile disposable pipettes, syringe filters, Cryovials -
SARSTEDT Inc. (Montreal, Quebec, Canada)

Dialysis Bag, Molecular cutoff (MCO) 4000-6000 – Spectrum Laboratories (Toronto,
ON)
2.3.2 Instruments employed

2.3.2.1 Synthesis of a coloured nitrite adduct from sinapinic acid and NO⁺ donors: potential applications

Agilent 8453 UV-visible spectrometer – Agilent Technologies Inc. (Palo Alto, CA)

Spectra Max Gemini XS Multiplate Reader Spectrophotometer -Molecular Devices (Toronto, ON)

Orion 420A model pH meter-VWR Scientific (Mississauga, ON)

Mettler AJ 100 balance- Mettler Instrument Inc. (Highstown, NJ)

LABCONCO freeze dryer 3- VWR Scientific (Mississauga, ON)

2.3.2.2 Bovine serum albumin nanoparticles as carriers of NO

Zeiss Axivert 200 microscope, Empix (Mississauga, ON)

Benchtop centrifuge, non refrigerated EBA 21- Hettich Zentrifugen (Germany)

VWR stirrer-VWR scientific (Mississauga, ON)

Sonicator- Transonic T420, Mandel Scientific (Toronto, ON)

Autoflow CO₂ water-jacketed incubator-NUAIRE-Scientific surplus (Plymouth, MN)

NUAIRE biological safety cabinets-class II type- NUAIRE-Scientific surplus (Plymouth, MN)
2.4 Methods

2.4.1 Synthesis of a coloured nitrite adduct from sinapinic acid and NO$^+$ donors: potential applications

2.4.1.1 Synthesis of peroxynitrite: Peroxynitrite was synthesized in a quenched-flow reactor as described by Beckman et al. (1990). Solutions of (i) 0.6 M NaNO$_2$ and (ii) 0.6M HCl/0.7 M H$_2$O$_2$ were pumped at 26 ml/min into a tee-junction and mixed in a 3-mm diameter by 2.5-cm glass tube. The acid catalyzed reaction of nitrous acid with H$_2$O$_2$ to form peroxynitrous acid was quenched by pumping 1.5 M NaOH at the same rate into a second tee-junction at the end of the glass tubing. Excess H$_2$O$_2$ was removed by passage of peroxynitrite solution over MnO$_2$ powder. The solution was frozen at $-20^\circ$C for up to one week. The concentration of peroxynitrite was determined spectrophotometrically at 302 nm ($e = 1670$ M$^{-1}$Cm$^{-1}$) (Hughes and Niklin, 1968).

2.4.1.2 Synthesis of dihydroSA: DihydroSA was a gift from Dr. Jim Green, University of Windsor. It was synthesized according to the procedure described earlier (Smith et al., 2000)

2.4.1.3 Kinetic studies of the peroxynitrite reactions: SA was dissolved in acetonitrile and diluted in 0.1 M sodium phosphate buffer pH 7.4 to get a final concentration of 10 $\mu$M. (final conc. of acetonitrile was 1%) in a cuvette. The reaction was started by the addition of 100 $\mu$M peroxynitrite. Time-dependent spectra were monitored immediately after the addition of peroxynitrite.
2.4.1.4 Reaction of SA in acidified nitrite: SA was dissolved in 90% acetonitrile 0.1 M HCl/10% acetic acid to a final concentration of $2.67 \times 10^{-4}$ M. SA solution in 60% ACN 10% acetic acid was prepared in the same way to a final concentration of $5.35 \times 10^{-4}$ M. The reaction was initiated by the addition of equimolar sodium nitrite. The UV/vis spectrum of the solution was monitored as a function of time.

2.4.1.5 Assay of SIN-1 mediated oxidation of dihydrorhodamine: Stock solutions were freshly prepared (1 mM SIN-1 in phosphate buffer; 10 mM SA in acetonitrile; 5 mM dihydrorhodamine (DHR) 6G and 5 mM rhodamine 6G in DMF), and diluted using phosphate buffer (100 mM, pH 7.4) to the desired concentrations in a total volume of 200 µl. Concentrations of SIN-1 and DHR-6G were fixed at 20 µM and 5 µM respectively. Readings were taken in a multiplate fluorescence reader in triplicate and averaged. Data were normalized by setting the value of fluorescence intensity of rhodamine-6G to 100 %, and that of DHR-6G at time zero to 0%. Excitation and emission wavelengths were set at 528 nm and 554 nm, respectively. These reactions were carried out in air-equilibrated solutions under covered condition. The chemicals were added in the order of DHR, phosphate buffer, SA, and lastly SIN-1 to initiate reaction. Percentage of all the organic solvents in the final mixture was in the range of 0.3% to 0.1% (for acetonitrile it was less than 0.1% and for DMF it was less than 0.2%).

2.4.1.6 O-nitrosoSA formation from GSNO and SA: GSNO solutions (0.3 to 1.5 µM) were incubated with 0.5 mM SA for 15 min. The absorption spectra of the mixtures were recorded at $25^\circ$C within the wavelength range of 450-600 nm after the incubation period.
2.4.1.7 Isolation of the red product: 0.2 g SA was dissolved in 15 ml methanol and diluted in 125 ml of 0.1 M acetic acid pH 2. 0.11 g Sodium nitrite was added to the SA solution followed by the addition of 4.8 ml of 2M HCl. The reaction was instantaneous. The pH of the reaction mixture was adjusted back to 7.4 with 2M NaOH after the completion of the reaction and the reaction mixture was freeze dried immediately and stored at -80°C.

2.4.1.8 Spectroscopic measurement of S-nitrosoBSA (BSA-SNO): 375 μM BSA solution was prepared in phosphate-buffered saline, pH 7.4. Freeze-dried neutral red product (O-nitroSO-SA) was dissolved in minimal volume of methanol and added in equimolar amounts to the BSA solution. The reaction mixture was incubated at room temperature for 35 min under stirring. At the end of the incubation period the product was purified using Sephadex G-25 column pre-equilibrated with the same buffer.

2.4.2 Bovine serum albumin nanoparticles as carriers of NO

2.4.2.1 Synthesis of S-nitrosooctadecane (SNOD) thiol: 0.38 M 1-octadecanethiol solution in chloroform or hexane was reacted with equimolar tertiary butyl nitrite at room temperature. The concentration of SNOD formed was determined by measuring the absorbance at 340 nm (ε_{340nm} = 980 M⁻¹cm⁻¹) (Wood et al., 1996).

2.4.2.2 Removal of fatty acid from bovine serum albumin (BSA): 5 g of bovine serum albumin was dissolved in 50 ml distilled water at room temperature. 2.5 g of activated charcoal was added to the BSA solution and mixed thoroughly by stirring and the pH of
the solution was adjusted to 3 by the addition of 0.2M HCl. The mixture was stirred for one hour in an ice-bath. The charcoal was removed by centrifugation at 20,000xg for 20 min. The pH of the supernatant was then adjusted back to 7 by the addition of 0.2M NaOH. The defatted BSA solution (supernatant) then obtained was freeze-dried and stored at 4°C.

2.4.2.3 Synthesis of dansylated bovine serum albumin (Dns-BSA): 0.5 g BSA was dissolved in 10 ml of freshly made 0.1 M NaHCO₃ buffer pH 9. 10 mg dansyl chloride were dissolved in 2 ml acetone and added to the BSA solution in dropwise under constant stirring. The reaction was performed for 4 hours at 4°C under stirring at moderate speed. Following completion of the reaction, the product was dialyzed (dialysis bag, molecular cutoff, 6000-8000) with 0.01 M NaHPO₄ pH 7.4 at least three times to remove the excess reactants. The product then obtained was freeze dried and stored at 4°C.

2.4.2.4 Conjugation of lissamine rhodamine B-sulfonyl chloride (LRSC) to BSA: 0.3 g BSA was dissolved in 6 ml of 0.1M NaHCO₃ buffer pH 9. Five-fold molar excess of LRSC (10 mg/ml stock solution in DMF) was added in aliquots to the BSA solution under stirring. The reaction was performed for 2 hours in an ice-bath in the dark. After completion of the reaction, the product was immediately purified using Sephadex G-25 column. Conjugation of the BSA with LRSC was confirmed by measuring the absorbance of the eluted sample both at 280 nm and 575 nm (Schreiber and Haimovich, 1983). The purified product was freeze-dried immediately and stored at -80°C.
2.4.2.5 Synthesis of S-dansyloctadecane (Dns-Octadecane): 1-octadecane thiol was dissolved in chloroform. Approximately, 2-3 drops of triethylamine were added to the 1-octadecanethiol solution to make the solution basic. The reaction was started by the addition of equimolar dansyl chloride (DnSCl) to the 1-octadecanethiol solution. The reaction was carried out at room temperature under moderate stirring overnight. At the end of the reaction period, the product, which was formed in the chloroform layer, was washed with water at least three times successively using a separatory funnel. The purity of the product in the chloroform layer following washing was confirmed by thin layer chromatography using n-propanol: chloroform: water (60:30:10) as a mobile phase.

2.4.2.6 Cell Culture: Human foreskin fibroblasts were cultured in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, and antibiotic antymycotic reagent and maintained in a humidified incubator under 5% CO₂ at 37°C.

2.4.2.7 Subculturing/freezing: When the fibroblasts became confluent, the media was removed and the cells were detached from the flask by treating with trypsin-EDTA (0.25% trypsin, 1 mM EDTA) for 30 seconds. Following trypsinization, the cells were resuspended in fresh media and were aliquoted into petri dishes in desired concentrations depending on the experiment. If the cells were required to be stored for a long time for later use, the cells were resuspended in 95% complete media (DMEM, 10% fetal bovine serum, antibiotic antymycotic) and 5% DMSO and stored in cryovials at −80°C.
2.4.2.8 *Preparation of liposomes from SNOD:* 50 μl 0.35 M S-nitrosooctadecane solution in chloroform was sonicated in the presence of 1 ml 1x PBS for 5 minutes. The liposome suspension formed in the PBS layer was transferred to another vial and neocuproine solution (10 mM stock solution in phosphate buffer) was added to the liposome suspensions immediately to a final concentration of 0.1 mM.

2.4.2.9 *Preparation of the defatted BSA nanoparticles:* Nanoparticles were prepared according to the procedure described by Langer *et al.*, (2000) with minor adjustments in the concentration of BSA and glutaraldehyde. Briefly, 200 mg defatted bovine serum albumin was dissolved in 1 ml of 0.1 M Na₂HPO₄ pH 7.4. Desolvation of the 5% (w/v) defatted BSA solution was achieved by dropwise addition of 2 ml anhydrous ethanol under continuous stirring until the solution became turbid. The BSA nanoparticles so formed were crosslinked by adding 0.1% aqueous glutaraldehyde solution under stirring. Crosslinking was performed for 2-3 hrs under stirring at room temperature. Following crosslinking, the reaction mixture was centrifuged at 15,000 rpm at 25°C for 10 min, and the supernatant was removed. The nanoparticle pellet then obtained was washed three times by threefold centrifugation and redispersion in water at 15,000 rpm. Following final wash, the nanoparticles were resuspended in PBS prior to the experiment. Nanoparticles were prepared fresh prior to each experiment.

2.4.2.10 *Determination of nanoparticle size:* An aliquot of the BSA nanoparticle suspension in PBS was spread over a microscope slide and imaged under a fluorescence
microscope. The diameter of all the spheres in each field was calculated with the aid of
Zeiss Axiovert 200/Empix Northern Exposure imaging software.

2.4.2.11 Labelling of BSA nanoparticles with Dns-Cl: In order to label the
nanoparticles, dansyl chloride-conjugated, defatted BSA was dissolved in phosphate
buffer at pH 7.4. The solution was desolvated and the particles were crosslinked
following the same procedure described previously (section-2.4.9). Following
crosslinking, the particles were washed at least three times as described earlier (section-
2.4.2.9) and were viewed under a fluorescence microscope.

2.4.2.12 BSA nanoparticle loading with S-dansyloctadecane/S-nitrosooctadecane: S-
dansyloctadecane/SNOD was loaded into the BSA nanoparticles by following two
different procedures.

In the first method, S-dansyloctadecane/SNOD was added to the BSA solution
and stirred for roughly 10 min. Excess lipid was removed by passing the mixture through
a Sephadex G-25 column pre-equilibrated with 0.1 M Na₂HPO₄ pH 7.4. The sample was
eluted with 0.1 M Na₂HPO₄ pH 7.4. The equilibration buffer and elution buffer both
contained 0.1 mM neocuproine, while SNOD was loaded instead of S-dansyloctadecane.
The purified BSA-S-dansyloctadecane/BSA-SNOD solution obtained from the Sephadex
G-25 column was subsequently used to prepare the nanoparticles following the procedure
described above.

In the second method, S-dansyloctadecane/SNOD was added following the
desolvation of BSA containing 1% rhodamine conjugated BSA solution with ethanol
under stirring. The mixture was then stirred for an additional 10 minutes. The nanoparticles were crosslinked with glutaraldehyde following the same procedure described above. Excess S-dansyloctadecane/SNOD was removed by three-fold centrifugation and redispersion in water.

2.4.2.13 SNOD mediated effect on GSH upon exposure to light

*In the presence of oxygen:* An aqueous solution of 0.7 mM GSH and 10 mM S-nitrosooctadecane solution in hexane was taken into a quartz cuvette and the content of the cuvette was saturated with oxygen by bubbling O₂ through the solution for 5 minutes. The SNOD containing hexane layer was then irradiated with 100 pulses (50 mJ/shot) of a Nd:YAG laser. Following irradiation, the content of the cuvette was sonicated for less than 30 seconds and the aqueous layer was separated by centrifugation. The UV/vis spectrum of the aqueous layer was taken.

*In the presence of oxygen and α-tocopherol:* Experimental setup in this experiment was the same as described above, except that in this case the hexane layer also contained 8.6 mM α-tocopherol. The content of the cuvette was saturated with oxygen as before and the rest of the procedure was followed exactly as described above.

*In the presence of nitrogen:* Another experiment was performed in which the cuvette containing SNOD solution in hexane and aqueous solution of GSH, was saturated with nitrogen instead of oxygen by bubbling N₂ through the contents of the solution for 5 minutes. The hexane layer containing SNOD was then irradiated with the laser as described above. The rest of the procedure was followed exactly as described above.
2.4.2.14 *Effect of S-nitrosooctadecane on GSH in the absence of light:* A 1 mM aqueous GSH solution containing 0.1 mM neocuproine, a Cu$^{1+}$ chelator, was incubated with equimolar SNOD liposomes in 1x PBS at room temperature under constant stirring. The reaction was performed for 180 minutes in the dark. An aliquot of the mixed suspension was removed after 180 min and the spectrum was taken.

2.4.2.15 *Dansyl labelled BSA (Dns-BSA) nanoparticle uptake by normal human fibroblast (NHF):* Freshly subcultured NHF cells were grown overnight in medium petri dishes to allow for cell attachment. 200 µl Dns-BSA nanoparticle dispersion in 1x PBS was added to the cells in the medium and was incubated for 5 hours. At the end of the incubation period, the medium was removed and the cells were washed three times with fresh media. The cells were then viewed and imaged under a fluorescence microscope in 1x PBS.

2.4.2.16 *Delivery of SNOD using BSA nanoparticles:* Freshly subcultured NHF cells were grown overnight in medium petri dishes to allow for cell attachment. Cells were then incubated with 200 µl SNOD-loaded BSA nanoparticle suspension (in 1x PBS) for 5 hours at 37°C in the presence of 5% CO$_2$. During that incubation period the petri dishes were taken out and shaken gently at 30 min intervals. At the end of the incubation period, the excess nanoparticles were removed by washing the cells three times with fresh media. Following the wash, the cells were exposed to visible light (27.15Mw/cm$^2$) in PBS for 15 min. After exposure to light, PBS was replaced with fresh media and the cells were
incubated further for another 24 hrs at 37°C in the presence of 5% CO₂. At the end of the incubation, the cells were stained with Hoechst dye and imaged under the fluorescence microscope.

In the control experiment, rhodamine-labelled BSA nanoparticles were used instead of SNOD loaded BSA nanoparticles.

2.4.2.17 Hoechst staining: Hoechst dye was added to the media of the cells to a final concentration of 10 μg/ml and incubated for 10 minutes at room temperature. Following incubation, the image of the cells was captured by a fluorescence microscope.

2.4.2.18 Surface modification of BSA (defatted) nanoparticles: BSA nanoparticles were prepared according to the procedure described before (section-2.4.2.9). LRBC solution (1mg/ml in DMF) was added to the desolvated BSA solution to a final concentration of 20 μg/ml prior to glutaraldehyde crosslinking to label the nanoparticles. Glutaraldehyde mainly reacts with ε-amino group of lysine in proteins. Some free amino groups were reported to remain on the surface of the BSA nanoparticles subsequent to the particle formation due to the low molar ratio of glutaraldehyde to lysine used to crosslink the particles. These remaining groups could be utilized to link to other compounds (Langer et al., 2000). Following purification, the nanoparticles were reacted with 100-fold molar excess of SMCC in 0.1M NaHCO₃ buffer pH 9 for an hour at room temperature. Excess SMCC was removed by threefold centrifugation of the nanoparticle suspension and redispersion in 1x PBS pH 7.4.
2.4.2.19 Targeting the nanoparticles to the cell surface: Semiconfluent NHF cells were incubated with 300 µl SMCC-conjugated, rhodamine-labelled BSA nanoparticle suspension (in 1x PBS) in the medium for 3 hours at 37°C in the presence of 5% CO₂. At the end of incubation, excess particles were removed, and cells were washed extensively with 1x PBS pH 7.4, and images of the cells were captured using a fluorescence microscope.

For a negative control, NHF cells grown in another petri dish was treated with 10 mM iodoacetamide in 1x PBS at pH 8 for 15 minutes at 37°C in the presence of 5% CO₂ to block their surface thiols. Following incubation, the excess iodoacetamide was removed, and the cells were washed three times with fresh medium. The cells were then treated with SMCC conjugated rhodamine labelled BSA nanoparticles as described above. The cells were imaged following the removal of excess SMCC by washing with 1x PBS.
2.5 RESULTS

2.5.1 Synthesis of a coloured nitrite adduct from sinapinic acid and NO$^+$ donors: potential applications

2.5.1.1 Reaction of peroxynitrite with SA: Reaction of SA (10 μM) with 10-fold molar excess of peroxynitrite in 0.1M phosphate buffer at pH 7.4 resulted in the formation of a red compound. The formation of the product was characterized by appearance of a 532 nm peak (Figure1). The second-order rate constant extracted from the data performed under the pseudo-first order experimental condition was estimated to be 812 M$^{-1}$s$^{-1}$. The extinction coefficient of the red product was found to be 2074.5 M$^{-1}$cm$^{-1}$ at 532 nm under these experimental conditions. Decomposed peroxynitrite also resulted in identical product formation.

2.5.1.2 Acidified nitrite also forms a red compound with SA: UV/vis spectra were recorded following the mixing of equimolar amounts of SA (5.35 x 10$^{-4}$ M) and sodium nitrite in 60% acetonitrile (ACN) and 10% acetic acid as a function of time. The spectra were characterized by time-dependent increase of 490 nm and 520 nm absorbance peaks and a concomitant ~ 40 nm red shift and decrease of the SA absorbance peak at 324 nm (Figure 2). The formation of the 520 nm peak was well fitted to a first order process. The rate constant of the reaction was determined to be 0.0004 s$^{-1}$. The extinction coefficient of the 510 nm peak was estimated to be 8419 M$^{-1}$cm$^{-1}$ (in 90% ACN 10% acetic acid).
Figure 2.5.1: Assay of peroxynitrite with SA. 100μM peroxynitrite solution was added to 10 μM SA in 0.1 M sodium phosphate buffer pH 7.4. The spectra were recorded immediately after the addition of peroxynitrite solution over the wavelength region between 200 to 650 nm as a function of time. Final spectrum of the red product is plotted.
Figure 2.5.2: Time course of O-nitrosoSA formation in the presence of acetic acid. Reaction of $5.35 \times 10^{-4}$M SA with equimolar NaNO$_2$ in 60% acetonitrile and 10% acetic acid. Spectra were taken at 7, 60, 240, 900, 1500, 2400, 3000, 4000, 4600, 5400 and 6600s.
In order to further examine the reactivity of sodium nitrite towards SA, the reaction was carried out in 0.1 M HCl instead of 10% acetic acid. Reaction of SA under these conditions with equimolar nitrite led to the formation of an identical red product as evidenced by the growth of a peak at 454 nm along with a decrease of SA peak at 323 nm as a function of time (Figure 3). The rate constant was determined to be 8.32 M$^{-1}$ s$^{-1}$ in the presence of HCl.

There is a possibility that the shift observed for the absorption maximum from 532 nm (in the presence of 0.1 M Na$_2$HPO$_4$ pH 7.4) to 520 nm (in the presence of 10% acetic acid 60% acetonitrile) to 454 nm could be due to the differences in the solvent system. To check for that possibility, an aliquot (50 μL) of the product formed through the reaction of SA with acidified (10% acetic acid/0.1M HCl) nitrite was immediately transferred into two different cuvettes containing 0.1M Na$_2$HPO$_4$ buffer pH 7.4. As expected, both products exhibited peaks at ~ 532 nm in the phosphate buffer (Figure 4). In order to further investigate whether this observed effect was due to the change in pH or due to the presence of a different solvent, I conducted two different experiments. In one experiment I transferred the products to a solution of 10% acetic acid, while in the other experiment the products were transferred to 0.1 M NaHCO$_3$ buffer pH 9. In both cases the absorption peaks were observed at ~ 532 nm (Figure 4). On the other hand, when the product was transferred to a cuvette containing 100% ACN, the absorption peak was observed at 510 nm (Figure 4).
Figure 2.5.3: The spectrum of the red product after 300 s (at completion) of reaction between $2.67 \times 10^{-4}$M SA in 90% acetonitrile 0.1M HCl and equimolar sodium nitrite (A). Time-dependent increase of O-nitrosoSA formation from $2.67 \times 10^{-4}$M SA in 90% acetonitrile 0.1M HCl upon addition of equimolar sodium nitrite (B).
Figure 2.5.4: Spectra of O-nitrosoSA under different solvent conditions. Spectra of S-nitrosoSA in the presence of (Δ) 100% acetonitrile (□) 0.1 M Na₂HPO₄ pH 7.4/0.1 M NaHCO₃ pH 9/10% acetic acid
2.5.1.3 Characterization of the red compound: There have been several suggestions in the literature that the reaction of aryloxide ion with alkyl nitrites always occurs through the oxygen atom to yield an unstable O-nitrosocompound. This intermediate is likely to undergo an internal rearrangement of the NO group to give the corresponding C-nitrosoprod.., competing with homolysis of the O-NO bond to yield nitric oxide. Phenols are ambient nucleophiles and their reaction with the nitroso group can also take place at the oxygen atom. Challis and Higgins (1973) suggested that the nitrosation of the 2-naptholate ion at pH <5 occurs by reversible formation of an unstable aryl nitrite. This same species has been proposed as an intermediate in the nitrous acid catalyzed nitration of phenols (Leis et al, 1998).

To explore the possibility that SA might react with peroxy..itrite or acidified nitrite according to the proposed mechanism, another set of experiments was designed using analogues of SA. Sodium nitrite was added to 4-hydroxycinnamic acid, 3,4,5-trimethoxycinnamic acid, 3,4,5-trimethoxyhydrocinnamic acid, dihydroSA or 3-(3,4,5-trimethoxyphenyl) propionic acid solution in 60% ACN 10% acetic acid instead of SA and spectra were recorded immediately after the addition of equimolar sodium nitrite. Among the SA analogues used, only dihydroSA gave rise to an identical peak characteristic of the red compound upon reaction with sodium nitrite under the conditions used. These results indicate the involvement of –OH group of SA in the reaction. These results further indicate that the methoxy groups in the two ortho positions and the side carbon chain in para position of the SA are required for the nitrite adduct formation.
2.5.1.4 Evidence for involvement of NO$^+$ in the O-nitrososinapinic acid formation: In order to determine the reacting species derived from peroxynitrite or acidified nitrite, SA was mixed with NO (aq) or NO$_2^-$ solution in PBS (200 mM pH 7.4) or aqueous solution of NOBF$_4$ (a NO$^+$ donor). The spectrum with identical absorption properties ($\lambda_{\text{max}}$, 532 nm) as the red product formed with ONOO$^-$ was obtained only in the presence of NOBF$_4$ (Figure 5). The reaction rate of NOBF$_4$ with SA was also comparable to the reaction rate of peroxynitrite with SA. These results suggest the involvement of NO$^+$ in the reaction with SA.

The UV/vis spectrum of the O-nitroSA solution following the adjustment of pH to 7.4 was monitored hourly for 5 h. No considerable change was observed in the spectrum of O-nitrosoSA. This observation indicates that O-nitrosoSA is stabilized at pH 7.4.

2.5.1.5 SA protects DHR from SIN-1 mediated oxidation: Dihydrorhodamine (DHR) has been frequently used for assaying the formation of cell- and tissue-derived peroxynitrite. The two- electron oxidation of dihydorhodamine to rhodamine (RH; $\lambda_{\text{ex}}$ = 500 nm, $\lambda_{\text{em}}$ = 536 nm) results in the formation of highly fluorescent products. (Kooy et al., 1994) This method allows for the detection of submicromolar levels (e.g. 50 nM) of peroxynitrite. SIN-1 is frequently used to generate peroxynitrite in situ (Uppu et al., 1996).

In the present study, SIN-1 (20 $\mu$M) was mixed with DHR (5 $\mu$M). The fluorescence at 554 nm increased with time and reached a plateau in 100 min. The addition of SA (0.01 to 7 $\mu$M) was able to quench DHR oxidation by SIN-1 (Figure 6).

This experiment was done in Dr. Thatcher's lab (Queen's University, Ontario).
**Figure 2.5.5:** $O$-nitrosoSA formation from SA and nitrosonium tetrafluoroborate (NOBF$_4$). The spectrum of aqueous sinapinic acid solution following its reaction with nitrosonium tetrafluoroborate at 25°C.
**Figure 2.5.6**: Inhibition of SIN-1-mediated dihydorhodamine oxidation by SA. SIN-1 (20 μM) was added to 5μM dihydorhodamine (DHR) in the presence of increasing SA concentration at 37°C and pH 7.4. Data were normalized by setting the value of fluorescence intensity of rhodamine-6G to 100 %, and that of DHR-6G at time zero to 0%. Excitation and emission wavelengths were set at 528 nm and 554 nm, respectively. Values are mean ± SD, n = 3.
2.5.1.6 *O-nitrosoSA formation upon incubation with GSNO:* GSNO is well known to be able to *S*-nitrosate thiols by NO⁺ transfer in biological systems (Arnelle and Stamler, 1995). Since my previous experiments suggested the involvement of NO⁺ in the reaction with SA, I examined whether GSNO could promote *O*-nitrosoSA formation by NO⁺ transfer. These reactions were performed in 60% ACN with 10% acetic acid. The *O*-nitrosoSA formation was monitored spectrally at 510 nm as a function of [GSNO]. As can be seen from Figure 7, the yield of *O*-nitrososinpinic acid was also increased as a function of [GSNO].

2.5.1.7 *O-nitrosoSA as a potential S-nitrosating agent:* Since *O*-nitrosoSA is a direct analog of *S*-nitroso compounds, it should be able to *S*-nitrosate thiols or amines at physiological pH. To test this possibility, neutralized *O*-nitrosoSA was incubated with L-Trp, L-Tyr, DL-Hcys, GSH and bovine serum albumin. As can be seen from Table 1, only thiol-containing amino acids and BSA resulted in rapid decrease in the 520 nm peaks with comparable rate constants.

Evidence for *S*-nitrosation of BSA was obtained by the characteristic S-NO absorption peak (343nm) of BSA subsequent to its reaction with *O*-nitrosoSA (Figure 8).
Figure 2.5.7: O-nitrosoSA formation from SA and GSNO. O-nitrosoSA formation in the presence of \(\circ\) 0.3 \(\mu\)M, \(\Delta\) 0.6 \(\mu\)M, \(\Box\) 1 \(\mu\)M and \(\diamond\) 1.5 \(\mu\)M GSNO in 60% acetonitrile 10% acetic acid after 15 min incubation at 25\(^{\circ}\) C.
Table 2.5.1: Rate constants for transfer of NO\(^+\) from O-nitrosoSA

<table>
<thead>
<tr>
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<th>Rate constant (M(^{-1})S(^{-1}))</th>
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<tbody>
<tr>
<td>GSH</td>
<td>23.6 ± 1.6</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>15.2 ± 1.2</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\)Decomposition of 0.25 mM SANO solution was monitored at 520 nm as a function of time in the presence of 10 fold molar excess of GSH/Homocysteine/Tryptophan/Tyrosine in phosphate buffered saline. Data are mean ± SD, n= 3
Figure 2.5.8: $S$-nitrosoBSA formation from BSA and $O$-nitrosoSA. UV/vis spectrum of BSA (375 μM) upon incubation with equimolar $O$-nitrosoSA in PBS pH 7. The spectrum of the product was taken using same concentration of BSA as blank.
2.5.2 Bovine serum albumin nanoparticles as carriers of NO

2.5.2.1 Synthesis of S-nitrosooctadecane (SNOD): The reaction of 1-octadecane thiol with equimolar amount of tert-butyl nitrite gave rise to a red product instantaneously. This product was thought to be, S-nitrosooctadecane (SNOD) since it had two UV/vis peaks, at 340 and at 548 nm, which are characteristic of S-nitrosothiols. SNOD was stable in quartz for two weeks at room temperature when kept in the dark.

2.5.2.2 Effect of SNOD on GSH in the presence of light: Irradiation of the SNOD solution in the presence of oxygen and subsequent mixing with aqueous glutathione solution as described in methods (section 2.4.2.13) resulted in the formation of S-nitroso glutathione (GSNO) as indicated by the formation of a characteristic peak of S-NO at 340 nm (Figure 9, blue line).

α-tocopherol is known to quench N₂O₃. Irradiation of S-nitrosooctadecane solution in hexane in the presence of α-tocopherol followed by incubation with aqueous GSH solution reduced the extent of S-nitrosation by ~ 85% as indicted by the reduction of the –SNO peak at 340 nm (Figure 9, light green line).

In order to examine whether this observed S-nitrosation of GSH by the irradiated SNOD solution was dependent on oxygen, SNOD solution was irradiated in the absence of oxygen and sonicated with aqueous GSH solution following irradiation. However, irradiated SNOD solution failed to cause any S-nitrosation of GSH in the absence of oxygen as evidence by the absence of the characteristic peak of S-NO at 340 nm (Figure 9, purple line).
**Figure 2.5.9:** Effect of irradiated $S$-nitrosooctadecane solution on aqueous GSH solution. Spectra of the aqueous GSH solution following mixing with laser irradiated (100 shots, 50 mj/shot) $S$-nitrosooctadecane solution in hexane (blue line) saturated with oxygen; (light green line) containing $\alpha$-tocopherol and saturated with oxygen; (purple line) saturated with nitrogen.
2.5.2.3 Effect of SNOD liposome on GSH in the absence of light: SNOD liposomes incubated with aqueous solution of GSH in the presence of oxygen under dark condition, failed to cause any S-nitrosation of GSH even after incubation for 180 minutes (Figure 10).

2.5.2.4 Characterization of the dansyl labelled defatted BSA nanoparticles: The size of the dansyl labelled BSA nanoparticles was estimated to be between 300-500 nm (Figure 11). These particles were stable in water, 0.1 M Na₂HPO₄ pH 7.4 and PBS buffer. The diameter of the particles remained constant when stored in PBS at -20°C for a week.

2.5.2.5 The uptake of Dns-BSA nanoparticles: The normal human fibroblasts were incubated with dansyl labelled defatted BSA nanoparticle suspension for 5 hours in culture medium. Following incubation, free Dns-BSA nanoparticles were washed away. Cell uptake of fluorescent labelled nanoparticles was indicated by both the phase contrast (Figure 12A) and fluorescent image (Figure 12B) of the cells captured following the incubation period upon removal of the excess nanoparticles as described in the methods.

2.5.2.6 Rhodamine labelled BSA nanoparticle loading with S-dansyloctadecane: BSA nanoparticles were formed following incubation of rhodamine labelled BSA solutions with S-dansylooctadecane according to the procedure described in the methods. Following purification of the nanoparticles, the S-dansyloctadecane was detected using a dansyl filter (Figure 13A) and the rhodamine labelled BSA nanoparticles was detected using a rhodamine B-isothiocyanate (RITC) filter (Figure 13B) in the fluorescence microscope. The S-dansyloctadecane loaded BSA nanoparticles were depicted by the yellow regions in the superimposed image (Figure 13C) following overlapping of the
Figure 2.5.10: The spectrum of GSH following incubation with SNOD in the dark. The spectrum of the separated aqueous GSH solution (1 mM) was taken following 180 min incubation with S-nitrosooctadecane liposome containing 0.1 mM neocuproine in the dark. Same concentration of aqueous GSH solution was used as blank.
Figure 2.5.11: Fluorescent image of dansyl labelled BSA nanoparticles taken under fluorescence microscope with 20 x objective using dansyl filter (Ex 335 nm; Em 500 nm).
**Figure 2.5.12:** Uptake of dansyl labelled BSA nanoparticles by fibroblasts. Images A) phase contrast image; B) fluorescent image taken using dansyl filter (Ex 335 nm Em 500 nm) of the fibroblasts following incubation with Dns-BSA nanoparticles for 5 hours.
**Figure 2.5.13:** S-dansyloctadecane loading into the LRBC labelled defatted BSA nanoparticles. Following loading of the particles with S-dansyloctadecane, fluorescent image of the particles were taken using A) a dansyl filter (Ex-335 nm, Em 500 nm) B) a rhodamine B-isothiocyanate filter (Ex 570 nm, Em 595 nm). Image C is the superimposed image of image A and B.
two images obtained with dansyl and RITC filters. Almost 100% of the nanoparticles were shown to be loaded with the S-dansyl-octadecane.

2.5.2.7 Delivery of SNOD to the fibroblasts: The S-NO bond in SNOD was observed to be photolabile as in other S-nitrosothiols. SNOD is capable of S-nitrosating small molecular weight thiols in the model membrane system in the presence of oxygen. In order to assess the potential of the BSA nanoparticles to deliver a large amount of SNOD to fibroblasts, cells were incubated with rhodamine labelled BSA nanoparticles loaded with SNOD. NO has been reported by many researchers to cause apoptosis in different types of cells (Marshall and Stamler, 2002; Simeone et al., 2002). Therefore, SNOD-induced apoptosis could be utilised as a marker for the successful delivery of SNOD to fibroblasts.

Following the uptake of the nanoparticles, the fibroblasts were illuminated with visible light for 15 minutes. The fibroblasts were then incubated for an additional 24 h at 37°C in the presence of 5% CO₂. Fibroblasts that took up the SNOD loaded nanoparticles and were exposed to light, underwent cell death by apoptosis as indicated by the phase contrast image and fluorescent image of hoechst stained cells captured 24 hours after exposure to visible light. Apoptosis was evidenced by the shrinkage of cell volume in phase contrast image (Figure 14A) and their condensed nuclei that appear bright in the fluorescent image (Figure 14B). On the other hand, the fibroblasts that took up S-dansyl-octadecane loaded particles appeared to be healthy following 24 hours incubation as shown both in phase contrast (Figure 15A) and fluorescent images (Figure 15B).
Figure 2.5.14: Effects of S-nitrosooctadecane loaded BSA nanoparticles along with visible light exposure on the fibroblasts. A) phase contrast B) fluorescent images of the nanoparticles and phototreated fibroblasts taken subsequent to their staining with hoechst dye.
Figure 2.5.15: Effects of rhodamine labelled BSA nanoparticles on the fibroblasts. A) phase contrast B) fluorescent images of the nanoparticles treated fibroblasts taken subsequent to their staining with hoechst dye.
Treatment of the cells with SNOD-loaded particles followed by irradiation caused 56% of the fibroblasts to undergo cell death by apoptosis. However, when the cells were protected from light following the nanoparticle uptake, apoptosis occurred only in ~5% of the fibroblasts (Figure 16, treated).

On the other hand, incubation of the fibroblasts with rhodamine labelled BSA nanoparticles caused only 2% of the cells to undergo cell death by apoptosis irrespective of light exposure (Figure 16, control).

**2.5.2.8 Targeting of the BSA nanoparticles to the cell surface:** Since the cell surface is rich in thiols, modification of the particles with a heterobifunctional linker, SMCC, that reacts with an amino group in one end, and reacts with a thiol group in the other end should be more efficient way to attach larger numbers of BSA nanoparticles to the cell surface. Incubation of fibroblasts with SMCC-conjugated BSA nanoparticles led to the attachment of nanoparticles to 28% of the fibroblasts in the culture (Figure 17).

In contrast, when the cells were pre-treated with iodoacetamide to block their surface thiols, incubation with the same concentration of SMCC conjugated BSA nanoparticles resulted in the covalent attachment of the particles to 15% of the fibroblasts in culture (Figure 18).
**Figure 2.5.16:** Effect of S-nitrosooctadecane loaded BSA nanoparticle treatment on the fibroblasts. Fibroblast treated with S-nitrosooctadecane loaded rhodamine labelled BSA nanoparticles. Control cells were treated with rhodamine labelled BSA nanoparticles. Following treatment and hoechst staining the number of apoptotic cells were counted under the microscope to get the percentages. The data represent the mean ± SD, n = 3.
Figure 2.5.17: Fibroblasts coincubated with rhodamine labelled SMCC conjugated defatted BSA nanoparticles. The phase contrast image (A); and the fluorescent image (B) of the fibroblasts taken following the incubation with SMCC conjugated defatted BSA nanoparticles.
**Figure 2.5.18:** Fibroblasts having their surface thiols blocked coincubated with rhodamine labelled SMCC conjugated defatted BSA nanoparticles. The phase contrast image (A); and the fluorescent image (B) of the fibroblasts taken following the incubation with SMCC conjugated defatted BSA nanoparticles.
2.6 Discussion

2.6.1 Synthesis of a coloured nitrite adduct from sinapinic acid and NO⁺ donors: potential applications

Peroxynitrite was previously demonstrated to oxidize a wide variety of biomolecules in vitro either by direct reactions or by secondary radicals (CO₂⁻*, *NO₂, *OH). In the present study, peroxynitrite was observed to react with SA leading to the formation of red coloured compound (O-nitrosoSA) (λₘₕₐₓ = 532 nm), with an estimated second order rate constant of 812 M⁻¹ s⁻¹. Decomposed peroxynitrite resulted in the formation of apparently identical product. These observations suggested that peroxynitrous acid was not involved in the reaction but rather some species derived from peroxynitrite through decomposition.

Reaction of equimolar SA (5.35 x 10⁻⁴ M) in 60% acetonitrile 10% acetic acid with sodium nitrite also gave rise to a red product (λₘₕₐₓ = 520 nm) with identical characteristics as the one obtained with peroxynitrite (Figure 2). The growth of the 520 nm peak appeared to be a first order process with a rate constant of 0.0004 s⁻¹. This red product had an extinction coefficient (in 90% ACN 10% acetic acid) that was ~ 495-fold larger than the 543 nm extinction coefficient reported for small molecular weight S-nitrosothiols such as S-nitrosoglutathione. Furthermore, while the reaction was carried out between equimolar sodium nitrite and SA in 0.1 M HCl instead of 10% acetic acid an identical red product was formed (Figure 3). However, the rate constant determined under
this condition was found to be 20,800-fold larger in comparison to that observed in the presence of 10% acetic acid.

It is well established that in the presence of mineral acids sodium nitrite is converted to nitrous acid. In aqueous phase direct protonation of nitrous acid occurs to form nitrosonium ion. SA can react with the newly formed NO\(^+\) from nitrous acid leading to the formation of the red compound. Nitrosation by nitrite in aqueous solution is enhanced in acidic pH, which favor the formation of nitrosonium, NO\(^+\).

The apparent difference in reaction rate of SA toward sodium nitrite observed in acetic acid vs. HCl can therefore be attributed to the nature of the acid used. There could be a few possible explanations behind the slower rate of red product formation observed in the presence of acetic acid. One of the possible reasons could be the difference in the rate of reactivity of nitrosonium ion in the presence of different solvents. Nitrosation has been suggested to also occur through different carriers of NO\(^+\) depending on the acidity and other species present. The reactivity of the NO\(^+\) carriers toward the target also varies a lot. In the presence of hydrochloric acid nitrosyl chloride (NOCl) can serve as a carrier of NO\(^+\), which in turn, can nitrosate the target. Similarly nitrosation of the target can occur through nitrosyl acetate (NOOAc) in the presence of acetic acid. The order of reactivity of the three nitrosating species has been suggested as NO\(^+\)\textgreater NOCl\textgreater NOOAc (Ridd, 1971). Another possible reason could be slow deprotonation of acetic acid, since the pK\(_a\) of acetic acid is 4.75. Slow deprotonation renders the rate of HNO\(_2\) formation also lower and that will ultimately lead to the slower production of NO\(^+\) from HNO\(_2\). Since NO\(^+\) has a very short half-life in water (t\(_{1/2}\) \(3\times10^{-10}\)s), slow formation of NO\(^+\) in the presence of acetic acid increases the percentage of NO\(^+\) reacting with SA instead of being
lost through the reaction with water. Thereby, slow formation of \( \text{NO}^+ \) promotes more efficient reaction between SA and nascent \( \text{NO}^+ \). Therefore, the extinction coefficient of the red product was determined to be higher in acetic acid even though the reaction rate was lower.

However, the rate of the red product formation was much faster in the presence of HCl than in the presence of acetic acid. We suggest the following explanations for this observation. HCl dissociates readily to produce \( \text{H}^+ \). As a result, the formation of \( \text{NO}^+ \) from \( \text{HNO}_2 \) is fast. Although a high concentration of \( \text{NO}^+ \) is produced, only a low percentage of the newly formed \( \text{NO}^+ \) will react with SA owing to its high reactivity with water. Therefore, the yield of the red product was lower even though the reaction rate was much faster in the presence of HCl.

A shift in the absorption maximum was also observed while the reaction was performed under different reaction conditions. This observed shift is most probably due to the different solvents. This suggestion was supported by our experimental results that the products formed in the presence of acetic acid and 0.1 M HCl gave rise to absorption peak in the same wavelength region \( (\lambda_{\text{max}} = 532 \text{ nm}) \) (Figure 4) following their transfer to the 0.1M Na\textsubscript{2}PO\textsubscript{4} buffer pH 7.4. However, at this point we were not certain whether this observed effect was due to the change in pH or due to the presence of a different solvent. When both products were transferred separately to aqueous solutions of different pHs the absorption peaks remained at \( \sim 532 \text{ nm} \) (Figure 4) refuting the possibility of pH effect. These findings confirm the fact that the use of different solvents was indeed responsible for the observed shift in the absorption maximum of the nitrite adduct of SA.
Involvement of an unstable intermediate, O-nitrosocompound in the reaction of aryloxide ion with alkyl nitrites has been reported in the literature. This intermediate was suggested to undergo an internal rearrangement of the NO group leading to the formation of corresponding C-nitrosoprodut. Phenols were also demonstrated to react through their oxygen atom by following a similar mechanism. Nitrosation of the 2-naphtholate ion at pH <5 had also been suggested to occur via reversible formation of an unstable aryl nitrite. In addition, nitrous acid catalyzed nitrification of phenols had also been proposed to undergo through the intermediate formation of aryl nitrite (Leis et al., 1998).

I suggest that NO\(^+\) derived from peroxynitrite or acidified nitrite reacts with the \(-\text{OH}\) group of SA forming an aryl nitrite, \(3,5\)-dimethoxy-4-nitrosooxycinnamic acid. Since the ortho positions are blocked by the two methoxy groups and the para position is blocked by the carbon side chain in SA, the rearrangement of the NO group of aryl nitrite is probably not feasible as in any other phenols. Therefore, I could observe the formation of an aryl nitrite by spectroscopy, unlike other phenols.

To check for that possibility, sodium nitrite was reacted with different SA analogues namely 4-hydroxycinnamic acid, 3,4,5-trimethoxycinnamic acid, 3,4,5-trimethoxyhydrocinnamic acid, dihydroSA or 3-(3,4,5-trimethoxyphenyl) propionic acid solution instead of SA in 60% ACN 10% acetic acid, only dihydroSA gave rise to an identical peak characteristic of the red compound. Both phenolic \(-\text{OH}\) and the two methoxy groups appears to be essential for the formation of the red compound as neither 3,4,5-trimethoxycinnamic acid nor 4-hydroxycinnamic acid could result in the formation of the red product. These findings also correlate well with the suggested formation of \(O\)-nitroSA from the reaction between SA and peroxynitrite or acidified nitrite. The absence
of the red product formation with 4-hydroxycinnamic acid was probably due to the absence of the two methoxy groups in the ortho positions which could block the quick rearrangement of NO group of the aryl nitrite (O-nitrosoSA). Therefore, aryl nitrite formation could not be detected. DihydroSA gave identical red product as SA, but the yield of the product was considerably lower compared to SA. This difference in reactivity between SA and dihydroSA towards sodium nitrite could be attributed to the difference in conjugation system in these two compounds. DihydroSA has a less extended conjugated system compared to SA due to the reduction of the side chain double bond. Extinction coefficient tends to increase as the extension of conjugation system increases. Therefore, the lower yield upon reaction with sodium nitrite is likely due to the less extended conjugation in dihydroSA.

Upon mixing with SA, neither NO (aq) nor NO₂⁻ solution in PBS (200 mM pH 7.4) resulted in the formation of the red product. Only aqueous solution of NOBF₄ (a NO⁺ donor) led to the formation of a red product (Figure 5) with identical absorption properties (λ_max = 532 nm) as the product formed with ONOO⁻ and acidified nitrite through the reaction with SA. These observations suggest the involvement of NO⁺ in the reaction with SA.

Dihydrorhodamine has been frequently used for assaying the formation of cell and tissue derived peroxynitrite. The two-electron oxidation of dihydrrhodamine to rhodamine (RH; λ_exc = 500 nm, λ_em = 536 nm) results in the formation of highly fluorescent products (Koooy et al., 1994) This method allows for the detection of submicromolar levels (e.g. 50 nM) of peroxynitrite.
In the present study, SA (0.01 to 7 μM) successfully prevented the oxidation of DHR (5 μM) by SIN-1 (20 μM) as monitored by the quenching of rhodamine fluorescence (Figure 6). This observation indicates the potential of SA to be used as an inhibitor of peroxynitrite-mediated damage.

O-nitrosoSA appeared to be stable following adjustment of the pH to 7.4 for hours since no apparent change in the UV/vis spectra was observed while monitored in hourly basis.

In the present study, GSNO resulted in the formation of O-nitrosoSA upon reacting with sinapinic acid by transferring NO\(^+\). GSNO is known to nitrosate thiols through NO\(^+\) transfer. The yield of O-nitrosoSA was also increased as a function of [GSNO] (Figure 7). This result provides another line of evidence for the high reactivity of NO\(^+\) with SA.

Upon incubation of neutralized O-nitrosoSA with L-Trp, L-Tyr, DL-Heys, GSH and bovine serum albumin, only thiol containing amino acids and BSA resulted in rapid decrease in the 520 nm peak (Table I) with comparable rate constants. S-nitrosation of BSA was also demonstrated by the characteristic S-NO absorption peak (343nm) of BSA subsequent to its reaction with O-nitrosoSA (Figure 8). The fact that O-nitrosoSA can promote S-nitrosation of thiols demonstrates that it holds the potential of being used as a S-nitrosating agent in vitro.

In conclusion, I have shown in this study that SA could be utilized as an analyte for the spectroscopic detection of peroxynitrite and NO\(^+\) donors in submicromolar range in physiological conditions or in vitro. Furthermore, the demonstrated ability of O-nitrosoSA to serve as a S-nitrosating agent for thiol containing amino acids, peptides and
proteins, makes it potentially very useful in the study of S-nitrosothiol biochemistry and physiology.

2.6.2 Bovine serum albumin nanoparticles as carriers of NO

Nedospasov et al. (2000) have shown recently that hydrophobic pockets of proteins act as micellar catalysts for the nitrosation of their own thiol groups and thiol groups outside the protein hydrophobic pockets. It was demonstrated that these protein hydrophobic cores could concentrate hydrophobic molecules including \(^*\)NO and \(O_2\) thereby facilitating the formation of \(N_2O_3\). Hydrophobic pockets also protect the \(N_2O_3\) from the aqueous environment. It was suggested that these hydrophobic interiors in the major plasma protein albumin can act as a reservoir of \(NO\) that can play an important role in regulating NO mediated processes in vasculature (Nedospasov et al., 2000, Rafikova et al., 2002). Ramachandran et al. also demonstrated the pivotal role of \(N_2O_3\) formation in the membrane in cell surface PDI mediated extracellular NO influx into the cytosol. The \(N_2O_3\) formed in the membrane can S-nitrosate thiols in the membrane cytosol interface (Ramachandran et al., 2001).

The S-NO bond in S-nitrosothiols is photolabile. Upon exposure of S-nitrosothiols either to 340 nm or 545 nm light, this bond breaks, leading to the formation of nitric oxide. In this study, I report the synthesis of a novel S-nitrosothiolipid, S-nitros octadecane (SNOD) that is stable in quartz for 14 days at room temperature, while kept in the dark. S-nitrosation of the 1-octadecanethiol was confirmed from the characteristic peaks of S-nitrosothiols, which were 340 nm and 548 nm. S-NO bond in S-nitros octadecane were also observed to be photolabile, like other S-nitrosothiols. We
were able to S-nitrosate low molecular weight thiols (GSH) upon photolysis of the SNOD in the model membrane system (hexane:PBS). This S-nitrosation was found to be N$_2$O$_3$ mediated as this process required the presence of oxygen and was attenuated in the presence of α-tocopherol (Figure 1). Thus my study supports the interphasial S-nitrosation mechanism reported by others earlier (Rafikova et al., 2002; Ramachandran et al., 2001).

NO is known to be cytotoxic when produced in large amounts (Hibbs et al., 1998). Coworkers from my laboratory demonstrated previously that thionein-NO synthesized through the nitrosation of apo-metallothionen was capable of killing human adenocarcinoma cells in vitro upon photolysis. The tumoricidal activity was increased in the presence of H$_2$O$_2$ (Tannous et al., 1997).

There are a growing number of studies implicating NO as a mediator of apoptosis. NO has been demonstrated to cause apoptosis through different pathways depending on the cell line and physiological conditions (Kawahara et al., 2001; Oyadomari et al., 2001). One of the pathways through which NO has been shown to cause apoptosis, is by the S-nitrosation of key enzymes or transcription factors involved in the transcription of antiapoptotic proteins. Recently it has been demonstrated that NO induces apoptosis in A549 cells by S-nitrosating a critical cysteine residue in the antiapoptotic transcription factor NF-kB (Marshall and Stamler, 2002). Therefore, delivering a large amount of NO to cells should be potentially effective in triggering cell death upon exposure to light.

Delivery of free RSNO is not feasible since NO will be diverted to other low molecular weight thiols or protein thiols in the serum through transnitrosation before finding its target. Extensive research had been carried out in the last several years to
design a suitable delivery method for different types of drugs. Nanoparticles had been demonstrated to offer a protective environment for the delivery of several drugs and antisense RNAs in vitro studies. The use of nanoparticles had also been shown to facilitate slow, controlled release of the drug over a long period a time (Santhi et al., 2000). In vitro studies performed with methotrexate-loaded BSA nanoparticles showed the release of around 50% of the drug within the first 12 hours followed by a slow release of the remaining drug over a 24 hours period. Nanoparticles have also shown the potential for the delivery of antisense RNA for antiviral therapy (Schwab et al., 1994).

Therefore, encapsulating RSNO in the particles was expected to eliminate the problem of premature loss of NO through transnitrosation. In addition, the time of NO release could be controlled because NO would be released only upon exposure of light following particle uptake by the cells. Furthermore, the nanoparticles would not impose additional stress to the cells because they have been shown to be non-toxic and non-antigenic.

My newly synthesized S-nitrosooctadecane appeared to be a good candidate for the delivery of large amount of NO to the cytosol due to its relative stability and NO releasing properties as other RSNOs. In this study, I prepared BSA (defatted) nanoparticles that were stable when stored in buffer or dry at -20°C. The size of the particles was not observed to change upon exposure to the buffer. The average size of the nanoparticles was in the range of 300-500 nm (Figure 11) as determined by the Zeiss Anxiovert Empix imaging software in the fluorescence microscope. I was also able to label the nanoparticles with different fluorophores namely dansyl chloride and rhodamine B acid chloride to monitor particle uptake.
Loading nanoparticles with S-nitrosooctadecane was successful as confirmed by labelling the particles with rhodamine and labelling thiolipid with dansyl chloride. The overlaid image of the rhodamine labelled particle and dansyl-thiolipid depicted yellow regions (Figure 13C) that were indicative of dansylthiolipid loaded rhodamine labelled BSA nanoparticles.

BSA nanoparticles showed the potential to deliver large amount of NO to the fibroblasts as evidenced by using NO induced apoptosis as a marker for delivery. Shrinkage of cell volume and chromatin collapse are two of the main characteristic features of apoptosis (Kerr et al., 1972). The Hoechst-stained NHF cells following their treatment with SNOD loaded nanoparticles and exposure to visible light depicted shrinkage of cell volume in the phase contrast image (Figure 14A) and bright condensed nuclei in the fluorescence image (Figure 14B) which were indicative of apoptotic cells. The apoptosis was caused by the release of NO from the delivered SNOD by the particles. SNOD loaded nanoparticles could induce 58% of the cells in culture to undergo apoptosis upon exposure to light (Figure 16). However, further complementary studies are required to confirm SNOD induced apoptosis in the fibroblasts.

In order to employ these S-nitrosooctadecane-loaded nanoparticles for efficient delivery of NO \textit{in vivo} a method had to be developed to target these particles to the site of interest. Therefore, the nanoparticles surfaces were covalently linked with SMCC, a heterobifunctional linker. This heterobifunctional linker is capable of linking a molecule with an amino group to a molecule with a thiol group. Fibroblast surfaces are rich in thiols. Therefore, the nanoparticles covalently linked with SMCC through their amino
groups would be able to link these particles to the fibroblast surface by reacting with the
–SH groups in the surface.

Around 28% of the fibroblasts in culture were able to bind the BSA nanoparticles
upon incubation with SMCC-conjugated BSA nanoparticles (Figure 17). I did not obtain
high percentage of particle attachment to the cells probably because all the nanoparticles
were not successfully conjugated with the heterobifunctional linker. On the other hand,
when surface thiols of the fibroblasts were blocked with iodoacetamide, the particles
could still bind to 15% of the fibroblasts in culture (Figure 18). Thus, even after blocking
the surface thiols attachment of particles to lower percentage of fibroblasts occurred. My
inability to block all the thiols in the cell surface by iodoacetamide treatment could be the
possible reason for the observed attachment of SMCC conjugated nanoparticles to 15%
of the fibroblast in culture. If all the thiol groups on the cell surface have not been
blocked efficiently nanoparticles would attach to the surface using the remaining thiol
groups on the cell surface.
PART III

Molecular aspects of $S$-nitrosothiols
3.0 Abstract

3.0.1 Exploration of potential roles of tyrosines in rat calmodulin

Previous studies from our laboratory demonstrated that upon incubation with peroxynitrite the functional activity of bovine calmodulin, including activation of phosphodiesterase, activation of calcineurin phosphatase and binding of MLCK peptide, reduces dramatically. However, Ca\(^{2+}\)-binding ability of the modified calmodulin was retained. It was suggested that the nitration of the tyrosine residues in calmodulin by peroxynitrite was responsible for these observed inhibitions of calmodulin function (Pathrose, 1997).

In order to study the role of the two tyrosine residues at the 99 and 138 positions respectively in the activity of rat calmodulin, three different mutants were created using site-directed mutagenesis namely CaM Y\(_{99A}\), CaM Y\(_{138A}\) and CaM Y\(_{99A}Y_{138A}\). Tyrosine residues were replaced with alanine residues in those specific positions of the mutants. All the mutants and the native fibrinogen protein were expressed on *E. coli* BL21(DE3) cells. The yield of the protein was decreased dramatically in the double mutant, CaM Y\(_{99A}Y_{138A}\). The introduction of alanine residue at 99 position reduced the \(\alpha\)-helical content by \(\sim 6\%\). On the other hand \(\alpha\)-helical content was increased by \(\sim 4\%\) following substitution of tyrosine at 138 position with alanine. All the mutants were capable of binding Ca\(^{2+}\) however a great variability was exhibited in the extent of Ca\(^{2+}\) binding. CaM Y\(_{138A}\) demonstrated the highest Ca\(^{2+}\) binding ability as evidenced by CD spectroscopy. CaM Y\(_{99A}\) had the lowest Ca\(^{2+}\) binding ability. CaM Y\(_{99A}Y_{138A}\) also showed relatively high Ca\(^{2+}\) binding capability close to CaM Y\(_{138A}\). Thus, tyrosine residues in
calmodulin appear to play an important role both in maintaining the structural integrity and in Ca^{2+} binding.

In another approach, an attempt was made to explore the effects of the close proximity of tyrosine 138 on the lability of S-NO bond in S-nitrosocysteine. Creation of two calmodulin mutants by substituting valine 142 and isoleucine 85 with cysteine was attempted. Valine 142 and Isoleucine 85 were chosen for mutation owing to their close proximity (3-5 Å) to the Tyr^{138} in CaM. Cysteine was chosen as a new residue to replace tyrosines to allow for their S-nitrosation following purification of the mutant protein. I could successfully introduce the mutations however, I was unable to transform it to *E. coli* BL21(DE3) cells to express the mutated proteins.

### 3.0.2 Photoactivated cleavage of DNA to decontaminate platelet rich plasma

Even though blood and blood products from the donors are tested for viral contamination using different techniques in the laboratory, there is still a potential risk of viral transmission by these products. In order to further ensure the safe use of these products, intensive studies have been going on recently to develop photosensitizers that will be able to photoinactivate any trace amounts of viral DNA remaining in the blood or blood products upon illumination with light at a particular wavelength.

I wanted to determine whether fluorescent RSNO's could be used for light dependent destruction of DNA. To this end, *N*-dansyl-*S*-nitrosoglutathione (Dns-GSNO) and *N*-dansyl-*S*-nitrosohomocysteine (Dns-HCysNO) were tested in the presence and absence of light for their ability to cleave plasmid DNA.
Plasmid DNA treated with 3.75 and 15 mM Dns-GSNO and illuminated with visible light for 30 minutes aggregated completely. On the other hand, DNA treated with 0.9 mM Dns-GSNO and illuminated with visible light for the same period of time degraded the DNA. However, these effects did not seem to require light, since the same effects were observed, when the plasmid was incubated with the same compound in the dark.

Treatment of plasmid DNA with 2.46-3.16 mM Dns-HCysNO and illumination with visible light for 60 minutes resulted in its complete degradation. On the other hand 1.54 mM Dns-HCysNO and light exposure for 60 minutes did not have any effect on plasmid DNA. In contrast, maintaining the DNA samples in the dark following the addition of Dns-HCysNO (1.54-2.48 mM) for the same period of time could cause partial degradation of plasmid DNA only when it was treated with 2.48 mM Dns-HCysNO. Furthermore, it was determined that the Dns-HCysNO mediated effect on plasmid DNA was light illumination time dependent (60-120 min).

I was unable to examine whether these compounds could be utilized to decontaminate platelet rich plasma due to our inability to attract funding from Consortium for Plasma Science, our potential sponsors.
3.1 Introduction

3.1.1 Exploration of potential roles of tyrosines in rat calmodulin

3.1.1.1 Calmodulin

Calmodulin is a small calcium-binding protein found in all eukaryotic cells. This 16.7 kDa protein is very acidic (isoelectric point ~ 4) and thermostable. It has 148 amino acid residues and the majority of its hydrophobic residues are buried within the protein in the absence of Ca\(^{2+}\) (Kuboniwa et al., 1995; Zhang et al., 1995). One distinct feature of this protein is the relative abundance of methionine residues in its amino acid sequence compared to the other proteins. All of its methionine residues are located in the hydrophobic surfaces (Babu et al., 1988a). Calmodulin contains only two tyrosine residues both of which are located in the C terminal half of the molecule, one at position 99 and another one at position 138 (Dedman et al., 1977; George et al., 1993). It contains four Ca\(^{2+}\)-binding sites per molecule (Haiech et al., 1981).

A high degree of homology is observed among the calmodulin isotypes isolated from different sources by comparison of their amino acid sequences. Therefore, all the calmodulin isotypes are suggested to have diverged from the same ancestor. Variation in only 7 out of 148 residues is revealed when the amino acid sequences of calmodulin from 6 higher animals are compared. Differences in the amide assignment contribute to 3 of those variations. Domain I is entirely conserved, and there is only a single substitution in domain II among all the isotypes of calmodulin. Domain III and domain IV contain most
of the variable amino acids. There is also some variation at positions 143 and 147 outside the binding loop of IV. However, many of these substitutions are conservative namely Phe instead of Tyr at position 99 is actually observed in all invertebrate calmodulins (Smith et al., 1987). It has been hypothesized that this high degree of conservation in the amino acid sequence observed among the calmodulin isotopes may be required for the interaction with such a diverse family of CaM-binding proteins (Crivici and Ikura, 1995). Calmodulins isolated from diverse organisms are remarkably similar in their biological, chemical, and physical properties (Lin, 1982; Means et al., 1982).

\begin{center}
\begin{tabular}{ll}
1 & \quad 37 \\
\alpha & ADQLTEEQIAEFKAFSLF[DKDGDTITTKE]LGVVRMRL \\
40 & \\
\beta & GQNPTEAEQLDMINEVDADGNFTDFPEFLTMARK \\
76 & \\
\alpha' & MKDTESEEEIREAFRVDKDGNYISAAELRHVMNTNL \\
106 & \\
\beta' & GEKLTDEEVDEMIREDIDGDGQVNYEEFVQMMTAK \\
113 & \\
148 & \\
\end{tabular}
\end{center}

**Figure 3.1.1:** Amino acid sequence of rat calmodulin. Four Ca$^{2+}$ binding sites are highlighted with boxes. Each Ca$^{2+}$ binding site is 12 amino acid residues long. The sequence has been divided into four segments: $\alpha$ (residues 1-39) includes site I; $\beta$ (40-75) includes site II; $\alpha'$ (76-112) includes site III; $\beta'$ (113-148) includes site IV. (Madeline et al., 1996)
The function of calmodulin is to amplify intracellular calcium signals as well as to mediate a wide range of cellular responses required for muscle contraction, synaptic plasticity, energy metabolism, and maintenance of cellular homeostasis (James et al., 1995). Upon calcium binding, calmodulin is capable of activating as many as 30 different target proteins including myosin light chain kinase, cyclic nucleotide phosphodiesterase, CaM kinases, calcineurin, caldesmon etc. (Means et al., 1991). Both hydrophobic and electrostatic interactions are involved in the binding of calmodulin to its targets (Afshar et al., 1994). It is believed that the flexible central linker region and the two methionine rich hydrophobic surfaces of calmodulin enhance the binding of a wide range of substrates. Since the central linker region is flexible, it has the ability to unwind to different magnitude to accommodate various target proteins. In addition, the highly flexible surface provided by methionine residues enables distinct target proteins to bind to calmodulin (Gellman, 1991).

There is a little sequence homology in the CaM binding domains of various proteins. Nevertheless, these target proteins all share some common characteristic features, such as the presence of hydrophobic and aromatic residues, mainly tryptophan or phenylalanine. These residues are often located at the peptide N- or C- termini, which have a tendency to form amphiphilic helices upon binding to CaM (O’Neill et al., 1987; O’Neill and DeGrado, 1990). A 20-residue peptide segment of the target proteins is usually involved in the interaction with CaM (Means et al., 1991). Synthetic peptides corresponding to this domain peptide have demonstrated the same binding ability to calmodulin in the presence of Ca$^{2+}$ as the native protein. These peptides form basic
amphipathic helices upon calmodulin binding thus facilitating the interaction between the hydrophobic phase of the peptide with the hydrophobic surfaces of calmodulin (Ikura et al., 1992; Meador et al., 1992; Meador et al., 1993)

Both of the domains of calmodulin namely amino and carboxyl terminal domains are demonstrated to be capable of activating target proteins independently (Suko et al., 1985). It has been observed later that there is considerable interaction between the amino and carboxyl terminal domains in native apo-calmodulin. Both the calcium binding ability and energetics of the amino terminal domain has been observed to be modulated by the calcium binding properties of the carboxyl terminal. The $T_m$ of the amino terminal domain has been indicated to increase in apo-CaM compared to the isolated amino terminal domain (Sorensen and Shea, 1998). Calmodulin has been demonstrated to form a dimer through non-covalent interactions (Lafitte et al., 1999)

Calmodulin has been known to undergo different types of posttranslational modification including N-methylation carboxymethylation and other reactions (Murtaugh et al., 1983). In rat liver, a fraction of calmodulin has been reported to be constitutively phosphorylated (Quadroni et al., 1994). Phosphorylation of calmodulin has been demonstrated to reduce its ability to activate target enzymes considerably. On the other hand, activation capacity has been observed to increase upon its phosphorylation (Quadroni et al., 1998).

3.1.1.1 $Ca^{2+}$ Binding to calmodulin: Calmodulin-$Ca^{2+}$ complex resolved by X-ray crystallography has indicated that calmodulin is composed of two independently folded domains connected by a long $\alpha$-helical linker thus adopting a dumbbell shape structure.
Each globular domain contains two-helix loop helix Ca\(^{2+}\) binding motifs or EF hands connected by a small β sheet (Babu et al., 1988a, Figure 2).

Calmodulin is capable of binding up to four calcium ions in a partially cooperative manner (Anderson et al., 1983; Thulin et al., 1984; Forsén et al., 1986). The binding of Ca\(^{2+}\) to calmodulin is a sequential process. It initially binds Ca\(^{2+}\) though the two high affinity binding sites (domains III and IV, numbered from the amino terminus), followed by binding at two low affinity sites (domains I and II) (Wang, 1985). This binding induces a substantial conformational change in calmodulin over a narrow range of intracellular Ca\(^{2+}\) concentration (10\(^{-7}\) – 10\(^{-6}\) M).

![Figure 3.1.2: Crystal structure of Ca\(^{2+}\) bound form of vertebrate calmodulin taken from Protein Data Bank (structure code 1CLL)](image_url)
Two hydrophobic surfaces become exposed upon this conformational change (La Porte et al., 1980; Vogel et al., 1983). These hydrophobic domains are rich in methionine residues and involved in target protein binding (Babu et al., 1988a).

Even though the central linker region of CaM is a helix in X-ray structure (Babu et al., 1988a), it has been demonstrated to be flexible in solution (Barbato et al., 1992). Upon the addition of five interdomain residues, the thermostability of the rat calmodulin (r-CaM) amino terminal domain fragment of calmodulin has been observed to increase whereas the calcium-binding affinity was decreased. Therefore, it was suggested that the interdomain linker might also play a regulatory role in the control of amino terminal function (Sorensen et al., 2002).

The affinity of calmodulin to Ca\(^{2+}\) increases dramatically following its binding to target proteins, CaM binding domain peptides and hydrophobic drugs (Mills et al., 1985; Yagi and Yazawa, 1989; Kasturi et al., 1993). It has been demonstrated that at physiological ionic strength, both apo and Ca\(^{2+}\) bound form of calmodulin possess structurally distinct conformations of the central helix. The structure of the central helix has been observed to be dependent on ionic strength and pH indicating the presence of ionizable groups in the central linker. These ionizable groups probably alter the stability of the central linker thus helping in the sequential and ordered binding of target proteins (Sun et al., 1999).
3.1.1.2 Interaction of calmodulin with peptides

Calmodulin can interact with diverse peptides. Peptides are usually identified as calmodulin binding ligands depending on four different criteria: 1) They bind to calmodulin and compete with each other at one or two high affinity Ca$^{2+}$ dependent sites, 2) Binding of the peptide increases the affinity of calmodulin for Ca$^{2+}$, 3) they inhibit the calmodulin stimulation of the enzymes under calmodulin control, 4) their interaction with calmodulin is prevented by anticalmodulin drugs. The propensity of the calmodulin binding peptides to form amphiphilic basic helices is proposed to be responsible for their ability to bind calmodulin (Cox et al., 1985). Upon the addition of Ca$^{2+}$, hydrophobic surfaces of calmodulin are exposed, and the two domains come closer and wrap themselves around the peptide. The peptide, which is initially unstructured in solution, adopts an α-helical conformation upon binding to calmodulin (Klevit et al., 1985, Figure 3). However, induction of α-helices is not observed for all peptides following interaction with calmodulin. Calmodulin binding domain of troponin I fails to exhibit an increase in α-helical content upon interaction with calmodulin (Cachia et al., 1986).

O’Neil and DeGrado (1985) suggested that the plausible peptide binding sites are located between the E and F helices of domains II and IV of calmodulin. These sites would be complementary to the target peptide to allow for hydrophobic and electrostatic interactions with it. Melittin has been reported to form a 1:1 complex with calmodulin by interacting with two different sites of calmodulin simultaneously (Maulet and Cox, 1983). The high resolution X-ray and NMR structure of several Ca$^{2+}$–CaM peptide complexes have been determined (Ikura et al., 1992). Ca$^{2+}$–CaM binding removes a
pseudosubstrate inhibitory domain from many enzymes active sites. Removal of the inhibitory domain consequently leads to the activation of smooth muscle myosin light chain kinase (smMLCK), CaM Kinase II, calcineurin, plasmalemma Ca\textsuperscript{2+}-ATP ase and cyclic nucleotide phosphodiesterase (James et al., 1995).

Figure 3.1.3 MLCK peptide binding to Ca\textsuperscript{2+}-CaM. The calmodulin amino terminal domain is coloured light gray, carboxyl terminal domain is coloured medium gray and the linker between the two domains coloured dark grey; and the MLCK peptide is coloured black (Nelson and Chazin, 1998).
3.1.1.3 Calmodulin-protein interactions

The interaction of calmodulin with target proteins is similar to its interaction with peptide in many respects. Calmodulin interacts with and activates enzymes only in its Ca$^{2+}$ bound form, and the effect is readily reversed upon removal of Ca$^{2+}$.

Calmodulin activates different protein kinases that catalyze the transfer of phosphate from ATP to serine, threonine or tyrosine residues in protein substrates. Myosin light chain kinases are one type of kinases that are activated by calmodulin (Stull et al., 1986). These kinases catalyze calmodulin dependent phosphorylation of P-light chain in myosin. Myosin light chain kinases from vertebrate striated smooth muscles require both Ca$^{2+}$ and calmodulin for activity. A study indicated that calmodulin forms 1:1 complex with myosin light chain kinase including enzymes from skeletal, cardiac and smooth muscles (Stull et al., 1986). Binding of four Ca$^{2+}$ to the four divalent metal binding sites on calmodulin is required for the activation of the kinases and the activation is a reversible process.

Following complex formation with Ca$^{2+}$, calmodulin binds to and activates the inactive catalytic subunit of rabbit skeletal muscle myosin light chain kinase to form active holoenzyme complex, Ca$_4^{2+}$-calmodulin-myosin light chain kinase. Activation of calmodulin-stimulated, cyclic-nucleotide phosphodiesterase follows a similar pathway (Huang et al., 1981). The activation of these two calmodulin-dependent enzymes is positively cooperative in regard to Ca$^{2+}$. Under physiological conditions, the enzyme activity can be regulated by the small changes in sarcoplasmic Ca$^{2+}$ concentration. The
actual concentration of Ca\(^{2+}\) required for the activation will not only depend on calmodulin concentration, but also on the ratio of calmodulin to myosin light chain kinase (Stull \textit{et al.}, 1981). Activation of the enzyme is associated with an increase in sarcoplasmic Ca\(^{2+}\) concentrations \textit{in vivo}, followed by Ca\(^{2+}\) binding to calmodulin, and Ca\(^{2+}\)-calmodulin binding to the enzyme thus leading to enzyme activation. The enzyme becomes inactivated in response to a decrease in sarcoplasmic Ca\(^{2+}\) concentration.

Both the amino and carboxyl terminal lobes of CaM must bind Ca\(^{2+}\) and expose their hydrophobic pockets to activate target proteins (Persechini and Kretsinger, 1988). The RS20 and C\(_2\)K peptides represent the CaM interaction domain of smooth muscle MLCK and CaM Kinase II respectively. Upon binding of these two peptides to calmodulin, the affinity of its carboxyl terminal Ca\(^{2+}\) binding sites increases significantly. These sites may be 50-80\% occupied even at the resting levels of Ca\(^{2+}\) found in smooth muscle cells (Cornwell and Lincoln, 1989). The higher affinity carboxyl terminal lobe of CaM may be bound to some target proteins even at the resting Ca\(^{2+}\) level without resulting in the activation of the target protein (Johnson \textit{et al.}, 1995). Subsequent rapid binding of Ca\(^{2+}\) to the N terminal results in the activation of the target proteins.

A similar mechanism exists in skeletal muscle troponin C, where the higher affinity C terminal Ca\(^{2+}\)Mg\(^{2+}\) sites stabilize the troponin complex at resting state, and the rapid exchange of Ca\(^{2+}\) with the faster N-terminal sites regulate contraction and relaxation (Potter and Johnson, 1981). Ca\(^{2+}\) dissociates from CaM after cellular free Ca\(^{2+}\) levels fall, resulting in the disruption of most CaM-target protein complexes and hence
target protein inactivation. Since both halves of CaM are required for activation, the removal of Ca\(^{2+}\) from either the amino or carboxyl terminal Ca\(^{2+}\) binding site would result in enzyme inactivation (Johnson et al., 1995).

Calmodulin also modulates the activity of two ATPases involved in Ca\(^{2+}\) transport, including ATPases of the plasma membrane and cardiac sarcoplasmic reticulum. Calmodulin interacts with plasma membrane Ca\(^{2+}\)-ATPase directly. On the other hand, calmodulin modulates the activity of ATPases of cardiac sarcoplasmic reticulum indirectly. It has been demonstrated that the C-terminal half of calmodulin containing residue 78-148, has the ability to fully activate the Ca\(^{2+}\) transporting ATPase, whereas the N terminal half containing 1-77 cannot activate the Ca\(^{2+}\) transporting ATPase. However, at high concentrations the fragment containing 1-106 amino acid residues can activate ATPase fully thus indicating the third Ca\(^{2+}\) binding site is involved in the interaction with Ca\(^{2+}\)-ATPase (Guerini et al., 1984).

Initial binding between CaM and target proteins, including Ca\(^{2+}\)-ATPase, involves the association of carboxyl terminal chain. Subsequent association of the amino terminal domain is aided by the reduced volume available for diffusion of the amino terminal domain (Persechini et al., 1994; Bayley et al., 1996). Therefore, the concentration of the amino terminal domain available for binding is equal to that of the carboxyl-terminal domain of CaM bound to the PM Ca\(^{2+}\)-ATPase.
3.1.1.1.4 *Interaction of calmodulin with other proteins*

Even though most of the interactions of calmodulin with proteins are dependent on Ca$^{2+}$, few interactions have been demonstrated to be independent of Ca$^{2+}$ binding. One of the isoforms of nitric oxide synthase, iNOS whose expression is induced by cytokines and bacterial endotoxin, remains active, independent of calcium ion concentration. In addition, CaM binds tightly to iNOS even in the absence of Ca$^{2+}$ (Cho *et al.*, 1992).

The CaM binding domain of iNOS contains three aromatic or long-chain hydrophobic side chains that are utilized by MLCK peptide to form key contacts with CaM in the Ca$^{2+}$–CaM-MLCK peptide complex. Although hydrophobic residues appear to be more important than charged residues in binding to CaM, basic residues are invariably found in CaM binding domains. Many of these residues contribute to CaM binding through electrostatic interactions between basic residues in the CaM binding protein and acidic regions in CaM. All seven basic residues in the CaM binding domain in MLCK make salt bridges with CaM. (Meador *et al.*, 1992). iNOS CaM binding domain contains eight basic residues and only one acidic residue for a net charge of +7. iNOS is predominantly in β conformation in solution and becomes helical upon interaction with Ca$^{2+}$-CaM (Yuan *et al.*, 1998).

3.1.1.1.5 *Interaction with drugs and other agents:* Following an extensive reagent screening, several laboratories reported that as many as 50 biologically unrelated peptide hormones, venoms and toxins can bind to calmodulin (Anderson and Malencik, 1986). Some peptide hormones, such as endorphin and corticotropin, bind CaM with 1000-fold
lower affinity compared to the affinity of calmodulin towards enzymes (Malencik and Anderson, 1982). A cytotoxic peptide from bee venom, melittin, forms a 1:1 complex with CaM (Comte et al., 1983). The helical content of melittin increases from 5 to 70% upon complexation with CaM (Maulet and Cox, 1983). The helix formed by melittin is highly basic and amphiphilic, i.e. the hydrophobic and hydrophilic residues project from the opposite faces (Terwilliger and Eisenberg, 1982; Kaiser and Kézdy, 1984). Membrane binding and cytotoxic effects of several lytic peptides require this conformational change (DeGrado, 1983; DeGrado et al., 1981). Mastoprans, another group of peptide toxins capable of forming highly basic amphiphilic helices, were shown to bind CaM with dissociation constant in the nanomolar range (Malencik and Anderson, 1983a). Furthermore a 1:1 complex with fairly high affinity is formed between CaM and either a vasoactive intestinal peptide secretin, or a gastric inhibitory peptide (Malencik and Anderson, 1983b). These homologous peptides are members of the glucagon family and share the conserved structural features of an amphiphilic helix in close proximity to basic residues (Bodansky, 1974).

Each half of calmodulin contains a drug binding site (Vogel et al., 1983; Brzeska et al., 1983; Newton et al., 1984) however, the affinity of the carboxyl terminal half is found to be higher than that of the amino terminal half as measured with the isolated halves (Vogel et al., 1983). Drugs bind to the two sites in the native protein cooperatively. The affinity of calmodulin for the Ca$^{2+}$ channel blocker, nifedipine, has been shown to increase in the presence of trifluoperazine (Mills et al., 1985). The cooperativity of the two drug binding sites was prevented by lanthanides, Cd$^{2+}$ and Pb$^{2+}$.
but not by Ca$^{2+}$. The presence of Ca$^{2+}$, Zn$^{2+}$ and Hg$^{2+}$ abolishes the cooperativity of the drug binding sites. It has been suggested that calmodulin contains an additional metal binding site that affects the interaction between the two halves of the protein (Mills and Johnson, 1985).

3.1.1.2 Site Directed mutagenesis

Site-directed mutagenesis is a technique often utilized for introducing a specific set of alterations in a cloned DNA sequence. This technique allows the creation of very specific changes in the amino acid sequence. Site-directed mutagenesis can introduce three types of mutations namely; the insertion of one or more amino acids; the deletion of amino acid residues, and the substitution of one amino acid residue with another (Gibbs, 1996). The substitution of individual amino acid residues in proteins by site-directed mutagenesis is a powerful tool for analyzing the functional role of specific residues.

To date, a variety of mutagenesis strategies are available and can be applied to study the relationship between the structure and function of a protein. The gene of interest has to be cloned and expressed in cells that do not normally express the corresponding protein in order to perform functional analysis of the mutant protein. Numerous methods are currently available for constructing site directed mutations in vitro. Oligonucleotide-directed mutagenesis is the most commonly used method. Synthetic primer DNA encoding the mutation promotes the synthesis of a mutant strand of the single stranded DNA template using this method (Gibbs, 1996).
The technique of PCR has demonstrated the potential to be utilized for mutagenesis during the early phase of its development (Scharf et al., 1986). A single base mismatch between the primer and the template could be easily incorporated into the template sequence as a result of amplification (Higuchi et al., 1988). It has eventually become possible to introduce all three different types of mutations mentioned above by PCR (Saiki et al., 1988).

3.1.1.3 Polymerase chain reaction

The polymerase chain reaction (PCR) is a reaction in which a fragment of DNA is rapidly amplified by successive rounds of DNA replication by means of a thermostable DNA polymerase enzyme. The basic principle is as follows: two oligonucleotide primers usually 17-30 nucleotides long hybridize to the opposite stands of the double stranded target DNA following its denaturation. Following hybridization, DNA synthesis by DNA polymerase occurs through the regions between the two primers. The DNA polymerase generates two double stranded target regions through the extension reactions. These target regions are again denatured, hybridized and extended by DNA polymerase. The third cycle generates two double-stranded DNA identical to the target sequence. The repeated cycles of heat denaturation, primer hybridization and extension lead to a rapid accumulation of target fragment DNA (White et al., 1989).

Initially, the Klenow DNA polymerase was used for the PCR reaction. This polymerase usually had to be replenished in each cycle due to the employment of very high temperature during the denaturation step (Mullis and Falloara, 1987; Mullis 1990).
Isolation, characterization and expression of *Taq* DNA polymerase from *Thermus aquaticus* in *E. coli*. allowed an easier performance of the PCR (Lawyer *et al.*, 1989). The *Taq* DNA polymerase is thermostable and thus eliminates the necessity to replenish the DNA polymerase in each cycle of the PCR reaction (Saiki *et al.*, 1988).

RBS
AGGAAACAG ATG GCT GAC CAA CTG ACT GAA
GAG CAG ATC GCA GAA TTC AAA GAA GCT TTC TCC CTA
TTT GAC AAG GA GGG GAT GGG ACA ATA ACA ACC AAG
GAG CTG GGG ACG GTG ATG CGG TCT CTG GGG CAG AAC
CCC ACA GAA GCA GAG CTG CAG GAC ATG ATC AAT GAA
GTA GAT GCC GAC GGT AAT GGC ACA ATC GAC TTC CCT
GAA TTC CTG ACA ATG ATG GCA AGA AAA ATG AAA GAC
ACA GAC AGT GAA GAA GAA ATT AGA GAA GCG TTC CGT
GTG TTT GAT AAG GAT GGC AAT GGC TAC ATC AGT GCA
GCA GAG CTT CGC CAC GTG ATG ACA AAC CTT GGA GAG
AAG TTA ACA GAT GAA GAG GTT GAT GAA ATG ATC AGG
GAA GCA GAC ATC GAT GGG GAT GGT CAG GTA AAC TAC
GAA GAG TTT GTA CAA ATG ATG ACA GCG AAG TAA

**Figure 3.1.4** DNA sequence of the rat calmodulin gene (taken from NCBI, accession number XM_236326) and the sequence of ribosome-binding site (RBS) incorporated to the 5’ site of the DNA sequence of rat CaM during incorporation on the multiple cloning site of pKK223-3 plasmid. The DNA sequence is shown in bold.
3.1.2 Photoactivated cleavage of DNA to decontaminate platelet-rich plasma

3.1.2.1 Plasma

Blood consists of particulate cell forms suspended in a fluid medium called plasma. It is a very complicated mixture of inorganic, as well as simple and complex organic materials dissolved in water. If blood is collected without anticoagulant, in vessels with siliconized or non-polar surfaces, the separating fluid is referred to as native plasma. This plasma closely resembles the plasma in the circulating blood. However, in practice, plasma specimens are collected with anticoagulants such as oxalate, citrate, EDTA and heparin for study or analysis. The plasma collected by using anticoagulant differs from native plasma because, it is modified by the addition of chemicals and by the partial loss of calcium which binds oxalate, if it is used.

Plasma contains about 93% water. The remaining 7% consists almost entirely of proteins (Grant and Kachmar, 1976). It contains an extremely complex mixture of simple proteins, lipoproteins, glycoproteins, and other conjugated proteins. Plasma proteins collectively serve a number of different functions. The plasma proteins can serve as storage of amino acids, which can be recycled upon demand for the synthesis of other proteins. They can also be deaminated to give keto acids, which can be mobilized to provide caloric energy, or they can be transformed into carbohydrates and lipids.

Plasma proteins play a crucial role in maintaining the colloidal osmotic pressure. Since they are large colloidal molecules, they are unable to pass through the thin capillary wall membrane as other plasma solutes. They are thus entrapped in the vascular system
and exert a colloidal osmotic pressure, which serves to maintain normal blood volume, and normal water content in the interstitial fluid and the tissues. Albumin plays the most important role in plasma in maintaining normal colloidal osmotic pressure in blood.

Plasma proteins are also involved in maintaining the acid-base balance in blood. They function as buffers to minimize sudden, gross changes in the pH of the blood. Plasma proteins are divided into two large classes, albumins and globulins, on the basis of the precipitate formed in a solution half-saturated with ammonium sulfate as first observed by the scientists. The precipitate is called globulin and the protein remaining in solution is albumin. Albumin and most of the globulins, including the blood clotting and transport proteins are synthesized by the liver, whereas antibodies are synthesized and secreted by the plasma cells in the lymph nodes, bone marrow, spleen and elsewhere. Plasma globulins include fibrinogen. Albumin is the most soluble of all the major serum protein components (Hoffman, 1970).

3.1.2.2 Platelets

Platelets are versatile fragments of cytoplasm whose major function is to cease bleeding. They travel singly as smooth-surfaced discs. Upon injury however, platelets adhere to the newly exposed subendothelial fibril, become sticky and adhere to each other to form a hemostatic plug. Vascular injury without transaction of the vessel wall can also produce platelet masses or white thrombi that may adhere to the endothelial walls, serving as niduses for the growth of more extensive red thrombi or promoting atherosclerotic changes. Platelets need to be activated prior to their aggregation.
The physiological stimuli that promote the activation of platelets are diverse. These stimuli are proteolytic enzymes, such as thrombin or trypsin, ADP, serotonin, or epinephrine. Plasma membrane contains receptors for all these agents (Zucker and Vivianne, 1985). Most stimuli cause a change in the shape of platelets. This change involves first the formation of very fine pseudopodia from the rim of the disc, followed by a general rounding up of the platelet, such that it becomes a spiny sphere, often with much broader pseudopodia (Nachmias, 1983).

3.1.2.2.1 Platelet Adhesion: Platelet adhesion plays a pivotal role in the formation of hemostatic plugs and thrombi, particularly arterial thrombi, although venous thrombi may be initiated by a mass of aggregated platelets that accumulate on adherent platelets in a valve pocket of an injured vein. When a small vessel is severed or when a large vessel is punctured, platelets accumulate rapidly at the site and form a plug composed of aggregated platelets (Mustard and Packman, 1979; Sixma, 1981). Fibrin forms around the plug stabilizing it within a few minutes. The adhesion of platelets to the injured site is one of the first events that follows the removal of the endothelial lining of a normal blood vessel which has not been injured previously.

Platelets are also necessary to maintain normal vessel wall integrity (Johnson, 1971). Around 10-15% of circulating platelets are consumed for the sake of maintaining vascular integrity each day in a normal subject (Hanson et al., 1983). In order to maintain the endothelial lining, platelets must adhere to the vessel wall. Electron micrographs
illustrate that platelets interact with the vessel wall, if there are gaps between the endothelial cells (French, 1969; Hovig et al., 1968).

Activation of platelets can be triggered by a variety of chemically and functionally unrelated compounds. However, the outcome is independent of the stimulating agent. A series of events take place following activation such as aggregation, prostaglandin and thromboxane synthesis, specific secretion of substances from storage organelles, and manifestation of contractile and procoagulant activities. These events usually, but not always, precede the change of platelet shape (White, 1974). Some of these events may proceed independent of the others. Since response is identical following platelet activation, all the agents probably activate the same effector system that determines further the course of events. Several studies have demonstrated later that this effector is Ca²⁺ (Bygrave, 1978; Carafoli et al., 1975).

3.1.2.3 Restriction Endonucleases

Restriction endonucleases are enzymes that have the ability to cut DNA molecules at specific sites. These enzymes were first isolated from bacteria. These restriction enzymes are composed of three subunits: a specificity subunit, which recognizes specific sequences in DNA, a modification subunit and a restriction subunit. Restriction enzymes require magnesium ions and both cofactors ATP and S-adenosylmethionine (SAM) for activity (Wilson and Murray, 1991).

In the presence of SAM, the enzyme binds to the bipartite recognition sequence irrespective of its methylation state. If the recognition sequence is methylated in both strands, the ATP stimulates the dissociation of the enzyme. However, if the site is
hemimethylated ATP stimulates the methylation of the other strand using SAM as the methyl donor. On the other hand if the site is unmethylated, cleavage occurs (Yuan et al., 1975; Bickle et al., 1978).

These enzymes can be divided into three major classes namely class I, class II and class III. Class I restriction endonucleases recognize specific nucleotide sequences, but cleavage occurs at sites other than the recognition sites (Yuan et al., 1980). Class II enzymes recognize specific sequences of four to six nucleotide pairs in a duplex DNA molecule and cleave the polynucleotide chains within, or near to that sequence. Thus type II mediated cleavage gives rise to discrete DNA fragments of defined length and sequence. Class III enzymes recognize asymmetric sequence of DNA and the cleavage occurs by nicking one strand at a measured distance to one side of the recognition sequence. Two sites in opposite orientations are necessary to cleave the DNA duplex (Kelly and Smith, 1970; Smith and Wilcox, 1970).

3.1.2.4 Photocleavage of DNA

Many chromophores that are capable of initiating specific or non-specific cleavage of DNA upon photoillumination have been developed over the years. The cleavage of DNA upon photoactivation of the chromophores can occur through different pathways. Following light absorption by a DNA associated photosensitizer, the excited state can: i) abstract a hydrogen atom from ribose; ii) abstract an electron from the nucleic acid base; iii) transfer energy to molecular oxygen to produce singlet oxygen; or iv) initiate hemolytic or heterolytic bond cleavage elsewhere in the chromophore to generate reactive oxygen species (Armitage, 1998).
If the photocleavage agent is covalently or non-covalently bound to DNA, the initial site of DNA damage will be site specific (Rogers et al., 2001). DNA strand scission can result from the production of a carbon-based radical following hydrogen atom abstraction from deoxyribose. In general, the scission is initiated by the reactive intermediates produced in bulk solution leading to non-specific cleavage of the phosphodiester backbone. Hydroxyl radicals, generated from the Fenton reaction, and nπ* triplet excited states have been shown to initiate hydrogen atom abstraction (Rokita and Romero-Fredes, 1992; Breslin and Schuster, 1996).

Among the classes of compounds that have been shown to potentiate photocleavage through the oxidation of nucleic acid bases are riboflavins (Ito et al., 1993), imide (Saito et al., 1996) and anthraquinone derivatives (Armitage et al., 1994; Breslin et al., 1997). Adam and his coworkers (1997) showed for the first time that alkoxy and benzoyloxy radicals are also capable of causing strand cleavage in DNA. Upon UVA irradiation, N-alkoxypyridinethiones, N-isopropoxy pyridine-2-thione and N-tert-butoxypyridine-2-thione induced strand cleavage in supercoiled pBR322 DNA through the generation of alkoxy radical. (Adam et al., 1997).

3.1.2.4.1 DNA photocleavage arising from deoxyribose oxidation: Oxidation of deoxyribose due to hydrogen atom abstraction from the sugar furanose ring is quite often the crucial step in DNA cleavage. The resulting sugar radicals (Tullius, 1998) can decompose by a variety of pathways yielding small molecule byproducts and DNA fragments (Papavassiliou, 1995; Pratviel et al., 1995). Since, deoxyribose residues are found at every step along the DNA duplex, this method of cleavage is inherently not
sequence specific. The selectivity depends on the local structure of DNA and physiochemical properties of the abstraction agent. The H-abstraction can be highly selective if the photocleavage agent binds to only one or a few sequences (Armitage, 1998).

3.1.2.4.2 Photocleavage through electron transfer: Electronically excited states of some photosensitizers are powerful enough to oxidize nucleic acid bases. Oxidation of guanine (G) by an excited state photosensitizer (P) produces the G radical cation and the P radical anion. The subsequent reactivity of these ions is complex and depends on the secondary structure of the DNA resulting in the decomposition of both 8-Oxoguanine and oxazolone (Angelov et al., 1997).

\[ P^* + G \rightarrow P^*+ G^{**} \]

The excited state electron transfer agents cleave B-form DNA specifically at guanines. However, the efficiency of cleavage at different G sites is quite variable depending on its flanking sequence. In particular, guanines located on the 5' side of at least one other G are strongly preferred over all other cleavage sites. Not all GC sites are cleaved equally (Armitage, 1998).

3.1.2.4.3 Photocleavage through singlet oxygen generation: Electronically excited compounds that are capable of intersystem crossing to the triplet state and possess sufficiently high triplet energy, which can generate singlet oxygen by energy transfer to
oxygen. Singlet oxygen is a highly reactive species that reacts preferentially with guanine leading to its oxidation (Lee and Rogers, 1987). The resulting oxidized guanine is sensitive to piperidine treatment, which induces a strand scission at different G containing sites. The yield of cleavage at different G sites does not vary a lot (Blazek et al., 1989; Devasagayam et al., 1991). However, in a biological environment that contain both nucleic acid and protein components, singlet oxygen mediated DNA cleavage is not desirable because singlet oxygen will react with proteins leading to secondary damage (Matheson and Lee, 1979).

Porphyrims are well-known singlet oxygen generators (Verlhac et al., 1984; Keir et al., 1987). They can selectively cleave DNA at guanine residues (Croke et al., 1993). Photocleavage of DNA by porphyrin derivatives has been reported by many research groups.

3.1.2.5 NO release from GSNO in the presence of photosensitizer: It has been shown previously that photosensitizers enhance the release of NO from the donor compounds (Singh et al., 1995). In the presence of a photosensitizer called Rose Bengal, the quantum yield for NO production from GSNO has been demonstrated to increase nine fold. A concomitant increase in the rate of thyl radical production has also been observed. Rose Bengal has an absorption peak with \( \lambda_{\text{max}} = 545 \text{ nm} \), which overlaps considerably with the GSNO absorption peak. The enhancement of the quantum yield was suggested to be due to the spectral overlaps. Hematoporphyrin also enhanced the rate of NO production by 2-3 fold (Singh et al, 1995).
3.1.2.6 Reacting nitrogen species and DNA damage: The major nitric oxide derived species that are involved in DNA damage in cells are nitrous anhydride and peroxynitrite (Tannenbaum et al., 1994; Koppenol et al., 1992). Nitrous anhydride is able to cause nitrosative deamination of nitrogen bases in DNA. Guanine and adenine residues are deaminated more readily than cytosine (Schmutte et al., 1994). N-nitrosoamine can be formed from the reaction of nitrous anhydride with primary amines (Lewis et al., 1995). These well-known chemical carcinogens are metabolized to strongly alkylating electrophiles that would directly react with DNA at several nucleophilic sites. This has been demonstrated in both cell cultures and animals, with preformed N-nitroso compounds.

Nitric oxide can react with molecular oxygen to form nitrous anhydride (N₂O₃). It may form tertiary species, reacting with amines to form nitrosamines, or with thiols to form S-nitrothiols. Nitric oxide can also react with superoxide to form the very potent oxidant, peroxynitrite. Tertiary products formed from peroxynitrite include glucose adducts (Moro et al., 1995), nitrosamines (Ohshima and Bartsch, 1994), and nitrosothiols (Mayer et al., 1995; Jia et al., 1996).

An increased level of deamination and oxidation products of DNA bases have been detected in macrophages which are activated with lipopolysaccharides and interferon γ. Peroxynitrite formed from macrophages has been implicated as one possible mechanism for oxidative DNA damage (deRojas-Walker et al., 1995). Guanine has been demonstrated to react rapidly with peroxynitrite under physiological conditions. 8-nitroguanine has been identified as a major product based on chromatographic analysis (Yermilov et al., 1995a). Other NO donating compounds or nitrating agents fail to form
identical products with guanine. 8-nitroguanine (Nitro\textsuperscript{8}Gua) is rapidly depurinated from DNA, thus yielding apurinic sites that are potentially mutagenic (Yermilov \textit{et al.}, 1995a). It has been suggested that Nitro\textsuperscript{8}Gua could be potentially utilized as a specific marker of DNA damage induced by NO and peroxynitrite in inflamed tissues, like oxo\textsuperscript{8}Gua serves as marker for oxidative damage (Yermilov \textit{et al.}, 1995a). Two compounds employed in this study to induce photocleavage of DNA are shown in Figure 5.

\[ \begin{align*}
\text{N-dansyl-S-nitrosohomocysteine} &\quad (\text{Dns-HcysNO}) \\
\text{N-dansyl-S-nitrosoglutathione} &\quad (\text{Dns-GSNO})
\end{align*} \]

\textbf{Figure 3.1.5} Structure of the two compounds, Dns-GSNO and Dns-HCysNO employed in this study
3.2 Purpose of study

3.2.1 *Exploration of potential roles of tyrosines in rat calmodulin*

Homolytic cleavage of the S-N bond in RSNO generates NO and thyl radical (RS*) (Josephy *et al.*, 1984). It has been demonstrated earlier that in aerated solution, RS* can either react with molecular oxygen to form RSOO* or can react with GSNO and water to form GSSG*. The formed GSSG* is capable of oxidizing oxygen thus generating superoxide (Wardman, 1988; Winterbourn, 1993), which can in turn react with NO to form peroxynitrite. Thus, homolytic cleavage of RSNO can potentially lead to the formation of peroxynitrite. We have shown this to be the case: photolysis of air-saturated GSNO solution resulted in peroxynitrite formation (Mutus *et al.*, 1999). Under appropriate conditions, peroxynitrite generation has also been reported from NO· (Hogg *et al.*, 1996) that can be formed by heterolytic cleavage of RSNO (Arnelle and Stamler, 1995).

Peroxynitrite is both a good nitrating and oxidizing agent (Beckman, 1996). Peroxynitrite has been demonstrated to cause partial or full inactivation of enzymes and proteins by nitrating their critical tyrosine residues (Ara *et al.*, 1998; Wong *et al.*, 2001). Pathrose, one of my coworkers has demonstrated previously that calmodulin loses its functional activity upon incubation with peroxynitrite. It was suggested that nitration of the tyrosine residues were responsible for the inactivation of calmodulin (Pathrose, 1997).

The goal of this study was to further evaluate whether the nitration of the tyrosine in calmodulin was indeed responsible for this observed loss of functional activity.
reported by Pathrose (1997). In addition, I wanted to identify the specific tyrosine residue that was the target of peroxynitrite modification.

3.2.2 Photoactivated cleavage of DNA to decontaminate platelet rich plasma

Restriction endonucleases purified from bacteria are generally used to initiate double strand cleavage in DNA in performing different molecular biological techniques. Although restriction enzymes are very useful tools in the manipulation of nucleic acids in many molecular biological techniques, most of them recognize specific sequences that limits their utility in many other applications. Furthermore, restriction enzymes have been isolated and identified for only a number of possible nucleic acid sequences. Therefore, synthesis of artificial nucleases is required that can be designed to cleave a sequence of choice.

Reagents that are capable of initiating cleavage of DNA irrespective of the sequence are utilized in many analytical applications. However, there is still a lot of interest to design compounds that will be able to cleave any sequence of DNA as well as controlling the initiation of the cleavage.

Viral transfusion through the blood is still a concern even though blood products are extensively tested for any possible contamination following their collection. The goal of our study was to synthesize compounds that would be capable of decontaminating platelet-rich plasma upon photoillumination. Peroxynitrite is known to cause DNA damage (Yermilov et al., 1995a; Gu et al., 2002). Aerated aqueous solution of GSNO has been demonstrated to lead to the formation of peroxynitrite following the release of NO
by photolytic decomposition (Mutus et al., 1999). Conjugation of a photosensitizer has been reported to increase the yield of NO generation form GSNO by nine fold. The goal of our study was to develop fluorophore-labelled RSNO compounds that will be able to inactivate any potential contaminating viruses by damaging their DNA in platelet rich blood plasma.
3.3 Chemicals, supplies and Instruments

3.3.1 Exploration of potential roles of tyrosines in rat calmodulin

3.3.1.1 Chemicals and supplies

3.3.1.1.1 General chemicals

PhenylSepharose CL-4B, Ampicillin, Sodium chloride, Trishydroxymethylethanolamine, Ethylenediaminetetraacetic acid (EDTA), Sodium dodecyl sulfate (SDS), phenylmethysulfonyl fluoride (PMSF), and Isopropylthiogalactoside (IPTG), β-mercaptoethanol were purchased from Sigma-Aldrich (Oakville, ON)

Bio-Rad protein assay reagent, Coomassie Brilliant Blue R-250, ammonium persulfate, polyacrylamide solution, N, N, N', N' Tetramethylethylenediamine (TEMED), Chelex 100 resin (50-100 mesh), and protein molecular weight standards for SDS-PAGE (Broad range, Mw 6900 to 205 000 Da) were purchased from BioRad (Hercules, CA)

Trypticase peptone, yeast extract, granulated agar-Beckton-Dickison Co. (Cockeysville, MD)

Trichloroacetic acid, glycerol were purchased from BDH Inc (Toronto, ON)

3.3.1.1.2 Supplies

FlexiPrep kit for plasmid DNA purification was purchased from Pharmacia Biotech (Baie d’Urfe, Quebec)

Nalgene™ 25 mm syringe filter (pore size 0.2 μm) was purchased from Sarstedt Inc. (Montreal, Quebec, Canada)

QuickChange site directed Mutagenesis kit was purchased from Stratagene (La Jolla, CA)
3.3.1.1.3 Plasmid construct of rCaM: Complete plasmid construct pKK223-3 (Pharmacia) containing the rat CaM open reading frame (ORF) was kindly provided by Dr. Arthur Szabo, University of Waterloo. The plasmid was designed to express rat calmodulin under the control of tac promoter. Rat CaM ORF was cloned into the multiple cloning site (MCS) of the vector using Smal and Hind III restriction sites. A new ribosome-binding site was also introduced into the construct.

3.3.1.1.4 Primers for site directed mutagenesis

Oligonucleotides used for PCR primers for creating CaMY₊

5'- GAT AAG GAT GGC AAT GGC GCG ATC AGT GCA GCA GAG C
3'- CTA TTC CTA CCG TTA CCG CGC TAG TCA CGT CGT CTC G

Corresponding amino acid sequence

Asp-Lys-Asp-Gly-Asn-Gly-Ala-Ile-Ser-Ala-Ala-Glu-

Oligonucleotides used for PCR primers for creating CaMY138

5'- GGG GAT GGT CAG GTA AAC GCG GAA GAG TTT GTA CAA ATG
3'- CCC CTA CCA GTC CAT TTG CGC CTT CTC AAA CAT GTT TAC

Corresponding amino acid sequence

Gly-Asp-Gly-Gln-Val-Asn-Ala-Glu-Glu-Phe-Val-Gln-Met

3.3.1.1.5 Buffers and solutions

3.3.1.1.5.1 General buffer

1x Phosphate buffered saline (PBS)- consisted of 0.137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.76 mM KH₂PO₄ pH 7.4.
3.3.1.1.5.2 Solutions for bacterial culture

Luri-Bertani Broth (LB broth): LB broth was prepared by dissolving 10 g trypticase peptone, 5 g NaCl and 5 g yeast extract together in 1 litre of water. The solution was autoclaved for 20 minutes following its preparation and used the same day or stored at 4°C for later use (Sambrook et al., 1989).

100 mM ampicillin stock was sterilized with 0.2 μm syringe filter

500 mM Isopropylthiogalactosidase (IPTG) stock-following preparation was sterilized with a 0.2 μm syringe filter and aliquoted for storage at -20°C.

3.3.1.1.5.3 Buffers and solutions for calmodulin purification

Buffer A: this buffer was composed of 20 mM Tris pH 7.4, 10 mM CaCl₂ and 5 mM β-mercaptoethanol

Buffer B: Buffer B was composed of 20 mM Tris pH 7.4, 10 mM CaCl₂ 5 mM β-mercaptoethanol, and 0.5 M NaCl

Buffer C: Buffer C was composed of 20 mM Tris pH 7.4, 1 mM EDTA, and 5 mM β-mercaptoethanol

Buffer D: 20% ethanol solution

25-50 mg/ml Phenylmethylsulfonyl fluoride (PMSF) stock solution

SDS-PAGE sample buffer: 0.1% bromophenol blue, 2% SDS, 10% glycerol, 50 mM Tris-HCl pH 6.8

Staining solution for SDS-PAGE: contains 0.1% Coomassie blue R250, 40% methanol and 10% acetic acid

Destaining solution: 40% methanol and 10% acetic acid
3.3.1.2 Photoactivated cleavage of platelet rich plasma

3.3.1.2.1 General chemicals and supplies

Ethidium Bromide, Glutathione, Homocysteine, Dansyl chloride, Agarose Tris-hydroxymethylethanolamine, ethylene diamine tetraacetic acid (EDTA), bromophenol\nblue - SIGMA Chemical Co. (Oakville, ON)\n
Ethanol, Acetic acid, glycerol - BDH Inc. (Toronto, ON)\n
FlexiPrep kit for plasmid DNA purification-Pharmacia Biotech (Baire d’Urfe, Quebec)

3.3.1.2.2 Plasmids used for purification: *E.coli* JM 109 transformed with plasmid DNA\nR773 containing the gene sequence for arsenic reductase and the ampicillin resistance\ngene was a generous gift from Dr. Lana Lee, Department of Chemistry and Biochemistry,\nUniversity of Windsor, Windsor. The size of the plasmid was approx 3200 bp.

3.3.1.2.3 Plasmid DNA purification: (Flexi-prep Kit, Amersham-Pharmacia Biotech)

Solution I: 100 mM Tris-HCl pH 7.5, 10 mM EDTA, 400 μg/ml RNAase I

Solution II: 0.18 M NaOH, 1% (w/v) Sodium dodecyl sulfate (SDS)

Solution III: 3M Potassium, 5 M acetate solution

Sephaglas™ FP: Sephaglas FP suspended in a buffered solution of guanidine-HCl, 50\nmM Tris-HCl (7M guanidine-HCl, 50 mM Tris-HCl pH 7.5, 10 mM EDTA)\n
Wash buffer: 20 mM Tris-HCl pH 7.5, 2 mM EDTA, 200 mM NaCl, 60% ethanol
3.3.1.2.4 Buffers for agarose gel electrophoresis

50x TAE buffer stock solution: 2M Tris-hydroxymethylethanolamine, 50 mM EDTA, 1M acetic acid

Agarose gel running buffer: 50x TAE buffer was diluted to 1x for the agarose gel electrophoresis. 300 ml of the running buffer was required for each run.

6x gel loading dye stock: 0.25% bromophenol blue containing 30% glycerol

Tris-EDTA (TE) buffer: 10 mM Tris base pH 8, 1 mM EDTA
3.3.2 Instruments employed

3.3.2.1 Exploration of potential roles of tyrosines in rat calmodulin

BioRad HPLC system-BioRad (Hercules, CA)

Aviv CD spectrometer model 62A (Aviv Associates, Lakewood, NJ)

SDS-PAGE electrophoresis chamber- BioRad (Hercules, CA)

Fisher Water bath-Fisher Scientific (Toronto, ON)

Scanner-Hewlet Packard, Scanjet 4C

PCR-48-well Perkin Elmer Cetus DNA thermal cycler (Perkin Elmer, Norwalk, CT)

Orbit Labline Incubator shaker - Lab-Line instruments (Melrose Park, Ill)

Orbit Shaker-Lab-Line instruments (Melrose Park, Ill)

VWR Scientific Incubator- VWR (Mississauga, ON)

3.3.2.2 Photoactivated cleavage of DNA to decontaminate platelet rich plasma

MINI-SUB cell GT-DNA electrophoresis chamber-BioRad (Hercules, CA)

Fisher Vortex Genie 2-Fisher Scienitfic (Toronto, ON)

Kodak Camera-Kodak

Benchtop ultraviolet Transilluminator-VWR Scientific (Mississauga, ON)

Benchtop centrifuge-non refrigerated EBA 21- Hettich Zentrifugen-Rose Scientific (Mississauga, ON)

Eppendorf Mixer 5432-Brinkmann Instruments (Mississauga, Ontario)

Visible lamp (20W)

Orbit Shaker-Lab-Line instruments (Melrose Park, Ill)
3.4 Methods

3.4.1 Exploration of potential roles of tyrosines in rat calmodulin

3.4.1.1 Preparation of $\text{Ca}^{2+}$ free buffers: A slurry of chelex-100 (approximately 30 ml) was prepared by mixing chelex-100 with a minimal amount of the buffer to be decalcified and packed into a column. The buffer that needs to be decalcified was run though the column and the decalcified buffer then obtained was stored in polycarbonate bottles for up to three days at 4°C. A 30 ml chelex column was used for the decalcification of each litre of buffer.

3.4.1.2 Trichloroacetic acid precipitation of calmodulin variants: 1/6 volume of 100% trichloroacetic acid solution was added to the purified calmodulin solution and vortexed to mix the solution thoroughly. The mixture was incubated on ice for 10 minutes and then centrifuged at 10,000x g for 1 min in a benchtop centrifuge. Following centrifugation, the supernatant was discarded and the pellet was washed twice with water. The clean pellet was finally resuspended in the $\text{Ca}^{2+}$ free buffer.

3.4.1.3 SDS-PAGE analysis: Purified calmodulin samples obtained by affinity chromatography were diluted 2x in sample buffer and boiled 5 minutes in a hot water bath. The boiled protein samples were then loaded along with a protein standard onto a 15% polyacrylamide gel prepared according to the standard protocol and protein bands were separated by electrophoresing the gel for ~50 minutes at 150 V.
3.4.1.4 Staining/Destaining: Following electrophoresis, the protein gels were stained overnight with staining solution at room temperature under moderate shaking. The gels were then destained by incubating with destaining solution for 3 hours at RT under gentle shaking. The destained gels were then scanned using Hewlet Packard Scanjet to obtain a permanent image of the gel.

3.4.1.5 Creation of the calmodulin mutants: The calmodulin mutants were created by using the QuickChange™ Site-Directed mutagenesis kit from Stratagene. The kit uses the thermostable DNA polymerase, Pfu DNA polymerase that can replicate both plasmid strands with high fidelity without displacing the mutant oligonucleotide primers.

A supercoiled, plasmid pKK223-3 containing the rat calmodulin gene and two oligonucleotides (27-28 bp long) complementary to the both strands of calmodulin gene containing mutations at 99 or 138 position were utilized. The oligonucleotides contained the coding sequence for alanine instead of tyrosine at position 99 or 138. PCR amplification by Pfu polymerase led to the generation of a mutated plasmid containing staggered nick through the incorporation of the oligonucleotide primers.

Following temperature cycling, the parental DNA was digested with Dpn I, which was specific for methylated, and hemimethylated DNA. Since all plasmid DNA isolated from E. coli strains were methylated, Dpn I only digested parental DNA leaving the double stranded mutated PCR product intact. Following digestion of the parental plasmid, Epicorian coli XL1-Blue supercompetent cells were transformed with nicked vector DNA containing the desired mutations.
For the generation of the double calmodulin mutant following introduction of the first mutation, the mutated plasmid was used again as a template and another cycle of PCR was run following the procedure described above to introduce the second mutation.

3.4.1.6 Transformation of Epicurian Coli XL1-Blue cells: A 50 µl aliquot of Epicurian coli XL-1-Blue supercompetent cells were aliquoted to prechilled Falcon 2059 polypropylene tubes. A volume of 1 µl of Dpn-1 treated DNA was then added to the aliquots of the supercompetent cells, gently mixed, and incubated on ice for 30 minutes. The cells were then incubated in a water bath for 45 seconds at 42°C. Following heat shock, the cells were further incubated on ice for 2 minutes. Approximately 0.5 ml preheated LB broth was then added to the polypropylene tubes, which were further incubated at 37°C for 1 hour under moderate shaking. The LB broth containing the cells was finally plated on agar plates containing ampicillin.

3.4.1.7 Purification of mutant rCaM from Epicurian Coli XL1-Blue cells: Plasmids containing the mutated rCaM sequences were purified from transformed Epicurian coli XL-1-Blue cells using a Flexi-Prep kit from Pharmacia as described above.

3.4.1.8 Preparation of competent E. coli BL21(DE3): E. coli BL21 (DE3) cells were grown until the absorbance reading reached 0.6. At this point, the cells were pelleted by centrifuging at 10,000 rpm for 5 minutes at 4°C, and the supernatant was discarded. The pellet was then resuspended in cold 50 mM CaCl₂ and incubated on ice for 45 minutes.
The cells were then centrifuged again and the supernatant was poured off. The pellet was resuspended in 1 ml of cold CaCl₂ solution containing 8% glycerol and stored at -80°C.

3.4.1.9 Transformation of *E.coli* BL21(DE3): Approximately 1-2 μl of plasmid DNA purified from *Epicurian coli* XL1-Blue cells was added to 200 μl of competent *E. coli* BL21(DE3) cells and mixed gently. The mixture was then incubated on ice for 45 minutes. Following incubation, the bacteria were heat shocked at 42°C for 90 seconds. After heat shock, the suspension was spread over on agar plates containing ampicillin using a glass spreader and grown overnight at 37°C in the incubator.

3.4.1.10 Overexpression of rat calmodulin in *E. coli*. *BL21(DE3)* cells: *E.coli* BL21(DE3) colonies transformed with rCaM were inoculated into 10 ml sterile LB medium containing 0.1 mM ampicillin and were grown overnight under moderate shaking at 37°C. The following day, 2 ml of the overnight culture was transferred to 2 litres of fresh LB medium containing 0.1 mM ampicillin and grown further at 37°C until the absorbance reading reached 0.6-0.7 at 600 nm. At this point, 1mM IPTG was added to the growing culture to induce protein synthesis and the cells were grown further for an additional 4 hours at 37°C to maximize protein synthesis. *E. coli.* cells were finally harvested by centrifugation at 5,000 rpm for 20 minutes at 4°C, and the bacterial pellet was stored at -20°C.

3.4.1.11 Lysis of *E. coli*. *BL21(DE3)* cells: The frozen cell pellet was thawed and resuspended in 10 ml of buffer C containing 5 units of DNAase and 0.2-0.3 μg/ml PMSF.
The cell pellet suspension was then sonicated for 5 minutes to obtain the cell lysate. The cellular debris was pelleted by centrifuging the suspension at 15,000 rpm for 20 minutes. The supernatant was used for the purification of the calmodulin mutants.

### 3.4.1.12 Purification of rat calmodulin:

Wild type and the three calmodulin mutants were purified in two steps by using a phenyl Sepharose-CL 4B affinity column. Calmodulin has large hydrophobic regions that become exposed only upon calcium binding. This structural feature of calmodulin was utilized to purify the three mutants and the native rat calmodulin by using affinity chromatography according to the procedure described by Pedigo and Shea (1995).

In the first step, Ca$^{2+}$ was not included in the crude extract of the proteins or the buffers; therefore the hydrophobic region of CaM was buried and could not bind to the phenyl Sepharose affinity column. CaM was thus washed right through, whereas the other proteins were bound to the column. In the second step, Ca$^{2+}$ was added to the CaM containing fractions eluted from the first column to uncover the hydrophobic domains. All the buffers used also contained 0.1 mM Ca$^{2+}$. In this step, CaM bound to the column though hydrophobic interactions, while other proteins were washed out.

#### A. First Step:

Phenyl Sepharose CL-4B was equilibrated with buffer C overnight at $4^\circ$ C prior to its use. Approximately 50 ml of phenyl Sepharose column was used for the cell lysate obtained from 2 litre culture. The supernatant obtained following centrifugation of the crude cell lysate was loaded onto the column at a flow rate of 0.75 ml/min and the effluents were collected at 1 min intervals immediately after loading of the sample. The
fractions were checked for proteins using protein spot tests. Then the column was washed with buffer C using the same flow rate, until no more spots could be detected. The column was washed with buffer E followed by buffer D to regenerate the column for the second purification step.

The fractions depicting positive in the spot test were electrophoresed using a 15% polyacrylamide gel to identify calmodulin-containing fractions. The fractions that exhibited bands corresponding to the size of calmodulin following gel staining were pooled together for further purification in the second step.

**B. Second step:** The regenerated column from step one was used to perform the second step of purification. CaCl$_2$ was added to the pooled fractions of calmodulin to a final concentration of 10 mM, and the contents were mixed gently. The Ca$^{2+}$-containing calmodulin solution was then loaded onto the phenyl Sepharose column pre-equilibrated with buffer A. The column was washed with buffer A, followed by buffer B, and then buffer A again using a flow rate of 0.75 ml/min following loading the calmodulin solution. The bound calmodulin was then eluted from the column with buffer C in 1 min fractions using the same flow rate. The column was then washed again with buffer C, followed by buffer D. The purity of calmodulin fractions were examined by electrophoresing the samples with 15% polyacrylamide gel.

**3.4.1.13 Determination of calmodulin concentration:** The concentration of calmodulin in the samples was determined by following the Bradford assay (Bradford, 1976). Briefly, the protein samples were diluted in 800 µl of water, followed by the addition of 200 µl of Bradford reagent from Sigma. The protein-dye mixtures were then incubated for 10 minutes at room temperature and the absorbance was read at 595 nm at the end of
the incubation period. A mixture of water and Bradford reagent in the same ratio as the protein sample was used to blank the spectrophotometer. The unknown concentration of the protein sample was calculated from a standard curve generated with standard protein solutions of wild type rat calmodulin.

3.4.1.14 Circular dichroism measurements: CD spectra of native rat calmodulin and different calmodulin mutants were acquired in an AVIV CD spectrometer model 62A DS. Trichloroacetic acid precipitated calmodulin samples were dissolved in Ca$^{2+}$-free PBS buffer and the pH of the protein samples was adjusted with filtered NaOH. The CD measurements were taken by using 10-20 μg/ml solution of calmodulin in Ca$^{2+}$-free PBS containing either 0.1 mM EDTA or 0.1 mM CaCl$_2$. All experiments were performed at room temperature using an all side clear quartz cuvette with a 1 cm path-length. The CD spectra were collected over the wavelength region of 190 to 260 nm at 1 nm intervals with an integration time of 10 sec. Each scan was the average of three different scans. Each spectrum presented in the figures was the average of four independent experiments.

3.4.2 Photactivated cleavage of platelet rich plasma

3.4.2.1 Agarose gel electrophoresis of DNA: 0.35 g agarose was mixed with 50ml of 1x TAE buffer and the agarose was melted in a microwave for 90 sec. The melted agarose was then poured into a gel-casting tray containing teflon combs to solidify. Following solidification the comb was removed. The DNA samples were loaded onto the gel after
diluting it (1:6 volume) with the 6x gel loading dye. The gel was run at 90 V until the dye front reached two third of the gel length (Sambrook et al., 1989).

3.4.2.2 Staining of the agarose gel: The agarose gel containing the resolved DNA bands were incubated with 0.6 μg/ml EtBr containing 1x TAE buffer solution for 30 min at room temperature with moderate agitation (Sambrook et al., 1989).

3.4.2.3 Purification of Plasmid DNA: DNA was purified from E. coli JM 109 cultures using a Flexi-prep kit from Amersham Pharmacia Biotech. A 10 ml bacterial culture grown in LB containing 0.05 mM ampicillin was used. The culture was grown at 37°C under shaking at 220 rpm overnight. Bacterial pellet was collected by centrifuging 1.5 ml of bacterial culture for 1 minute at 14,000 rpm in a benchtop centrifuge.

Following centrifugation the pellet was resuspended in 200 μl of solution I by vigorous vortexing. A 200 μl of solution II, followed by 200 μl of solution III was added to the suspension and mixed thoroughly. After mixing, the suspension was centrifuged for 5 minutes at 14,000 rpm at room temperature. The supernatant was transferred to a new microfuge tube and the DNA was precipitated by the addition of 420 μl isopropanol. The precipitated DNA was collected by centrifuging the suspension again for 10 minutes at 14,000 rpm. The DNA pellet was then resuspended in 150 μl of the Sephaglas FP slurry and incubated for 1 min at room temperature to allow binding of DNA to the Sephaglas. The Sephaglas pellet with bound DNA was collected by centrifugation and washed with 200 μl of wash buffer followed by 300 μl of 70% ethanol. The washed
pellet was then allowed to dry in the air for 10 minutes. Finally, the Sephaglas bound DNA was eluted with 50 μl TE buffer at room temperature and stored at 4°C for later use.

3.4.2.4 *Synthesis of S-nitroso-N-dansylhomocysteine (Dns-HCysNO) and S-nitroso-N-dansylglutathione (Dns-GSNO):* Synthesis of both Dns-HCysNO and Dns-GSNO were performed in two steps. In the first step, GSNO was synthesized according the procedure described in the method in Part I (section-1.3.1) of the thesis. In the second step, 20 mg dansyl chloride was dissolved in 2 ml of acetone and mixed with 4 ml of 50 mM Li₂CO₃ buffer pH 9.5. The reaction was started by the addition of equimolar GSNO to the dansyl chloride solution and the reaction was allowed to progress overnight at room temperature under moderate stirring in the dark. The product obtained was then purified with a Sephadex G10 column, freeze-dried and stored at -80°C.

Dns-HcysNO was synthesized according to the same procedure using S-nitrosohomocysteine instead of S-nitrosogluthathione.

3.4.2.5 *The effect of Dns-GSNO/ Dns-HCysNO and light on the plasmid DNA:* Equal amounts (0.3 μg) of plasmid DNA in TE buffer were aliquoted into two sets of microfuge tubes. Appropriate amounts of Dns-GSNO or Dns-HCysNO were added to both sets of microfuge tubes containing the plasmid DNA, except for the control experiment. Following addition, the content in the microfuge tubes were mixed thoroughly by gentle vortexing and incubated for 30 minutes. During incubation, one set of the microfuge tubes were exposed to visible light using a 20 W lamp from 1 inch distance and the other set was protected from light by wrapping the tubes with aluminium foil. At the end of the
incubation period, all the DNA samples were electrophoresed on a 0.7% agarose gel. Following electrophoresis the gel was stained with 0.6 μg/ml EtBr solution to visualize DNA under UV using a UV transilluminator. The pictures of the gel were captured using a Kodak camera.

3.4.2.6 Effect of light exposure time: To evaluate the effect of exposure time with visible light on the Dns-HeysNO treated plasmid DNA, (1.08-1.95 mM) Dns-HeysNO were mixed with to 0.3 μg plasmid DNA aliquoted in microfuge tubes. Each sample was illuminated with visible light for different periods of time. The microfuge tubes exposed to shorter intervals of time were stored in the dark until the exposure time of all the microfuge tubes had been completed. All samples were electrophoresed on a 0.7% agarose gel, which was stained with 0.6 μg/ml EtBr solution to visualize the DNA bands under a UV illuminator.
3.5 Results

3.5.1 Exploration of potential roles of tyrosines in rat calmodulin

3.5.1.1 SDS-PAGE of the wild type calmodulin (wt-CaM) and calmodulin mutants: SDS-PAGE of both native and mutant rat calmodulin fractions purified by phenyl Sepharose affinity column was performed to assess the purity and the yield of these purified proteins. All the CaM variants were obtained with almost 98% purity as confirmed by SDS-PAGE (Figures 1-4). Among the mutants, CaM Y138A had the highest yield (Figure 3) and CaM Y99A Y138A had the lowest yield (Figure 4) as depicted by SDS-PAGE. wt-CaM and CaM Y99A both had relatively moderate yields (Figures 1, 2).

3.5.1.2 Effect of Ca²⁺ on the secondary structure of wt-CaM: Calmodulin is a calcium-binding protein, and Ca²⁺ binding is known to increase the percentage of its α-helical content (Killhofer et al., 1981). The secondary structural changes occurring upon the formation of the Ca²⁺–wt-CaM complex were examined by CD spectroscopy. CD spectra of 10 µg/ml recombinant rCaM solution in Ca²⁺ free PBS were collected either in the presence of 0.1 mM CaCl₂ (squares, Figure 5) or 0.1 mM EDTA (diamonds, Figure 5). As can be seen in Figure 5, the addition of Ca²⁺ to the CaM solution increased the negative ellipticity at 210 and 222 nm and positive ellipticity at 193 nm (diamonds). The deconvolution of the obtained CD data of rCaM was translated into 14 % increase in the α-helix content upon Ca²⁺ binding (Table 1 and 2).
Figure 3.5.1: SDS-PAGE of purified wt-CaM. Purified fractions collected from phenylSepharose CL-4B column were electrophoresed in 15% polyacrylamide gel. The separated bands on the gel were then stained following the procedure described in the materials and methods. Protein molecular weight marker and bands corresponding to wt-CaM were indicated with arrows.
Figure 3.5.2: SDS-PAGE of purified CaMY\textsubscript{99A}. Purified fractions collected from phenylSepharose CL-4B column were electrophoresed on 15\% polyacrylamide gel. The separated bands on the gel were then stained following the procedure described in the materials and methods. Protein molecular weight marker and bands corresponding to CaMY\textsubscript{99A} were indicated with arrows.
**Figure 3.5.3:** SDS-PAGE of purified CaMY$_{138A}$. Purified fractions collected from phenylSepharose CL-4B column were electrophoresed on 15% polyacrylamide gel. The separated bands on the gel were then stained following the procedure described in the materials and method section. Protein molecular weight marker and bands corresponding to CaMY$_{138A}$ were indicated with arrows.
**Figure 3.5.4:** SDS-PAGE of purified CaM-Y99A-Y138A. Purified fractions collected from phenyl-Sepharose CL-4B column were electrophoresed on 15% polyacrylamide gel. The separated bands on the gel were then stained following the procedure described in the materials and method section. Protein molecular weight marker and bands corresponding to CaM-Y99A-Y138A were indicated with arrows.
Figure 3.5.5: CD spectra of Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound wt-CaM. CD spectra of 10 \(\mu\)g/ml (0.6 \(\mu\)M) wt-CaM in Ca\(^{2+}\) free PBS in the presence of \(\diamond\) 0.1 mM EDTA, \(\square\) 0.1 mM CaCl\(_2\). Spectra were collected over the wavelength region of 190 to 260 nm. Each spectrum presented in the figure was the average of four independent experiments. The spectrum acquired in each experiment was the average of three independent scans.
Table 3.5.1: Secondary structural components of CaM variants in the absence of Ca\(^{2+}\)

<table>
<thead>
<tr>
<th></th>
<th>wt-CaM</th>
<th>CaM Y(_{99})</th>
<th>CaM Y(_{138})</th>
<th>CaM Y(<em>{99}) Y(</em>{138})</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helix</td>
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</tr>
<tr>
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<tr>
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<td>32.6%</td>
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<td>35.0%</td>
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</tbody>
</table>

Table 3.5.2: Secondary structural components of CaM variants in the presence of Ca\(^{2+}\)

<table>
<thead>
<tr>
<th></th>
<th>wt-CaM (X-ray)</th>
<th>wt-CaM</th>
<th>CaM Y(_{99})</th>
<th>CaM Y(_{138})</th>
<th>CaM Y(<em>{99}) Y(</em>{138})</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helix</td>
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<td>27.9%</td>
<td>55.6%</td>
<td>46.2%</td>
</tr>
<tr>
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<td>23.8%</td>
<td>9.0%</td>
<td>11.7%</td>
</tr>
<tr>
<td>β turn</td>
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<td>18.0%</td>
<td>13.4%</td>
<td>14.7%</td>
<td></td>
</tr>
<tr>
<td>Random coil</td>
<td>31.8%</td>
<td>30.4%</td>
<td>22.0%</td>
<td>27.6%</td>
<td></td>
</tr>
</tbody>
</table>

Secondary structural components were determined by deconvoluting the obtained CD spectral data using the deconvolution software [http://bioinformatik.biochemtech.uni-halle.de/cdnn](http://bioinformatik.biochemtech.uni-halle.de/cdnn). Secondary structural components from X-ray crystal structure were obtained from Protein Data Bank (Structure code 1CLL).
3.5.1.3 Effect of Ca\textsuperscript{2+} on the secondary structure of CaMY\textsubscript{99A}: In the presence of Ca\textsuperscript{2+}, the negative ellipticity at both 210 and 222 nm and the positive ellipticity at 193 nm were increased as depicted in Figure 6 (squares). Deconvolutions of the obtained CD data were translated into an 11.6 % increase in α-helix (Table 1 and 2).

3.5.1.4 Effect of Ca\textsuperscript{2+} on the secondary structure of CaMY\textsubscript{138A}: Both the negative ellipticity at 210 and 222 nm and positive ellipticity at 193 nm were increased following the exposure of the CaMY\textsubscript{138A} solution to 0.1 mM Ca\textsuperscript{2+} (Figure 7, squares). Upon deconvolution of the CD data of CaMY\textsubscript{138A} a 29.6 % increase in α-helix was achieved (Table 1 and 2).

3.5.1.5 Effect of Ca\textsuperscript{2+} on the secondary structure of CaM CaMY\textsubscript{99A-Y\textsubscript{138A}}: Exposure to Ca\textsuperscript{2+} increased the negative ellipticity at 210 and 222 nm as well as the positive ellipticity at 193 nm (Figure 8, squares). The deconvolution of the CD data obtained for CaMY\textsubscript{138} demonstrate a 24.6 % increase in α-helix (Table 1 and 2).
Figure 3.5.6: CD spectra of Ca$^{2+}$-free and Ca$^{2+}$-bound CaM Y$_{99A}$. CD spectra of 16 μg/ml (0.96 μM) CaM Y$_{99A}$ in Ca$^{2+}$-free PBS in the presence of 0) 0.1 mM EDTA □ 0.1 mM CaCl$_2$. Spectra were collected over the wavelength region of 190 to 260 nm. Each spectrum presented in the figure was the average of four independent experiments. The spectrum acquired in each experiment was the average of three independent scans.
Figure 3.5.7: CD spectra of Ca$^{2+}$-free and Ca$^{2+}$-bound CaM Y$_{138}$A. CD spectra of 15 µg/ml (0.9 µM) CaM Y$_{138}$A in Ca$^{2+}$-free PBS in the presence of •) 0.1 mM EDTA □) 0.1 mM CaCl$_2$. Spectra were collected over the wavelength region of 190 to 260 nm. Each spectrum presented in the figure was the average of four independent experiments. The spectrum acquired in each experiment was the average of three independent scans.
Figure 3.5.8: CD spectra of Ca$^{2+}$-free and Ca$^{2+}$-bound CaM Y$_{99A}Y_{138A}$. CD spectra of 21 μg/ml (1.27 μM) CaM Y$_{99A}Y_{138A}$ in Ca$^{2+}$-free PBS in the presence of ◇ 0.1 mM EDTA □ 0.1 mM CaCl$_2$. Spectra were collected over the wavelength region of 190 to 260 nm. Each spectrum presented in the figure was the average of four independent experiments. The spectrum acquired in each experiment was the average of three independent scans.
3.5.2 Photoactivated cleavage of DNA to decontaminate platelet rich plasma

3.5.2.1 Effect of Dns-GSNO and visible light on the plasmid DNA: In order to assess the ability of Dns-GSNO to cleave plasmid DNA upon exposure to light, purified plasmid DNA (0.3 µg) aliquoted in microfuge tubes was treated with Dns-GSNO and exposed to visible light for 30 minutes or kept in the dark for the same period of time. The concentrations of Dns-GSNO employed were between 0.9 to 15 mM. The treatment with 0.9 mM Dns-GSNO and exposure to light degraded the plasmid DNA completely whereas the treatment with a 3.75 mM Dns-GSNO and 15 mM Dns-GSNO and illumination with light led to the complete aggregation of plasmid DNA as depicted in the EtBr stained agarose gel (Figure 9).

When the plasmid DNA was treated with the same concentration range of Dns-GSNO and maintained in the dark, only 3.75 mM and 15 mM Dns-GSNO resulted in the complete aggregation of plasmid DNA. The 0.9 mM Dns-GSNO led only to partial degradation of the plasmid DNA (Figure 9).

3.5.2.2 Effect of Dns-HCysNO and visible light on the plasmid DNA: In order to examine the potential of Dns-HCysNO to cleave plasmid DNA upon exposure to light, 0.3 µg DNA aliquoted in microfuge tubes was incubated with increasing concentrations of Dns-HCysNO (150-600 nM) for 60 minutes under visible light. No degradation of plasmid DNA was observed both with and without exposure to visible light (Figure 10).
Figure 3.5.9: Photo-induced damage of R 773 plasmid DNA by Dns-GSNO. Samples for lane 2-4 were treated with 0.94, 3.75 and 15 mM Dns-GSNO respectively. Reactions in lane 2-4 were incubated in the dark. Samples for lane 5-7 were treated with 0.94, 3.75 mM, and 15 mM Dns-GSNO respectively. Reactions in lane 4-6 were irradiated with visible light for 30 minutes. Lane 1 contains non-treated DNA.
Figure 3.5.10: Phototreatment of R 773 plasmid DNA with Dns-HcysNO. Photograph of an ethidium bromide stained agarose slab gel containing R773 plasmid DNA upon incubation with Dns-HcysNO. The concentration of Dns-HcysNO employed to treat the plasmid DNA samples were indicated for each lane on top of the figure. The samples irradiated with light were indicated with a + sign and the samples incubated in the dark were indicated with a – sign on top of the image. Light treated samples were exposed to light for 60 minutes.
The plasmid DNA was then incubated with higher concentrations of Dns-HCysNO (1.54 mM to 3.16 mM) following the same experimental protocol as above. All the concentrations employed above 1.54 mM caused the degradation of plasmid DNA upon exposure to light as evidenced by agarose gel electrophoresis of the treated and irradiated samples following staining with EtBr solution (Figure 11). In contrast, only a Dns-HCysNO concentration of 2.48 mM caused a partial degradation of plasmid DNA in the absence of light. Treatment with lower concentration of Dns-HcysNO and incubation in the dark did not have any effect on plasmid DNA (Figure 11). Figure 10 and Figure 11 together shows the range of Dns-HCysNO employed to induce photocleavage of plasmid DNA.

3.5.2.3 Effect of illumination time on Dns-HCysNO treated plasmid DNA: In order to evaluate whether duration of light exposure had any influence of the observed Dns-HCysNO mediated effect on plasmid DNA, 0.3 μg of plasmid DNA were treated with (1.08-1.95 mM) Dns-HcysNO and irradiated for different time periods (60-120 min). The degree of degradation upon incubation with Dns-HcysNO increased as a function of exposure time when DNA was treated with either 1.08 or 1.95 mM Dns-HcysNO (Figure 12).
<table>
<thead>
<tr>
<th>Non-treated DNA</th>
<th>1.54 mM</th>
<th>1.54 mM</th>
<th>2.48 mM</th>
<th>2.48 mM</th>
<th>2.46 mM</th>
<th>3.16 mM</th>
<th>[Dns-HcysNO]</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Light</td>
</tr>
</tbody>
</table>

1 2 3 4 5 6 7

**Figure 3.5.11:** Photo-induced damage of R 773 plasmid DNA by Dns-HcysNO. Photograph of an ethidium bromide stained agarose slab gel containing plasmid DNA upon incubation with Dns-HcysNO. The concentration of Dns-HcysNO used to treat the plasmid DNA samples were indicated for each lane on top of the figure. Samples irradiated with light were indicated with a + sign and the samples incubated in the dark were indicated with a – sign on top of the image. Light treated samples were exposed to light for 60 minutes.
Figure 3.5.12: Effect of irradiation time in the photoinduced damage of R773 plasmid DNA by Dns-HCysNO. Lane 1, untreated DNA, Lanes through 2-4, DNA treated with 1.08 mM Dns-HcysNO. Reactions in lanes 2-4 were irradiated for 30, 60 and 120 min respectively. Lanes through 5-7, DNA treated 1.95 mM Dns-HcysNO. Reactions in lane 5-7 were irradiated for 30, 60 and 120 min respectively.
3.6 Discussion

3.6.1 Exploration of potential roles of tyrosines in rat calmodulin

One of my laboratory coworkers has reported previously that the functional activity of bovine calmodulin is inhibited remarkably upon its incubation with peroxynitrite. The modified calmodulin had been demonstrated to be unable to activate its known substrates including phosphodiesterase, calcineurin, phosphatase and other enzymes. The binding affinity to MLCK peptide had also been reported to be reduced considerably. However, the Ca$^{2+}$ binding ability of this modified calmodulin was retained. The nitration of the two tyrosine residues in calmodulin by peroxynitrite had been reported to be responsible for these observed inhibitions of calmodulin functions (Pathrose, 1997). Therefore, my study attempted to elucidate the putative roles of the tyrosine residues in the structural integrity and the function of calmodulin.

A number of studies have reported inactivation of several enzymes in the presence of peroxynitrite. Nitration of their tyrosine residues by peroxynitrite had been reported to be responsible for this inactivation (Francescutti et al., 1996; Ara et al., 1998; Yamakura et al., 1998). The detection of 3-nitrotyrosine is widely used as a marker of peroxynitrite production under pathological conditions (Lymer et al., 1996). The loss of functional activity of glutathione-S-transferase by peroxynitrite has also been reported recently. The reported loss of activity was due to the nitration of tyrosine residues as evidenced by the increase of the formation of protein associated nitrotyrosine. The increase in the yield of nitrotyrosine correlated well with the loss of functional activity of the enzyme (Wong et al., 2001).
To date, mutation of specific amino acid residues by site directed mutagenesis is a widely used method for studying the relationship between the structure and the function of a protein. Replacing an amino acid residue with another using site directed mutagenesis can also be utilized to assess the role of that particular residue in the function of the protein. However, the DNA sequence encoding that protein has to be known in order to employ this technique and to obtain the desired mutants. In addition, the protein has to be expressed in a host that normally does not express it, in order to study the characteristics of mutant proteins. Bacteria are most commonly used to express mammalian gene products. Bacteria are widely used as a host, because they are relatively easy to grow and can produce the protein of interest in large amounts due to their fast growth rate (Georgiou, 1996).

In order to assess the role of the two tyrosine residues located at position 99 and 138 on the structure and function of rat calmodulin, three calmodulin mutants were created by replacing tyrosine with alanine residues. One of the mutants contained alanine at position 99 leaving the other tyrosine intact; the second mutant contained alanine at position 138 leaving the other tyrosine intact; and the third mutant had both tyrosines replaced with alanines. The Quick-Change site directed mutagenesis kit from Pharmacia enabled me to obtain the three plasmids containing the desired mutated sequences of CaM. All three mutant proteins were expressed in *E. coli* BL21(DE3) cells and were successfully purified in two steps using phenyl Sepharose CL-4B affinity column. The three mutants, namely CaM Y$_{99}$A, CaMY$_{138}$A, CaM Y$_{99}$A Y$_{138}$A, and the native CaM, purified using affinity chromatography, were almost completely pure as demonstrated by the SDS-PAGE (Figures 1-4). The yield of the purified protein was the highest for CaM
Y_{138A} and the lowest for CaM Y_{99A}Y_{138A}. This lower yield is probably due to the improper folding of the mutant protein, which may cause lower protein stability. The replacement of both tyrosine residues probably causes significant perturbations in the CaM structure thus contributing to the reduced stability of the protein. On the other hand, CaM Y_{138A} had a relatively high yield, probably due to structural modulations induced by the mutation at position 138 which could lead to higher protein stability.

Calmodulin is a Ca^{2+}-binding protein in which the \( \alpha \)-helix content is known to increase upon Ca^{2+} binding (Kilhoffer et al., 1981). Therefore, increases in the \( \alpha \)-helical content could be utilized as an indirect marker for Ca^{2+} binding. The effect of replacing tyrosine residues on the calcium-binding profiles of the mutated proteins was analysed using circular dichroism spectroscopy. Mutation of the tyrosine residue at position 99 reduced the \( \alpha \)-helical content by approximately 6\% (Figure 2 and Table 1), while mutation of the tyrosine residue at position 138 increased the \( \alpha \)-helical content by approximately 4\% (Figure 3 and Table 1) in the absence of Ca^{2+}. However, substitution of both tyrosine residues resulted in only 0.6\% decrease in \( \alpha \)-helical content. This observed effect in the double mutant was not exactly the sum of the effects observed in the single mutants. This discrepancy was not unexpected, since there could be always interactions between the residues within each half of the calmodulin domains.

The side chain of tyrosine 99 residue is directed towards the surface of the protein (Babu et al., 1988b). Therefore, Tyr\(\rightarrow\)Ala substitution at position 99 would expose a hydrophobic residue in the polar environment. This could contribute to the unfolding of some \( \alpha \)-helical segments that may result in the observed reduction in the \( \alpha \)-helical content in CaMY_{99A} in the absence of Ca^{2+}. On the other hand, Tyr_{138} interacts with two
other hydrophobic residues (Babu et al., 1988b). Hydrophobic interaction is one of the major driving forces of protein folding. The replacement of tyrosine at position 138 with the more hydrophobic alanine will strengthen the hydrophobic interaction thus resulting in better folding of the mutant protein. This could be the reason for the observed higher helical content observed in CaM Y_{138} in the absence of Ca^{2+} (Table 1).

All the mutants retained their ability to bind Ca^{2+}. However, the extent of Ca^{2+} binding was affected by the mutation as demonstrated by the fact that the increase in the $\alpha$-helical content of the mutants upon Ca^{2+} binding was different from that of the wt-CaM. Binding of Ca^{2+} to wt-CaM resulted in 14% increase in helix as evidenced by the deconvolution of the collected CD data of CaM in the presence of Ca^{2+}. On the other hand, the $\alpha$-helical content of CaM Y_{99A} was increased by 11.6% upon binding to Ca^{2+}. This observation was not unexpected, since Tyrosine 99 is located in the third calcium binding loop of calmodulin and its carbonyl oxygen is involved in the coordination of Ca^{2+} (Babu et al., 1988b). Therefore, the substitution of that tyrosine will eliminate a coordination site for Ca^{2+} that would not likely be compensated by alanine. As a result, the overall Ca^{2+} binding capacity of the mutant protein might decrease as observed in our study due to less tight Ca^{2+} binding in the third Ca^{2+} binding loop. A more dramatic increase in the $\alpha$-helical content was observed in CaM Y_{138A} and CaM Y_{99A} Y_{138A} where Ca^{2+} binding induced 29.6% and 24.6% increase in the $\alpha$-helix respectively (Table 1 and 2). Tyrosine at 138 position interacts with two other hydrophobic residues in the protein (Babu et al., 1988b). Since alanine is relatively more hydrophobic and less bulky than tyrosine it might be able to interact in a better way with the other hydrophobic residues. This interaction probably induces some conformational change in the third Ca^{2+} binding
loop thus assisting in binding more Ca$^{2+}$ as observed in our study (Table 1 and 2). Since, replacement of tyrosine 99 residue had the opposite effect on the Ca binding ability of the protein, the double mutant CaM Y$_{99A}$ Y$_{138A}$ will not bind as much as Ca$^{2+}$ as the CaM Y$_{138A}$.

Mutation of tyrosine at 99 position lowered the $\alpha$-helical content of apo-calmodulin as compared to the native calmodulin. However, the Ca$^{2+}$ binding capability appeared not to be affected significantly since the $\alpha$-helical content was increased by $\sim$ 11.6% upon Ca$^{2+}$ binding thus being very comparable to the native calmodulin ($\sim$ 14%). Mutation of residue at 138 position induced 3.8% increase in the $\alpha$-helix content in the absence of Ca$^{2+}$. The Ca$^{2+}$ binding ability of rCaM was also increased significantly (28.6%) following mutation of Tyr residue 138.

In this study, we have demonstrated that the substitution of two tyrosine residues affect the secondary structural components and Ca$^{2+}$ binding ability of the calmodulin. Therefore, tyrosines might play a significant role both in the folding and Ca$^{2+}$ binding ability of calmodulin.

3.6.2 Photoactivated cleavage of DNA to decontaminate platelet rich plasma

Blood donors are carefully selected and even after obtaining the blood, products are carefully screened for any viral transmission by extensive laboratory testing in order to ensure the safety of the recipient. Even though all these precautions are taken, there is still the possibility of viral transmission through blood products due to the lower sensitivity of the instruments to detect low level of viral or latent infection. Therefore, other methods of decontamination are required to destroy the possible remaining viruses
in the blood or blood products, which could potentially be missed by traditional screening procedures. A large number of photosensitized compounds have been developed over the last decade in order to photo deactivate viruses in blood and blood products. These compounds are called photonucleases, since they cleave DNA following absorption of light.

These compounds are usually capable of cleaving DNA according to one of the four different mechanisms discussed (Armitage, 1998). One group of compounds cleave DNA by abstracting H from the deoxyribose or ribose moiety. The compounds that demonstrated DNA cleaving potential by this pathway include metal binding complexes such as uranyl salts (Nielsen et al., 1988; Mollegard et al., 1994), organometallic complexes of rhodium (Chow and Barton, 1992), cobalt (III) bleomycin (Saito et al., 1989), anthraquinone derivatives (Breslin et al., 1997), nitrosubstituted imides (Saito, et al., 1995) and other compounds. A study reported nicking of supercoiled DNA in the presence of uranyl salt following irradiation with long wavelength ultraviolet light. The cleavage was found to be dependent on the irradiation time and the concentration of the salt. The singlet oxygen quencher NaN₃ and free radical quencher dithiothreitol did not have any effect on uranyl salt mediated cleavage of DNA (Nielsen et al., 1988). Since deoxyribose moiety exist along the entire length of the DNA, this type of DNA cleavage is not sequence specific. The second group of compounds oxidize a nucleic acid base leading to the formation of a radical cation, which in turn, can eventually migrate to a GG site and get trapped thereby resulting in G specific cleavage. The compounds demonstrated to work by this pathway include riboflavin (Ito et al., 1993), anthraquinones (Breslin et al., 1996) etc. The third class of compounds does not directly
interact with DNA, but produces upon light absorption, reactive intermediates that would directly cleave DNA. Usually singlet oxygen is produced through the reaction between excited state of photosensitizer and molecular oxygen. Porphyrins have been observed to cause DNA scission by this pathway (Croke et al., 1993). Reactive oxygen species are generated by homolytic or heterolytic bond cleavage in the chromophore itself following absorption of light in the fourth class of compounds. The photolytically generated chromophore then interacts with DNA to cause its cleavage. One of the compounds that has been reported to cause plasmid DNA scission in this pathway is OH radical that is generated from aromatic hydroperoxides (Saito et al., 1990).

We synthesized two compounds namely Dns-GSNO and Dns-HCysNO both of which had a fluorophore conjugated to compounds with a photosensitive S-NO bond. Both GSNO and HCysNO are well known to release NO upon photoillumination (Sexton et al., 1994, Wood et al., 1996). The yield of NO production from GSNO has been reported to increase by 9 fold when coupled to Rose Bengal (Singh et al., 1995). Conjugation of dansyl chloride to HCysNO has been demonstrated earlier to potentiate the release of NO much more easily compared to HCysNO by coworkers from my laboratory. This is believed to be due to the spectral overlap of dansyl moiety (λ_max, 327 nm and λ_em 520 nm) and S-NO (abs peak 340 nm and 545 nm). Since the S-N bond breaks upon illumination with 340 and 543 nm light, the light emitted by the dansyl moiety gets absorbed by S-NO leading to the enhancement of NO release. Several minimum energy structures obtained through the molecular modeling of this compound (simulated annealing Tripos Sybyl) depicted very close proximity (3.5 -4.2 Å) between S-NO and dansyl ring (Ramachandran et al., 1999).
In the presence of hydrogen peroxide, HCysNO has been shown to cause strand scission in pBluescript plasmid DNA. The percentage of cleavage was increased with higher temperatures. The cleavage was suggested to be induced by *OH that could be formed via the reaction between thyl radical and hydrogen peroxide since *OH radical scavengers such as mannitol and azide could prevent the DNA damage (Park and Kim, 1994). Peroxinitrite has been reported to induce DNA strand scission and oxidative damage in vitro (King et al., 1992). However, it was observed by another group later that peroxynitrite interacts with DNA preferentially with guanine residues. They suggested that *NO2 species could be formed by homolytic cleavage of ONOO- thus reacting with DNA to form 8-nitroguanine (Nitro8Gua). Formation of Nitro8Gua was also dependent on the concentration of ONOO- employed. On the other hand, no significant formation of 8-oxoguanine was observed. Incubation of the DNA following treatment with ONOO- led to the removal of the 8-nitroguanine from the DNA (Yermilov et al., 1995b).

It has been reported recently that peroxynitrite reacts with guanine to form a very stable product, 5-guanidino-4-nitroimidazole. The modified DNA was not depurinated to any significant extent even after hot piperidine treatment. Synthetic oligonucleotide containing this 5-guanidino-4-nitroimidazole also appeared to be a poor substrate for formamidopyrimidine DNA glycosylase (Fpg glycosylase) repair enzyme. In addition, exonuclease III failed to cleave this site from the DNA. (Gu et al., 2002). We demonstrated earlier that ONOO- can be generated upon irradiation of air saturated GSNO solution. Following irradiation, the homolytic cleavage of S-NO bond generates NO* and GS* (Mutus et al., 1999). A complex series of reactions between molecular oxygen and GS* has been shown to ultimately form O2•- in aerated solutions of GSNO
(Winterbourn, 1993). It was suggested that the $O_2^{•−}$ formed could then further react with NO resulting in the formation of ONOO$^−$ (Mutus et al., 1999).

In this study, treatment with 0.9 to 15 mM Dns-GSNO along with illumination with visible light for 30 minutes was observed to cause complete aggregation of plasmid DNA as evidenced by the lack of movement of the DNA band in the agarose gel (Figure 9). In contrast, when the plasmid DNA was treated with the same concentrations range of Dns-GSNO but maintained in the dark, different results were obtained. Treatment with 3.75 and 15 mM Dns-GSNO under dark condition led to the aggregation of plasmid DNA (Figure 9). However, 0.9 mM Dns-GSNO did not have any significant effect on plasmid DNA in the absence of light exposure. Even though high concentrations of Dns-GSNO could aggregate plasmid DNA, the same effect was observed both with and without exposure light. Therefore, Dns-GSNO does not show the potential of causing light induced DNA cleavage.

Similar studies were then performed using Dns-HCysNO. Treatment with Dns-HCysNO had a different effect on plasmid DNA compared to the results obtained with Dns-GSNO. When the plasmid DNA was treated with 1.54 to 3.16 mM Dns-HCysNO and exposed to visible light for 30 minutes all the concentrations employed above 1.54 mM caused the degradation of plasmid DNA. The extent of degradation was observed to be dependant on the concentration of Dns-HCysNO employed (Figure 11). On the other hand, when the plasmid DNA was treated with 1.54 to 2.48 Dns-HCysNO without visible light illumination, only 2.48 mM Dns-HCysNO could cause partial degradation of plasmid DNA. Plasmid DNA did not appear to be affected when treated with 1.54 mM Dns-HCysNO without light exposure. This partial degradation obtained using Dns-
HCysNO in the absence of light could be due to the release of NO by trace amounts of metal ions present in Dns-HCysNO solution. When the studies were carried out using lower concentration of Dns-HCysNO (500-600 nM), no apparent damage in the plasmid DNA was observed both with and without exposure to light (Figure 10).

In order to evaluate the effect of illumination time on the Dns-HCysNO mediated damage on plasmid DNA, plasmid samples were treated with equal concentration of Dns-HCysNO while illuminating with visible light for different time intervals. The extent of DNA degradation was observed to increase as a function of light exposure time (Figure 12). This result was to be expected since increase of irradiation will also increase the yield of Dns-HCys* and NO*. The observed DNA damage could be due to the generation of ONOO− according to the mechanism described earlier (Mutus et al., 1999).

I was unable to extend our studies further to evaluate the potential of our compound to decontaminate platelet rich plasma due to our inability to attract funding from the Consortium for Plasma Science, a potential sponsor.

3.6.3 Exploration of potential effects of close proximity of tyrosine on the stability of the S-NO bond in S-nitrosocysteine

We further intended to study any potential effect of tyrosine proximity in calmodulin on the lability of the S-NO bond. Rat calmodulin has two tyrosine residues at 99 and at 138 position. By molecular modeling, Valine 142 and Isoleucine 85 were found be within the 5 Å distance from the tyrosine residue at position 138. Two different mutants were created by substituting either one of these two residues with cysteine. The
mutants were generated by site directed mutagenesis using the same Quick change site
directed mutagenesis kit and following the same procedure as mentioned for tyrosine
mutants of CaM. The following primers were used for creating the mutants

Oligonucleotides used for PCR primers for creating CaM I_85C

5'-CA GAC AGT GAA GAA GAA TGC AGA GAA GCG TTC CGT GTG
3'-GT CTG TCA CT T CTT CTT ACG TCT CTT CGC AAG GCA CAC

Corresponding amino acid sequence

-Asp-Ser-Glu-Glu-Cys-Arg-Glu-Ala-Phe-Arg-Val

Oligonucleotides used for PCR primers for creating CaM V_142C

5'- G GTA AAC TAC GAA GAG TTT TGC CAA ATG ATG ACA GCG AAG
3'- C CAT TTTG ATG CTT CTC AAA ACG GTT TAC TAC TGT CGC TTC

Corresponding amino acid sequence

-Val-Asn-Tyr-Glu-Glu-Phe-Cys-Gln-Met-Met-Thr-Ala-Lys

However, we were unable to express the mutant protein due to unexplainable
problems in transforming the plasmids containing the mutated rCaM sequence into the E.
coli. BL21(DE3) cells for their expression. The cysteine residue incorporated by site-
directed mutagenesis in calmodulin mutants were intended to be nitrosated following
their purifications and study the lability of S-NO by CD and UV/vis spectroscopy.
Conclusions

S-nitrosothiols (RSNO) have been demonstrated earlier to cause inhibition of platelet aggregation and blood clot formation. RSNOs also regulate the activity of many proteins. These RSNO mediated effects have been implicated to be due to their NO release thereby triggering cGMP pathway or S-nitrosation or S-thiolation of a critical cysteine residue in the enzymes or proteins involved in these processes. In my study, I have demonstrated that the S-nitrosothiols (RSNO) induce the inhibition of thrombin catalyzed fibrinogen polymerization, which does not involve any of the previously suggested pathways of regulation by RSNOs. A conformational change in fibrinogen induced by its allosteric interaction with GSNO is responsible for the observed inhibition. Thus, in my study I have demonstrated that RSNOs are capable of regulating important physiological processes by chemical as well as allosteric interactions with their targets. The new regulatory role of RSNO revealed by our study will contribute in future studies of identifying other proteins, which could be regulated by RSNO by a similar mechanism in physiological condition.

RSNOs mediate many of their functions by transferring NO$^+$ moiety to another thiol, which is also called transnitrosation reaction. However, there is no analyte currently available that is capable of detecting NO$^+$ in vitro or in physiological conditions. In my study, I have demonstrated that sinapinic acid (SA) can be potentially utilized for the spectroscopic detection of peroxynitrite and NO$^+$ donors in submicromolar range under physiological conditions or in vitro. Furthermore, the demonstrated ability of O-nitrosoSA to serve as a S-nitrosating agent for thiol containing amino acids, peptides and
proteins, makes it a potentially useful reagent in the study of \( S \)-nitrosothiol biochemistry and physiology. In addition, SA has been shown to be very effective in preventing the oxidation of diydorhodamine by peroxynitrite. Future studies could focus on evaluating the ability of SA to prevent peroxynitrite mediated oxidative damage \textit{in vivo}.

I have prepared defatted BSA nanoparticles, which have been successfully packed with \( S \)-nitrosothiolipid, \( S \)-nitosooctadecane (SNOD). I have also demonstrated that these packed nanoparticles could be potentially employed to deliver large amounts of SNOD into the cell. These delivered RSNOs have shown to be able to trigger a large intracellular influx of NO leading to the death of fibroblasts by apoptosis in the preliminary studies. However, further studies are required to ensure efficient targeting of the nanoparticles \textit{in vivo} and to confirm apoptotic cell death induced by large amounts of NO delivered by SNOD loaded particles by another independent technique.

Upon modification with peroxynitrite, calmodulin has been reported previously to partially lose its functional activity. The nitration of the protein's two tyrosine residues has been suggested as the cause of this inactivation. In my study, I have attempted to examine the significance of the tyrosine residues for the structure and function of calmodulin by mutating them using site directed mutagenesis. My studies have demonstrated that the tyrosine residues might play significant roles both in maintaining the structural integrity and calcium binding ability in calmodulin. Further studies could be carried out to evaluate the roles of tyrosines in rat calmodulin function.
We synthesized two photoactivated compounds in order to decontaminate platelet-rich plasma. One of the two compounds, Dns-HCysNO has demonstrated the ability to cleave DNA upon photoillumination and the extent of degradation has been shown to be a function of light exposure time. Thus, Dns-HcysNO could be potentially employed for testing its potential to decontaminate platelet-rich plasma in future studies. The other compound, Dns-GSNO demonstrated light-independent cleavage of plasmid DNA therefore could not be employed for further studies.
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