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Novel Conjugation Strategies for Hybrid Biomacromolecules

Mary Catherine King

Thesis submitted to the School of Graduate Studies and Research in partial fulfillment of the degree of Doctor of Philosophy in Chemistry

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...for my husband Ian King, my mother Catherine E. Keir, and my father Robert J. Keir

ABSTRACT

The work presented in this thesis describes the development of novel methodology for the preparation of macromolecular conjugates using proteins and dendrimers as scaffolds. This methodology is based on the discovery that chemical modification of proteins can be achieved *in vacuo*, in the absence of solvents, *viz.*, water. The results obtained represent the first attempts to use this methodology to achieve protein modifications that are otherwise difficult or impossible under aqueous conditions. While the inspiration for this research stemmed from practical objectives, much of the work undertaken developed into a proof of principle, not by design, but due to the fact that there is no precedence or established theoretical base for the *in vacuo* chemical modification of proteins.

Initially, the scope of the project involved the design and development of a novel detoxication construct for an artificial liver consisting of an enzyme coupled to lipophilic poly(propyleneimine) dendrimers as partitioning agents for the clearing of hydrophobic compounds from aqueous solution. While the lipophilic dendrimers proved to be feasible partitioning agents when tested with some drugs in aqueous solution, attempts to attach a model enzyme to the construct by established methods in solution met with limited success. The search for better, more efficient methods of tethering, immobilizing and cross-linking enzymes lead to the investigation of alternative conjugation methods.

p-Nitrophenylchloroformate is a common activating reagent used in organic synthesis and has been used to activate chemical modifiers for proteins. However, this reagent has not been used for direct chemical modification of proteins because of its insolubility in water. The results obtained show that this reagent, when used with the *in vacuo* procedure, can

activate and cross-link proteins through chemical modification of protein carboxyl groups. In the course of these studies it was observed that a covalent dimer was present in ribonuclease A heated in the absence of reagent. This observation lead to an investigation which demonstrated that proton transfers between interacting carboxylate and ammonium groups *in vacuo* results in the formation of amide bonds.

Compared to solution methods, the *in vacuo* methods developed are experimentally simple, and may also be carried out without the use of reactive chemical reagents. Glycation of protein amino groups with reducing monosaccharides can readily be achieved by the formation of stable ketoamine derivatives using the *in vacuo* methodology. Stable, multivalent monomeric and oligomeric neoglycoconjugates capable of binding a galactose-specific lectin were prepared by reaction between the reducing disaccharide lactose and bovine serum albumin under *in vacuo* glycation conditions. In order to achieve cross-linking via *in vacuo* glycation, compounds bearing two or more reducing sugars separated by branched and linear spacers were designed, synthesized and reacted with hemoglobin and cytochrome c. Various oligomeric cross-linked products were obtained, and the extent of cross-linking could be controlled by the addition of the non-reducing, disaccharide excipient, trehalose. Although similar attempts to generate glycodendrimers by glycating poly(propyleneimine) dendrimers with glucose and mannose yielded unstable products, the results provided further evidence for the role of proton transfer in the *in vacuo* glycation phenomenon and the role of hydrogen bonding in stabilizing the conjugate.

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-di-[3-ethyl-benzothiazoline-(6)-sulfonic acid
ACN	acetonitrile
ASA	acetylsalicylic acid
Asp	aspartic acid
BSA	bovine serum albumin
CE	18-crown-6 ether
DAB	polypropyleneimine dendrimer
DEPC	diethyl pyrocarbonate
DIPEA	diisopropylethylamine
dH_2O	distilled water
DMF	dimethylformamide
DMSO	dimethylsulfoxide
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
FPLC	fast protein liquid chromatography
G1.0	generation 1.0 dendrimer
HATU	O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluroniumhexafluorophosphate
Hb	hemoglobin
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
kDa	kiloDaltons
K_m	Michaelis constant

LpH	lyophilized pH
Lys	lysine
MeOH	methanol
MW	molecular weight
NADPH	nicotinamide adenine dinucleotide phosphate
NHS	N-hydroxysuccinamide
NMR	nuclear magnetic resonance
PAMAM	polyamidoamine dendrimer
PBS	phosphate buffered saline
pK_a	negative logarithm of the ionization constant
p-NPCF	*para*-nitrophenylchloroformate
r.t.	room temperature
RNase	ribonuclease
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
STI	soybean trypsin inhibitor
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TNBS	2,4,6-trinitrobenzenesulfonic acid
V_{max}	maximum velocity of enzyme
μCi	microCurie
α-CD	α-cyclodextrin

Chapter 1 Introduction

1.1 Thesis Aim and Scope

The objective of the research presented in this thesis is the development of alternative strategies for the construction of macromolecular conjugates with a view to practical applications. Pharmacologically important polymer-protein conjugates and polymer-drug conjugates are the focus of much current research dedicated to therapeutic and drug delivery applications (Duncan *et al.*, 1994). In this thesis some novel approaches are presented for the modification of existing synthetic and natural macromolecules, in which dendrimers and proteins respectively are used as the polymers or scaffolds for conjugation. Although many of the modifications to selected macromolecules give rise to products that may have value in therapeutic medicine or in the field of biomaterials, the novelty lies mainly in the modification techniques developed to produce these conjugates. Characterization of end products from the modification of some model proteins and dendrimers provided insights into the chemistry behind the techniques used, some of which involved the discovery of unexpected reactions in the dry state under *in vacuo* conditions.

Applications for modified proteins abound in many different fields, and the majority of this research endeavor involves exploration into new methods of protein modification and cross-linking. The new approaches developed have general applicability and are not exclusive to biomaterials or biomedical products. The appeal of these techniques is the overall simplicity and the lack of toxic reagents and solvents, which offers the possibility of reducing manufacturing costs and producing safer materials for therapeutic applications.

By way of introduction, this chapter provides background information regarding some of the materials and techniques used as well as state-of-the-art methods currently used in selected applications.

1.2 Dendrimers

Dendrimers are monodisperse, symmetrical, branched, macromolecules. They were first synthesized just over twenty years ago and have since become an increasingly popular focus of research and the subject of many review articles (Tomalia *et al.*, 1990; Tomalia, 1994; Astruc, 1996; Zeng *et al.*, 1997; Issberner *et al.*, 1994; Bosman *et al.*, 1999; Hecht *et al.*, 2001). Each iterative reaction step in the synthesis of dendrimers gives rise to a new 'generation' with increasing three-dimensional, fractal-like molecular architecture. Although the number of iterations is limited by steric congestion at the periphery, these macromolecules can reach nanoscopic dimensions (Tomalia, 1994). Dendrimers have been synthesized with various cores, branching units and end groups, the most commonly known and commercially available dendrimers are poly(amidoamine) (PAMAM) 'starburst' dendrimers and poly(propyleneimine) (DAB) dendrimers. The dendrimers selected for the purposes of the research reported here were poly(propyleneimine) dendrimers (Figure 1.1).

The availability of multiple functional groups at the periphery of dendrimers makes them amenable to covalent modification. The use of dendrimers as scaffolds upon which to graft functional molecules to develop macromolecular conjugates with specific properties and/or activities is growing in popularity. Applications arising from surface modification of dendrimers span the fields of biology, medicine and catalysis.

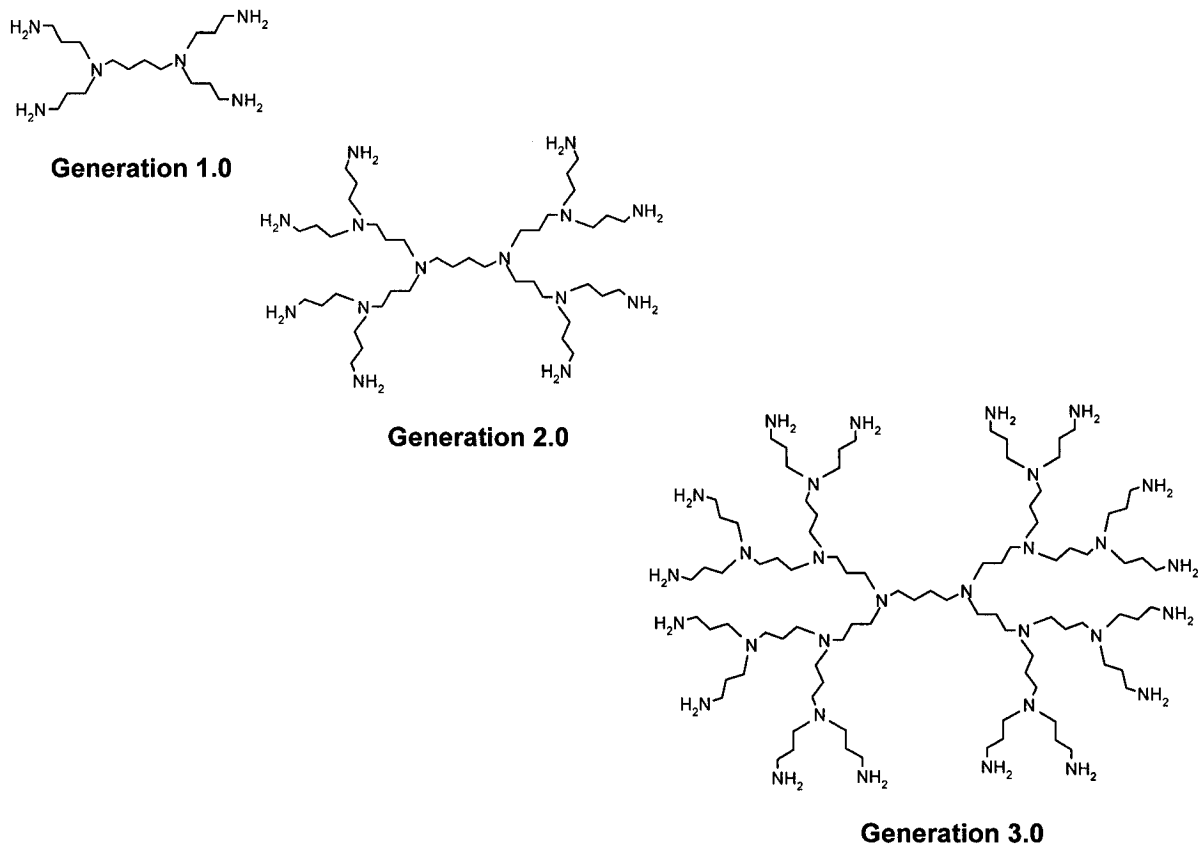


Figure 1.1: First three generations of polypropyleneimine dendrimers

Roy *et al.* pioneered the preparation of biologically active glycoconjugates by coupling a thiosialoside to a lysine core dendrimer (Roy *et al.*, 1993). Glycodendrimers are now a recognized, well-established class of biopolymers (Roy, 1996; Turnbull *et al.*, 2000). Weinmann *et al.* designed a magnetic resonance imaging (MRI) contrast agent by covalently attaching gadolinium complexes to lysine-based dendrimers (Weinmann *et al.*, 1997). Surface attachment of alkaline phosphatase and an antibody to form a multifunctional dendrimer reagent for immunoassays was developed by Singh *et al.* through activation of the peripheral functional groups in PAMAM dendrimers prior to reaction with the proteins (Singh *et al.*, 1994; Singh *et al.*, 1996; Singh, 1998). By biotinylating PAMAM dendrimers, Wilbur *et al.* developed biotin-radioactive streptavidin conjugates useful for the delivery of

high levels of radiation to cancerous tissue (Wilbur *et al.*, 1998). These are but a few of the many applications of dendrimer surface modifications that have been successfully carried out.

1.2.1 Dendrimer Conjugates for an Artificial Liver Application

The design and construction of macromolecular architectures using synthetic scaffolds, *viz.*, dendrimers, was investigated, and their potential for use as part of a detoxication chamber in a two-stage extracorporeal bridge-to-transplant artificial liver device was examined. The detoxication model design involves a lipophilic, peripherally modified dendrimer to which either cytochrome P450, the enzyme responsible for metabolizing xenobiotics in the liver, or a suitable P450 mimic can ultimately be tethered. The rationale behind the design was to create a membrane mimetic capable of partitioning and accumulating hydrophobic xenobiotics to be processed by a proximally tethered enzyme in a manner similar to the membrane-bound cytochrome P450 system.

The various cytochrome P450 species form a family within a superfamily of mixed function oxidases that operates in conjunction with the reductant nicotinamide adenosine dinucleotide (NADPH), and cytochrome P450 reductase, an electron carrier, as depicted in Figure 1.2. Cytochrome P450s are triangular and prismatic in shape with dimensions of 6.5 nm on each side and a thickness of 3.5 nm. They are classed as haem-thiolate monooxygenases because they insert one oxygen atom from molecular oxygen into exogenous xenobiotic substrates, such as drugs and carcinogens, or endogenous substrates, such as steroids and prostaglandins, making them water soluble to facilitate excretion by the kidneys. Expression of individual P450s is selectively induced in the liver by the presence of

specific substrates. The sparingly soluble substrates accumulate in cell membranes until they are acted upon by cytochrome P450.

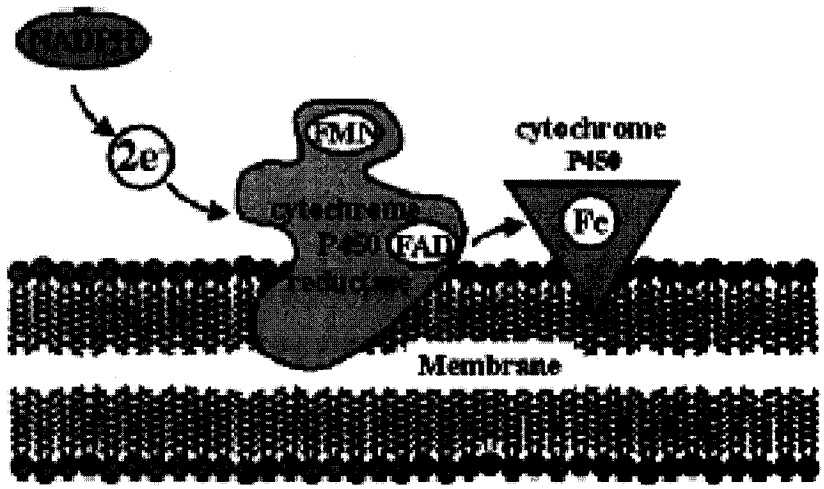


Figure 1.2: Schematic of the membrane-bound cytochrome P450 enzyme system (Feiters *et al.*, 2000).

Dendrimer modification for the purposes of generating macromolecular conjugates capable of sequestering substrates similar to the cell membrane is described. The ability of these modified dendrimers to partition some aqueous drugs was tested in order to assess the feasibility of the design. Although the design of the detoxication model was conceived as part of this thesis and partitioning studies showed some promise for the design, tethering of an enzyme was met with limited success. The difficulty involved in coupling the enzyme to the lipophilic macromolecular dendrimer conjugate stems from the extreme differences between the natures of the two entities. Aside from the slower reaction kinetics due to phase heterogeneity of the two reactants in solution, proteins are prone to denaturation and structural alterations in the presence of solvents other than water. This is important because biological activity is largely dependent upon the structural integrity of the enzyme. As a result of these challenges, alternative coupling strategies for future work towards integrated

enzyme conjugates were sought after. These pursuits lead to some very interesting novel research that involved protein modification and cross-linking under unusual reaction conditions.

The alternative coupling strategies investigated were based on the work of Taralp and Kaplan who developed a non-aqueous, *in vacuo* method to modify proteins whereby the protein tertiary structure and function are not compromised by the coupling procedure (Taralp *et al.*, 1997). In order to comprehend the premise behind this methodology and its inherent advantages, it is necessary to review the complex, multifunctional nature of proteins and current state-of-the-art solution techniques used for modifiying, cross-linking and immobilizing enzymes.

1.3 Proteins

Proteins are large, complex natural polymers consisting of combinations of 20 amino acid side chains bearing many potentially reactive functional groups. Functional groups such as amino, imidazole, thiol, hydroxyl and carboxylic groups give rise to intramolecular and solvent interactions that result in complex secondary, tertiary and quaternary molecular conformations. The amino acid sequence (primary structure) influences the protein structure by folding according to hydrogen bonding and hydrophobic interactions within the side-chains of the amino acids. Secondary α-helices and β-sheet structures arise which pack into tertiary three-dimensional compact structures called domains. Further association by the tertiary structures can bring about quaternary structures in some proteins.

Such molecular interactions bring otherwise distant functional groups together resulting in the formation of local microenvironments within the macromolecule. In

enzymes, the tertiary or quaternary structure affords a special microenvironment, or active site, which is a protected pocket containing an active functional group and a binding site; it is this functional region that determines the catalytic specificity for each enzyme. Other microenvironments can give rise to field and inductive effects caused by the spatial proximity of electron withdrawing groups to ionizable groups on amino acid residues in the backbone of the protein. This can give rise to significant shifts in pK_a of the ionizable groups involved, thus altering their chemical behaviour. Reactivity and accessibility of protein functional groups are largely determined by pH, steric hindrance, interaction with proximal functional groups as well as solvent, and the ionization state of the functional groups. Chemical modification of such convoluted, complex macromolecules, therefore, requires due consideration since the reactivities of functional groups are not readily predictable.

Usually only nine of the 20 side chains in proteins are chemically reactive under conditions where proteins retain their native structure: the thiol group in cysteine; the ε-amino group of lysine; the carboxyl groups of aspartic and glutamic acids; the imidazolyl group of histidine; guanidinyl of arginine; the thioether moiety of methionine; and the phenolic hydroxyl group of tyrosine (Tischer *et al.*, 1999). The α-amino group of the N-terminus and the α-carboxyl of the C-terminus are also reactive. The chemical modification reactions investigated in this thesis involve primarily the amino and carboxyl groups, which are usually the most abundant functional groups in protein.

1.3.1 Protein Immobilization and Cross-linking in Solution

Proteins are often modified for a number of reasons: 1) to study structure-function relationships; 2) to introduce a label or tag for tracking; 3) to alter physical

properties, such as solubility, or 4) to improve stability. New enzymes have been prepared by chemically modifying existing proteins to either introduce catalytic activity or alter existing catalytic properties (Qi *et al.*, 2001). Immobilized enzymes offer distinct economic and technical advantages, such as facile enzyme separation and recovery, reusability, and increased enzyme stability. Although immobilization generally leads to somewhat decreased enzyme efficiency due to impaired binding and mass-transfer effects (restricted sustrate diffusion and enzyme mobility), there have been reports of enhanced performance in some cases (Singh *et al.*, 1994; Akasaka *et al.*, 1993).

Several approaches to immobilizing proteins, both chemical and physical, on solid supports have been reported in the literature (Wehtje *et al.*, 1993; Dumitriu *et al.*, 1997; Arica *et al.*, 2000; Vianello *et al.*, 2000; Brunner *et al.*, 1979), and are the subject of many recent review articles (Katchalski-Katzir, 1993; Tischer *et al.*, 1999; Govardhan, 1999; Tischer *et al.*, 1999). Physical adsorption on solid supports is by far the simplest method and thus is widely used in commercial applications, particularly those involving non-aqueous systems. However, the complex network of hydrophobic and electrostatic interactions as well as Van der Waals forces involved in adsorption is not easy to control and is somewhat reversible depending on the environment. Another non-covalent immobilization method involves the encapsulation of the enzyme within a membrane bound device such as a hollow fibre, a polymeric network, or a microcapsule. Covalent immobilization, which is the main focus of interest in this thesis, can be achieved by the use of a variety of cross-linking reagents.

While there are as many reasons to cross-link proteins as there are cross-linkers for proteins, research in this area remains an ongoing endeavour. Although this is due in part to

the specific physical properties or biological activity required of the cross-linked product, it is also due to the individuality and complexity of reactive functional groups within the proteins themselves. The main objective for cross-linking proteins, regardless of the application, is to impart stability. Cross-linked enzyme crystal technology (CLEC) is one of the most widely studied cross-linking methodologies (Govardhan, 1999; Margolin *et al.*, 2001). It is a two-step chemical cross-linking methodology whereby proteins are first crystallized and then cross-linked using one of the typical, commercially available protein cross-linkers shown in Figure 1.3. In a very recent review article, Margolin *et al.* reported increased chemical and mechanical stability as well as enhanced performance with many of the crystallized proteins they generated by cross-linking enzymes and proteins using some of the cross-linkers shown in Figure 1.3 (Margolin *et al.*, 2001).

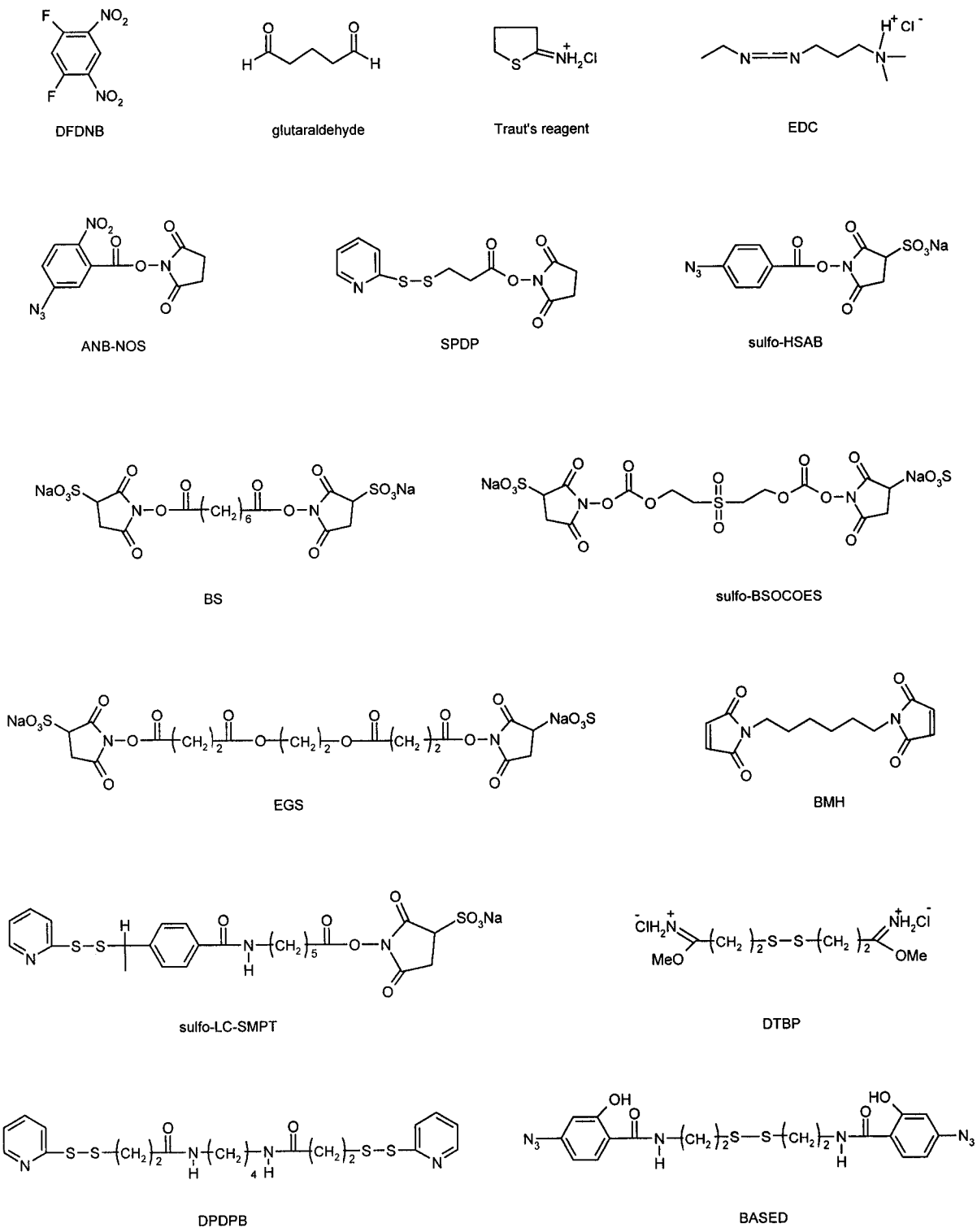
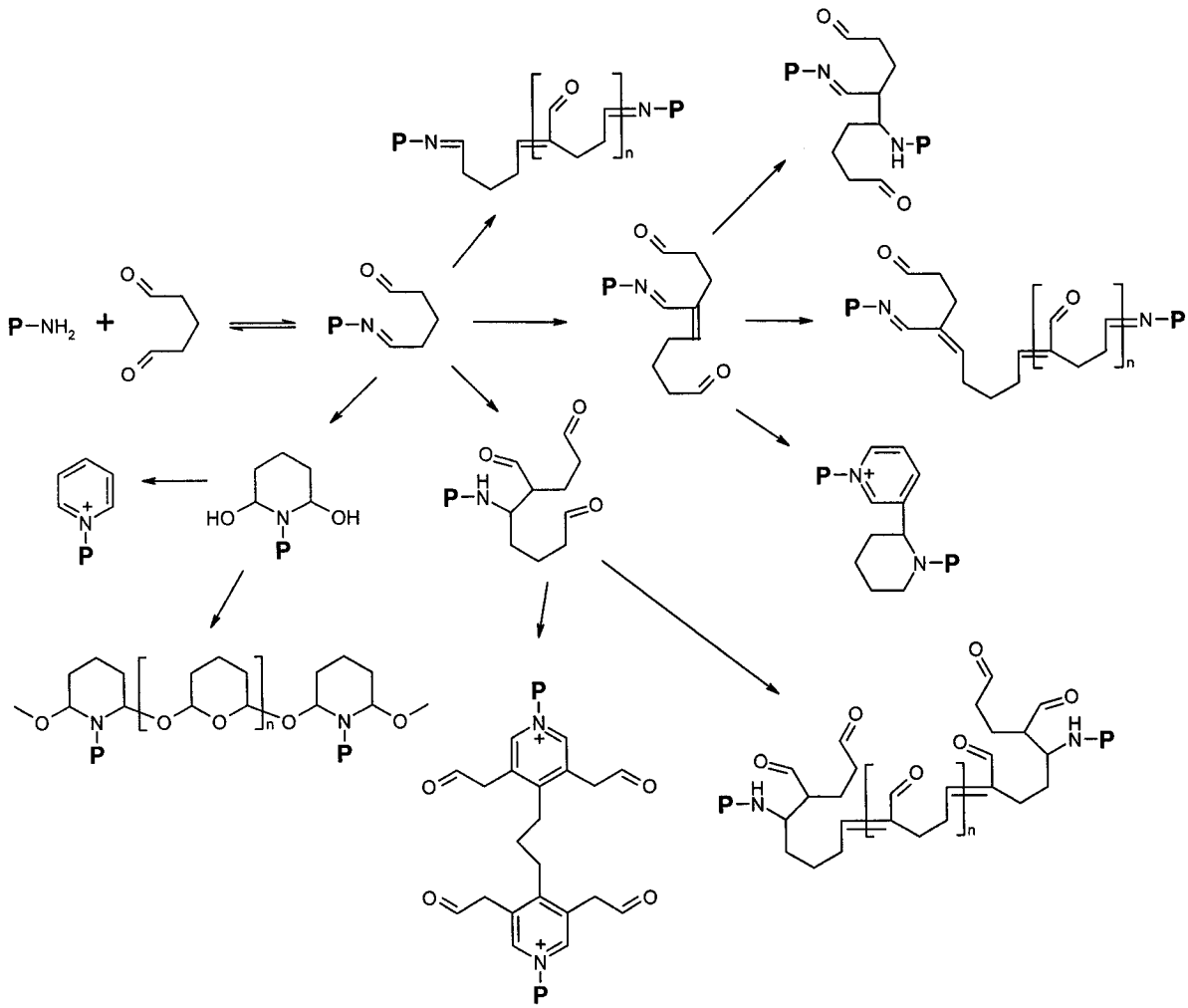


Figure 1.3: Some commonly used protein cross-linking reagents (Margolin *et al.*, 2001).*

* Abbreviations: DFDNB – 1,5-difluoro-2,4-dinitrobenzene; EDC – 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ANB-NOS – N-5-azido-2-nitrobenzoyloxysuccinimide; SPDP – N-succinimidyl-3-(2-pyridyldithio)propionate; Sulfo-HSAB – N-hydroxysulfosuccinimidyl-4-azidobenzoate; BS – bis(sulfosuccinimidyl)suberate; Sulfo-BSOCOES – bis[2-(sulfosuccinimidooxycarbonyloxy)ethyl]sulfone; EGS – ethyleneglycol bis(succinimidylsuccinate); BMH – bis(maleimidohexane); Sulfo-LC-SMPT – sulfosuccinimidyl-6-[α-methyl-α-(2-pyridyldithio)toluamido]hexanoate; DTBP – dimethyl-3,3'-dithiobispropionimidate • 2 HCl; DPDPB – 1,4-di-[3'-(2'-pyridyldithio)propionamido]butane; BASED – bis[β-(4-azidosalicylamido)ethyl]disulfide.

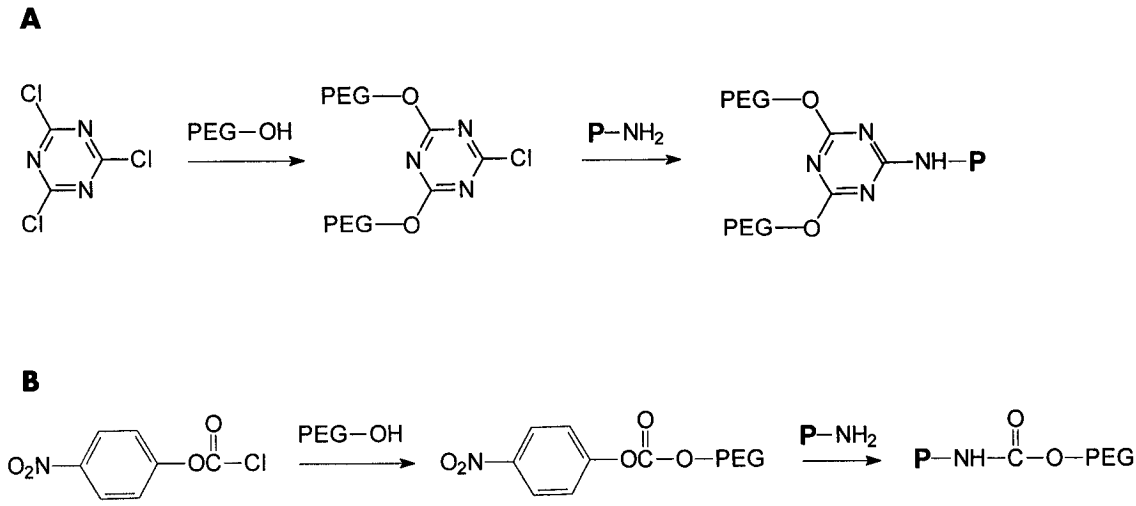
Since it is relatively inexpensive, readily available and efficient, glutaraldehyde is one of the most widely used reagents for cross-linking proteins. Cross-linking using this reagent is a very complicated process that is believed to occur through a variety of pathways, as shown in Scheme 1.1, beginning with the rapid formation of a Schiff base via reaction between the aldehyde groups of glutaraldehyde and the ε–amino group of lysine on proteins. However, since low doses of glutaraldehyde have been found to be toxic, it is an undesirable reagent for use in the preparation of implantable biomaterials or therapeutic macromolecular conjugates. Although glutaraldehyde continues to be used as a cross-linking reagent, the need for safer, alternative reactive bifunctional cross-linking reagents or techniques is clearly evident to keep up with the ever-increasing demand for biomedically significant macromolecular conjugates.



Scheme 1.1: Cascade of cross-linking reactions initiated by reaction between glutaraldehyde and protein (Olde Damink *et al.*, 1995).

Polyethylene glycol (PEG) is another reagent that is commonly used to prepare protein conjugates, particularly for biomedical applications since it has been approved by regulatory agencies for use in pharmaceuticals (Duncan *et al.*, 1994). However, unlike the reactive homo- and hetero-bifunctional reagents shown in Figure 1.3, PEG requires pretreatment with either *p*-nitrophenylchloroformate or cyanuric chloride in order to activate its terminal hydroxyl groups for reaction with the functional groups of protein in aqueous solution (Scheme 1.2). Hernaiz *et al.* activated PEG using *p*-nitrophenylchloroformate,

yielding the carbonate derivative precursor which was then used to study the catalytic activity and stability of PEGylated lipase (Hernaiz *et al.*, 1999). Despite the high toxicity of cyanuric chloride it is sometimes utilized for the preparation of conjugates intended for use in biomedical applications (Lundblad *et al.*, 1997).



Scheme 1.2: Activation of polyethyelene glycol with **A**) cyanuric chloride and **B**) *p*-nitrophenylchloroformate prior to reaction with protein (DeSantis *et al.*, 1999).

1.3.2 *In Vacuo* Protein Modification

The *in vacuo* protein modification methodology developed by Taralp and Kaplan (Taralp *et al.*, 1997) is a non-aqueous technique in which protein derivatization occurs in the lyophilized state at elevated temperatures under vacuum. The main advantage of this method is that modification can be carried out without regard for problems such as proteolysis, denaturation, reversibility, or conformational dynamics, which can potentially arise under aqueous conditions. Furthermore, the selection of modifying reagents is limited in aqueous

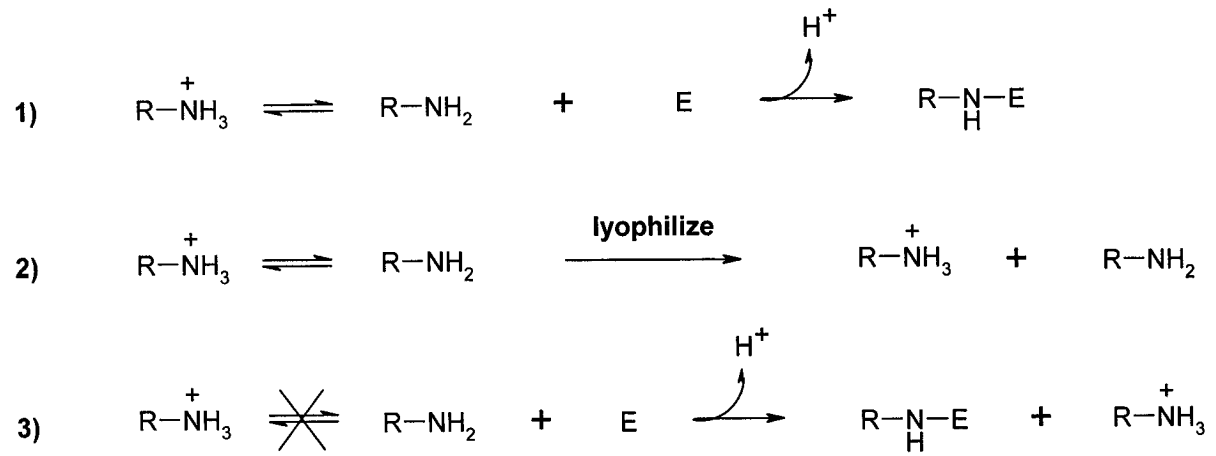
solution since many of them are insoluble in water, are reactive with water, or react with protein functional groups to form labile derivatives in water.

One of the potential disadvantages is that, unlike protein crystallization which preserves the native state of the protein, lyophilization induces reversible changes in the secondary structure of proteins. Griebenow *et al.* observed an increase in the β-sheet content resulting in a more ordered structure along with a concomitant decrease in α-helices (Griebenow *et al.*, 1995). Excipients are often employed as lyoprotectants to prevent or limit problems associated with lyophilization in sensitive proteins (*vide infra*). Accessibility of functional groups may also be limited by the surface area of the protein (Rees *et al.*, 2001) and diffusivity of the modifying reagent through the lyophilized protein. The number of protonated vs. unprotonated ionizable functional groups in the lyophilized state is fixed and is largely dependent on the pH of the solution immediately prior to lyophilization.

1.3.3 pH Memory Effect

In aqueous solution, all ionizable species exist in both protonated and unprotonated forms in equilibrium; the degree of protonation being determined by the pK_a of the ionizable group. During a reaction in solution, the equilibrium will shift to produce that species which is consumed by the reaction in accordance with Le Chatelier's principle, provided no equilibrium exists between the products formed and the reactants. Upon dehydration during lyophilization of an aqueous solution containing ionizable groups in equilibrium, the protonated and unprotonated species are 'frozen out' and a dynamic equilibrium no longer exists between them. This is know as the 'pH memory effect', a term coined by Alexander Klibanov in the 1980's to explain the catalytic activity of lyophilized enzymes in organic solvents (Zaks *et al.*, 1985). According to theoretical precedence established from the known

chemistry of nucleophilic reactions in solution, it is expected that after lyophilization, in the presence of a suitable electrophile, reaction with only the unprotonated amine will occur as shown in Scheme 1.2.



Scheme 1.3.: Reaction of amino groups in solution *vs.* the lyophilized state
1) Reactivity of amine in equilibrium in aqueous solution toward electrophile **E** - depletion of protonated amine by Le Chatelier's principle. 2) Both amine species (existing in equilibrium in solution) are 'frozen' out by lyophilization. 3) Dry state reaction under vacuum – electrophile **E** reacts only with a free amine.

1.3.4 The Use of Excipients

The reasons for using excipients with proteins in the lyophilized state are two-fold: 1) separation of protein molecules by the inclusion of excipient molecules prevents protein-protein interactions in the solid phase and 2) lyoprotection by stabilizing the tertiary structure. Although there are a number of compounds that can be used as potential excipients, carbohydrates provide the best stabilization protection and are thus widely used, particularly in pharmaceutical protein preparations (Yu, 2001). It is believed that during the

dehydration process the hydrogen bonds of water, which are essential for maintaining the protein structure in its native form, are replaced by those of the carbohydrate excipients (Souillac *et al.*, 2002; Arakawa *et al.*, 2001; Yu, 2001; Griebenow *et al.*, 1995).

Excipients should remain amorphous and should not crystallize out during the freeze-drying process in order to be most effective for use both as protein stabilizers and as solid spacer molecules. For the purposes of *in vacuo* modification, vitrification of a protein with or without an excipient during the freeze-drying process is undesirable, since the rigidity of a glass precludes diffusion of small molecules through the protein molecules (Rees *et al.*, 2001). Therefore, in order to activate functional groups on more than just the exposed protein molecules on the surface, excipients with a high glass transition temperature, T_g, are desirable.

In light of these criteria, sorbitol is a potentially good excipient and is widely used in the pharmaceutical industry, unlike its stereoisomer mannitol which has a strong tendency to crystallize out (Yu, 2001). Although lactose has been utilized in commercial lipase preparations to modulate hydrolysis, it is generally a poor choice as a protein excipient since, as with all reducing sugars, it can participate in the Maillard reaction with lysine residues (*vide infra*) ultimately resulting in the degradation of the protein. The non-reducing disaccharide, trehalose has been shown to have superior protein stabilization properties (Colaco *et al.*, 1994) and a relatively high T_g. Thus, trehalose was selected as the excipient of choice for the purposes of this research.

1.4 Glycation of Proteins and Dendrimers

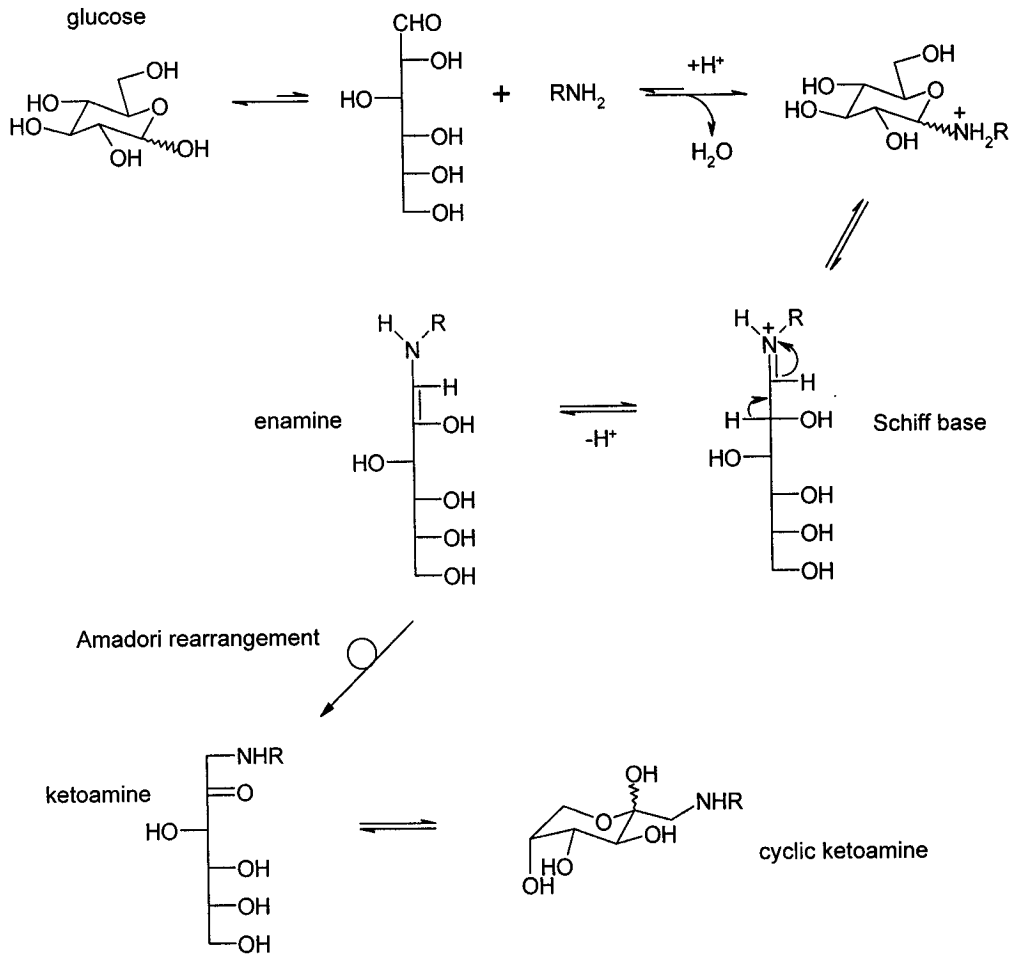
Studies have shown that modification of proteins with sugars imparts thermal stability to proteins, and a variety of solution methods have been employed to covalently link carbohydrates to proteins. For example, using reductive alkylation, Sundaram *et al.* attached high molecular weight carbohydrate polymers to chymotrypsin to minimize thermal and urea-induced degradation (Sundaram *et al.*, 1998). Venkatesh and Sundaram prepared a 'thermophilic enzyme' by covalent modification of trypsin using oxidized polymeric sucrose (Venkatesh *et al.*, 1998). Through covalent attachment of glucosamine to ribonuclease A using carbodiimide chemistry, Baek *et al.* generated a proteolytic resistant glycoprotein with enhanced thermal stability (Baek *et al.*, 1997).

Another reason for attaching carbohydrates to proteins is to prepare neoglycoconjugates for use as artificial vaccines (Kamicker *et al.*, 1977), tumour markers (Roy *et al.*, 2001), diagnostic reagents, and immunogens (Mammen *et al.*, 1998); the predominant method of attachment is through chemically modified carbohydrate moieties (Roy, 1996). Macromolecular conjugates using dendrimers instead of proteins having similar multivalent biological properties have also been prepared using similar methodology (Roy, 1996; Andre *et al.*, 1999).

1.4.1 The Maillard Reaction

In solution, reducing sugars exist in cyclic hemiacetal and open chain conformations in an equilibrium favouring the cyclic form. Glycation of a protein occurs by way of a condensation reaction between the aldehyde of the aldose reducing sugar and the amine groups of a protein, resulting in a cascade of reactions known simply as the Maillard

reaction. The typical amine-aldehyde chemistry in solution involves an initial nucleophilic attack of the open chain sugar aldehyde by the unprotonated amine which condenses to form a Schiff base (aldimine) (Scheme 1.3). Unless the Schiff base is trapped by a suitable reducing agent such as sodium cyanoborohydride, product accumulation is slow because of the reversible nature of glycation in solution. In the absence of a reducing agent, under acidic conditions the glycosylamine rearranges to the more stable ketoamine. In the case of aldoses, this rearrangement process is known as the Amadori rearrangement, while in the case of ketoses such as fructose, it is known as the Heyns rearrangement.



Scheme 1.4: Reaction scheme for amine glycation in solution (early Maillard reaction).

Glycation of proteins is a process that is not exclusive to a solvent environment; the formation of Amadori rearrangement products and their degradation products is also known to spontaneously occur under dry conditions. The brown colour and aroma of baked foods, for example, is attributable to advanced glycation products (Yaylayan *et al.*, 1994; Wrodnigg *et al.*, 2001).

Although there have been many recent dry state glycation studies (Boratynski *et al.*, 1998; Boratynski, 2002; Boratynski, 1998; Quan *et al.*, 1999; Morgan *et al.*, 1999; French *et al.*, 2002), all were performed under atmospheric pressure in the presence of oxygen and most were conducted under conditions of relatively high humidity over extended time periods. It was previously discovered in the Kaplan group that co-lyophilizing a protein with a reducing sugar followed by the application of heat under conditions of reduced pressure results in extensive glycation of the protein (Kaplan *et al.*, 2002). This discovery fostered further research into the possibility of developing a novel class of cross-linkers bearing reducing sugar moieties, and is the subject of a good part of this thesis. The cross-linkers were designed such that the reducing functionality of the sugars are separated by a linear or branched spacer and are readily accessible for facile reaction with the protein.

1.5 Thesis Structure

The lipophilic modification of dendrimers and their potential for use in an artificial liver construct was ascertained by way of aqueous drug partitioning studies. The synthesis and partitioning results are covered in Chapter 2. Issues that arose as a result of the experimental design for building an artificial liver module gave rise to the work presented in Chapters 3 and 4. Chapter 3 deals with the investigation and characterization of a non-

aqueous, *in vacuo* protein activation method that involves the use of *p*-nitrophenylchloroformate as an activator. This novel method has been extensively studied; a mechanism of action is proposed, and the practicality of using it as a means to cross-link proteins is examined in this part of the thesis. In Chapter 4 the design of a new class of protein cross-linkers based on the Maillard reaction between reducing sugars and amines conducted under unusual conditions is presented. The results obtained from that work spawned the idea that perhaps the same methodology could be extended to synthesize multivalent neoglycoconjugates for use in medicinal treatments and therapies by coupling immunologically relevant reducing sugars to the amine periphery of polypropyleneimine dendrimers or the amines of multiple lysine residues in such proteins as bovine serum albumin. Efforts towards this end and the subsequent mechanistic insights gained are presented in Chapter 5. Chapter 6 provides conclusions for the thesis by way of an integral look at the work presented in the preceding chapters and recommendations for future work.

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Chapter 2 A Detoxication Model for an Artificial Liver Module

2.1 Design and Rationale

The liver is responsible for the detoxification of xenobiotics and the synthesis of many proteins and enzymes critical to maintaining fundamental body functions. In order to treat patients suffering from fulminant hepatic liver failure, there is much interest in developing an extracorporeal, bridge-to-transplant, bioartificial liver assist system, or artificial liver. Many of the current artificial liver systems are hybrid in design, involving the use of bound porcine hepatic cells to perform the functions of the liver (Flendrig *et al.*, 1997; Rozga *et al.*, 1995; Berry *et al.*, 1997; Kanematsu *et al.*, 1998; Jauregui, 1997; Ohshima *et al.*, 1997; Gerlach *et al.*, 1996; Yamashita *et al.*, 2002). One of the biggest shortcomings in these models is poor-to-fair functional efficiency resulting in premature death of the cells.

It is conceivable that the lifespan and efficiency of these hepatic cells could be greatly improved by 'pre-cleaning' the incoming blood in a preceding module, thereby effectively reducing the cellular 'workload' and prolonging the lifetime of the system. To this end, removing toxins from the blood prior to passage through the cellular component of the device is not a new idea and indeed has been attempted in many of the existing artificial liver designs (Rozga *et al.*, 1995; Cao *et al.*, 1998). Unlike the toxins associated with kidney failure, most of the xenobiotics normally cleared by the healthy liver are hydrophobic and are thus not amenable to removal by hemodialysis. Therefore, a blood purification technique known as adsorbent hemoperfusion which utilizes a charcoal filter is often incorporated into artificial liver devices, and has resulted in considerable improvements in hepatocyte bioreactor function and viability. The cytotoxic effects of the toxins present in the blood and

serum on the hepatocytes of patients with fulminant hepatic liver failure have been shown to decrease significantly with the use of charcoal filtration (Shi, 2000).

Modified dendrimers may provide a more efficient medium than charcoal by which to partition and accumulate hydrophobic xenobiotics such as drugs. Dendrimers inherently possess a high surface-to-volume ratio, and modification of the dendrimer periphery with lipophilic components can be readily achieved, thus providing the hydrophobicity required for partitioning. Futhermore, the unique properties of symmetry, multivalency and monodispersity of synthetic dendrimers make them attractive candidates for a uniform detoxication infrastructure of nanoscale proportions whose properties can be easily modeled for optimization.

From a bioreaction engineering perspective, dendrimers offer great design flexibility since the 'catalyst pellet' size of the detoxication bioreactor can be precisely controlled simply by utilizing the generation corresponding to the most desirable radius determined by a mathematical model; the volume of a dendrimer molecule is cubed with each generation. It can be assumed that almost all of the resistance to mass transfer occurs within the stagnant fluid layer next to the solid boundary of the pellet, where the change in concentration of the toxic solute from the bulk fluid phase concentration to the dendrimer phase concentration takes place. The thickness of this hydrodynamic boundary layer, and therefore the resistance to mass transfer, is proportional to both the size of the catalyst pellet, or dendrimer generation and the bulk fluid velocity. Under conditions of low fluid velocity, the thickness of this boundary layer is significant and the partitioning and subsequent reaction of the toxin will be diffusion-limited and the rate of partitioning and reaction is no longer limited by mass transfer across the layer. Under conditions of increased fluid velocity, the system becomes

reaction-limited. Optimization of the bioreactor configuration can be achieved by manipulation of these parameters to create a diffusion-limited or reaction-limited system, which would impact equilibrium effects of the xenobiotic substrates and their products from the dendrimer periphery.

Hypothetically, the accumulated hydrophobic xenobiotics can be effectively cleared from the dendrimer by the catalytic action of a suitable covalently attached enzyme and 'washed' away with the bulk flow. Detoxication of xenobiotics in a non-diseased mammalian liver is achieved mainly by the activity of the membrane-bound microsomal enzyme cytochrome P450, which transforms hydrophobic, lipophilic compounds into more hydrophilic and water-soluble compounds in order to facilitate excretion in urine by the kidneys. A bioreactor packed with 'catalytic pellets' consisting of cytochrome P450 or a suitable replacement enzyme covalently tethered to a lipophilic dendrimer membrane mimetic, could effectively mimic the detoxicating activity of P450 in hepatic cells.

2.1.1 Mechanism of P450 Detoxication in Hepatocytes

Although the exact mechanism of the catalytic activity of cytochrome P450 is not known, it is believed that the axial thiolate of a cysteine residue proximal to the ferriprotoporphyrin IX ring, acts as the fifth ligand which plays a significant role in the stabilization of the iron IV oxidation state that mediates oxygen transfer (Poulos, 1996; Rietjens *et al.*, 1996). The steps involved in the cytochrome P450 catalytic cycle, beginning with displacement of water by substrate binding to release of the oxygenated product, are depicted in Figure 2.1 below:

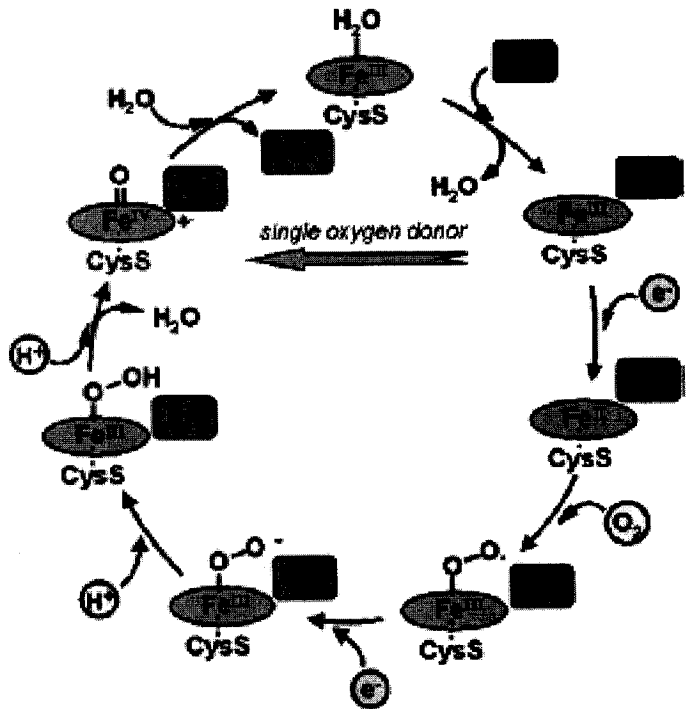


Figure 2.1: Cytochrome P450 catalytic cycle (Feiters, 2000)
RH = toxin substrate; CysS = cysteine thiolate.

The use of immobilized enzymes in artificial liver support systems is not in itself a novel initiative. Brunner *et al.* reported some success in glucuronidating *p*-nitrophenol using immobilized UDP-glucuronyltransferase on an agarose matrix with an entrapped cofactor (Brunner *et al.*, 1979). Although these authors acknowledge the superiority of cytochrome P450 as a liver-detoxifying enzyme, it was notably excluded from their experiments. While Lamb *et al.*, have successfully immobilized P450 (Lamb *et al.*, 1998) in polyaphrons (oil-in-water macro-emulsions), to the best of our knowledge, there are no reports of covalent attachment of this important enzyme to date.

2.1.2 Selection of a P450 Model

As with many membrane-bound enzymes, the integrity of P450 enzyme system is compromised when removed from its native environment. Therefore, the use of a model or a suitable mimic for the cytochrome P450 system may actually prove to be a more practical alternative. Indeed, many P450 models have been investigated, reported and reviewed in the literature (Feiters *et al.*, 2000; Tinoco *et al.*, 1998; Nakamura *et al.*, 1992; Wiseman, 1997), albeit not in the context of facilitating an artificial liver. In order to improve the activity and stability of the P450 enzyme system, catalytically self-sufficient systems consisting of P450 fused to its electron donor NADPH-P450 reductase have been successfully genetically engineered (Yabusaki, 1995). Lamb and co-workers immobilized a genetically engineered fusion protein which consisted of plant cytochrome P450-71B1 fused to NADPH-cytochrome P450 reductase (Lamb *et al.*, 1998). Some researchers have sought after alternative electron donating and carrying systems to improve the electron transfer process. Wiseman *et al.* co-immobilized P450 reductase and a dehydrogenase capable of regenerating NADPH *in situ* (Wiseman, 1993). In a P450-based biosensor consisting of a gelatin-entrapped microsomal rabbit liver fraction attached to an amperometric sensor, Schubert *et al.* utilized co-immobilized glucose oxidase (GOD), a fungal flavo-protein, to facilitate activity of the naturally occurring P450 in the absence of both NADPH and P450 reductase (Schubert *et al.*, 1980). Hydrogen peroxide is a by-product of the conversion of glucose to gluconic acid by GOD, which provides the necessary hydrogen ions and oxygen *in situ* to drive the P450 catalysis, and precluded the need for NADPH or P450 reductase in their application.

Incorporation of P450 by immobilization onto a dendrimer scaffold for a detoxication module in an artifical liver may not only prove to be an overwhelming challenge but may also have deleterious overall effects. Cytochrome P450 can generate reactive oxygen-centered free radicals that can trigger free radical chain reactions which are detrimental to cellular components as well as the enzyme itself (Castell *et al.*, 1997), and can potentially degrade the structural integrity of the dendrimer construct by hydrogen abstraction of the alkyl periphery. In the cell, protection against such damaging effects by reactive oxygen species is afforded by glutathione. Furthermore, P450-mediated biotransformations of some isoforms of P450 can actually produce carcinogenic compounds, exemplified by P450-1A1 which converts benzo[a]pyrene to the more toxic diol epoxide, 7,8-dihydro 7,8-dihydroxybenzo[a]pyrene-9,10 oxide (Wiseman, 1993), which may jeopardize the ensuing hepatocyte module.

Akasaka *et al.* discovered that in the presence of hydrogen peroxide, cytochrome c catalyzes N-demethylation, S-oxidation and epoxidation of olefins in much the same manner as cytochrome P450 (Akasaka *et al.*, 1993). Although other heme proteins such as chloroperoxidase and horseradish peroxidase (HRP) are capable of similar catalytic activity, their mode of action is different, even though they share structural resemblance to cytochrome c in that the fifth ligand of the heme group is the imadazole group of a histidine. Upon immobilization of cytochrome c, Akasaka *et al.* also observed an increased V_{max} for N-demethylation and S-oxidation reactions as well as an increase in efficiency of olefin epoxidation compared to free cytochrome c (Akasaka *et al.*, 1993). They believe that this increase in P450-like activity is due to a conformational change specifically brought about by immobilization of the enzyme.

Thus it would appear that cytochrome c may serve as a useful model for the cytochrome P450 enzyme. However, a source of electrons and an electron carrier must be included with this model enzyme in the artificial liver system design in order to effectively mimic the activity of the cytochrome P450/NADPH-P450 reductase membrane-bound system in hepatic liver cells. Cytochrome c co-immobilized with GOD on the hydrophobically terminated dendrimer may provide a feasible substitute system, as the activity of GOD would provide the necessary H_2O_2 to drive the xenobiotic transformations by cytochrome c in much the same manner as cytochrome P450.

2.1.3 Hydrophobic Modifications of Dendrimers

Hydrophobic surface modification of dendrimers has been the subject of much research over the past decade, mostly for the purposes of examining any changes in properties that such a modification will impart but also to generate symmetrical functional branched polymers. Sakthivel *et al.* studied the self-organization properties of the helical lipophilic polyamide dendrimers they synthesized using a lysine-based core to which they attached lipoaminoacids having long alkyl chains (Sakthivel *et al.*, 1998). Another research group (Piotti *et al.*, 1999) sought to reproduce the catalytic environment of enzymes for selected E1 and SN_2 reactions by preparing an aromatic dendritic core enriched with hydroxyl groups to provide transition state stabilization sites for hydrogen bonding and cation-π interactions, capped with an external corona of tetradecyl groups to encourage non-polar product migration away from the "active site".

Two of the most common dendrimers used for surface modification purposes are the commercially available, polyamidoamine (PAMAM) and polypropyleneimine (DAB)* dendrimers, and there are a few ways reported in the literature in which the terminal amine

groups of these dendrimers have been modified to generate an apolar periphery. Stevelmans *et al.* amidated the terminal amines of DAB dendrimers with palmitoyl chloride which yielded unimolecular, inverted micellar products having interesting host-guest properties (Stevelmans *et al.*, 1996). Baars *et al.* studied the liquid-liquid extraction properties of the same amidated dendrimers using dyes of varying pK_a values (Baars *et al.*, 1997; Baars, 2000) under conditions of variable pH. Froehling and Linssen prepared alkyl surface modified DAB dendrimers by a direct amidation method using stearic acid, in less than the stoichiometric amount, under azeotropic distillation conditions with xylene to investigate the distribution of partially modified products as well as the spatial heterogeneity of substituent groups on the dendrimer (Froehling *et al.*, 1998). Similarly, Ramzi *et al.* utilized the same azeotropic distillation strategy to covalently bind stearic acid to DAB dendrimers in order to quantify size and shape of the fatty acid-modified dendrimer in bulk and in dilute solution (Ramzi *et al.*, 1999).

In a liquid-liquid extraction experiment similar to that of Baars *et al.*, Stephan *et al.* studied the capabilities of alkyl urea functionalized dendrimers, prepared by reacting DAB dendrimers with alkyl isocyanates (Scheme 2.1), to bind nucleotides and other oxyanions (Holger *et al.*, 1999). Another modification strategy used to affix a lipophilic periphery onto dendrimers involved reaction of PAMAM dendrimers with various epoxyalkanes (Sayed-Sweet *et al.*, 1997). Tomalia's research group used these modified dendrimers as nanoscopic containers to transport $CuSO_4$ from an aqueous solution into a toluene organic phase.

In the work presented in this chapter, some of the synthetic strategies found in the afore-mentioned literature accounts were attempted in order to test whether such lipophilic modified dendrimers would indeed partition xenobiotics such as common analgesics and

drugs normally biotransformed in a healthy liver. While some degree of success was achieved to this end and the results show that lipophilic modified DAB dendrimers may indeed be of value as partitioning scaffold material in a detoxication module, some practical problems were encountered with the synthesis and are reported herein. The results of an initial attempt to tether a heme-based enzyme to a partially modified alkyl urea-functionalized DAB dendrimer are also reported in this chapter.

2.2 Materials and Methods

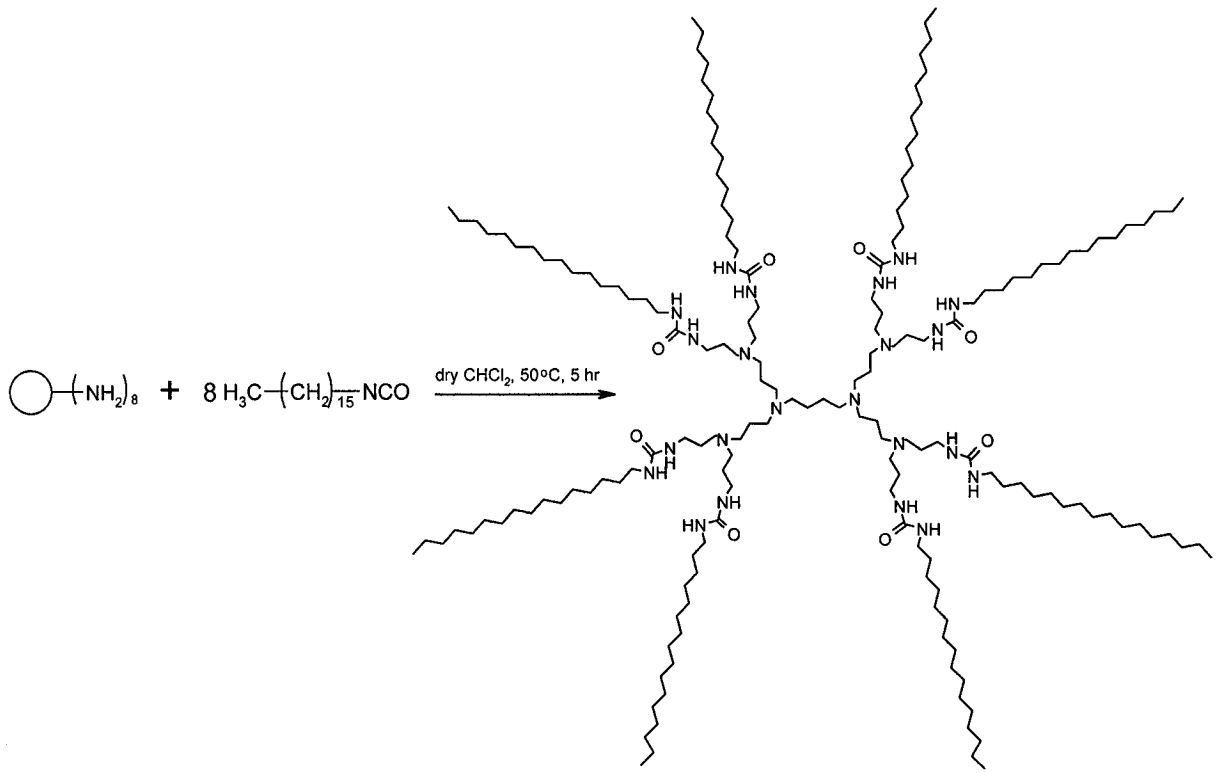
2.2.1 Synthesis of Lipophilic Modified DAB Dendrimers

Alkyl Urea - functionalized DAB dendrimers:

Approximately 100 mg. of G2.0 DAB dendrimers (Aldrich Chemical Co.) was dried overnight under vacuum and placed in a 50 mL two-necked round-bottom flask with 15 mL of dry dichloroethane and refluxed at a temperature of 50°C with stirring under dry nitrogen. To this solution, one equivalent plus an additional 5 - 10% excess of isocyanate* (Sigma Aldrich) per DAB dendrimer amine equivalent was slowly added dropwise. The dropper was rinsed well with dry dichloroethane and the mixture was left to react between 5-12 hrs. The reaction was stopped when the solution turned milky white and a ninhydrin test (approximately 0.6 mL of a 3% ninhydrin (Aldrich Chemical Co.) solution in ethanol was added to approximately 0.3 mL of reaction mixture and then heated) was no longer positive. The modified dendrimer solution was precipitated by dropwise addition to a saturated aqueous sodium chloride solution. The product was washed well with dH_2O, filtered by suction, and placed under vacuum overnight at room temperature. The typical yield of the isocyanate modified dendrimers was approximately 60%. All products were pale yellow,

waxy solids that were soluble in $CHCl_3$ except for the hexadecylisocyanate and octadecylisocyanate-modified dendrimers, which were white amorphous solids that were insoluble in common solvents such as DMSO, $CHCl_3$, MeOH, EtOAC, DMF, THF, and toluene. Modified dendrimers were characterized by Fourier transform infrared spectroscopy (FTIR) and solid state NMR in the case of octadecylisocyanate and hexadecylisocyanate-dendrimer products. The procedure was repeated for G3.0 and G4.0 DAB dendrimers.

*- isocyanates used were t-butyl, dodecyl, tetradecyl and hexadecyl.



Scheme 2.1: Modification of DAB G2.0 dendrimer with hexadecylisocyanate.

Alkyl Amide - functionalized DAB dendrimers:

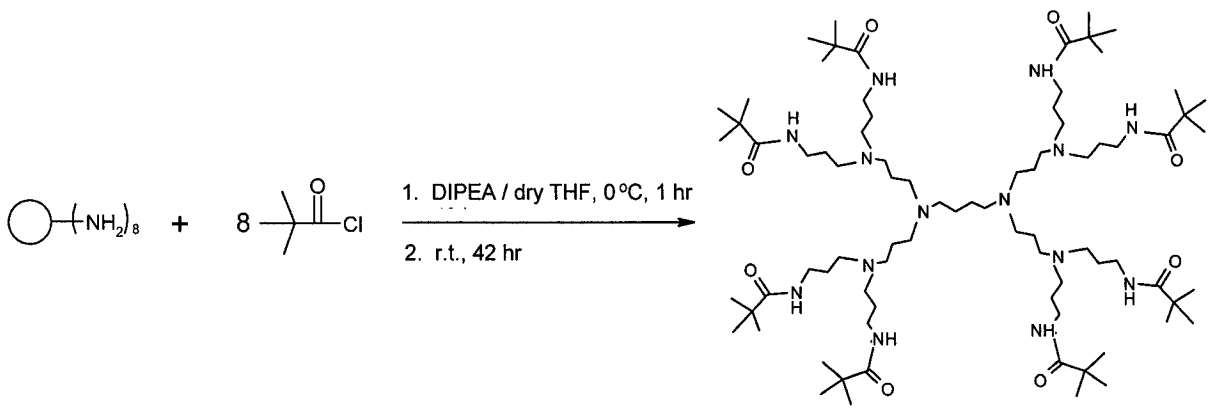
Method A:

Approximately 200 mg of accurately weighed DAB G2.0 dendrimer was placed in a round-bottomed flask and azeotroped with 3 x 7 mL aliquots of toluene on a roto-evaporator at 30°C. The flask was attached to a vacuum pump for 0.5 hr to remove water and other solvents. Approximately 1.05 equivalents of palmitic acid (C16) per equivalent of dendrimer amine were reluxed in 25 mL of xylene in a 50 mL round-bottom flask fitted with a Dean-Stark trap. After 4 hours, the reaction tested ninhydrin negative and xylene was then rotoevaporated off. The residue was dissolved in 20 mL of CH_2Cl_2 and the solution was transferred to a 125 mL separatory funnel into which 15 mL of 1M NaOH was also added. The separatory funnel was shaken and the resulting emulsion was broken with 10 mL of a saturated NaCl solution. The CH_2Cl_2 layer was filtered over anyhydrous Na_2SO_4 and the rotoevaporated to remove the solvent. The product was dried under vacuum overnight to afford a pale yellow waxy solid product in 63% yield.

Method B:

Approximately 100 mg of accurately weighed DAB G2.0 dendrimer was placed in a round-bottom flask, azeotroped with 3 x 5 mL aliquots of toluene at 30°C, and then placed under vacuum for 0.5 hr to remove water and other solvents. The flask was flushed with dry nitrogen and 10 mL of ultra-dry THF was added with stirring. After the dendrimer was completely dissolved (about 1 hour), 2.2 equivalents of diisopropylethylamine (DIPEA) per dendrimer amine equivalent was added to the flask, and the mixture was placed in an ice bath to cool. A solution containing 2.2 equivalent of trimethylacetylchloride (Aldrich) in 8 mL of

ultra-dry THF was added dropwise slowly over a 30 minute period and was left in the ice bath for another 1hr before allowing the mixture to continue stirring at r.t., under dry nitrogen conditions until a ninhydrin test showed negative after about 24 hrs. The THF was removed using a Bucchi rotoevaporator and then the product was refluxed in 15 mL of anyhydrous ether under dry nitrogen for 0.5 hr to react any excess trimethylacetylchloride. The product was filtered, washed with anhydrous ether, and dried under vacuum. The pale yellow waxy solid product, characterized by NMR (Figure 2.2), was soluble in CH_3Cl and the yield was about 70%.



Scheme 2.2: Modification of DAB G2.0 dendrimer with trimethylacetyl chloride.

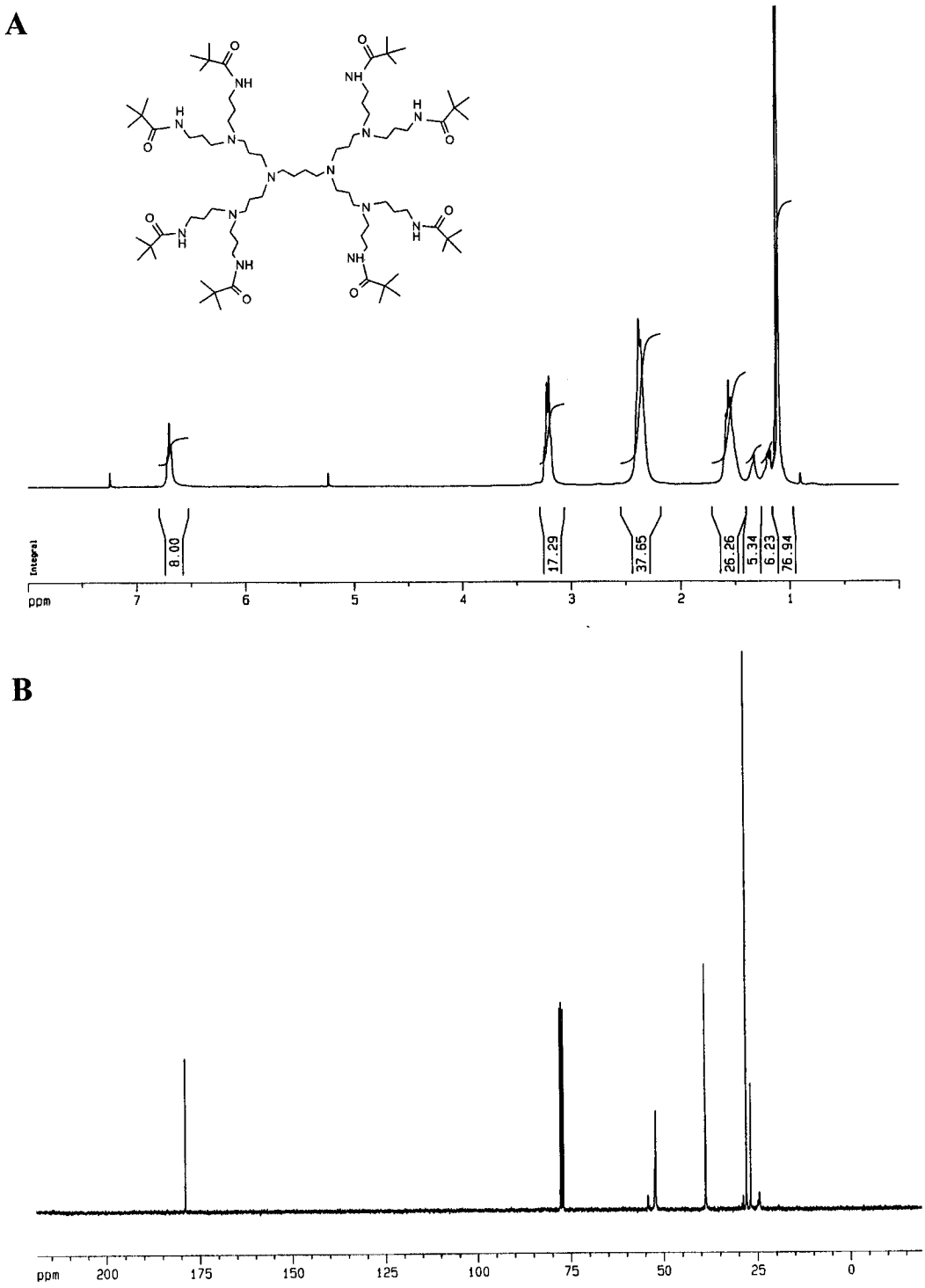


Figure 2.2: **A** - ^{1}H NMR ($CDCl_3$, 300 MHz) and **B** - ^{13}C NMR ($CDCl_3$, 300 MHz) spectra of DAB G2.0 dendrimer modified with trimethylacetylchloride.

Palmitoyl Chloride Modified Dendrimers:

Since large amounts of purified modified dendrimer were required to conduct the partitioning studies, purified and fully characterized DAB dendrimers (Generations 1.0-5.0) modified with palmitoyl chloride (C18) were provided as a kind gift from Dr. E.W. Meijer., Laboratory of Macromolecular and Organic Chemistry, Eindhoven University of Technology, Eindhoven, The Netherlands. A representative structure of a palmitoyl chloride modified dendrimer is shown in Figure 2.3 below:

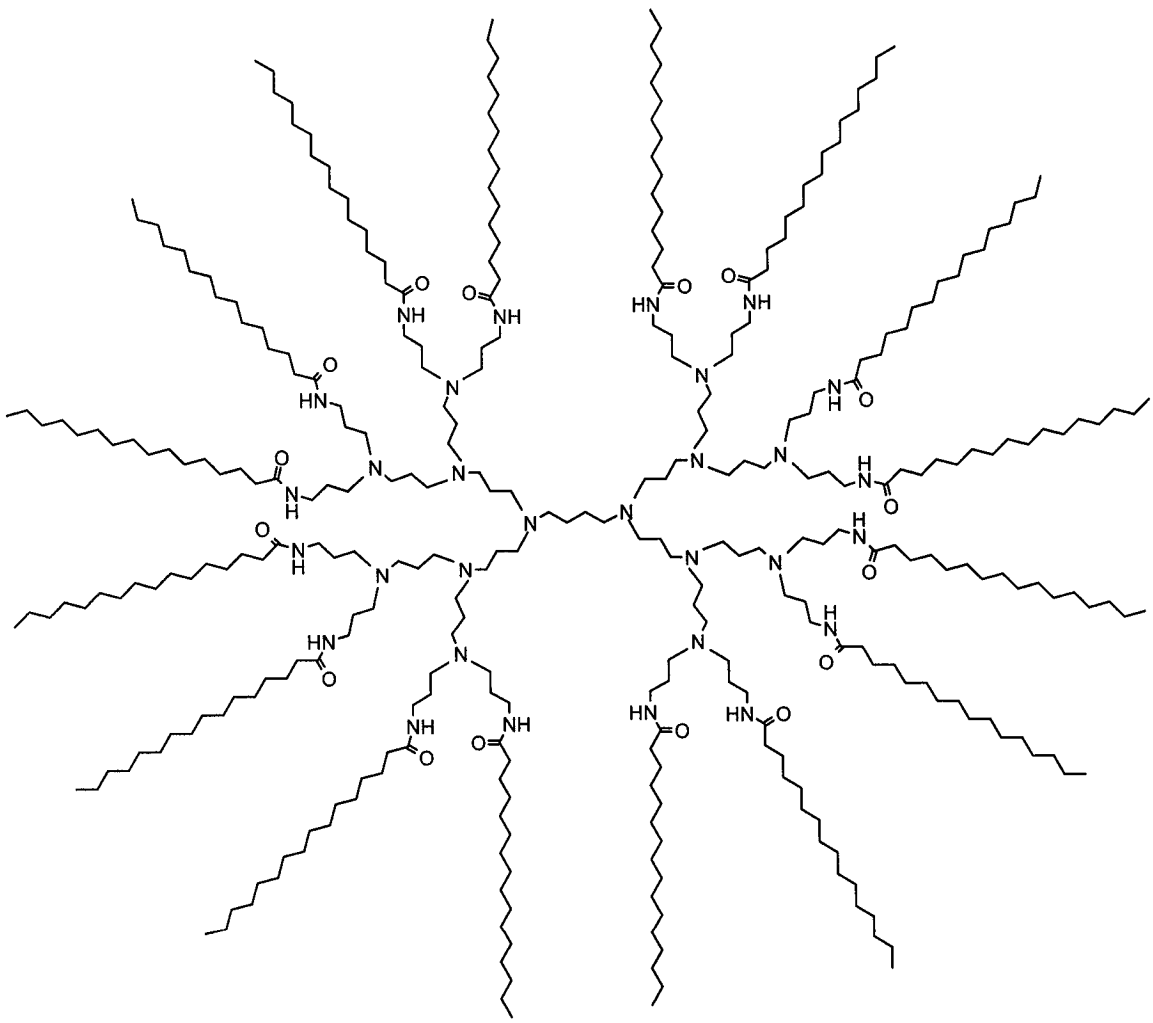


Figure 2.3: Chemical structure of G4.0 palmitoyl acid modified dendrimer.

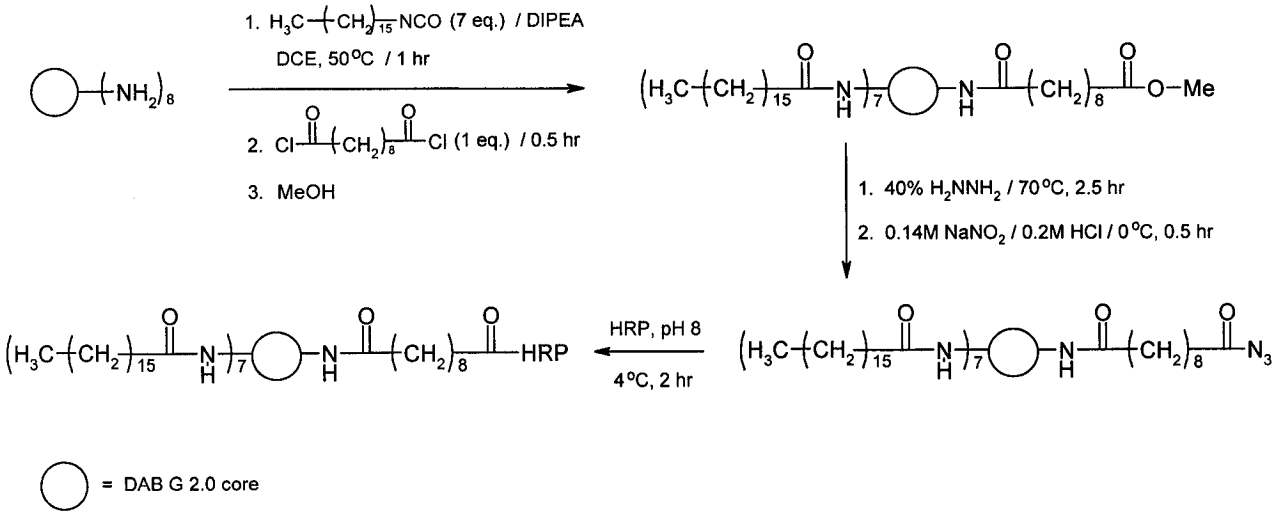
2.2.2 Partitioning Studies

The drugs selected for the partitioning study included erythromycin (Sigma Aldrich), acetylsalicylic acid (Sigma-Aldrich), acetaminophen (Sigma Aldrich), ibuprofen (Fluka Chemical Co.), caffeine (Sigma Aldrich), and lidocaine (Sigma Aldrich). An accurately weighed amount (approximately 10 mg) of dendrimer and 1.5 mL of the stock drug solution, with a concentration between 20 and 120 μg/mL (adjusted according to optimum UV absorbance reading), were placed in a 2 mL Eppendorf tube. For samples with lower water solubility, sonication was necessary to make a solution of adequate concentration for the partitioning studies. The Eppendorf tube containing the dendrimer and drug mixture was placed on a mechanical shaker for 30 minutes. The solution was then centrifuged and the supernatant was filtered using a syringe fitted with a 0.2 μm Acrodisc (Millipore) to remove any remaining dendrimer. The absorbance of this solution was then measured on a Beckman DU 640 UV-vis spectrophotometer and the amount of solute partitioned from the modified dendrimer was determined for each of the drugs via a standard calibration curve. To examine the kinetics of the partitioning reaction, the amount of acetylsalicylic acid (ASA) partitioned to the modified dendrimers was monitored as a function of time.

2.2.3 Enzyme attachment to Alkyl Urea–Linked Dendrimer

Approximately 1.03 equivalents of hexadecylisocyanate was added slowly dropwise to a round bottom flask containing 60 mg of DAB G2.0 dendrimer and 2.1 equivalents of DIPEA in 20 mL dichloroethane (DCE), and the mixture was left to reflux at 60°C for one hour. The flask was allowed to cool and placed in an ice bath whereupon about 1/8 of the stoichiometrically required amount of sebacoyl chloride (bis acid chloride C10) was slowly

added and left to stir at r.t. for 0.5 hr. An excess of MeOH was added and the flask was placed on a Bucchi rotoevaporator to remove all solvent. The solid residue was washed well with dH_2O and ether to remove DIPEA salt, urea hydrolysate of hexadecylisocyanate and sebacic acid. The remaining solid was transferred to a two-necked round bottom flask along with 50 mL of ice cold 0.2 M HCl. To this solution, 10 mL of ice cold 6% $NaNO_2$ was added dropwise slowly, generating nitrous acid *in situ,* and was left stirring at 0°C for 0.5 hr. The heterogeneous mixture was then filtered by suction and the solid was filtered first with ice cold 50% ethanol in dH_2O and then with 0.1 M pH 8 phosphate buffer. The solid product was immediately transferred to a 25 mL round bottom flask containing 2.5 mg of horseradish peroxidase in 10 mL of ice cold 0.1 M phosphate buffer pH 8. The mixture was gently rotated in a water-ice bath at approximately 4°C for 2 hr. The product was then filtered by suction and washed well with approximately 100 mL of 0.1 M phosphate buffer pH 7 to remove excess enzyme. Approximately 3 mL of dH_2O was added to a vial containing the solid white product to facilitate lyophilization. The final product was characterized by solid state ^{13}C NMR (Figure 2.13). Activity of the freeze-dried product was tested using ABTS as substrate (see Chapter 4, Scheme 4.6).



Scheme 2.3: Modification of DAB G2.0 dendrimer and attachment of HRP.

2.3 Results and Discussion

2.3.1 Preparation of Lipophilic Modified DAB Dendrimers

Several approaches to dendrimer modification were attempted, including some of those published in the literature. One of the biggest difficulties encountered with all of the methods used was determining when to terminate the reaction. A ninhydrin colorimetric spot test, which usually indicates the presence of unreacted amine groups instantaneously, proved to be unreliable for the dendrimer modifications. In many cases, especially when dealing with the higher generation dendrimers, a ninhydrin test performed after a published reaction time period, would show negative and then turn positive after about 10 minutes. Thus it became necessary to wait at least 15 minutes to determine the extent of reaction. It is possible that some of the amine groups are rendered inaccessible for reaction with ninhydrin due to some measure of backfolding within the dendrimer. Boris and Rubinstein confirmed the results of several other computer model simulations which showed that the core of a

starburst dendrimer with flexible spacers, such as that of the DAB dendrimers, is dense, with the free ends of the dendrimer dispersed throughout the molecule as depicted below in Figure 2.4 (Boris *et al.*, 1996).

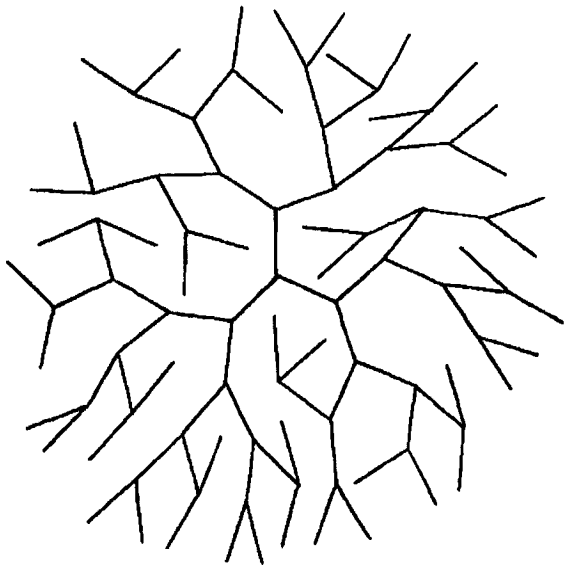


Figure 2.4: Boris and Rubinstein's schematic representation of backfolding in a trifunctional G5.0 dendrimer (Boris *et al.*, 1996).

Although there is some controversy in the literature regarding whether hollow spaces or cavities exist in dendrimers (Bosman *et al.*, 1999), backfolded end-groups, as depicted in Boris and Rubinstein's model (Figure 2.4), would explain the curious behaviour of the DAB dendrimer amine groups towards the ninhydrin test. As the reaction proceeds and the amine groups become functionalized with long chain alkyl groups, the inner core amine groups become less accessible to the modifiying reagent as well as ninhydrin. While initially the ninhydrin cannot 'see' these unreacted amine groups, movement of the flexible alkyl chains eventually allows the ninhydrin to permeate through closer to the core to react with these buried amines.

Another problem encountered in the preparation of urea-functionalized lipophilic dendrimers was that of solubility. The insoluble products obtained by reacting long alkyl chain (C16 and C18) isocyanates with DAB dendrimers made purification and characterization extremely difficult. In dendritic architectures, urea-functionalized dendrimers tend to form a strong network of intramolecular hydrogen bonds (Figure 2.5) which has been shown to be stronger for flexible long alkyl chains than for a more rigid structure such as adamantane (Baars, 2000), and has a significant impact on the solubility of such modified dendrimers

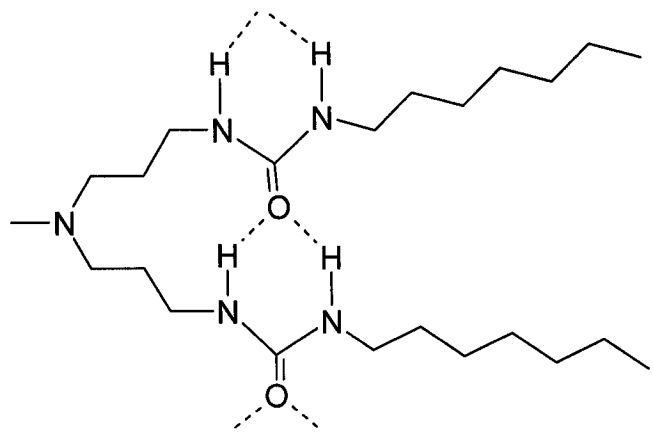


Figure 2.5: Intramolecular hydrogen bonding network in urea-functionalized DAB dendrimers.

Of all the methods attempted, Method A, which utilized a long chain fatty acid to generate an amide-functionalized lipophilic periphery proved to be the most inefficient. Separation of the product from the excess fatty acid starting material was rendered difficult by the formation of emulsions during the reaction work-up of this method. On the other hand Method B, which utilized an acid chloride strategy, was straightforward and efficient, yielding a soluble, easily characterized product in good yield.

2.3.2 Partitioning Studies

The results of this study show that model hydrophobic xenobiotics can indeed be partitioned from an aqueous suspension of hydrophobically terminated DAB dendrimers. Amounts partitioned were on the order of mole/mole of dendrimer, and not surprisingly, partitioning capacity increased with the increasing dendrimer generation. ASA showed the highest level of partitioning of all of the drugs studied, and a single generation 5.0 dendrimer molecule was capable of removing hundreds of ASA molecules.

Since all of the studies were conducted in unbuffered aqueous systems, the pH of the system was determined solely by the ionization properties of the drug in solution, and this in turn probably affected the protonation state of the tertiary amines in the dendrimer core. The results show evidence of a correlation between the pK_a of the substance in solution and the amount that is partitioned, indicating that partitioning may be dependent upon interactions within the core of the modified dendrimer in addition to hydrophobic adsorption interactions at the periphery. Interestingly, partitioning of those drugs having higher pK_a values, *viz.* acetaminophen, lidocaine, caffeine, and erythromycin, was in general significantly less than the partitioning of ASA and ibuprofen with lower pK_a values. In their investigation of the liquid-liquid extraction capabilities of palmitoyl acid modified DAB dendrimers, Baars *et al.* saw that dye extraction was related to the pH of the aqueous layer and the ionization state of the dye being extracted (Baars *et al.*, 1997). Their results suggested that modified dendrimers behave as basic extractants and the tertiary amines of the core dictate a high selectivity for solutes based on their pK_a as well as hydrophobicity. Moreover, they found that the amount of dye extracted was directly proportional to the number of tertiary amines in the core, i.e., dendrimer generation.

Partitioning of ASA and ibuprofen from aqueous, unbuffered solution to palmitoyl chloride modified dendrimer is shown in Figure 2.6.

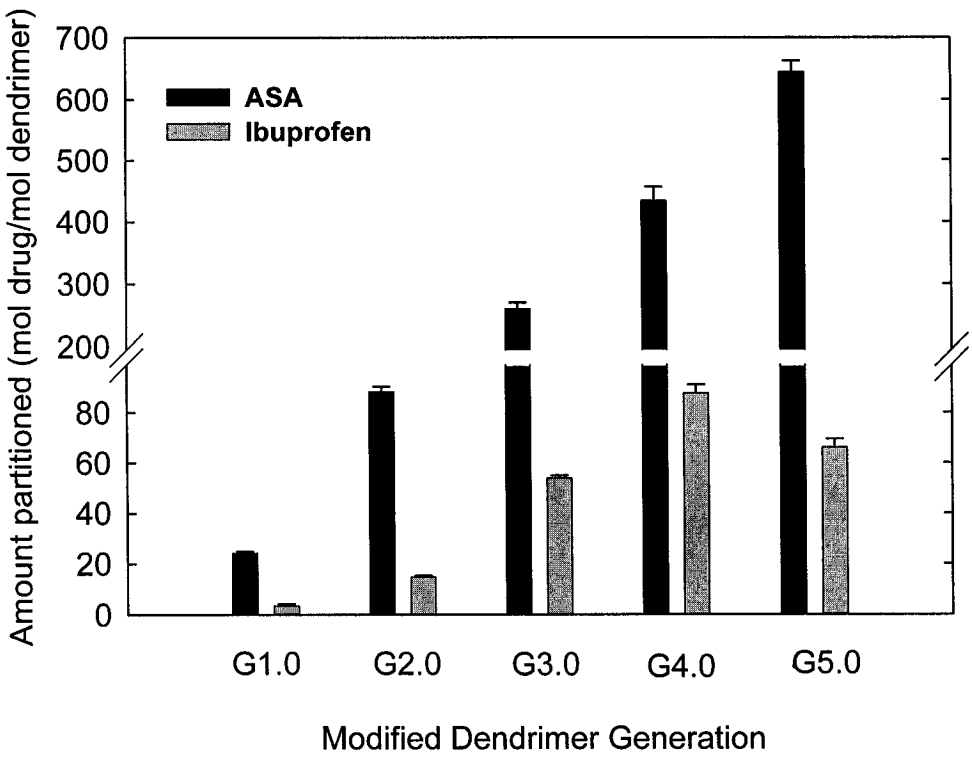
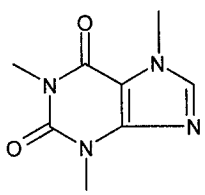


Figure 2.6: Partitioning of compounds with acidic pK_a values to modified dendrimers.

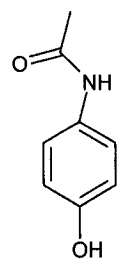
The water solubility data given in Table 2.1 shows that ASA is more soluble in water than ibuprofen by two orders of magnitude, yet ASA was removed from aqueous solution in much greater quantities.

Table 2.1: Physical properties of the drugs used in partitioning studies (data extracted from SRC Physical Properties Database).

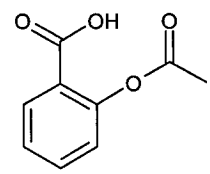
Drug	Molecular Weight (g/mol)	pK_a	Water solubility (mg/mL)	Octanol-Water Partition Coeff.
Caffeine	194.19	10.4	21.74	-0.07
Acetaminophen	151.16	9.38	14	0.46
Acetylsalicylic acid	180.15	3.49	4.6	1.19
Lidocaine	234.33	8.01	4.1	2.44
Ibuprofen	206.27	4.91	0.021	3.97
Erythromycin	733.92	8.88	0.00144	3.06



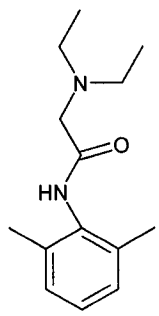
caffeine



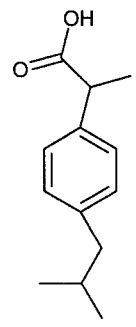
acetaminophen



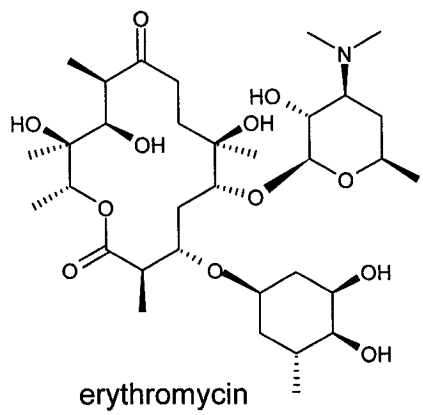
acetylsalicylic acid



lidocaine



ibuprofen



erythromycin

Figure 2.7: Structures of drugs used for partitioning studies shown in unionized form.

This is unusual also because ASA and ibuprofen are both capable of a similar ionic interaction with the protonated tertiary amine groups at the core. A comparison of their respective structures (Figure 2.7) points to the possibility that the partitioning enhancement seen with ASA may be due to hydrogen bonding between the carbonyl of the ester moiety of ASA and the hydrogen of the amide linkage at the dendrimer periphery-core interface as depicted in Figure 2.8 below.

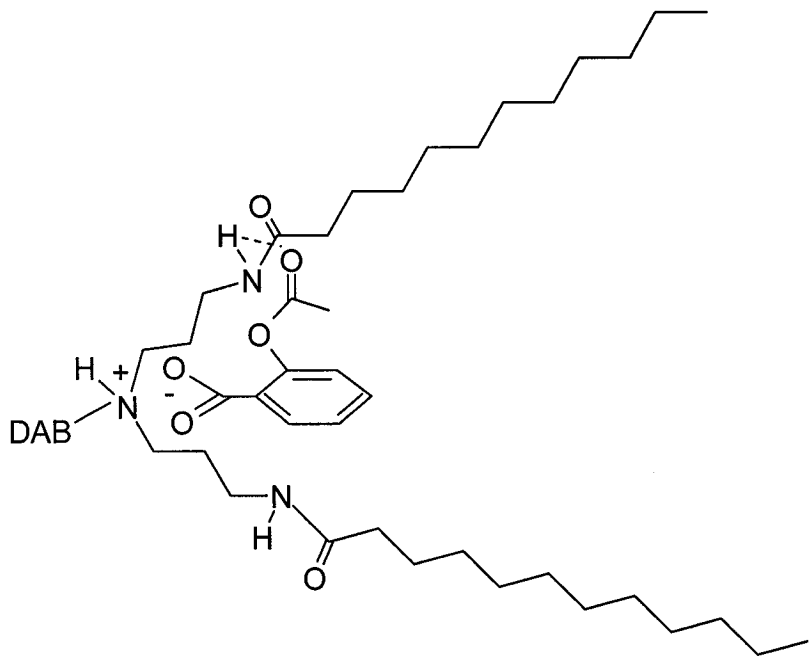


Figure 2.8: Ionic interaction and hydrogen bonding involvement of the amide hydrogen within the hydrophobically modified DAB dendrimer and intercalated ASA.

Baars *et al.* exploited these multiple interactions to generate novel supramolecular architectures consisting of peripherally rigid modified dendrimer scaffolds self-assembled with reversibly bound glycinylurea derivatives (Baars *et al.*, 2000).

Partitioning of acetaminophen, lidocaine, caffeine, and erythromycin, which in unbuffered, deionized water should exist in their protonated states, is summarized in Figure

2.9. Acetaminophen, with a relatively high water solubility and low octanol-water partition coefficient, showed minimal partitioning into the dendrimer phase in all cases as might be expected. However, erythromycin, with extremely low water solubility and a relatively high octanol-water partition coefficient, also showed very little partitioning. This low level of erythromycin partitioning may be due to the sheer bulk of the molecule relative to the other drugs examined (see Figure 2.7). The levels of caffeine and lidocaine partitioning were similar and considerably higher than those observed with the other protonated compounds, despite the very high reported water solubility of caffeine. With properties very similar to acetaminophen, it is surprising that caffeine partitions as well as it does, although it may be possible that with two potential hydrogen bonding sites, caffeine is able to orient itself such that hydrogen bonding is favoured with the amide linkage in a manner comparable to ibuprofen (without the ionic core interaction).

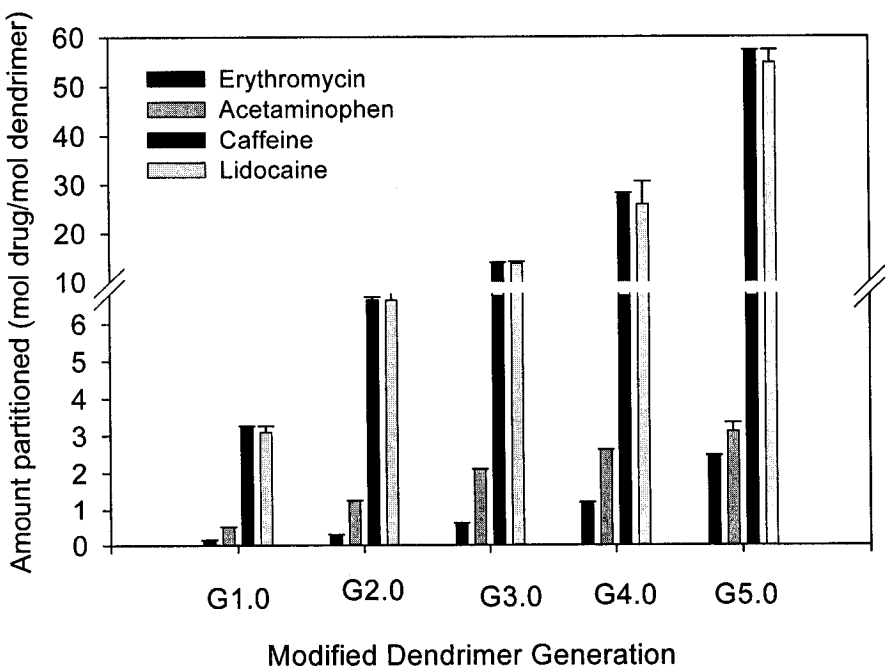


Figure 2.9: Partitioning of compounds with basic pK_a values to modified dendrimers.

The results shown were obtained consistently and the standard errors represented in the figures are for $n > 6$ samples and several trials. It therefore seems clear that many factors influence partitioning in this system, including the chemistry of the molecules and their molecular size, as well as their water solubility and octanol-water partition coefficient, and that the contributions of these factors may be greater in substances with a basic pK_a. A better understanding of the molecular mechanisms for xenobiotic partitioning may be required before it is possible to accurately predict levels of removal for specific compounds.

2.3.3 Kinetic Analysis

In order to assess the time required for partitioning of the solutes, kinetic analyses were performed using ASA and acetaminophen with the palmitoyl chloride modified dendrimers in two separate trials. Measurements were made after 0.5, 2, 5, 7, 10 and 60 minutes of exposure. The results for ASA, shown in Figure 2.10, demonstrate that while there is some time dependence, the change over time is relatively small and most of the partitioning occurs in the first 5 minutes. Similar results (not shown) were obtained with acetaminophen. Such rapid equilibration between the aqueous solution and the dendrimer phase suggest that under conditions of adequate mixing, the partitioning of the solutes from aqueous solution to the modified dendrimers is not kinetically limited, and these modified dendrimers may effect the partitioning required efficiently enough to be useful in the desired bioartificial liver application.

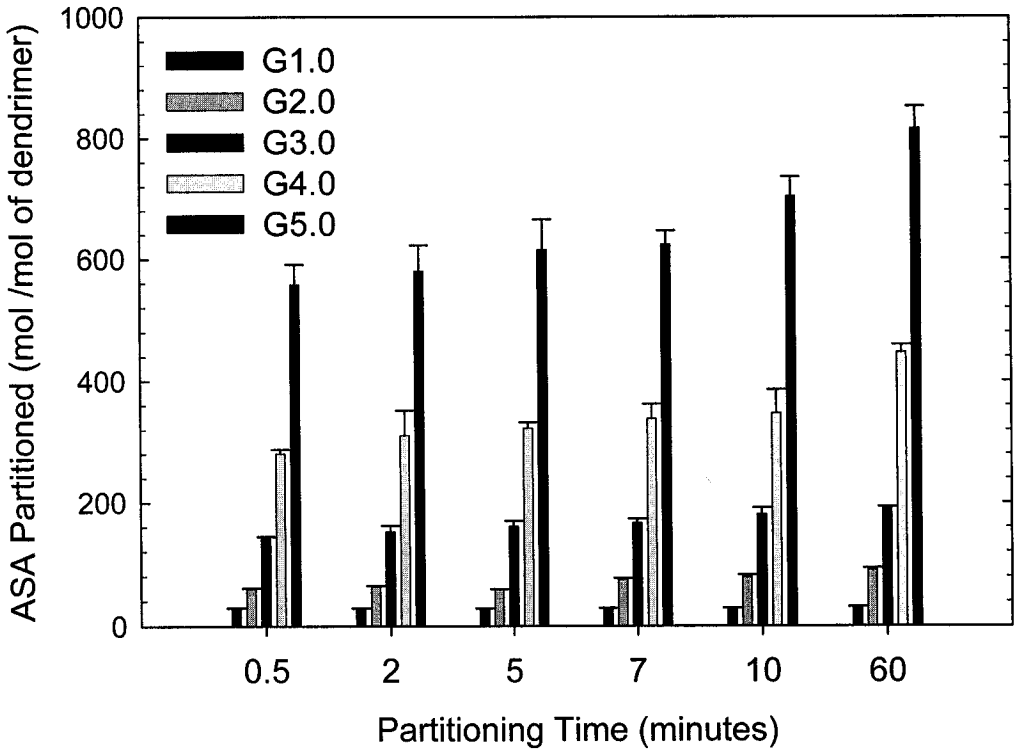


Figure 2.10: Effect of time on partitioning of ASA from aqueous solution to palmitoyl chloride modified DAB dendrimers.

2.3.4 Effect of Chain Length and Core-Periphery Linkage on Partitioning

A comparison of the partitioning of the analgesics, ASA and ibuprofen, to the modified dendrimers having linear long chain alkyl groups and short, branched alkyl groups is shown in Figure 2.11. A similar level of drug partitioning was noted with the C14 isocyanate modified dendrimers relative to the palmitoyl chloride modified dendrimers. Random testing with C10 and C16 isocyanate modified dendrimers demonstrated that the levels of drug partitioned to these molecules were similar to those with the C14 and C15 modified molecules (results not shown). However, when the periphery of the dendrimers

was modified with short, branched t-butyl groups, the amount of analgesic partitioned was significantly decreased as shown below (Figure 2.11).

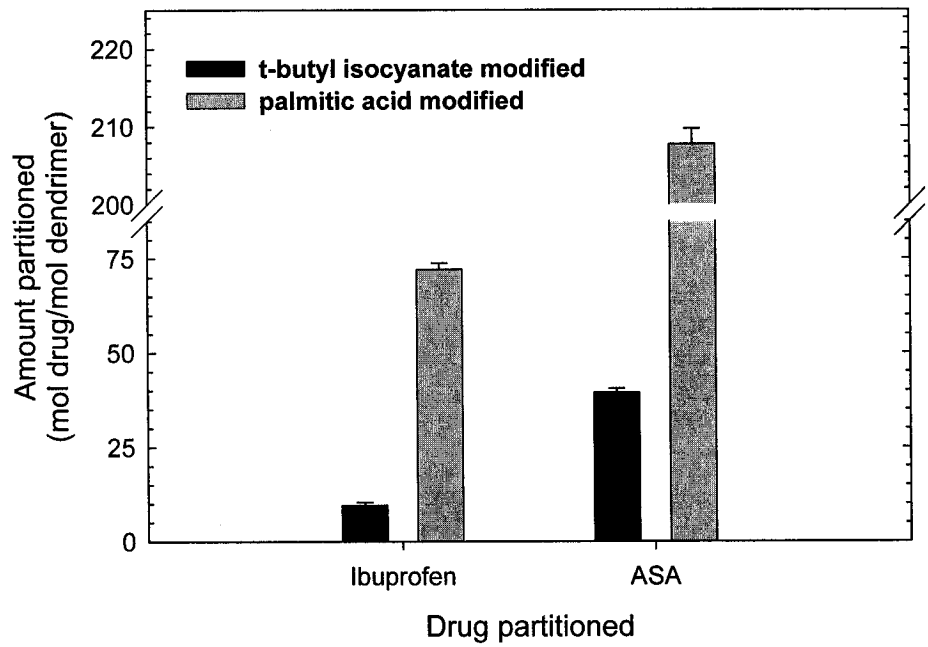


Figure 2.11: Partitioning of ibuprofen and ASA using short branched alkyl DAB G3.0 dendrimers and long chain alkyl DAB G3.0 dendrimers.

From these preliminary results, it appears that the linkage between the core and the periphery, be it an amide or a urea, does not affect the ability of the dendrimer to partition hydrophobic analgesics. While an increase in the linear chain length greater than C10 had little effect on partitioning, modification of the periphery with shorter, branched chain molecules significantly decreased the ability of the molecules to partition hydrophobic drugs from solution.

2.3.5 Enzyme Attachment to a Lipophilic Modified Dendrimer

The attempt at enzyme attachment to the lipophilic modified DAB dendrimer resulted in an amorphous, insoluble product that was difficult to characterize. The modified dendrimer with attached HRP was characterized by solid state ^{13}C NMR and compared to modified dendrimer with no HRP attached. Although there was no apparent difference between the two in a routine solid state NMR analysis, a ^{13}C NMR with dipolar dephasing showed a definite decrease of the methylene peak at ~33 ppm in Figure 2.12 **B** (modified dendrimer) and splitting of this similarly diminished peak in Figure 2.13 **B** (modified dendrimer with HRP attached). In this experiment, the dephasing delay of 40 μsec, where the high power proton decoupler is briefly turned off, was sufficient to rapidly dephase the C-H dipolar coupling of the methylene carbons resulting in a significantly diminished signal at ~33 ppm, but not enough to dephase the C-H coupling of the methyl groups (which tend to dephase more slowly) at ~15 ppm. The splitting of the methylene peak in Figure 2.13 **B** may indicate the presence of the CH_2 groups of the sebacoyl spacer used to link the enzyme to the dendrimer. Although the presence of HRP is not evident in the NMR, it was confirmed by a qualitative assay with the substrate ABTS; a distinct, characteristic green colour was detected indicating enzyme activity.

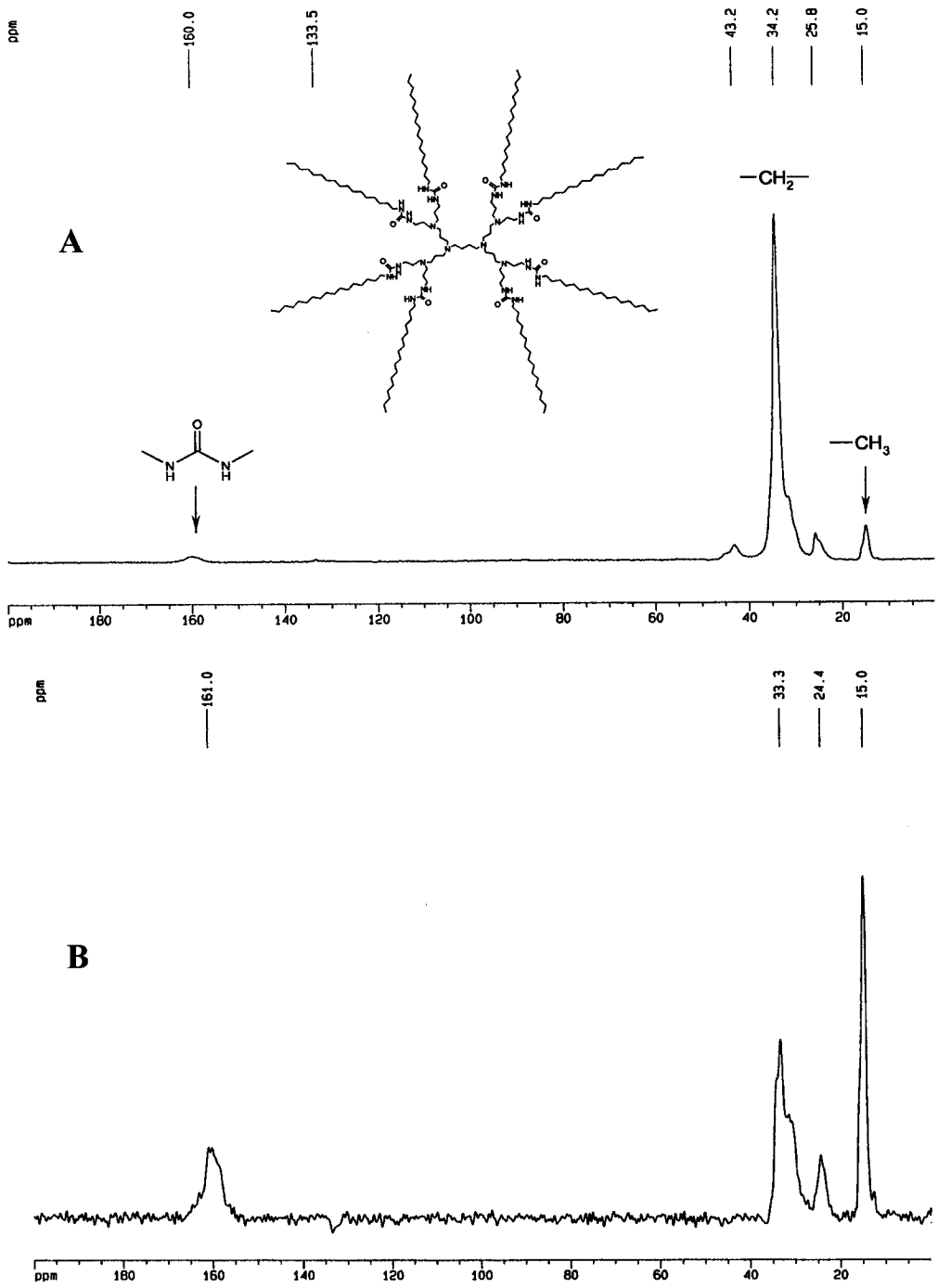


Figure 2.12: Solid state ^{13}C NMR (50.3 MHz, CPMAS spin rate of 4 Hz)) of DAB G3.0 dendrimer modified with hexadecylisocyanate

A – without dipolar dephasing; **B** – with 40 μsec dipolar dephasing.

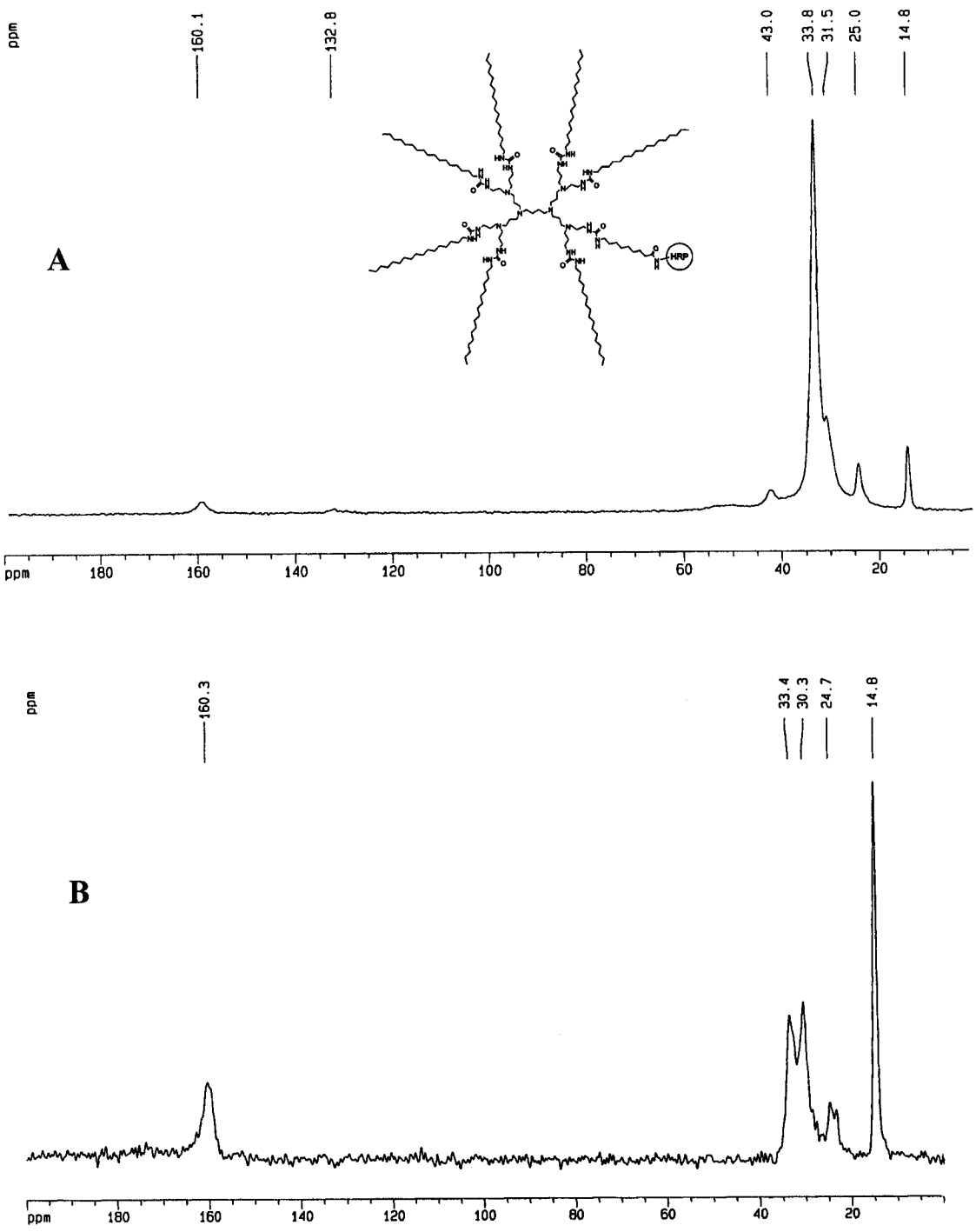


Figure 2.13: Solid state ^{13}C NMR (50.3 MHz, CPMAS spin rate of 4 Hz) of DAB G3.0 dendrimer modified with hexadecylisocyanate and HRP.
A – without dipolar dephasing; **B** – with 40 μsec dipolar dephasing.

2.4 Conclusions

The results of this preliminary study show that DAB dendrimers modified with a lipophilic periphery composed of linear, long chain alkyl groups can partition model hydrophobic xenobiotics and show promise as a replacement for charcoal in a detoxication module of a bioartificial liver. The kinetic study demonstrated that partitioning is rapid and as such, would be mass transfer rather than kinetically limited. Since rates of enzymatic conversion are also rapid, it can be assumed that mass transfer will remain the limiting factor for xenobiotic clearance.

Since the dendrimer core appears to play such a significant role in partitioning, it may prove beneficial in stabilizing the polar transition states of substrates as they undergo transformation by the bound enzyme. On the other hand, a core that is ionizable and capable of ionic interactions may also cause problems with product accumulation and subsequent enzyme inhibition. Clearly, more work needs to be done to in order to fully assess the role of the core with modified dendrimers that have the enzyme attached.

Lipophilic modified dendrimers with attached enzyme must also be more amenable to characterization. The recommended strategy for dendrimer modification which gives rise to an easily characterized product is the alkyl acid chloride method (Method B); it was the most efficient synthetic method and partitioning ability showed no apparent dependence on core-periphery linkage. Based on the preliminary results of the partitioning study, a G3.0 dendrimer with a C10-C12 linear alkyl chain appears to be a good candidate for enzyme attachment; the smaller hydrophobic corona afforded by a C10-12 linear alkyl chain may

allow for more rapid expulsion of the polar products into the aqueous surroundings compared to the larger corona of the palmitoyl modified dendrimers.

Although heterogeneous reactions are in general not ideal for achieving good yields and expedient reactions times, they are unavoidable when it comes to coupling very hydrophobic moieties, *viz.* lipophilic modified dendrimers, with extremely water-soluble, labile compounds such as enzymes. The multi-step biphasic reaction used to couple HRP to a modified DAB dendrimer here proved to be extremely inefficient and generated a product which could not be easily characterized. A better method of enzyme immobilization and attachment was sought and became the driving force behind the work presented in the next few chapters of this dissertation.

Acknowledgements

The technical assistance of Elizabeth Srokowski, who helped with the partitioning studies and syntheses of urea-functionalized dendrimers, and Lisa Wilkinson, who also helped with the partitioning studies, is greatly appreciated.

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Chapter 3 Protein Activation by *p*-Nitrophenylchloroformate

3.1 Introduction

In order to impart some degree of biocompatibility, materials designed for use with living tissues often involve chemically modified proteins. Biomaterials may consist entirely of cross-linked proteins, such as cross-linked collagen for contact lenses (Miyata *et al.*, 1992), or they may be a hybridization of protein grafted onto polymeric materials, such as fibronectin immobilized on polystyrene to promote endothelialization of vascular implants (Imanishi *et al.*, 1996). The work presented in this chapter is an investigation of a new strategy toward the development of hybridized materials involving cross-linked or immobilized proteins.

Regardless of the application or composition of the biomaterial, the linkage between molecular components is a particularly important consideration in the design and synthetic strategy employed. The linkage must be stable and resistant to attack by physiological enzymes in order to avoid degradation. Two of the most stable linkages commonly used for the synthesis of biomaterials are amide and urea bonds, which can be facilely generated by reaction between nucleophilic amines and carboxyl or amino groups, respectively. In order to facilitate attack by an amine, it is necessary to derivatize the carboxyl or amino group to increase its reactivity towards the nucleophile. While it is common practice to activate the functional group of a modifier or polymer prior to coupling with a protein in aqueous solution, this method of achieving protein modification is not without limitations: the extent of modification is often difficult to control, there may be issues of solubility, and large

amounts of protein are required in order to ensure reaction and to recover the modified product.

3.1.1 Carbodiimide-Mediated 'Zero-length' Protein Modification

A common means of achieving covalent chemical modification or immobilization of proteins in aqueous solution without the involvement of a reactive bifunctional modifier, is through the use of water-soluble carbodiimides, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). This reagent is often used to effect a 'zero-length' amide linkage between proteins and a synthetic polymer in biomaterials (Gratzer *et al.*, 2001; Imanishi *et al.*, 1996; Lundblad, 1994, pp 249-261). The major disadvantage associated with this approach is that the success and yield of the reaction is strongly dependent not only on carbodiimide concentration, but also on pH (Nakajima *et al.*, 1995). Paradoxically, while formation of the activated acyl urea intermediate is favored at lower pH (3.5 - 4.5), a higher pH is required to suppress ionization of the nucleophilic amine groups in order for the reaction to proceed. Moreover, hydrolysis of water-soluble carbodiimides occurs at pH values lower than 3.5. The limitations associated with pH also preclude the use of carbodiimides for cross-linking or modifying proteins that are irreversibly denatured under acidic conditions. While water-soluble carbodiimide is commonly used to modify proteins, there are few reports of carbodiimide-mediated protein cross-linking in the literature (Nakajima *et al.*, 1995).

3.1.2 The *In Vacuo* Protein Modification Method

An alternative method for protein modification developed by the Kaplan research group, involves derivatization of the functional groups of a lyophilized protein by heating

under reduced pressure in the presence of a modifying activating reagent. Vakos *et al.* successfully esterified the carboxyl groups of various lyophilized proteins using vapourized methanol and ethanol in the presence of HCl gas under *in vacuo* conditions (Vakos *et al.*, 2001). Similarly, Taralp and Kaplan used the same *in vacuo* method to acetylate and methylate amino groups of lyophilized proteins using acetic anhydride and iodomethane, respectively (Taralp *et al.*, 1997). They discovered that methylation with iodomethane by this procedure yields a quaternary trimethylated amino derivative, whereas reductively methylating proteins using formaldehyde and $NaBH_4$ in solution yields at most, the dimethyl derivative (Means *et al.*, 1971). Using the same *in vacuo* method to methylate various lyophilized peptides, Stewart *et al.* observed a significant increase in the sensitivity of detection by MALDI mass spectrometry, which they attributed to the permanent positive charge of the trimethylated amino groups (Stewart *et al.*, 2002).

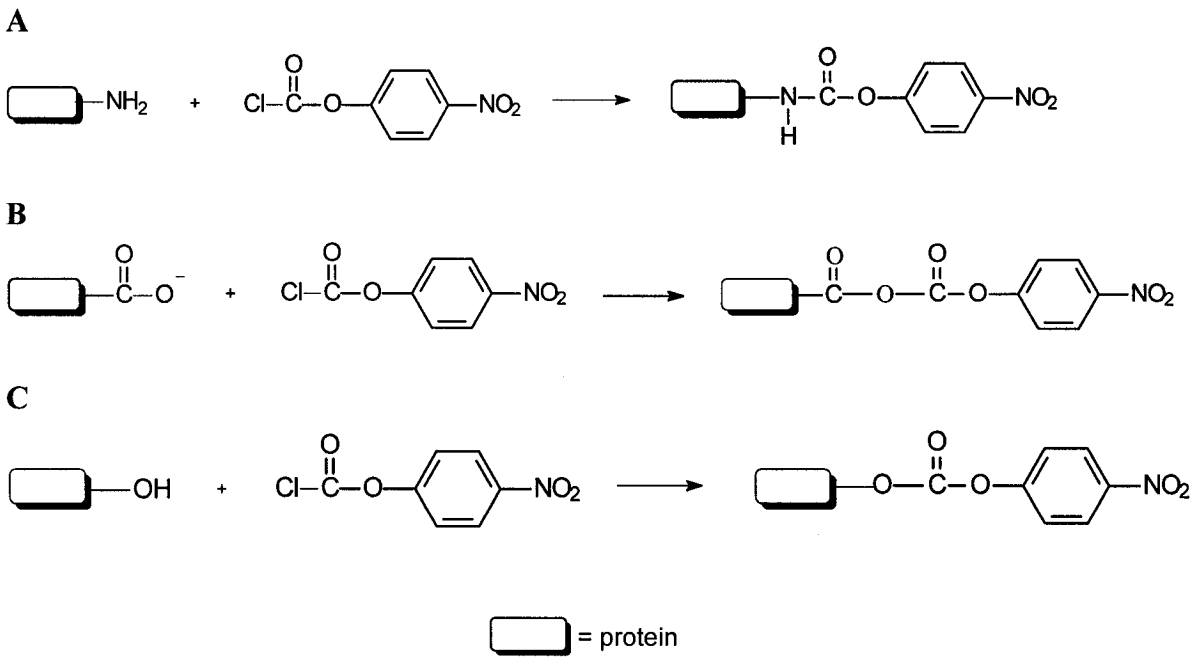
Protein modification by this novel method has proved to have many practical advantages over the current solution methods: 1) chemical modification of microscale quantities of protein can be carried out in a controlled manner with minimal risk to the integrity of the protein structure; 2) smaller amounts of reagents can be used, thereby reducing potential costs; 3) insolubility in water is not a limiting factor; and 4) reaction temperatures are not restricted to the boiling point of the solvent. These advantages as well as the prior successes of the *in vacuo* method in protein modification studies, inspired efforts to determine the feasibility of this novel process as a means to activate protein functional groups for subsequent cross-linking or modification reactions in solution.

Most proteins that are capable of withstanding lyophilization and elevated temperatures in the freeze-dried state should be amenable to the mild conditions associated

with this method. However, due to the nature of the method, the selection of appropriate activating reagents is limited somewhat by the following criteria: 1) it must be reactive with functional groups targeted for modification; 2) it must be stable as well as volatile at the activation temperature (which is pre-determined by the thermal stability of the protein); 3) it should have limited solubility in water during subsequent reaction with the modifier in aqueous solution; and 4) reaction of the activated functional group with water should result in hydrolysis back to the original functional group of the protein.

3.1.3 Reactivity of *p*-Nitrophenylchloroformate

With a melting point of 78°C, *p*-nitrophenylchloroformate (*p*-NPCF) fulfills all of the above selection criteria, is capable of reacting with functional groups found in proteins to form activated products and has the added benefit of being non-toxic (Hernaiz *et al.*, 1999). According to classical solution chemistry, reaction with *p*-NPCF can conceivably occur with three functional groups in the protein molecule, amino, carboxyl or hydroxyl groups, as shown in Scheme 3.1, which are found in Lys, Asp and Glu, and Tyr, Ser, and Thr amino acid residues, respectively. The use of *p*-NPCF as a means of activating alcohols (especially polyethylene glycols for use in biomaterial synthesis), carboxylic acids, and amines is very common in organic synthesis, and indeed it has been utilized extensively for activating reagents to be used for chemical modification of proteins (DeSantis *et al.*, 1999; Hernaiz *et al.*, 1999). Because it is insoluble in water, *p*-NPCF cannot be used to activate protein functional groups directly in aqueous solution; however it is a good candidate for use as a protein activator under the dry state conditions employed here.



Scheme 3.1: Reaction of *p*-NPCF with amine, carboxyl, and hydroxyl groups **A** – amine groups to form carbamates, **B** – carboxyl groups to form anhydrides, and **C** – hydroxyls to form carbonates in proteins.

3.1.4 Experimental Design

Ribonuclease A was selected as a model protein for activation by *p*-NPCF under *in vacuo* conditions because its structure, function and activity have been well-characterized, it is readily available, and it is rich in reactive functional groups (Raines, 1998). With a molecular weight of 13,686 Da, ribonuclease A is a small protein composed of 124 of all of the naturally occurring amino acid residues except tryptophan. The active site of this kidney-shaped enzyme is situated in the cleft where cleavage of the phosphodiester bond of adjacent pyrimidine nucleotides in RNA takes place.

Most of the experiments for this work involved activating ribonuclease with *p*-NPCF under various conditions, adding dH_2O to the activated protein, and analyzing the soluble

products by gel electrophoresis, FPLC or HPLC. Several of these experiments were designed to determine which functional groups were involved in the activation step, and while most of the information was obtained by blocking key functional groups, *viz.*, amines and carboxyls, other experiments such as co-lyophilizing ribonuclease with another protein (soybean trypsin inhibitor) prior to activation, confirmed the results. In order to determine the optimum pH of lyophilization at which activation occured, the extent of activation as a function of LpH, was measured by incorporation of ^{14}C from radio-labeled glycine. The reactivity of the activated protein in the absence of a competing nucleophile was also examined by reaction with a fluorescent label in an organic solvent.

Since the dimer of RNase A has practical value as a cancer therapeutic (Leland *et al.*, 2001; Antignani *et al.*, 2001; Kim *et al.*, 1995), attempts to optimize the cross-linking reaction in order to increase the yield of the homodimeric protein were also undertaken. Varying the concentration of *p*-NPCF and the use of excipients such as trehalose, sorbitol, α-cyclodextrin, and 18-crown-6 ether were explored to this end. The feasibility of using the *in vacuo* activation method to cross-link proteins without compromising their structural integrity, was assessed by measuring the activity of the RNase dimer and comparing it to that of the monomer by means of a widely used assay based on the method of Kunitz (Kunitz, 1946). This method relies on the premise that digestion of a polynucleic acid substrate (in this case Poly-A-Poly-U) by ribonuclease is accompanied by a hypsochromic shift in the absorption maximum of the UV spectrum. Activity is determined by measuring the increasing rate of change of absorption as a function of time during the initial stages of digestion. Since the potential for cytotoxicity is largely dependent upon dimeric structure

(Antignani *et al.*, 2001; Kim *et al.*, 1995) the activity of the dimer was also measured in the presence of RNase inhibitor (cRI).

The results presented in this chapter show the efficiency and mechanism of protein activation by *p*-NPCF, as well as the extent to which cross-linking can be achieved using this method. Some surprising observations were made during the course of this investigation which point to the possibility that proton transfer may occur in the dry state during the *in vacuo* activation process. A mechanism is proposed to explain the curious results obtained by this novel procedure.

3.2 Materials and Methods

Materials:

Ribonuclease A (RNase A) (contains a small amount of its glycosylated counterpart, ribonuclease B), lysozyme, and soybean trypsin inhibitor (STI) were purchased from Sigma Chemical Company and used without further purification. Trehalose was purchased from Fluka, glycinamide, monodansylcadaverine (MDC), glycine and a 5% w/v solution of 2,4,6,trinitrobenzenesulfonic acid (TNBS) in water were purchased from the Sigma Chemical Company, and 18-crown-6 ether (CE), α-cyclodextrin (α-CD), sorbitol, *para*-nitrophenylchloroformate (*p*-NPCF), succinic anhydride (SA), formaldehyde (37% in H_2O), and sodium borohydride were purchased from Aldrich and were used as received. ^{14}C-enriched glycine (50 μCi/mL, item # FD5651, Batch # B90), was purchased from Amersham, Arlington, IL, and scintillation cocktail, Aquasol-2 was purchased from Dupont. All buffers were prepared with distilled water and reagent grade quality chemicals were purchased from commercial sources. In all experiments where required, dialysis was performed using

Spectra/Por cellulose membrane dialysis tubing with a 3,500 molecular weight cut-off which was soaked in distilled water for one hour prior to use.

pH Measurement and pH-Stat:

A Radiometer Copenhagen type PHM26 pH meter fitted with an ORION semi-micro gel-filled combination electrode model 91-15 was used to measure pH in all experiments. For experiments where pH control was maintained using a pH stat, the pH meter was coupled to a Titration 11/ Ole Dich autotitrator assembly equipped with an adjustable recirculating water bath to maintain constant temperature when necessary.

3.2.1 *In Vacuo* Activation Method

Typically, a stock solution of 10 – 20 mg of protein/mL of dH_2O was accurately prepared and the pH was adjusted with either 1N NaOH or 1N HCl. Aliquots of the pH-adjusted protein stock solution (minimum volume of 500 μL) were transferred to borosilicate glass disposable culture tubes (13 x 100 mm) and then lyophilized using either a VIRTIS-24 commercial freeze dryer or a home-made freeze dryer equipped with an ethanol/dry ice trap and a 20 mTorr vacuum pump. The tubes containing the freeze-dried enzyme were scored and cut down to about 50 mm (just above the enzyme level) and were placed inside larger diameter (16 x 150 mm) pyrex borosilicate glass disposable culture tubes containing 0.00126 g of *p*-NPCF (prepared by transferring a 50 μL aliquot of stock solution containing 0.13 g of *p*-NPCF in 5.0 mL of dry benzene (dried over Na) and evaporating the benzene by blowing N_2 over the tubes). Using an oxygen-enriched flame, a short, very narrow constriction was made in the large test tube. The mouth of the tube was then carefully fitted with a vacuum hose, and suction was applied for 1 minute to evacuate the tube prior to flame-sealing under vacuum. The sealed tubes were then placed in an aluminum block in an oven set at 85°C for

about 15 - 30 minutes. At the end of the incubation time, the chloroformate vapour was trapped out onto the walls of the tubes by immediately submerging the tubes in crushed ice or liquid N_2. The vacuum seal was then broken and the protein removed for immediate processing and analysis, otherwise the tubes were stored unbroken in the freezer (-32°C) until required.

3.2.2 SDS-PAGE Analysis of Cross-linked Proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli (Laemmli, 1970) using a Bio-Rad Mini-PROTEAN II dual slab cell apparatus. All reagents were electrophoresis grade quality purchased from Sigma or BioRad (buffers were prepared as required using dH_2O). All of the SDS-PAGE analyses were conducted under discontinuous gel conditions with the stacking gel and the samples containing Tris•Cl at pH 6.8 and the resolving gel containing Tris•Cl at pH 8.8. The running buffer was prepared from Tris and glycine with a pH of 8.3 and reducing conditions were provided by β-mercaptoethanol, which was added to the sample buffer just prior to heating. The sample buffer, gels, and running buffer all contained 2% SDS. The molecular weight marker was purchased from BioRad, a broad range of SDS-PAGE standards consisting of aprotinin (MW = 6.5 kDa), lysozyme (MW = 14.4 kDa), soybean trypsin inhibitor (MW=21.5 kDa), carbonic anhydrase (MW = 31 kDa), ovalbumin (MW = 45 kDa), bovine serum albumin (MW = 66.2 kDa), phosphorylase B (MW = 97.4 kDa), β-galactosidase (MW = 116.3 kDa) and myosin (MW = 200 kDa). Approximately 5 - 20 μg (10 μL volume) was loaded into each of the wells of a 1.0 mm thick, 12% polyacrylamide gel and electrophoresis was carried out at a constant current of 20 - 25 mA/gel (with a voltage

gradient of 70 - 150 mV). Protein bands were visualized by staining with Coomassie Brilliant Blue R-250.

3.2.3 Size Exclusion Chromatography by FPLC Analysis

A Pharmacia FPLC gel filtration system consisting of a Pharmacia HiLoad 16/10 Superdex 75 preparative grade column connected to a high precision pump P-500 fitted with a 200 µL sample injection loop controlled by a LCC-500 Plus liquid chromatography controller was used to separate and isolate protein fractions. Peaks were detected by a Pharmacia Single Path Monitor UV-1 Optical Unit with a wavelength of 214 nm and were recorded on a Dual Channel Chart Recorder REC-482. The eluant phosphate buffer pH 7.0 prepared with 0.15 M NaCl in dH_2O was filtered through 0.45 micron cellulose acetate by suction and degassed prior to use. Fractions were manually collected and dialyzed to remove buffer and salt as required. RNase A was dialyzed against distilled water at 4°C for at least 24 hours with four changes of dialysate and then lyophilized overnight.

3.2.4 HPLC Analysis of Activated Ribonuclease A

Analytical reverse phase high performance liquid chromatography was carried out using a Varian Prostar quaternary pump HPLC system equipped with a 20 µL sample injection loop and a BioRad cationic exchange column. Peaks were detected by a sequential UV-vis detector set at a wavelength of 220 nm. The instrument was controlled and data was recorded by Varian Star Chromatography Workstation software version 5.3. Samples were manually injected and analyzed under ambient, isobaric, non-gradient, elution conditions with a flow rate of 1 mL/min. using 0.15 M sodium phosphate buffer pH 7.0 as the eluant.

3.2.5 Fluorescent Labeling of Activated Protein

Duplicate aliquots of an aqueous solution containing RNase A (5 mg/mL) adjusted to pH 8.0 with 1M NaOH, were transferred to two test tubes (13 x 100 mm) such that each tube contained 2.3 mg of RNase. Following lyophilization, one of the RNase A samples was activated with *p*-NPCF using the activation procedure above, while the other was subjected to the same conditions in the absence of *p*-NPCF. After heating under vacuum, the tubes were opened and the contents of the tubes were carefully transferred to Eppendorf tubes by suspension of the protein in dry benzene. The excess chloroformate was extracted with successive aliquots (1 mL) of dry benzene (upon addition of benzene, the Eppendorf tubes were vortexed for about 10 seconds, centrifuged for two minutes, and then the benzene was carefully suctioned out of the tubes) until the yellow colour of p-nitrophenol was no longer discernable with the addition of 1M NaOH (300 μL) to the benzene extract. An aliquot of 1.0 mL of a solution of monodansylcadaverine (2.2 mg/mL) in DMF was transferred to each of the tubes. The contents of the tubes were vortexed to disperse the protein and left to react for approximately 30 minutes, after which time the tubes were centrifuged for two minutes and the DMF was carefully suctioned off. The residual protein in both tubes was rinsed well with several aliquots of DMF until the DMF was no longer fluorescent under UV light. The Eppendorf tubes were then photographed under normal incandescent light and ultraviolet light for comparison purposes.

3.2.6 Reaction of Activated Protein with ^{14}C-Glycine

A solution containing 0.25 g of accurately weighed glycine and 10 μCi of ^{14}C-enriched glycine (200 μL from stock solution containing 50 μCi/mL) was added to 20 mL of dH_2O. After adjustment to pH 10.0 with 6M NaOH, the volume was diluted to 25.0 mL with

dH_2O. An aliquot of 500 μL of the ^{14}C-enriched glycine solution was added to each of eight borosilicate glass disposable culture tubes (13 mm x 100 mm) containing 2.0 mg of activated RNase A (RNase A was previously activated for 1 hour with *p*-NPCF) having pre-activation LpH values of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0. The reaction mixture was stirred using a glass rod and allowed to react for about 15 minutes and then transferred quantitatively, to dialysis tubing. The samples were dialyzed against two changes of NaOH (5 g/4 L) for 4 hours and then against two changes of dH_2O (4 L) for 8 hours. After dialysis was complete, the contents of the tubing was split into two equal volumes and lyophilized in test tubes. Urea (8M, 250 μL) was added to each test tube to dissolve the RNase and this solution was then quantitatively transferred to scintillation vials to which dH_2O (100 μL) and Aquasol-2 scintillation cocktail (2.5 mL) (Dupont) were added. The contents of the vials were mixed thoroughly to homogeneity and radioactivity was measured using a Beckman LS 6500 multi-purpose scintillation counter with the ^{14}C window.

3.2.7 Activation in the Presence of Excipients

Aliquots of a stock solution (300 μL) of RNase A (10 mg/mL in dH_2O with pH adjusted to 10.0 with 1M NaOH) were added to each of 17 tubes (13 x 100 mm) such that each tube contained 3 mg of RNase. Stock solutions of the excipients (each containing 10 mg/mL in dH_2O), trehalose, D-sorbitol, 18-crown-6 ether, and α-cyclodextrin were prepared. Four sets of four samples were prepared by transferring individual aliquots containing 0.01 mg, 0.1 mg, 0.5 mg and 1.0 mg of each excipient to the tubes containing RNase A solution. Appropriate adjustment with dH_2O was made to each tube such that the volumes of all tubes were equal, including one control sample tube where no excipient was added to the RNase solution. The tubes were allowed to stand for 30 minutes before freeze-drying. The freeze-

dried RNase samples were activated with *p*-NPCF (including the control) according to the activation procedure outlined above. The RNase in each tube was reconstituted with an aliquot of dH_2O (500 µL) and was analyzed by SDS-PAGE using the procedure described above.

3.2.8 Carboxylic Acid Group Blocking

Carboxyl groups were blocked by conversion to amides using a carbodiimide-mediated derivatization with glycinamide according to the method of Hoare and Koshland (Hoare *et al.*, 1966). The use of a denaturant such as urea was avoided in order to obtain limited modification of only the exposed, very reactive carboxyl groups. A solution of glycinamide hydrochloride (1.0 M) and EDC (0.1 M) in 25 mL was prepared and transferred to a temperature controlled, water-jacketed vessel. The pH was adjusted to 4.75 using 6M NaOH and the temperature was maintained at 25°C. To this solution, 133 mg of RNase A was added and the pH was adjusted and kept constant at 4.75 with 1M NaOH for a period of about 6 hours using a pH-Stat. The solution was then transferred to a dialysis bag (3,500 MW cut-off) and dialyzed extensively with six changes of dH_2O (4 L) for 48 hours at 4°C. The pH was adjusted incrementally to 3.0, 4.5, 6.0, 7.0, 8.0, 9.0, and 10.0 using 1M HCl and 1M NaOH accordingly. Prior to making the next successive pH adjustment, duplicate aliquots of 625 µL of the pH-adjusted protein solution were transferred to test tubes (13 x 100 mm) such that each tube contained approximately 2.5 mg RNase A. The tubes were then lyophilized without delay, and both pH-adjusted sets of tubes were subjected to *p*-NPCF activation followed by reconstitution with dH_2O (300 µL). SDS-PAGE analysis was carried out on one set and FPLC analysis was performed on the other set according to the procedures outlined above.

3.2.9 Amino Group Blocking

Method A: Reductive Methylation

Using the method of Means and Feeney (Means *et al.*, 1971), amino groups were blocked by reaction with aldehyde followed by reduction of the resulting Schiff base with sodium borohydride. A solution containing RNase A (125 mg) in 25 mL of 0.2 M borate buffer (pH 9) was transferred to a round bottom flask which was then placed in an ice bath. When the temperature of the solution reached 0°C, 12.5 mg of $NaBH_4$ was added and stirred by a magnetic stirring bar. Aliquots of 12.5 μL of formaldehyde (37 wt % solution in water) were added incrementally to the solution in the flask over a period of 30 minutes. The solution was then carefully transferred to a dialysis bag (3,500 MW cut-off) and dialyzed against six changes of dH_2O (4 L) over 48 hours at 4°C. The dialyzed solution was lyophilized to dryness and then quantitatively transferred to a 25 mL volumetric flask with dH_2O. The pH was adjusted incrementally to 3.0, 4.5, 6.0, 7.0, 8.0, 9.0, and 10.0 using 1M HCl and 1M NaOH accordingly. Prior to making the next successive pH adjustment, duplicate aliquots of 625 μL of the pH adjusted protein solution were transferred to test tubes (13 x 100 mm) such that each tube contained approximately 2.5 mg RNase A. Both sets of tubes containing the pH-adjusted protein were lyophilized without delay, and were then activated with *p*-NPCF. The protein in each tube was reconstituted by dH_2O (300 μL) followed by SDS-PAGE analysis on one set and FPLC analysis on the other set according to the procedures outlined above.

Method B: Succinylation

Amino groups of RNase A were blocked by reaction with succinic anhydride according to the method of Hollecker and Creighton (Hollecker *et al.*, 1980). A solution of

RNase A (125 mg/ 25mL) was prepared with a pH of 7.0 adjusted using 1M NaOH. Succinic anhydride (126 mg) was added incrementally over a period of 4 hours while stirring. The pH was continuously monitored and maintained at pH 7.0 by the addition of 1M NaOH using a pH-Stat. After the reaction was deemed complete (when the pH remained constant and stable for 30 minutes), the solution was carefully transferred to a dialysis bag (3,500 MW cut-off) and dialyzed against six changes of dH_2O (4 L) over 48 hours at 4°C. The solution was lyophilized to dryness and then quantitatively transferred to a 25 mL volumetric flask with dH_2O. The pH was adjusted incrementally to 3.0, 4.5, 6.0, 7.0, 8.0, 9.0, and 10.0 using 1M HCl and 1M NaOH accordingly. Prior to making the next successive pH adjustment, duplicate aliquots of 500 µL of the pH adjusted protein solution were transferred to two sets of test tubes (13 x 100 mm) such that each tube contained approximately 2.5 mg RNase A. In order to prepare a set of controls for this and the other blocking experiments, another solution of RNase was prepared, dialyzed, and lyophilized followed by reconstitution and pH adjustment in exactly the same way as the succinylated solution, omitting the step of modification by SA. All of the tubes containing the pH-adjusted protein were lyophilized without delay, and were then activated by *p*-NPCF. The protein in each tube was reconstituted with dH_2O (300 µL) followed by SDS-PAGE analysis on one set to determine the extent of cross-linking and a TNBS assay on the duplicate set using the following assay to determine the extent of lysine ε-amino group modification.

3.2.10 Amino Group Quantification by TNBS Assay

The extent of lysine ε-amino modification by reductive methylation and succinylation was determined by the reaction of 2,4,6-trinitrobenzenesulfonic acid (TNBS) (picric acid) with ε-amino groups in the protein to form a TNBS-lysine product according to the method

of Habeeb (Habeeb, 1966). To each pH adjusted tube containing 2.5 mg RNase/tube from the set of controls and both sets of blocked amino groups, was added 1 mL of pH 9 sodium bicarbonate buffer (4%). After the addition of TNBS solution (200 μL), the tubes were incubated at 37°C for 3 hours and the absorbance for each LpH sample was measured at 345 nm.

3.2.11 Protein Cross-Coupling

Equal aliquots (1 mL) of stock solutions containing RNase A (5.8 mg/mL) and STI (6.7 mg/mL) were mixed together in a test tube (10 x 75 mm) and the pH of the mixture was adjusted to 10.0 using 1M NaOH. One set of duplicate aliquots (450 μL) of the mixed protein solution and one set of duplicate aliquots (450 μL) of each of the protein stock solutions (which had also been adjusted to pH 10) were added to test tubes (10 x 75 mm). The protein in all of the test tubes was then lyophilized to dryness and activated using *p*-NPCF, except one of each of the duplicate samples which were used as controls, as outlined in the activation procedure above. The activated protein samples were then reconstituted with dH_2O (225 μL) and SDS-PAGE was performed on the samples as outlined above with loadings of approximately 20 μg protein/lane.

3.2.12 Ribonuclease Activity Assay

Two activity assays were performed according to the method of Kunitz (Kunitz, 1946) in the presence and absence of RNase inhibitor (cRI). To a tube containing ~2mg of *p*-NPCF-activated RNase A (LpH 7.0), 500 μL of dH_2O was added. Separation of dimer and monomer fractions was achieved by size exclusion chromatography (SEC) at r.t. using FPLC according to the procedure above. The resulting purified, dialyzed, and lyophilized sample

was reconstituted with 3 mL of diethylpyrocarbonate (DEPC)-treated water and dialyzed for 45 minutes in four changes of 500 ml of 0.015 M sodium citrate buffer prepared with 0.15 M NaCl, in DEPC-treated water, adjusted to pH 7.0 using acetic acid. In one set, aliquots of a stock Poly-A-Poly-U substrate in citrate-saline buffer (same as dialysate) were added to the wells of a UV microplate such that the substrate amounts ranged from 14, 16, 18, and 20 μg and the volume in each well was adjusted with citrate-saline buffer to a total of 150 μL. The RNase A concentration for monomer and dimer samples was determined using the BCA (bicinchoninic acid) protein assay (Lundblad, 1994, p 270). Aliquots of each RNase sample were added to the wells such that each contained 50 μg/mL of RNase in a volume of 50 μL, except in those wells reserved for buffer and substrate controls. The total volume in each well was 200 μL. In another set, 500, 1000, 2000, 3000 units of cellular RNase inhibitor (cRI) was added to the wells of a UV microplate along with 20 μg of Poly-A-Poly-U substrate in each well for a total volume of 150 μL to which 50 μg/mL of RNase A in a volume of 50 μL was also added. The absorbance of the solutions in the wells of the plates was read at a wavelength of 260 nm every 60 seconds for 14 hours.

3.3 Results and Discussion

3.3.1 Activation and Cross-linking

Results show that reactive functional groups in RNase can be successfully activated upon exposure to *p*-NPCF under the *in vacuo* conditions used here. When dH_2O was added to the activated protein, a significant amount of highly cross-linked, precipitated material that was impervious to denaturing conditions was observed in the resultant solutions, and the

presence of soluble oligomers was detected by SDS-PAGE analysis. A typical gel profile of such an analysis of the soluble oligomers is shown in Figure 3.1.

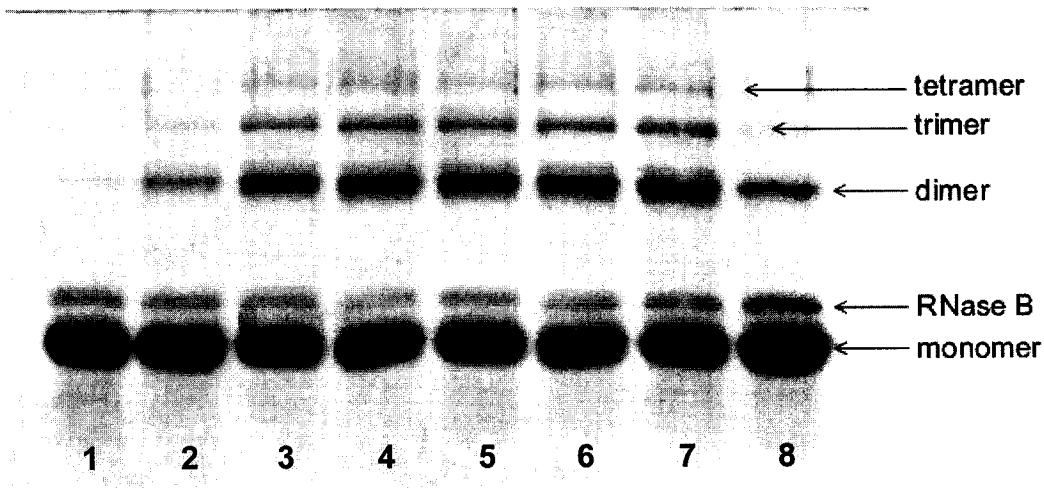


Figure 3.1: SDS-PAGE 12% polyacrylamide gel showing effect of *p*-NPCF concentration on amount of soluble oligomers of RNase (2.8 mg, LpH 9.8) observed with activation and reaction in dH_2O (500 μL). Various ratios of RNase A to *p*-NPCF are as follows: Lane 1:1:0; Lane 2: 1:1; Lane 3: 1:5; Lane 4: 1:10; Lane 5: 1:20; Lane 6: 1:30; Lane 7: 1:50; Lane 8: 1:300.

Commercially available RNase A obtained in great quantities from bovine pancreas contains a small amount of RNase B, a naturally occurring derivative of RNase A which has been glycosylated at the Asn 34 residue with a mixture of different glycoforms consisting of mannose (5 - 9) and N-acetylglucosamine (2) (Raines, 1998). For the purposes of this investigation, it was deemed unnecessary to further purify RNase A which was obtained from a commercial source, thus the presence of small amounts of RNase B gave rise to the faint band above the RNase monomer band that is apparent in all of the gels in this chapter.

A linear plot of migration distance of the bands though the gel *vs.* log of the molecular weight, shown in Figure 3.2, indicates that these bands have molecular weights corresponding to dimer, trimer, tetramer and higher oligomers.

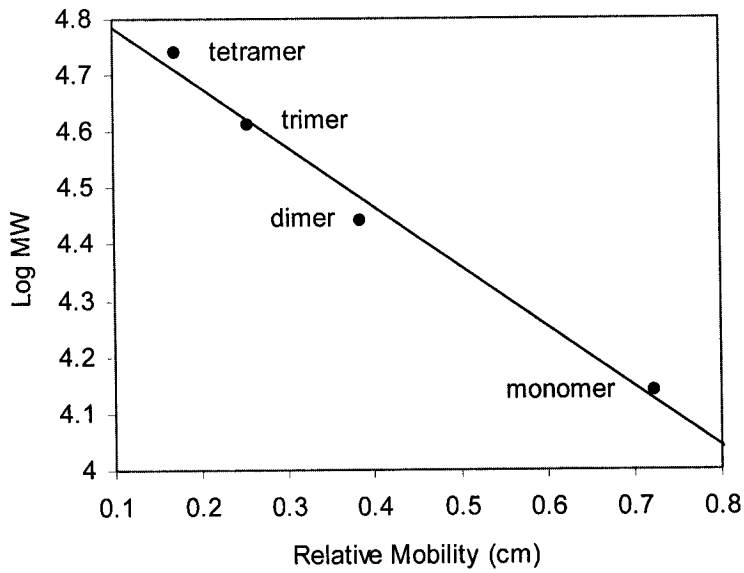


Figure 3.2: Plot of log of molecular weight (MW) *vs.* relative mobility (cm) of RNase oligomers
(data obtained from typical 12% polyacrylamide gels run under SDS-PAGE discontinuous gel conditions).

The amount of soluble oligomer vs. precipitated material appears to have some dependency on the concentration of chloroformate vapour relative to the amount of protein in the sealed tubes. The gel in Figure 3.1 shows that a maximum amount of dimer and other soluble oligomers is obtained with a mole ratio of protein to chloroformate that is somewhere between 1:10 and 1:50 (Lanes 4 - 7), for the volume available within the sealed tubes during incubation in the activation procedure. An order of magnitude higher than this amount of chloroformate induces extensive cross-linking within the activated protein resulting in large

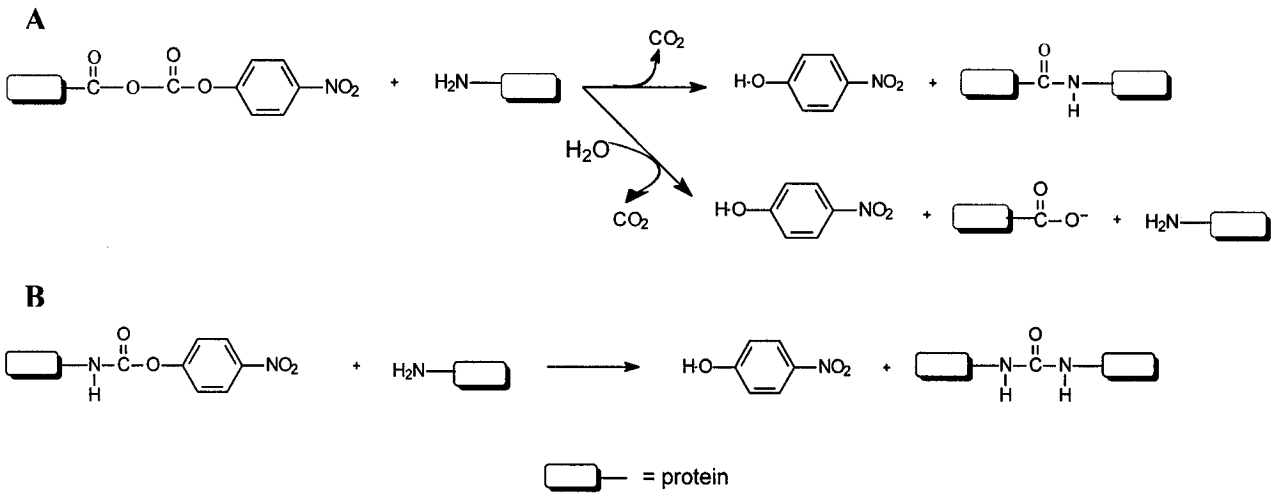
amounts of undesirable precipitated protein, while an order of magnitude lower than this is insufficient for adequate cross-linking.

3.3.2 Role of Various Classes of Functional Group

Since RNase has 11 amino groups (10 Lys, in addition to the amino terminus), 10 carboxyl groups (5 Glu and 5 Asp, in addition to the carboxyl terminus), and 31 hydroxyl groups (15 Ser, 10 Thr and 6 Tyr), there are many potential sites for activation by chloroformate under the *in vacuo* conditions used in this work. In order to ascertain the extent of functional group involvement as well as the extent of activation, three experiments were carried out which were designed to: 1) probe the effects of changing ionization states; 2) block the reactivity of carboxyl and amino groups; and 3) compare reactivity of a protein relatively deficient in two of the potentially reactive functional groups.

Due to the pH memory effect (discussed in Chapter 1, Introduction), changing the ionization state of available ionizable functional groups is easily achieved by lyophilizing a protein; in the lyophilized state the ionization states are essentially 'frozen out' and the reactivity towards chloroformate under the *in vacuo* conditions can be readily investigated. Preliminary experiments were conducted to investigate the effect of varying the pH of lyophilization prior to activation; SDS-PAGE analysis of the products upon subsequent addition of dH_2O showed an increase in the number and intensity of bands with increasing LpH (data not shown). Activation appeared to show some dependency upon the ionization state, thus implicating the involvement of the two groups with ionizable functionality, *viz.*, the carboxyl and amino groups. Even though both amino and carboxyl groups are equally capable of activation by chloroformate, it was initially assumed that the apparent pH dependence was due to activation of amino groups reacting with the remaining unprotonated

amines to form soluble oligomers. Reaction between a nucleophile in aqueous solution with activated carboxyl and amine groups can give rise to three different possible product outcomes depicted in Scheme 3.2.



Scheme 3.2: Reaction of *p*-NPCF activated species in dH_2O.
A) amide-linked dimer (upper) and hydrolysis of *p*-NPCF-activated protein carboxyl by water yielding initial protein (lower); **B)** urea-linked dimer.

Since the anhydride of the activated carboxylate group is more reactive compared to the carbamate of the activated amino group (Solomons *et al.*, 1998, pp 828-848), it is susceptible to hydrolysis by water in aqueous reactions resulting in the return of the protein's original carboxyl group, i.e., a null net reaction. Although water is a relatively weak nucleophile compared to the unprotonated amino group of protein in solution, and the rate constant for hydrolysis is much lower, its concentration of 55 M makes it a significant competitor, especially for the relatively unstable anhydride.

3.3.3 Modification by Radio-labeled Glycine

In an attempt to quantify the extent of activation by chloroformate, ^{14}C incorporation in samples of RNase of variable LpH which had been activated and then exposed to a basic, highly concentrated, radio-labeled glycine solution (pH 10), was measured. Although little to no ^{14}C was detected at the very low pH values, it was detected at pH values greater than 6. With pK_a values of 3 - 4.5 (Creighton, 1993, p 6) the acidic residues, Asp and Glu, would exist primarily in their ionized carboxylate state at most of the LpH values tested. On the other hand, a change in LpH would impact the number of amino groups capable of reacting with chloroformate. With a relatively high pK_a value of approximately 10 - 11 for lysine ε-amino groups (Creighton, 1993, p 6), significantly fewer unprotonated reactive amine groups would be available to react with chloroformate (at LpH values less than 10) compared to carboxylate groups, but their numbers would increase with each pH increment up to a ceiling of 50% at the pK_a.

Thus it was expected that a plot of ^{14}C incorporation *vs.* LpH of RNase would show a gradual incline in a positively sloping line as more amino groups became 'frozen out' in their reactive unprotonated state. Surprisingly, this was not the case, and the plot (Figure 3.3) actually shows a jump in ^{14}C incorporation at LpH values greater than 6. These results appeared to be counter-intuitive, and were initially dismissed as an artifact. However, the sigmoidal shape of this plot, reminiscent of an acid-base titration curve, suggests the strong possibility that carboxyl groups might indeed have been the predominant species being activated by *p*-NPCF in the *in vacuo* procedure.

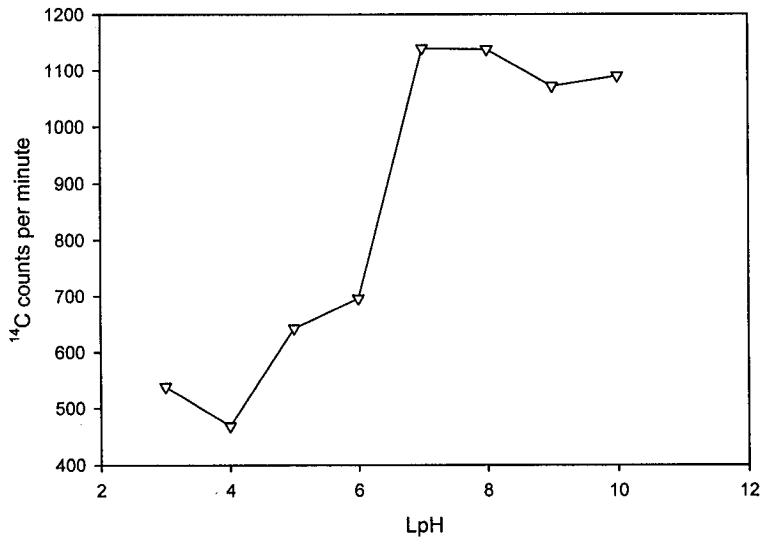


Figure 3.3: Plot of $^{14}C_1$-glycine counts per minute *vs.* LpH of RNase showing the effect of LpH on extent of activation by *p*-NPCF.

The number of glycine molecules incorporated into activated RNase as a function of LpH was calculated for each sample based on the amount of radioactivity per mole of glycine that was detected in the sample after extensive dialysis, is shown in Table 3.1. A threshold of between 5 and 6 molecules of incorporated glycine suggests a saturation of available reactive sites on the protein molecule. In their study to determine which carboxyl-bearing residues of RNase A were exposed and therefore susceptible to reaction with a water soluble carbodiimide, Reihm and Scheraga identified six possible candidates out of the 10 possibilities (Reihm *et al.*, 1966). Listed in order of decreasing susceptibility, they are: Asp 53, Glu 111, Glu 9, Glu 86 and Asp 38. Interestingly, Baek *et al.*, who utilized water soluble carbodiimide to glycate RNase with glucosamine hydrochloride, found only mono glycated and di-glycated RNase products (Baek *et al.*, 1997).

Table 3.1: Number of glycines per molecule of activated RNase at various LpH values.

LpH	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
# Glycine Molecules	2.6	2.3	3.1	3.4	5.1	5.5	5.2	5.3

3.3.4 Effect of Blocking Amino and Carboxyl Groups

Further evidence that carboxyl groups were the predominant species undergoing activation was provided by a study involving the mutually exclusive blocking of amino and carboxyl groups in RNase A prior to *in vacuo* activation with *p*-NPCF. When the carboxyl groups of RNase A were blocked by reaction with glycinamide, neither dimer nor higher soluble oligomers of RNase A was evident in either the FPLC or SDS-PAGE (not shown) analyses of the reconstituted aqueous protein solution following activation. However, when the amino groups were blocked by reductive alkylation, dimer and trimer peaks in the reconstituted protein solution were observed in the FPLC traces, and corresponding bands were evident in SDS-PAGE gels (not shown). Figure 3.4 shows a comparison of the FPLC traces for both RNase A blocking experiments. Succinylation of the protein, which results in the conversion of amino groups to carboxyl groups, also showed no evidence of cross-linking in SDS-PAGE analysis (Figure 3.5).

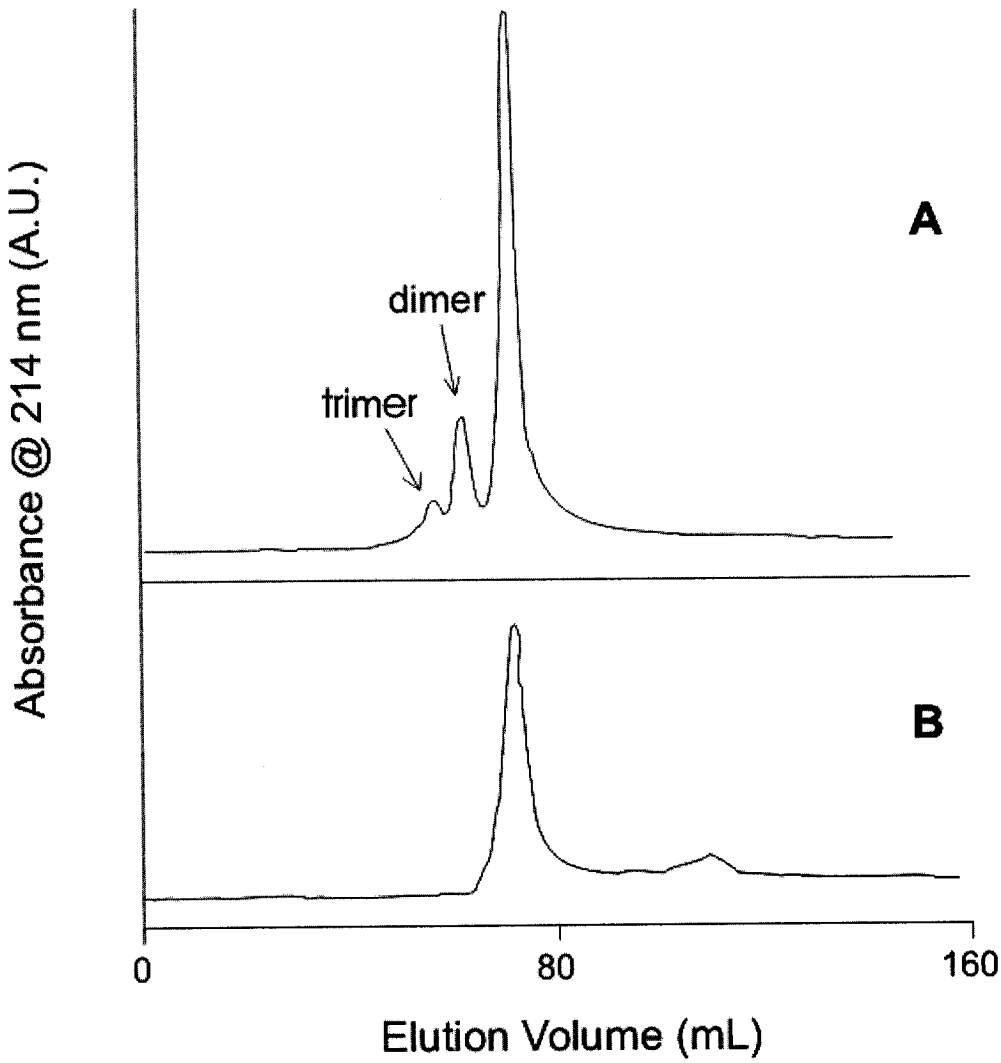


Figure 3.4: Effect of blocking amine and carboxyl groups prior to *p*-NPCF activation of RNase (LpH 10.0)

A – amino groups blocked by reductive methylation; B – carboxyl groups blocked by amidation.

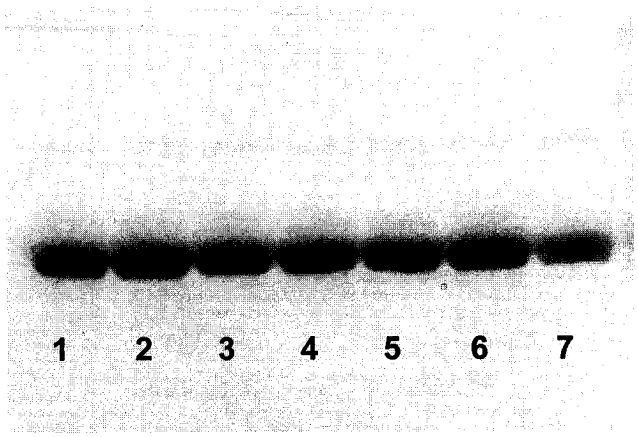


Figure 3.5: SDS-PAGE 12% polyacrylamide gel showing effect of blocking amino groups by succinic anhydride modification prior to *p*-NPCF activation at different LpH values.
Lane 1: LpH 3.0; Lane 2: LpH 4.5; Lane 3: LpH 6.0 activated; Lane 4: LpH 7.0; Lane 5: LpH 8.0 activated; Lane 6: LpH 9.0 control; Lane 7: LpH 10.

Although the results of the two methods for blocking amine groups, reductive alkylation and succinylation, appeared to be contradictory, an assay utilizing 2,4,6-trinitrobenzenesulfonic acid to determine the extent of amine conversion provided clarification and further insight into the activation mechanism. This assay revealed that blocking amino groups by the reductive alkylation method under the reaction conditions used here was incomplete compared to blocking by the succinylation method. Averaged results of a set of 6 reductively methylated samples of RNase lyophilized under various pH conditions relative to those of a set of control samples showed that 70% of the amine groups were unblocked, while the same treatment of data for the succinylated set of RNase samples showed that only 21% remained unblocked. While the procedure used here to reductively methylate lysines with formaldehyde and $NaBH_4$ is generally considered good and is widely used, one of the problems known to decrease the efficiency of the reaction is the presence of

paraformaldehyde in the formaldehyde reagent (Lundblad, 1994, p.144) which may explain the lack of amine derivatization here. In spite of the fact that less than 50% of the amine groups had been blocked by the reductive methylation procedure, the results obtained by these samples provided useful information about the functional groups involved in cross-linking.

Collectively, all of the results indicate that there is little, if any, involvement by hydroxyl-bearing side-chains. Hydroxyl groups activated with *p*-NPCF readily form esters with carboxylic acid groups and carbamates with amine groups in solution. However, no higher molecular weight cross-linked products are evident in the either the FPLC trace of RNase with blocked carboxyl groups (Figure 3.4) or in the gel of RNase with succinylated amines (Figure 3.5). The results of the functional group blocking experiments confirmed that carboxyl groups are the primary species activated by *p*-NPCF and that cross-linking of RNase following activation occurs via reaction with amino groups of protein.

3.3.5 Protein Cross-Coupling

Further evidence in support of this was provided by the results of an attempt to cross-couple two different proteins, RNase and soybean trypsin inhibitor (STI, MW = 21.5 kDa), by co-lyophilization and activation. STI was selected because it has a similar number of acidic residues, 7 Asp and 7 Glu, compared to RNase, 5 Asp and 5 Glu. Unlike RNase with 10 Lys, STI is relatively deficient in Lys residues, having only 2. The gel in Figure 3.6 shows that upon *in vacuo* activation with *p*-NPCF and reconstitution with dH_2O, STI by itself (Lane 8) yields no homodimer bands (MW = 43 kDa). There is a band visible at a position slightly lower than that expected for the homodimer which may be a heterodimer of STI and

one of the degradation products or impurities whose bands can be seen below the monomer band.

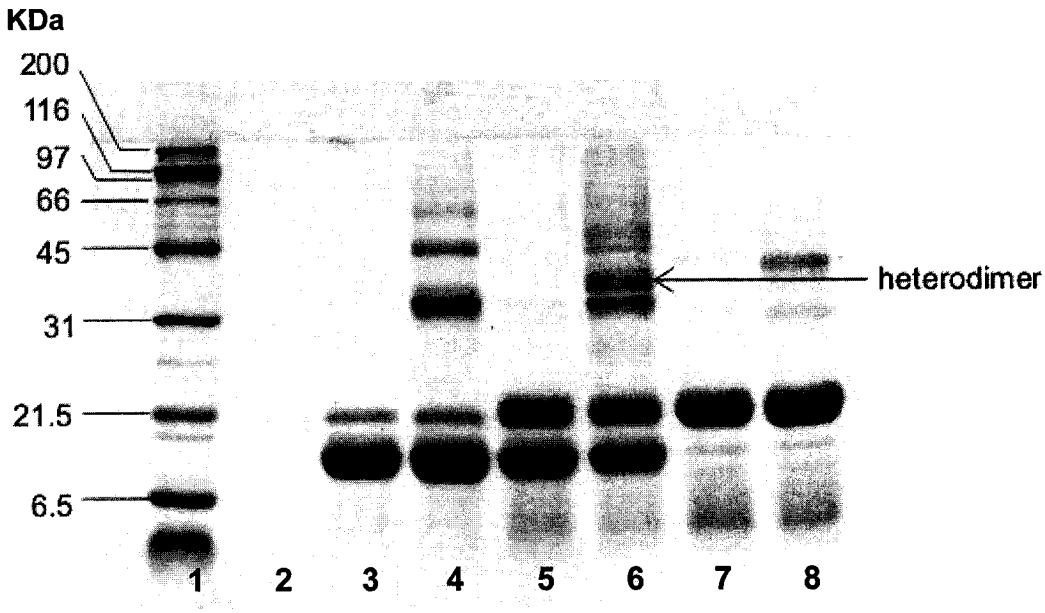


Figure 3.6: SDS-PAGE 12% polyacrylamide gel showing *p*-NPCF-induced heterogeneous cross-coupling between co-lyophilized STI and RNase A (1.3 mg/tube) at LpH 10.0.
Lane 1: MW marker; Lane 2: blank; Lane 3: RNase A (2.6 mg/tube) heated; Lane 4: RNase A (2.6 mg/tube) heated and activated; Lane 5: RNase A (1.3 mg/tube) and STI (1.5 mg/tube) heated; Lane 6: RNase A (1.3 mg/tube) and STI (1.5 mg/tube) heated and activated; Lane 7: STI (3.0 mg/tube) heated; Lane 8: STI (3.0 mg/tube) heated and activated.

On the other hand, Lane 4 shows comparatively more dimerization, typical of activated RNase A. Lane 6 shows a band at an intensity and molecular weight region consistent with a cross-coupled heterodimer of RNase and STI. The relative ratios of carboxyl to amino groups for each protein, in this case, 1:1 for RNase and 7:1 for STI, may be somewhat predictive of the cross-linking potential of a protein upon activation by *p*-NPCF.

3.3.6 Non-Aqueous Reaction of Activated RNase

Since the anhydride of activated carboxylate groups is so susceptible to hydrolysis in aqueous solution, it was difficult to assess the actual extent of *in vacuo* chloroformate activation by reaction of the activated RNase in water. In the absence of a competing nucleophile, extensive modification of RNase was evident when the activated protein (LpH 10) was exposed to monodansylcadaverine, a fluorescent amine, in organic solvent. An intense fluorescence was observable when the purified, modified protein was placed under ultraviolet light, while none was detected with the control sample which had been treated the same way, except without exposure to chloroformate (Figure 3.7). Unfortunately quantification was not possible because the modified protein product was not soluble in water, probably due to the presence of extensive cross-linked product (discussed in greater detail below) in addition to extensive modification by the hydrophobic fluorescent tag.

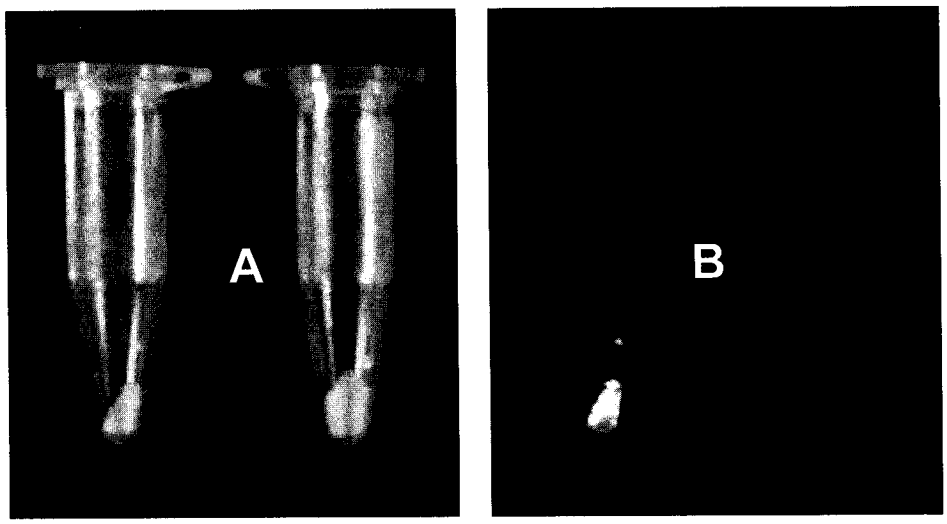


Figure 3.7: RNase exposed to monodansylcadaverine in DMF
RNase exposed to monodansylcadaverine in DMF: protein in the tubes on the left-hand side of both photos (**A** and **B**) was previously activated with *p*-NPCF, while protein in the tubes on the right was not: **A**) tubes photographed under visible light; **B**) tubes photographed under UV light.

3.3.7 Cross-linking as a Function of LpH

Interestingly, a similar trend to that in Figure 3.3 (^{14}C glycine results) arose from the results of a completely different study whereby the amount of soluble dimer of activated RNase reconstituted in dH_2O was examined as a function of LpH. Dimer and higher oligomer products are formed by the putative rapid reaction between activated carboxyl groups with nearby unprotonated amine groups upon addition of water. Figure 3.8 below shows a familiar sigmoidal shape with a steep incline at LpH greater than 6 for the relative amounts of dimer found in solution.

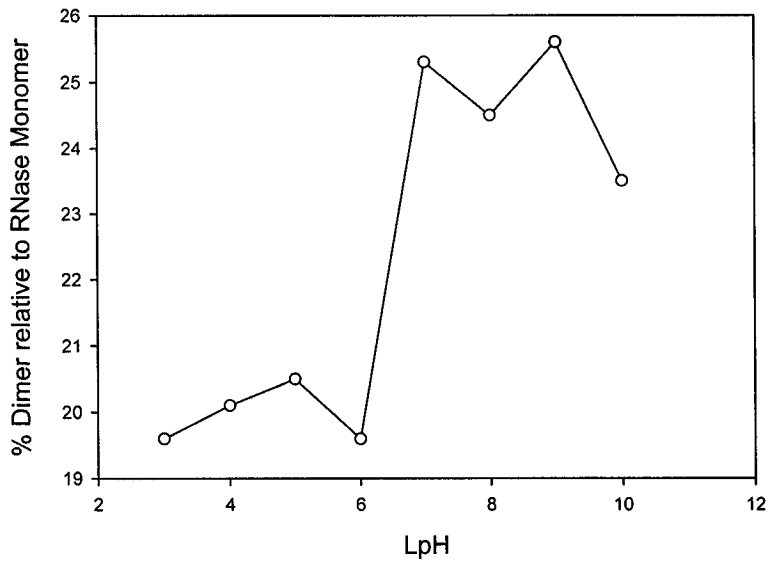


Figure 3.8: Plot of dimer peak area expressed as percent monomer peak area of ribonuclease A
(integrated peak areas taken from HPLC chromatograms).

The connection between these results and those observed with ^{14}C-incorporation, if any, is unclear. However, the extent of dimerization that occurs at LpH 7 and 8, where very few unprotonated amines (i.e., compared to LpH 10) are available in solution for reaction with

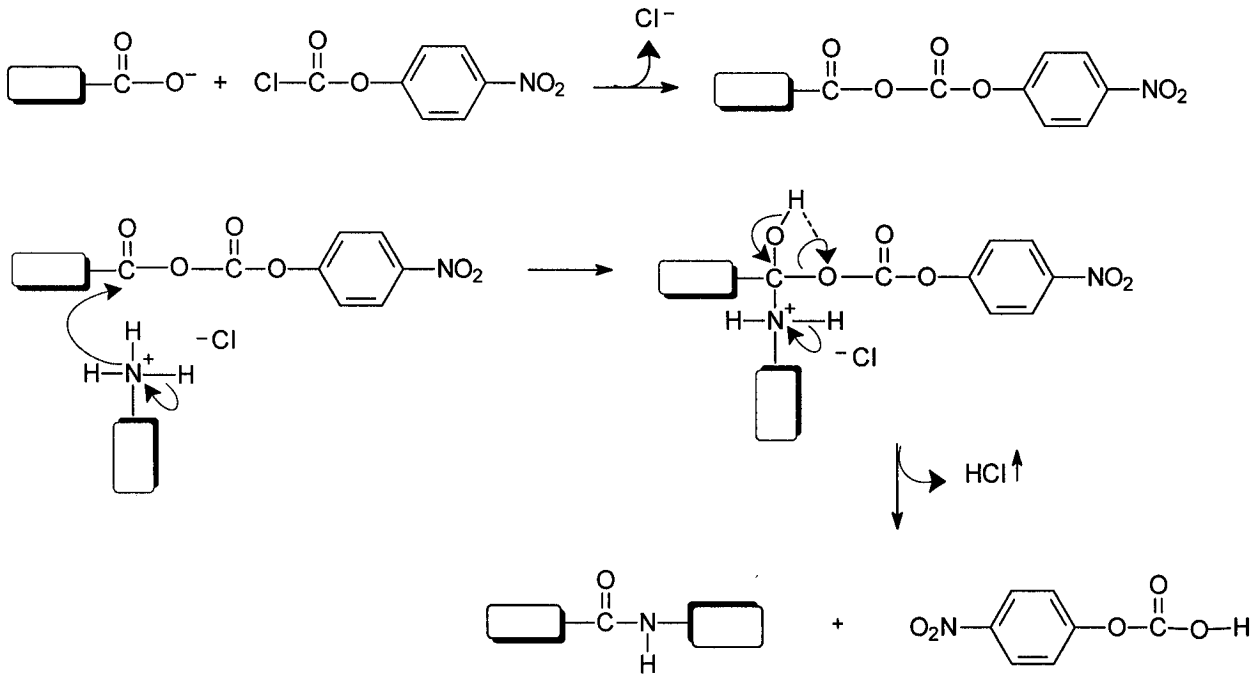
the activated carboxyl groups, is interesting and unexpected. Moreover, the amount of protein aggregation, indicative of extensive cross-linking, increased with increasing LpH. This is also unusual because at the lower LpH values, virtually all of the amines are protonated and as such would not be expected to react at all!

Based on observations from glycation results (Chapter 4, Results and Discussion) as well as other published studies using the *in vacuo* method (Taralp *et al.*, 1997; Vakos *et al.*, 2001; Kaplan *et al.*, 2002; Simons *et al.*, 2002), it is conceivable that to some extent, RNase A dimerization occurs between activated carboxylate groups and proximal ionized amine groups *via* a series of proton transfers in the dry state while under vacuum, as depicted in Scheme 3.2. The chloride ion released upon the interaction between chloroformate and the carboxylate is stabilized by the nearby protonated amine group which triggers a cascade of electron movement and proton transfers. Migration of the gaseous products away from the reaction site into the space of the evacuated tube provides the driving force behind this interaction. Moreover, the observation of tiny bubbles and the development of a yellow colour upon addition of dH_2O following activation indicated the formation of CO_2 and *p*-nitrophenol, consistent with the reactions in Scheme 3.2.

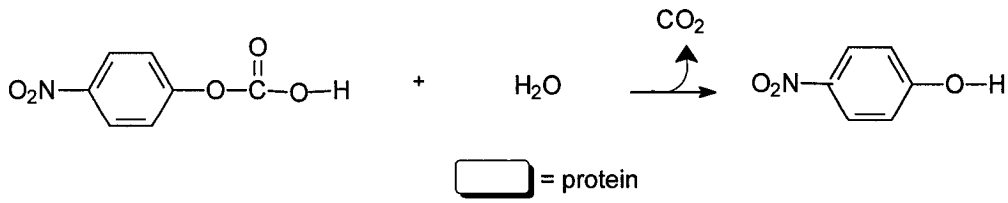
Such interactions in the dry state are not unprecedented; the ammonium and bicarbonate ions of dry ammonium bicarbonate undergo proton transfer under vacuum, ultimately resulting in the formation of gaseous ammonia, carbon dioxide and water. Townsend *et al.* showed that soluble aggregates and 'membranous precipitates' which formed in RNase that had been freeze-dried at pH 10 and stored under atmospheric pressure at 45 °C for 8 days and 30 days, respectively, were covalently bonded (Townsend *et al.*, 1991). Their results implicated lysine, aspartic and glutamic acids and, to a lesser extent,

alanine and valine, as participants in the aggregation and precipitation processes. Simons *et al.* showed that covalent dimers and other soluble oligomers are formed *via* a proton transfer mechanism between ionized carboxyl and amino groups in lyophilized proteins after incubating at 85°C under vacuum (Simons *et al.*, 2002). Although they observed significant amounts of dimer after 24 hrs, they found that maximum dimer formation in RNase (30% of the total protein) occured after 96 hrs.

In vacuo proton transfer:



In solution after activation:



Scheme 3.3: Proposed mechanism of *in vacuo* dimerization in the presence of *p*-NPCF.

From these studies and the results reported here, it appears that while the presence of a vacuum enhances the rate and efficiency of dry state proton transfer interactions/reactions, activation under vacuum has an even greater impact.

3.3.8 Activity of the RNase Dimer

The activity, under first order conditions, of the dimer product from *in vacuo* activation of RNase by *p*-NPCF as compared to the monomer is reflected in Figure 3.9, which shows the effect of different substrate concentrations on the rate of change of absorbance as a function of RNase digestion time. Initial velocities (V_0) were determined by the following relationship:

$V_0 = \frac{dA/dt}{\Delta A}$, where dA/dt is the slope of the linear part of the curve (taken after 11 minutes) and ΔA is the maximum absorbance (at the plateau).

The activity under first order conditions was calculated as follows:

$k_{cat}/K_M = V_0/[E]$, where k_{cat}/K_M is the first order rate constant and $[E]$ is mg RNase A.

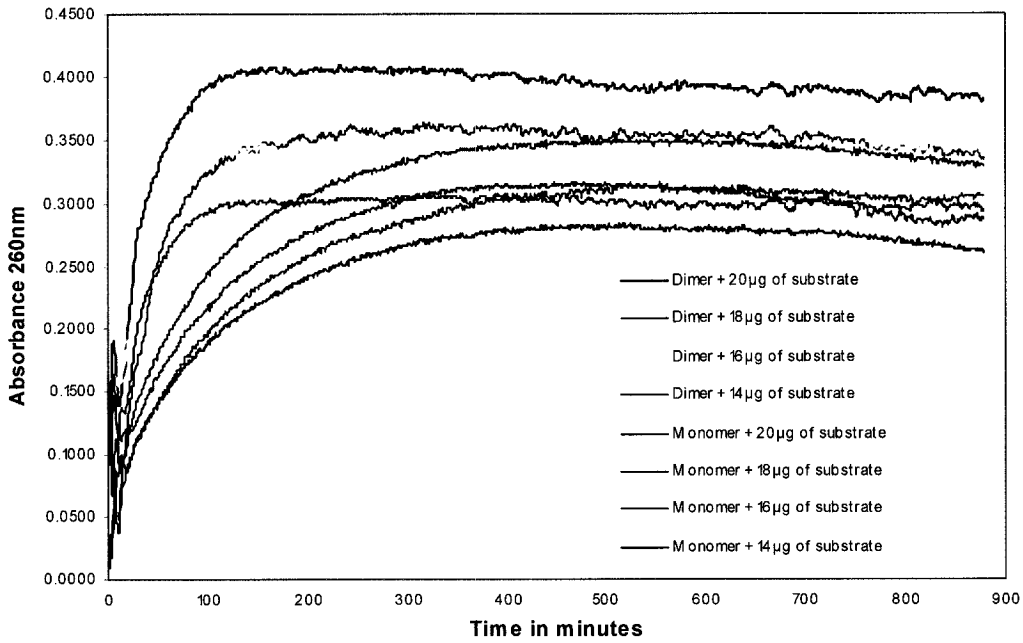


Figure 3.9: Kunitz activity assays for purified dimer and monomer fractions of *p*-NPCF - activated RNase A (LpH 8.0) in dH_2O (300 µL); [enzyme fraction] = 50 µg/ml with variable substrate (Poly-A-Poly-U) concentrations.

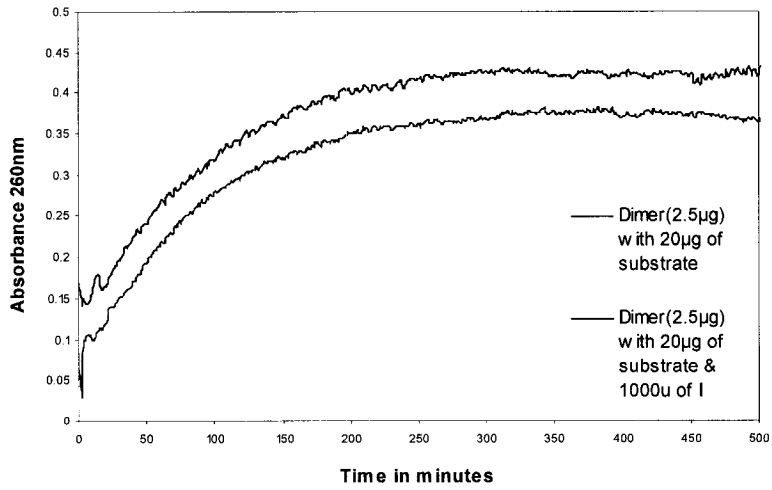


Figure 3.10: Kunitz activity assays with 2.5 µg of RNase A and 20 µg of substrate (Poly-A-Poly-U) in the presence and absence of cRI.

The specific activity per active site of the purified dimer produced by the *in vacuo* activation of RNase A (LpH 8.0) was found to be double that of the monomer and was unaffected by the presence of the cellular RNase A inhibitor (cRI) as shown in Figure 3.10 above and Table 3.2 below:

Table 3.2: RNase A Activity

RNase A Species	Specific Activity	Specific Activity with cRI
Monomer	0.16 ± 0.1%	none
Dimer	0.33 ± 0.2%	0.34 ± 0.2%

The retention of full catalytic activity demonstrates that the structure and function of RNase A are unimpaired by the conditions of the activation process. Interestingly, none of the covalently bound aggregates formed during the storage of freeze-dried RNase A examined by Townsend and DeLuca (mentioned previously) showed any activity by the same assay used here (Townsend *et al.*, 1991). In sharp contrast, the activity of covalently linked RNase A dimer formed by heating the lyophilized protein under vacuum is preserved (Simons, 2002). This suggests that over time, the presence of oxygen and moisture may adversely affect the conformation of the lyophilized protein monomer and hence the dimer that is formed.

3.3.9 Effect of Excipients

Excipients play a significant role in any application where freeze-drying proteins are involved, and the following results show that the *in vacuo* activation application is no exception. Some interesting results were obtained with the excipients, trehalose and α-

cyclodextrin (α-CD) (both separately and in combination), as well as 18-crown-6 ether (CE). However, since sorbitol produced a collapsed, glassy product upon co-lyophilization with RNase A under the conditions used here, it was excluded from further processing in the activation experiments.

Co-lyophilization of RNase A with trehalose and α-CD produced fluffy, white, voluminous products, but the excipient effects were quite different. Increasing amounts of trehalose co-lyophilized with RNase A resulted in a decrease in the amount of precipitated protein as well as soluble aggregates after activation and reconstitution with dH_2O (data not shown). The ratio of trehalose to RNase A that yielded maximum soluble dimer without concomitant precipitation was determined to be 2:3 (wt/wt). The amounts of soluble dimer generated by activated RNase A co-lyophilized with this optimal ratio of trehalose compared to the activated control (lyophilized without excipient) can be seen in Lane 6 and 8 of Figure 3.11 **A**, respectively. A comparison of Lane 7 with Lane 8 in Figure 3.11 **A** shows that the addition of α-CD had comparatively little impact on the cross-linking of RNase A. Curiously, co-lyophilization of RNase A with α-CD and trehalose together, gave rise to a synergistic action that resulted in the formation of significantly more soluble oligomeric products without the formation of precipitated aggregates. Lanes 1 to 5 in Figure 3.11 **A** show an increase in the amount of dimer and other soluble oligomers with increasing amounts of α-CD in the presence of the established optimum amount of trehalose.

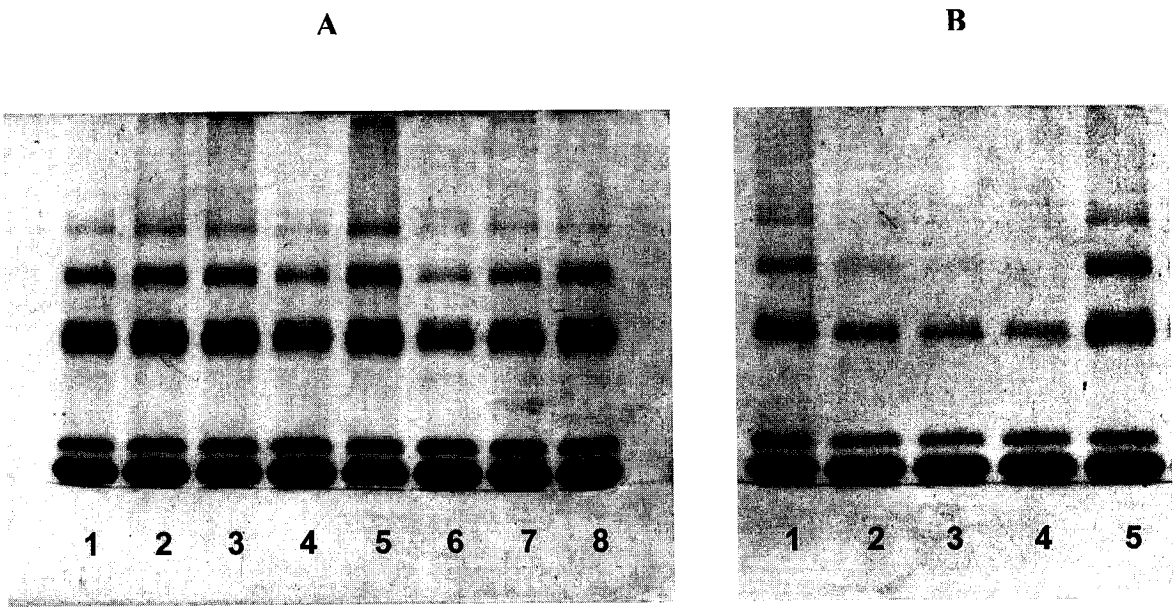


Figure 3.11: SDS-PAGE 12% polyacrylamide gels showing effect of excipients on *p*-NPCF-induced cross-linking of RNase A (3 mg, LpH 10).
A – effect of cyclodextrin and trehalose: Lane 1: CD (0.5 mg) and trehalose (2 mg); Lane 2: CD (1.0 mg) and trehalose (2 mg); Lane 3: CD (2.0 mg) and trehalose (2 mg) ; Lane 4: CD (3.0 mg) and trehalose (2 mg); Lane 5: CD (4.0 mg) and trehalose (2 mg); Lane 6: trehalose (2 mg); Lane 7: CD (2 mg); Lane 8: no excipient; **B** - effect of increasing concentrations of 18-crown-6 ether: Lane 1: CE (0.01 mg); Lane 2: CE (0.1 mg); Lane 3: CE (0.5 mg); Lane 4: CE (1 mg); Lane 5: no excipient.

The break in the trend that can be seen in Lane 4 can probably be ignored since the similarity of Lane 4 to Lane 6 where there is no α-CD, suggests that the addition of α-CD may have been mistakenly forgotten.

Figure 3.11 **B** indicates that as an excipient, crown ether behaves much like trehalose with respect to the nature and amount of RNase A aggregation formed. However, unlike α-CD there was no synergistic effect observed by co-lyophilization with trehalose. Although

these results proved to be interesting overall, the behaviour of these compounds as excipients was somewhat predictable and consistent with the literature. Aggregation, which is common in lyophilized proteins, is physically prevented by the spatial separation of protein molecules due to the presence of excipients. Trehalose and sorbitol are widely used as excipients and lyoprotectants to stabilize and protect the structure of proteins in pharmaceutical formulations (Yu, 2001; Bryn *et al.*, 2001; Cleland *et al.*, 2001) and to enhance the catalytic activity of lyophilized enzymes in organic media (Dabulis *et al.*, 1993). It is thought that stabilization of proteins is achieved through hydrogen-bonding between the sugar hydroxyl groups and residues of the protein, ultimately affording protection from lyophilization-induced conformational changes (Griebenow *et al.*, 1995).

Unlike trehalose and sorbitol, CE is not used in pharmaceutical preparations, but it has been found to dramatically improve the catalytic activity of lyophilized enzymes in dry and apolar solvents (van Unen *et al.*, 1998; van Unen *et al.*, 2002), up to two orders of magnitude above that found in water (van Unen *et al.*, 2001). Although the mechanism is not completely understood, van Unen *et al.* attributed the rate enhancement to the macrocyclic complexation of cationic lysines by 18-crown-6, thereby preventing the formation of salt bridges with anionic residues. 18-Crown-6 ether is known to selectively complex alkali metal ions, particularly K^+, as well as alkylated amines such as lysine. In proteins, protonated ε–amines of lysines that are not buried, sterically hindered, or involved in salt bridges, form hydrogen bonds with the ring oxygens. The three coplanar oxygens in CE are optimally positioned for hydrogen bonding with the hydrogens of the protonated amine in the cavity of the ring (Julian *et al.*, 2002). In the experiments reported here, the amount of soluble RNase A oligomers decreased proportionately upon addition of CE regardless of the

presence of trehalose, suggesting that the lysines of RNase A, rendered inaccessible by complexation with CE as depicted in Figure 3.12 **A**, were unavailable for reaction with the activated carboxyl groups. Thus, while CE effectively prevents the intermolecular interactions that give rise to aggregation during the *in vacuo* activation process, it does nothing to promote the formation of soluble oligomers.

The synergism exhibited by the combination of cyclodextrin and trehalose was most interesting and is worthy of further investigation. To date, the use of cyclodextrins as excipients or lyoprotectants for proteins has never been reported. However, combining different excipients as well as varying the ratio of the amount of excipient to protein has been shown to affect the degree of stabilization imparted to certain lyophilized proteins (Cleland *et al.*, 2001; Johnson *et al.*, 2002). The rationale behind using cyclodextrin in the research reported here pertains to the ability of these unique cyclic oligosaccharides to accommodate various organic compounds in their hydrophobic inner cavity. One research group (Leung *et al.*, 2000) prepared dimers of β-cyclodextrin separated by various spacers in order to prevent the aggregation of proteins by selectively binding hydrophobic side chains. Huh *et al.* exploited the host-guest properties of α-CD to construct a biodegradable polyrotaxane for drug delivery applications which involved the preparation of inclusion complexes in polylysine (Huh *et al.*, 2001). They found that the cavities of β- and γ-CDs were too large for the lysine side-chains. Harada *et al.* studied the inclusion complexes that form between cyclodextrins and pendant side chains on polymer backbones and found that of all the cyclodextrins, only α-CD was capable of forming complexes with n-butanol and did so with a K_a of 20 M^{-1} (Harada *et al.*, 1997). Based on these reports, it is conceivable that cyclodextrin forms a 'collar' around the pendant alkyl moiety of lysine, as depicted in Figure

3.12 **B**, in much the same way as it does with the n-butanol side-chain of the polymer in the study by Harada *et al.*.

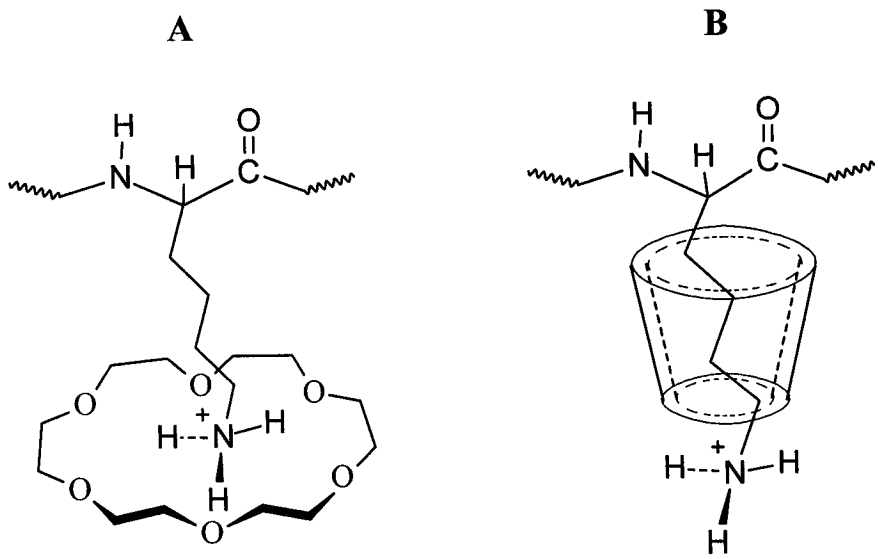


Figure 3.12: Effect of excipients on lysine: **A** – complexation of protonated ε-amine group with 18-crown 6 ether; **B** – α-CD-lysine host-guest inclusion complex

This would explain the observation that α-CD has little impact on the formation of oligomers during activation; since it is likely to be incorporated into the lysine side chains, it cannot provide the spatial separation required to prevent intermolecular interactions from occurring. Although the exact mechanism behind the increase in yield of soluble oligomers observed with the combination of co-lyophilized trehalose and with RNase A is unclear, it is plausible that the rigidity imparted by the α-CD collared lysine, coupled with the spatial separation afforded by the particular amount of trehalose used here, provides optimal spacing and molecular orientation to facilitate favourable interactions between reactive functional groups of adjacent RNase A molecules.

3.4 Conclusions

Facile preparation of high yields of homodimers and other cross-linked proteins is the subject of many current research endeavors. Based on the results of this investigation, it appears that proteins can indeed be activated with *p*-NPCF under conditions of reduced pressure to facilitate cross-linking or modification in solution. Since such unusual reaction conditions were employed to effect activation, several experiments were systematically carried out in order to ascertain reaction characteristics and limitations. The results of these experiments were somewhat surprising, and culminated in the proposal of a very interesting and novel mechanism involving proton transfers in the dry state.

Under the activation conditions employed here, the yields of soluble protein oligomers generated by this method are not sufficient to be considered practical. However, there are multiple variations of parameters, such as incubation time and temperature, LpH, excipients, other activating agents, etc., which can be manipulated in order to optimize yields. Furthermore, since activity measurements show that the monomer and dimer are structurally intact and active, the simplicity of the procedure and relatively short activation reaction time means that monomer can be easily recovered and cycled through the activation procedure in a series of sequential reactions.

Many interesting theoretical and practical questions have arisen from these preliminary experiments. Nevertheless, *in vacuo* activation with *p*-NPCF has many potential practical applications, and the results obtained so far indicate that further investigation aimed at elucidating the apparently novel theoretical aspects and optimizing reaction conditions would be worthwhile.

Acknowledgements

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Chapter 4 Protein Cross-linking by Glycation

4.1 Introduction

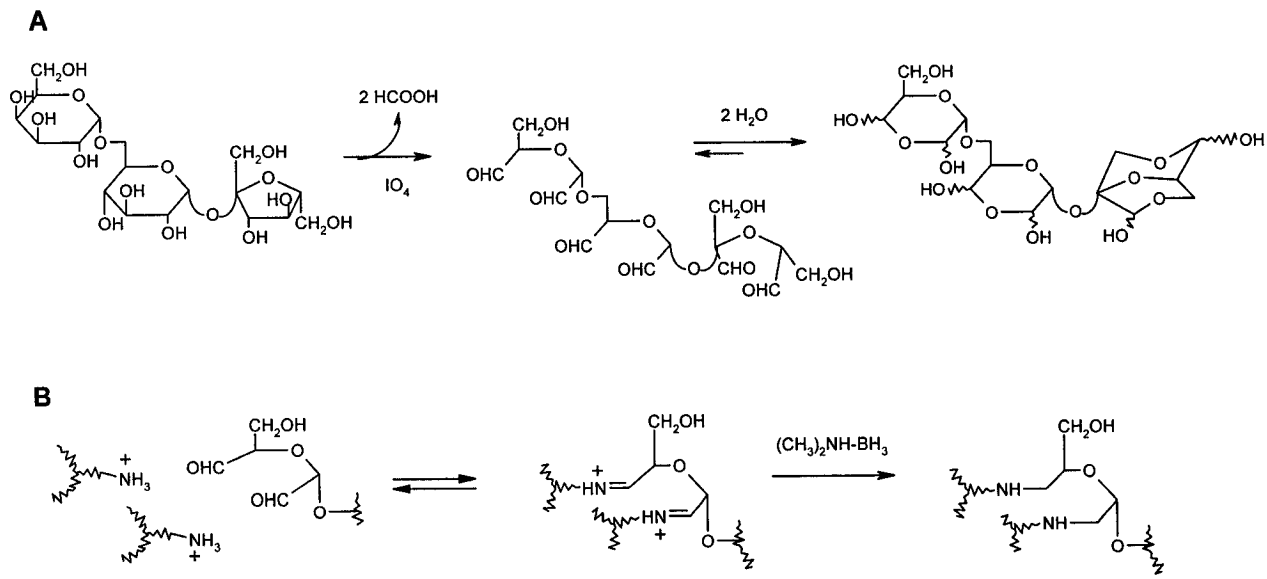
Protein cross-linking induced by post-translational glycation *in vivo* is a well known and widely studied naturally occurring phenomenon. α-Dicarbonyl compounds such as glyoxal and methylglyoxal are formed during the later stages of the Maillard reaction and are known to cause browning, denaturation, fluorescence and cross-linking in proteins (Marquie, 2001; Frye *et al.*, 1998; Shipanova *et al.*, 1997). Cross-linking by such advanced glycation end-products (AGEs) is often implicated in complications associated with glycemic disease states such as diabetes as well as the ageing of tissue proteins such as lens proteins and human skin collagen. Thus, many studies are directed towards the prevention or inhibition non-enzymatic glycation and AGE formation (Khalifah *et al.*, 1996; Frye *et al.*, 1998; Wrodnigg *et al.*, 2001 and references therein).

The benefits associated with intentionally cross-linking proteins using sugars are numerous. Sugars are readily available, inexpensive, easily modified, non-toxic and they are not expected to be immunogenic once incorporated into the cross-link. Although mono- and oligosaccharides have been employed to cross-link proteins by synthetic means, to date, there are no reported attempts to utilize the intrinsic aldehyde functionality of reducing sugars to intentionally cross-link proteins. The reason for this is largely based on the conformational dynamics of sugars in solution. Equilibrium favours the unreactive hemiacetal form in solution, but it is the acyclic aldehydic form of the sugar that is required for the initial nucleophilic condensation reaction with an amine to form a Schiff base. Migration of the Schiff base double bond, also known as Amadori rearrangement, results in the formation of a

ketoamine that can further react with protein amino groups resulting in cross-links *via* ketimine formation. Since this reaction is slow, reversible, and difficult to control (Acharya *et al.*, 1988), cross-linking with reducing sugars in solution is considerably inefficient. However, a simple modification to the sugar ring gives rise to improved reactivity and subsequent cross-linking efficiency.

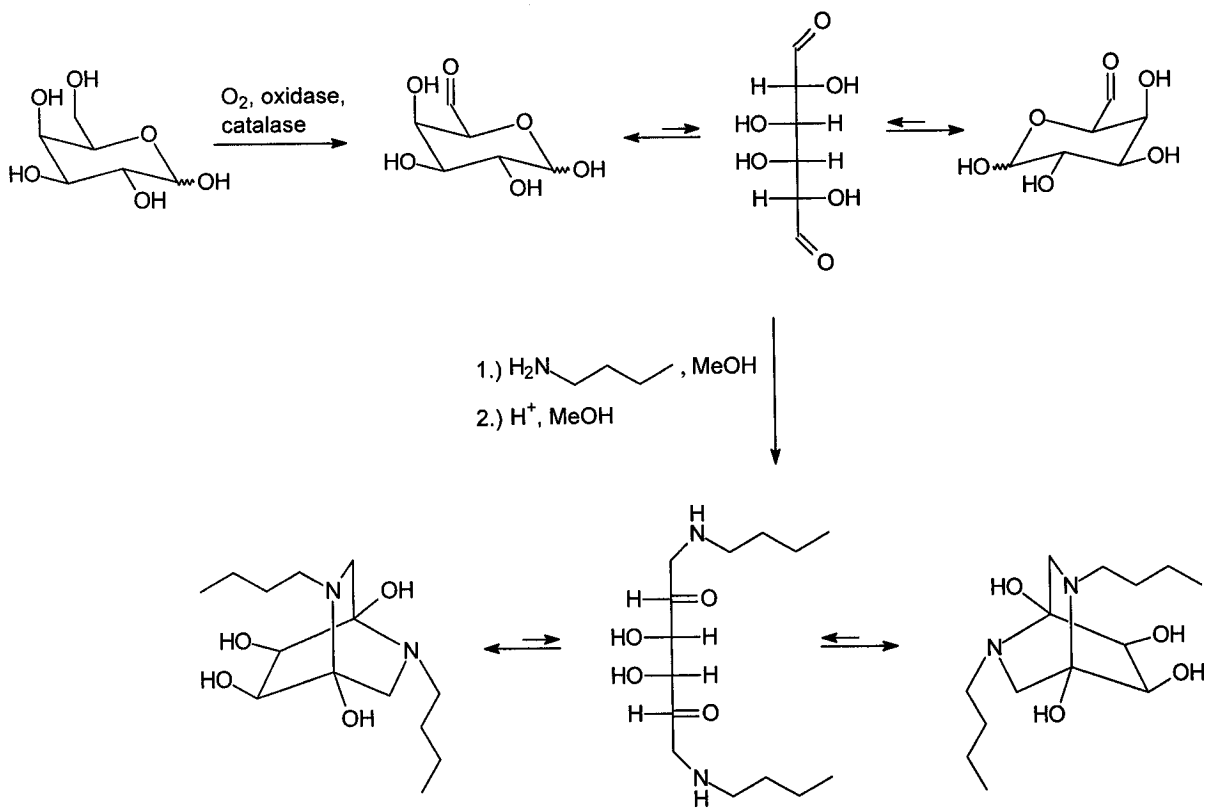
4.1.1 Cross-Linking Proteins using Modified Sugars

Oxidative ring-opening of sugars by sodium periodate generates two aldehyde groups per sugar monomer which are available for reaction with α- and ε-amino groups in order to introduce cross-links directly within proteins. The resulting Schiff bases are stabilized through reduction by a suitable reducing agent such as sodium borohydride, which drives the reaction forward. Periodate oxidized sugars are currently utilized to prepare cross-linked proteins of biomedical and pharmaceutical significance. Kazan *et al.* cross-linked penicillin G acylase, an enzyme used in the synthesis of penicillin, with periodate-treated dextran, a large oligosaccharide, in order to impart greater thermal stability to the enzyme (Kazan *et al.*, 1997) thus allowing the use of elevated temperatures during manufacturing processes. In doing so, these researchers achieved thermal stabilization not only through cross-linking the enzyme but also through the inclusion of a carbohydrate, which is known to protect proteins from thermal inactivation (discussed in detail in Chapter 5). Periodate ring-opening of the trisaccharide raffinose is used to cross-link hemoglobin for artificial blood preparations as depicted in Scheme 4.1, and is the basis of an industrial patent (Pliura *et al.*, 1998) and the Canadian company Hemosol.



Scheme 4.1: Hemosol patented hemoglobin cross-linking scheme.
A – periodate ring-opening of raffinose. **B** – cross-linking by reaction of raffinose aldehydes with lysines on hemoglobin (Pliura *et al.*, 1998).

Another interesting approach to using modified sugar as a cross-linker was recently published by Schevaart and Kieboom (Schoevaart *et al.*, 2002). They generated a dialdehyde through the enzymatic oxidation of the reducing sugar D-galactose by D-galactosidase in the presence of catalase, and studied its reactivity towards butylamine as a model for lysine groups. Unlike the periodate mechanism which forms two vicinal aldehydes, the two aldehydes in the galactose dialdehyde are at opposing ends of the molecule, which influences the reaction outcome dramatically. Instead of reducing the dual Schiff bases, Schevaart and Kieboom allowed double Amadori rearrangement to take place at both imines resulting in the interesting bicyclic linkage depicted in Scheme 4.2 below.



Scheme 4.2: Racemic mixture of cross-linked model lysine group (butylamine) *via* enzymatic oxidation of D-galactose (Schoevaart *et al.*, 2002).

Although low yields of cross-linked product in a reaction between galactose dialdehyde and gelatin precluded a conclusive determination of the nature of the cross-link, results of this study indicated that a similar double Amadori rearrangement also occurs in proteins. However, since solubility issues required the use of methanol for the reaction to proceed, and the yield of cross-linked material from both reactions with butylamine and gelatin were low, at present, this method lacks utility for widespread application.

4.1.2 Reducing Sugar Cross-Linker Design

In the research endeavor described in this chapter, the utility of cross-linkers composed of unmodified reducing sugars designed for reaction with proteins under *in vacuo*

glycation conditions was investigated. Glucuronic acid and glucosamine were selected as the active starting materials because they are plentiful, inexpensive glucose-based sugars containing reactive functional groups which are capable of coupling with spacers without impairing the reducing functionality of the sugar. The cross-linkers were synthesized by the formation of two amide linkages between diamine spacers and the carboxylic group in glucuronic acid, or diacid spacers and the amino group in glucosamine. A few coupling strategies which involved activating the carboxylic group (either on the spacer or on the sugar moiety) were attempted using carbodiimides or the coupling agent, O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HATU). Two different synthesis strategies were attempted to effect coupling of glucuronic acid to a diamine spacer. The first attempt involved a protect/deprotect strategy in order to purify the product by silica gel chromatography, while the second strategy involved direct coupling without protection and purification by size exclusion chromatography.

Selection of proteins suitable for cross-linking by these glycating cross-linkers requires the presence of available, unhindered amino groups. With 22 lysine groups, many of them on the surface, and 2 amino termini, globular dimeric hemoglobin was selected as one potential candidate, while cytochrome c which is a smaller protein having 14 surface lysines was selected as another (see structure in Figure 4.1).

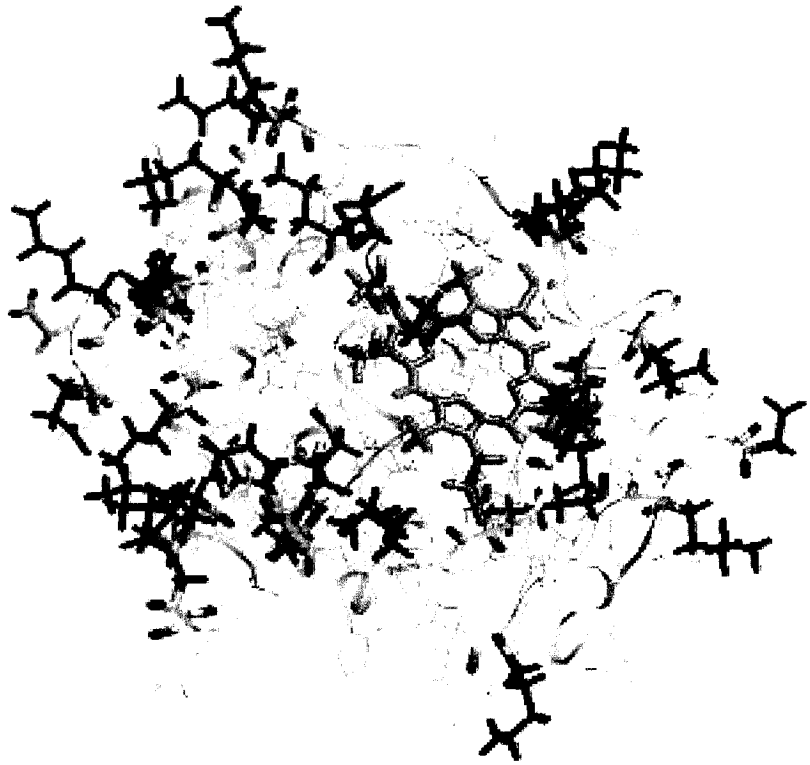


Figure 4.1: Structural depiction of cytochrome c showing availability of lysines (green) at the periphery of the molecule.

Cross-linking of cytochrome c is of interest for potential use in the detoxication module of the artificial liver construct described previously in Chapter 2. Cross-linking hemoglobin was also of interest because it is the subject of a growing number of commercial ventures and research articles in the literature pertaining to the production of artificial blood substitutes. The oxygen transport properties of hemoglobin are unparalleled, and in the absence of blood cell membranes there is no concern for blood group antigenicity. Inside red blood cells, hemoglobin exists as a globular tetrameric protein of the form $\alpha\alpha\beta\beta$; outside of the cell however, the tetramer spontaneously and reversibly dissociates into two non-covalently bound $\alpha\beta$ dimers. These dimers have a short plasma half-life of ~90 minutes and

thus can cause renal failure from massive hemoglobinuria. Thus, much research has been dedicated to the development of a suitable hemoglobin cross-linker in order to prevent dissociation of the tetramer.

4.1.3 Current Methods for Cross-linking Hemoglobin

There are numerous chemical and recombinant approaches to cross-linking hemoglobin reported in the literature, and the properties of the resultant cross-linked tetrameric products have been compared and assessed in review articles (Rudolph, 1998; Chang, 1997). One approach involves chemical modification to introduce intramolecular cross-links between the two αβ subunits of hemoglobin which results in a stabilized 64 kDa tetramer product having functionally efficient oxygen transport and delivery properties. Several small bifunctional cross-linkers have been specifically designed to react selectively with lysines at the diphosphoglycerate (DPG) binding site between two β chains (Walder *et al.*, 1994; Benesch, 1994; Kluger *et al.*, 1997). Analyses of the cross-links have shown that they typically form either between β-Lys-82 and β-Val-1 or β_1-Lys-82 and β_2-Lys-82. Interestingly, under deoxygenated conditions, one such cross-linker, bis(3,5-dibromosalicyl)fumarate, forms linkages between lysines of the two α chains (α_1-Lys-99 and α_2-Lys-99), thus imparting a lower oxygen affinity to the cross-linked tetramer (Walder *et al.*, 1994). The increased oxygen pressure at which the hemoglobin tetramer is half saturated (P_{50}) gives rise to oxygen transport properties that closely approximates those of whole blood. Kluger *et al.* found that varying the length of a series of unsaturated dicarboxylic acids, which selectively cross-link the two β-chains of hemoglobin under deoxygenated conditions, affected the oxygen affinity of the cross-linked product (Kluger *et al.*, 1996). Since the introduction of a cross-link restricts conformational movement within the protein

molecule, oxygen binding and cooperativity are affected. In order to cross-link hemoglobin for use as a potential blood substitute, the allosteric properties must not be impaired by the introduction of a cross-link.

Non-selective chemical reagents are also used to cross-link hemoglobin, producing heterogeneous mixtures of intermolecular cross-linked material. Compared to intramolecular cross-linked tetramers, soluble higher molecular weight, polymerized hemoglobin has the advantages of higher circulatory half-life and reduced colloid osmotic pressure (Chang, 1997; Rudolph, 1998). Although glutaraldehyde is commonly used to generate polymerized hemoglobin with intermolecular as well as intramolecular cross-links, polymerization is difficult to control, the product is unstable on storage and glutaraldehyde is toxic if released as a degradation product. A mixture of intra- and inter-molecular cross-linked hemoglobin products possessing good P_{50} properties can be generated from the use of a dialdehyde derived from oxidized raffinose (*vide supra*) (Pliura *et al.*, 1998). MacDonald and Pepper utilized glycolaldedyde, a small two carbon aldehyde, as a self-perpetuating cross-linker alternative to glutaraldehyde (MacDonald *et al.*, 1994). Upon reaction with protein amino groups, the resultant Schiff base rearranges to generate a second reactive aldehyde (*via* Amadori rearrangement) that can react with another proximal amino group to form the cross-link. The reaction proceeds more slowly, and lower molecular weight oligomers are produced.

The clinical advantages of oligomeric arrays of hemoglobin tetramers have spurred research groups towards the design of "smart" cross-linkers. Kluger *et al.* (Kluger *et al.*, 1999) designed such cross-linkers to generate a hemoglobin product having defined bis-tetrameric structures of the general type shown in Figure 4.2 **A** below. A computational

determination of the optimal intramolecular and intermolecular distances between amino groups required to achieve such bis-tetrameric arrays lead to their design of the cross-linker N,N'-5,5'-bis[bis(3,5-dibromosalicyl)-isophthalyl]terephthalamide (DBIT) shown in Figure 4.2 **B**.

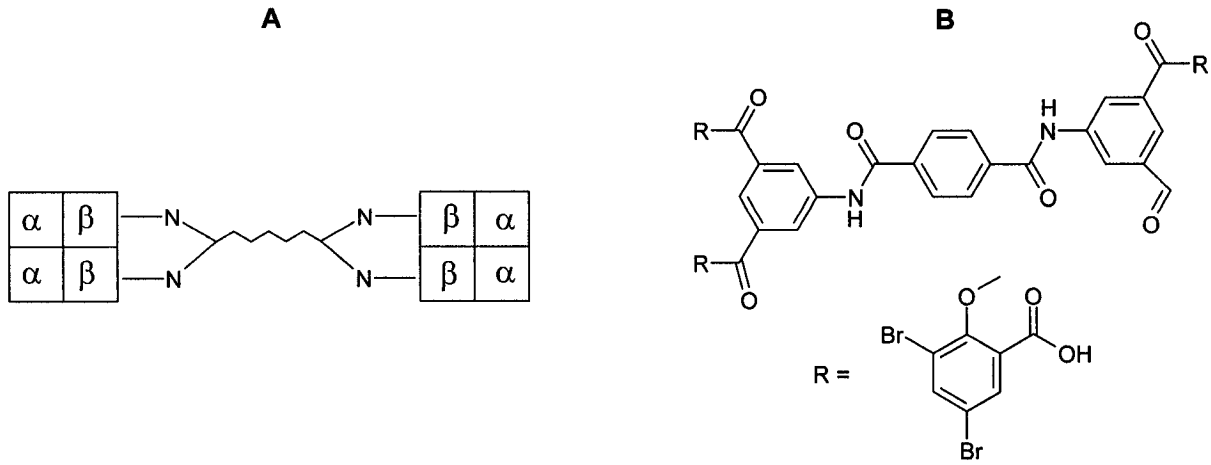


Figure 4.2: **A** - General structure of cross-linked hemoglobin bis-tetramers, **B** – 'smart' cross-linker, DBIT (Kluger *et al.*, 1999).

Hai *et al.* first cross-linked hemoglobin α-chains using a selective intramolecular bifunctional aspirin analogue and then polymerized the resultant tetramer using various water-soluble polyamide cross-linking agents (Hai *et al.*, 1999). Through manipulation of parameters such as pH, concentration of polyamide cross-linker and cross-linked hemoglobin tetramer, they were able to control the polymerization and oxygen binding properties of the oligomeric tetrameric products formed. In a later study, this same research group sought to extend the circulatory half-life of the polymerized tetramer even further by using an anionic non-immunogenic polysaccharide (chondroitin-4-sulfate) as the polymerizing reagent for the diaspirin cross-linked tetramer (Hai *et al.*, 2000).

4.2 Materials and Methods

pH Adjustment/Measurement:

The pH of aqueous solutions were measured using a Radiometer Copenhagen type PHM26 pH meter fitted with an ORION semi-micro gel-filled combination electrode model 91-15 and pH-stat titrations were performed using the above pH meter coupled with a Titration 11/Ole Dich autotitrator assembly.

4.2.1 Purification of Cross-linkers

Unless stated otherwise, preparative purifications of synthesized cross-linkers were performed by size exclusion chromatography at ambient temperature using Sephadex or Biogel columns connected to a Pharmacia LKB Peristaltic Pump P-1 and a Waters Differential Refractometer R403 detector. Traces were recorded on a Linear 1200 strip chart recorder and fractions were collected using a Pharmacia FRAC-100 fraction collector. Degassed, distilled water was used as the eluant in all cases.

4.2.2 NMR and Mass Analysis of Cross-Linkers

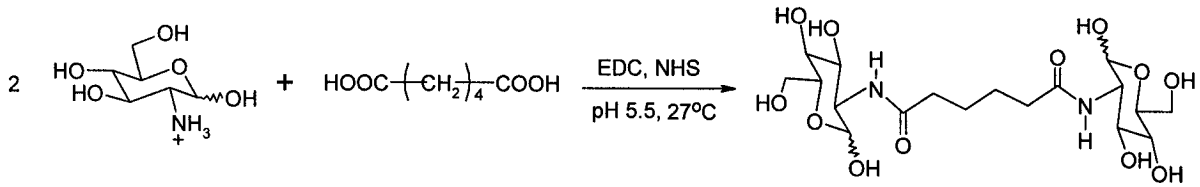
^{1}H- and ^{13}C-NMR were recorded on a Bruker Avance 300 spectrometer at 300 MHz and 75.48 MHz, respectively. Mass spectra were obtained using a VG platform electrospray-quadrupole mass spectrometer.

4.2.3 Synthesis of Linear Bifunctional Cross-linkers

4,7,10-trioxa-1,13-tridecanediamine was purchased from Fluka, 2-amino-2-deoxy-D-glucose (glucosamine), glucuronic acid, adipic acid were purchased from Sigma Chemical Company and DAB G1.0 dendrimer was purchased from Aldrich. All solvents and chemical

starting materials were of reagent grade quality and were used as received. Solvents were evaporated using a Bücchi rotary evaporator equipped with an ethanol/dry ice condenser and a water aspirator or a vacuum pump equipped with a liquid nitrogen trap.

4.2.4 1,4-Butanebisamido-2-glucopyranoside (1)



1

Scheme 4.3: Synthesis of a divalent glucosamine-based cross-linker (**1**)

Adipic acid (0.104g, 0.7 mmoles) was added to a water-jacketed vessel assembly along with 7.0 mL of dH_2O and was left to stir until the adipic acid had dissolved (~15 minutes) and the temperature had stabilized at 27°C (temperature was controlled by a recirculating water bath). To this solution, glucosamine hydrochloride (0.330g, 1.5 mmoles), NHS (0.172g, 1.5 mmoles) and EDC (0.289g, 1.5 mmoles) were added. The pH was adjusted to 5.5 with 1M NaOH (solution turned cloudy) and was maintained at this pH by a pH stat. After 8 hrs the solution turned clear and 750 µL was withdrawn from the solution and was purified by a Biogel P2 column (95 cm x 1.5 cm) with dH_2O as the eluant. The first fraction off the column was collected, freeze-dried and analyzed by NMR which indicated the presence of a contaminant. Another aliquot (2.0 mL) was withdrawn from the reaction

mixture and purified by SEC as before. This time the first fraction was collected and treated with strong cationic Dowex resin (which had been prepared by rinsing with 1M NaOH, then rinsing well with dH_2O followed by rinsing with 1M HCl) prior to freeze-drying, affording an off-white, fluffy, hygroscopic powder in ~60% yield. ^{1}H-NMR (DMSO-d_6) δ (ppm): ^{1}H-NMR (DMSO-d_6) δ (ppm): 7.73 (m, NH); 7.64 (m, NH); 7.54 (d, NH); 6.43 (m, OH-1); 4.90 (s, H-1); 4.60 (m, OH-2, OH-3, OH-4 (α,β), H-2, H-3, H-4, H-5 (α,β)); 2.18 (m, CO-CH_2); 2.04 (m, ?); 1.45 (m, CH_2). M.S. (ESI, formic acid): m/z: 491.1 [M+Na^+]

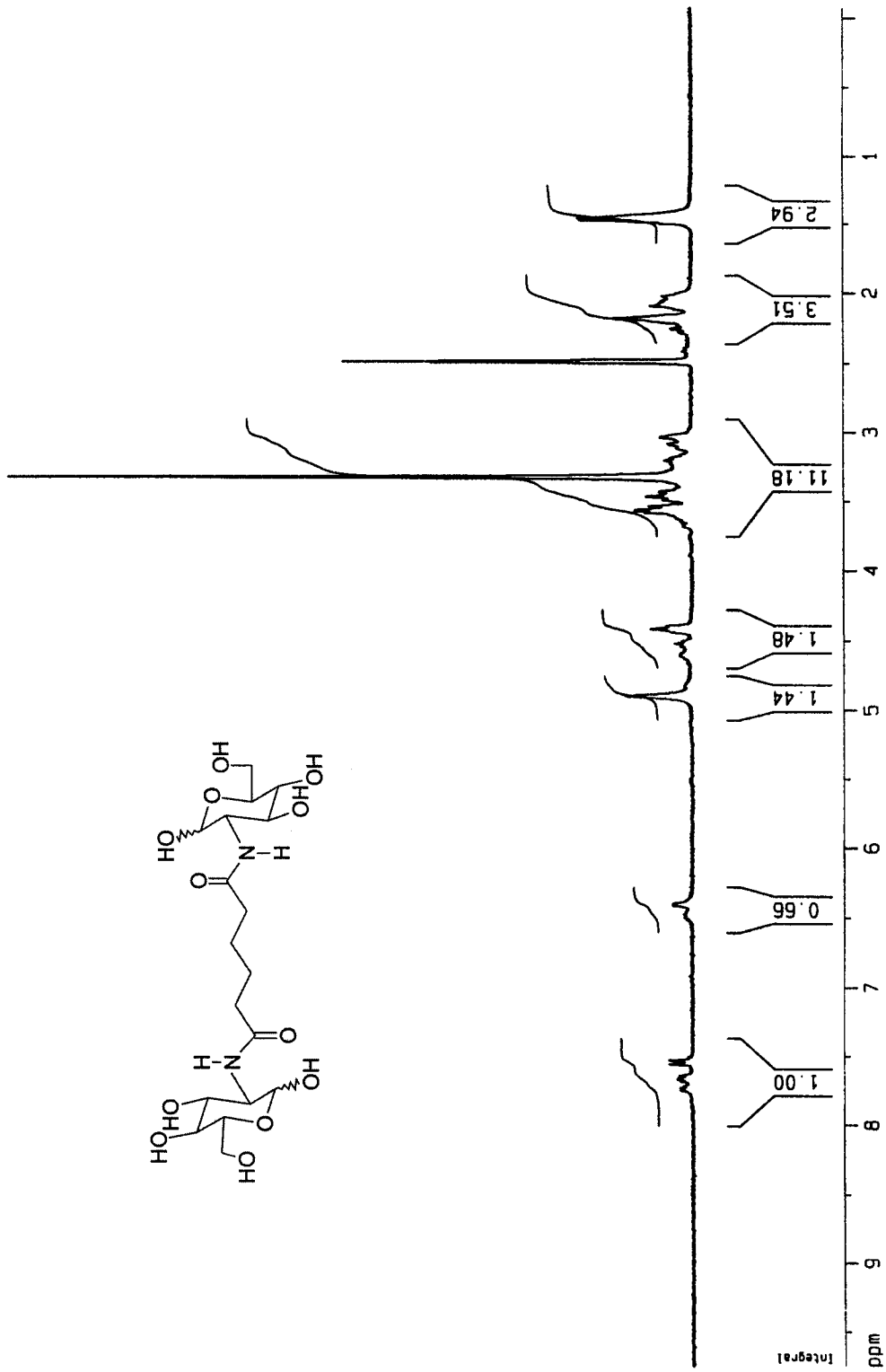
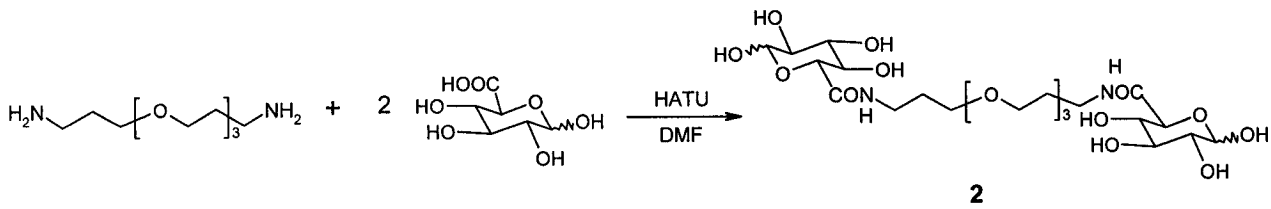


Figure 4.3: ^{1}H NMR (300 MHz, DMSO-d_6) of **1**.

4,7,10-trioxa-1,13-tridecanebisamido-5-glucopyranoside (2):



Scheme 4.4: Synthesis of a divalent glucuronic acid-based cross-linker (**2**)

Glucuronic acid (0.142 g, 0.73 mmoles) was added to 2 mL of DMF with stirring in a small round bottom flask. After the glucuronic acid had dissolved, 4,7,10-trioxa-1,13-tridecanediamine (77 μL, 0.35mmoles) and (~0.5 hr), HATU (0.277g, 0.73 mmoles) were added to the flask, and the mixture was left to stir for 1 hour. The reaction mixture (0.1 mL) was purified on a column packed with Biogel P2 (105 cm x 1.5 cm) with dH_2O as eluant. The product was collected in the first fraction and then lyophilized to dryness affording a hygroscopic, white fluffy powder. Product yield was estimated to be between 10-20%. ^{1}H-NMR (DMSO-d_6) δ (ppm):7.93 (t, NH); 6.77 (d, OH-1β); 6.48 (d, OH-1α); 4.97 (m, H-1α,β); 4.78 (d, OH-4α); 4.60 (d, OH-3α); 4.29 (t, H-3); 3.90 (d, H-5); 2.70-3.54 (m, OH-3, OH-4, H-2, H-3, H-4, H-5, O-CH_2); 1.61 (p, CH_2-CH_2,); 1.24 (t, ?); 0.85 (impurity). ^{13}C-NMR (DMSO-d_6) δ (ppm): 169.6, 170.6 (2 C=O); 98.1 (C-1β); 93.6 (C-1α); 77.2 (C-3β); 76.5 (C-5β); 75.3 (C-2β); 72.3 (C-3α); 70.6 (C-2,5α); 69.0 (C-4α,β); 36.6 (CH_2NH); 29.4 (CH_2). M.S. (ESI, formic acid): m/z: 595.2 [M + Na^+].

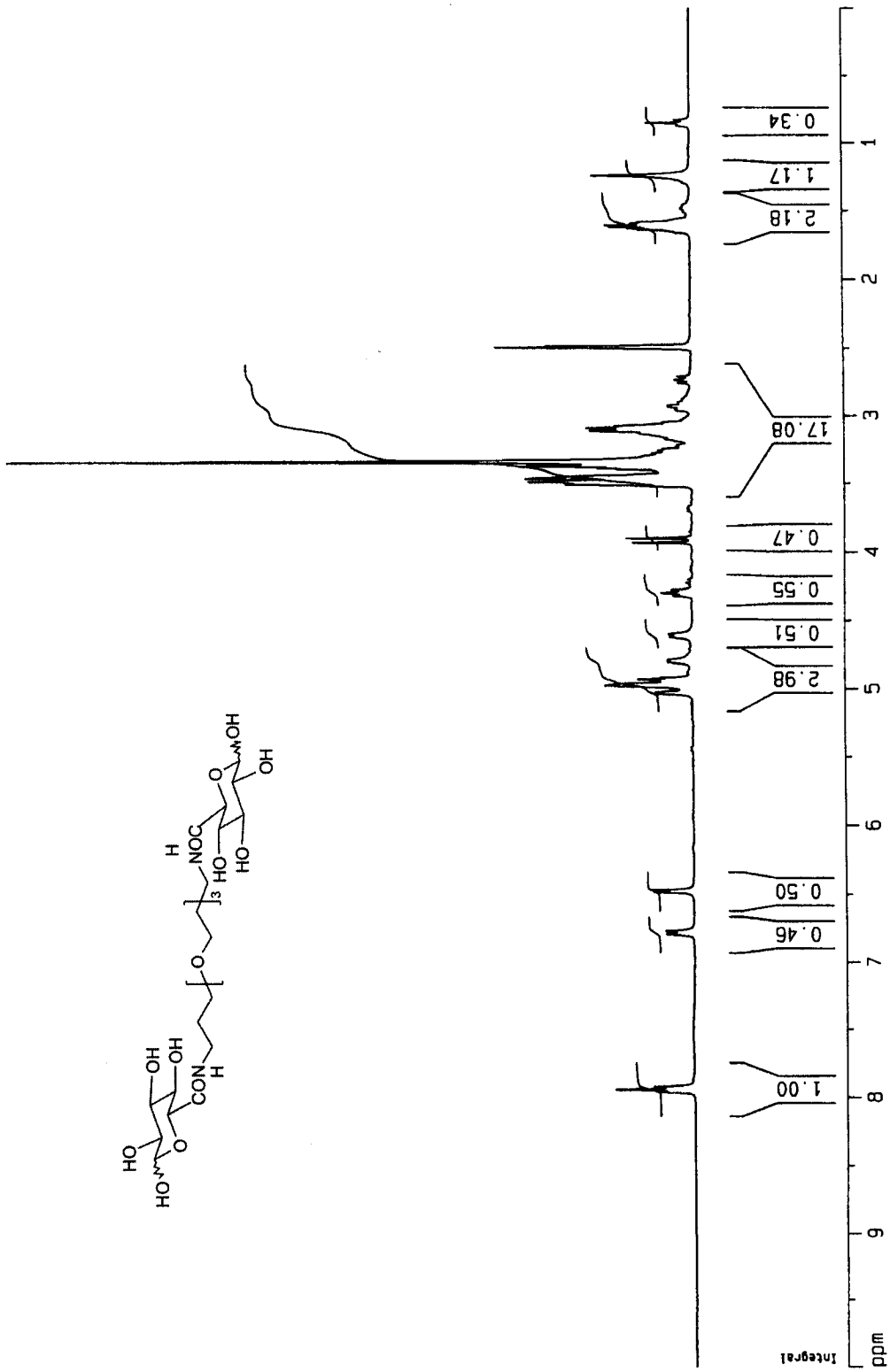
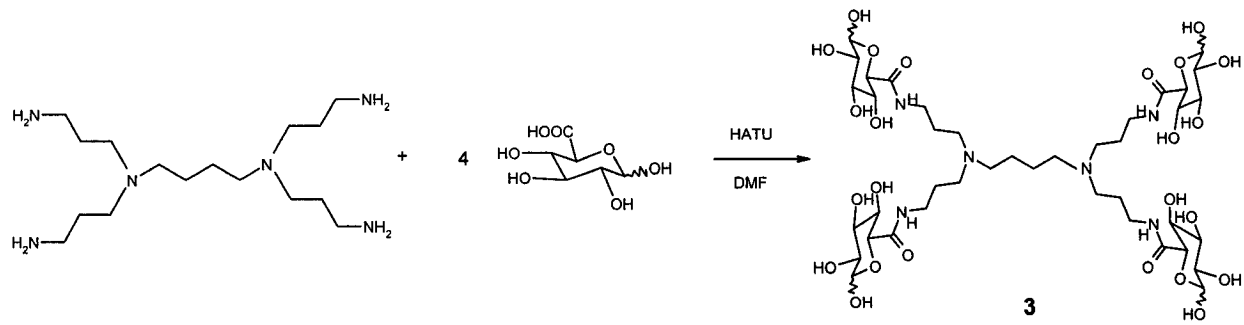


Figure 4.4: ^{1}H NMR (300 MHz, DMSO-d_6) of **2**.

4.2.5 Branched Tetrafunctional Cross-linker Synthesis

N,N,N'N'-Tetrakis(glucopyranose-3-amidopropyl)-1,4-butanediamine (3)



Scheme 4.5: Synthesis of a tetrafunctional sugar cross-linker (**3**)

Glucuronic acid (0.24 g, 1.2 mmoles) was added to 5 mL of DMF with stirring in a small round bottom flask. After the glucuronic acid had dissolved (~0.5 hr), HATU (0.450g, 1.2 mmoles) and DAB G1.0 dendrimer (0.35 mmoles) were added to the flask and the mixture was left to stir overnight. The reaction mixture (0.3 mL) was purified on a column packed with Sephadex G-15 (83 cm x 1.5 cm) with dH_2O as eluant. The product was collected in the first fraction and then lyophilized to dryness affording a hygroscopic, white fluffy powder that collapsed upon exposure to air. Product yield was estimated to be between 10-20%. ^{1}H-NMR (DMSO-d_6) δ (ppm): 7.98 (bs, NH); 6.76 (bs, OH-1β); 6.46 (bs, OH-1α); 4.93 – 4.78 (m, H-1, OH-4); 4.59 – 2.81 (m, OH-3, OH-5, H-2, H-3, H-4, H-5,); 1.56 - 1.39 (m, CH_2). M.S. (ESI, formic acid): m/z: 1,021.3 [M^+].

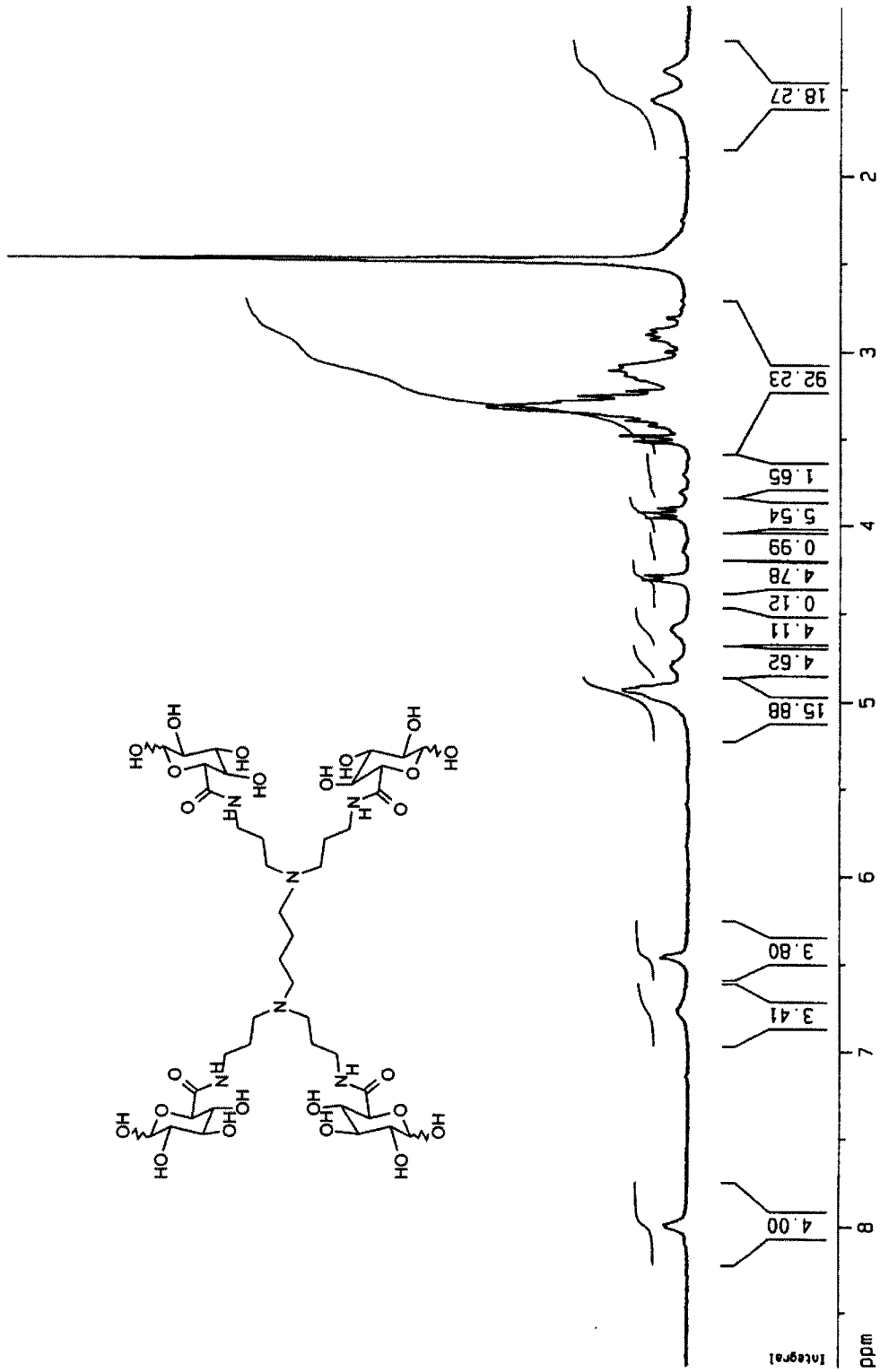


Figure 4.5: ^{1}H NMR (300 MHz, DMSO-d_6) of **3**.

4.2.6 *In Vacuo* Cross-linking Reaction Method

Typically, a stock solution of accurately weighed protein (cytochrome c or hemoglobin - see below for specific details for each protein) in dH_2O was prepared and, depending on the experiment, the pH was adjusted with either 1N NaOH or 1N HCl using a calibrated Radiometer Copenhagen type PHM26 pH meter fitted with an ORION semi-micro gel-filled combination electrode model 91-15. Aliquots of the protein stock solution of a minimum volume of 500 μL to yield between 2 - 5 mg protein/tube, were transferred to 13 x 100 mm borosilicate glass disposable culture tubes to which varying aliquots of a solution of cross-linker dissolved in dH_2O were added (to a maximum volume of ~1000 μL). The tubes were left to stand for approximately 10 - 15 minutes and were then flash frozen and lyophilized using either a VIRTIS-24 commercial freeze dryer or a home-made freeze dryer equipped with an ethanol/dry ice trap and a 70 mTorr vacuum pump. The tubes were then either sealed under vacuum using an oxygen-enriched flame and incubated at 60°C - 85°C or placed unsealed in a glass beaker in a vacuum oven set at 60°C - 85°C (the temperature was dependent upon the experiment) for a period of 12 - 20 hours. The protein was reconstituted with 0.5 mL of dH_2O and analyzed by SDS-PAGE and FPLC and/or HPLC as described below.

4.2.7 Cytochrome c with Linear Homobifunctional Cross-linkers

Various samples were prepared such that mole ratios of protein to linear homobifunctional cross-linker **1** were 1:0, 1:0.1, 1:0.5, 1:1, 1:5, 1:10. Aliquots of a cytochrome c stock solution (4.1 mg/mL, 500 μL), adjusted to pH 10.0, along with aliquots of a stock solution of **1** (4.2 mg/mL dH_2O) ranging from 0 to 200 μL, were added to six

pyrex borosilicate glass disposable culture tubes (16 x 150 mm) such that each tube contained 2 mg cytochrome c and **1** according to the mole ratios listed above. The volume in each tube was adjusted to a total of 700 μL with dH_2O and the mixture was lyophilized overnight. The tubes were then placed in a beaker in a vacuum oven with the temperature set at 60°C for 20 hrs. An aliquot of dH_2O (500 μL) was added to each tube and mixed well prior to analysis by SDS-PAGE and separation by FPLC. The procedure was repeated using stock solutions of **2** (4.2 mg/mL) and cytochrome c (2.1 mg/mL). An additional set of tubes was also prepared using the same amounts of protein and cross-linker **2** with trehalose (16.5 mg/mL, 50 μL) added prior to volume adjustment with dH_2O.

4.2.8 Hemoglobin with Linear Homobifunctional Cross-linkers

Various samples were prepared such that mole ratios of protein to linear homobifunctional cross-linker **1** were 1:0, 1:0.2, 1:1, 1:2, 1:10, 1:20. Aliquots of a hemoglobin stock solution (4.4 mg/mL, 330 μL), adjusted to pH 10.5, along with aliquots of a stock solution of **1** (4.2 mg/mL dH_2O) ranging from 0 to 200 μL, were added to six pyrex borosilicate glass disposable culture tubes (16 x 150 mm) such that each tube contained 1.4 mg hemoglobin and **1** according to the mole ratios listed above. The volume in each tube was adjusted to a total of 530 μL with dH_2O and the mixture was lyophilized overnight. The tubes were then placed in a beaker in a vacuum oven with the temperature set at 60°C for 20 hrs. An aliquot of dH_2O (500 μL) was added to each tube and mixed well prior to analysis by SDS-PAGE and HPLC. The procedure was repeated using stock solutions of **2** (4.2 mg/mL) and hemoglobin (2.1 mg/mL). Two additional sets of tubes were also prepared, each using the same amounts of protein and cross-linkers **1** in one set and **2** in the other set

with trehalose (16.5 mg/mL, 50 μL) added to both sets prior to volume adjustment with dH_2O.

4.2.9 Hemoglobin with Branched Homotetrafunctional Cross-linker

Various samples were prepared such that mole ratios of protein to branched tetrafunctional cross-linker **3** were 1:0, 1:0.2, 1:0.5, 1:0.7, 1:1, 1:2, and 1:1.5. Aliquots of a hemoglobin stock solution (7.2 mg/mL, 700 μL), pH 5.8 (unadjusted from bottle), along with aliquots of a stock solution of **3** (1.6 mg/mL dH_2O) ranging from 0 to 350 μL, were added to six pyrex borosilicate glass disposable culture tubes (16 mm x 150 mm) such that each tube contained 5.0 mg hemoglobin and **1** according to the mole ratios listed above. The volume in each tube was adjusted to a total of 1,050 μL with dH_2O and the mixture was lyophilized overnight. The tubes were then flame-sealed under vacuum and placed in an oven at 65°C for 19 hrs. An aliquot of dH_2O (1,000 μL) was added to each tube and mixed well prior to analysis by SDS-PAGE and HPLC.

4.2.10 Reduction of Branched Homotetrafunctional Cross-linked Hemoglobin

Three samples were prepared such that mole ratios of protein to branched tetrafunctional cross-linker **3** were 1:0, 1:0.4 and 1:0.8. Aliquots of a hemoglobin stock solution (7.2 mg/mL, 700 μL), pH 5.8 (unadjusted from bottle), along with aliquots of a stock solution of **3** (1.6 mg/mL dH_2O) ranging from 0 to 350 μL, were added to six pyrex borosilicate glass disposable culture tubes (16 x 150 mm) such that each tube contained 5.9 mg hemoglobin and **1** according to the mole ratios listed above (2 sets of three samples). The volume in each tube was adjusted to a total of 850 μL with dH_2O and the mixture was lyophilized overnight. The tubes were then flame-sealed under vacuum and placed in an

oven at 67°C for 17.5 hrs. An aliquot of dH_2O (1,000 µL) was added to each tube and mixed well. The samples were split into two equal aliquots of 500 µL and to one of these aliquots of each sample, $NaBH_4$ (1.9 mg/mL, 300 µL) was added and thoroughly mixed and allowed to react for 10 minutes. Both sets of tubes were adjusted to a total volume of 1,000 µL with dH_2O. The samples were immediately analyzed by SDS-PAGE.

4.2.11 FPLC Separation of Cross-linked Proteins

Preparative separation and analysis of cross-linked proteins were performed by size exclusion at ambient temperature using a Pharmacia FPLC gel filtration system consisting of a Pharmacia HiLoad 16/10 Superdex 75 preparative grade column connected to a high precision pump P-500 fitted with a 200 µL sample injection loop controlled by a LCC-500 Plus liquid chromatography controller. All samples were manually loaded and peaks were detected by a Pharmacia Single Path Monitor UV-1 Optical Unit with a wavelength of 280 nm and recorded on a Dual Channel Chart Recorder REC-482. The eluant phosphate buffer pH 7.0 with 0.15 M NaCl prepared with distilled water, was filtered through 0.45 micron cellulose acetate by suction and degassed prior to use. Fractions were manually collected and dialyzed to remove buffer and salt as required (*viz.*, for activity and analytical assays) using Spectra/Por cellulose membrane dialysis tubing with a 3,500 molecular weight cut-off soaked in distilled water for one hour prior to use. Proteins were dialyzed against distilled water (unless otherwise stated) at 4°C for at least 24 hours with a minimum of four changes of dialysate during the dialysis period.

4.2.12 HPLC Analysis of Cross-linked Hemoglobin

Analytical reverse phase high performance liquid chromatography was conducted using a Varian Pro-Star quaternary pump equipped with either a 20 μL or 50 μL sample injection loop and a Vydac Protein C_4 column (5 μm, 4.6 x 250 mm). Peaks were detected by a sequential UV-vis detector set at a wavelength of 220 nm. The instrument was controlled and data was recorded by Varian Star Chromatography Workstation software version 5.3. Samples were manually injected and analyzed under ambient isobaric gradient elution conditions according to the procedure of Masala *et al.* (Masala *et al.*, 1994). The mobile phase was pumped at a flow rate of 1 mL/min in a linear gradient of two solutions: Solution A (ACN:H_2O:TFA, 60:40:0.1) and Solution B (ACN:H_2O:TFA, 20:80:0.1) as per the following program in Table 4.1:

Table 4.1: Gradient elution program for hemoglobin analysis by HPLC.

Time (min)	% Solution A	% Solution B
0	50	50
60	37	63
80	14	86
82	14	86
84	50	50

4.2.13 UV-VIS Absorbance Measurements

Absorbance measurements were carried out using a Gilford spectrophotometer. In solutions known to contain only one protein, a wavelength of 280 nm was used and calibration curves were constructed of no less than five points when quantitative measurements were required.

4.2.14 SDS-PAGE Analysis of Cross-linked Proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli (Laemmli, 1970) using a Bio-Rad Mini-PROTEAN II dual slab cell apparatus. All reagents and molecular weight markers were purchased from Bio-Rad or Sigma, and were electrophoresis grade quality (buffers were prepared as required using dH_2O). Bio-Rad Broad Range prestained markers composed of myosin (203,000 Da); β-galactosidase (115,000 Da); bovine serum albumin (93,000 Da); ovalbumin (48,200 Da); carbonic anhydrase (34,700 Da); soybean trypsin inhibitor (28,200 Da); lysozyme (21,100 Da); aprotinin (7,200 Da), were used for the hemoglobin cross-linking SDS-PAGE analyses. Approximately 5 - 10 μg (10 μL volume) was loaded into each of the wells of a 1.0 mm thick, 12% polyacrylamide gel and electrophoresis was carried out at a constant current of 20 - 25 mA/gel (with a voltage gradient of 70 - 150 mV) under discontinuous gel conditions. Protein bands were visualized by staining with Coomassie Brilliant Blue R250.

4.2.15 Reducing Sugar Analysis of Cross-linked Cytochrome c

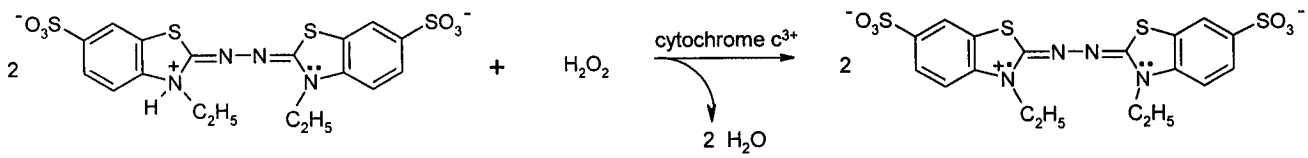
The amount of reducing sugar bound to cytochrome c was determined by the colorimetric method of Park and Johnson (Park *et al.*, 1949) after *in vacuo* reaction with sugar cross-linkers. Following FPLC purification, dialysis and lyophilization, selected

fractions containing monomer, dimer or trimer were reconstituted to 1.5 mL with dH_2O. The concentration of cytochrome c in each sample solution was determined by interpolating absorbance values at 280 nm on a calibration curve constructed from six standard solutions of cytochrome c ranging from 0.015 to 1.0 mg/mL. Aliquots of each fraction (1.0 mL) were transferred to test tubes (13 x 100 mm) to which $K_3Fe(CN)_6$ (0.5 g/L dH_2O, 1.0 mL) and a solution containing 5.3 g of Na_2CO_3 and 0.65 g KCN/L dH_2O (1.0 mL) were added. The test tubes were placed in a boiling water bath for 15 minutes, cooled to r.t., and a solution containing 1.5 g of $(NH_4)_2Fe(SO_4)_2 \cdot nH_2O$ and 1 g of SDS/L 0.05N H_2SO_4 (5 mL) was then added. After exactly 15 minutes, the absorbance was read at a wavelength of 690 nm. The reducing sugar content of a control sample of cytochrome c which had not been exposed to cross-linker was subtracted from that of the samples.

4.2.16 Cytochrome c Activity Assay

Cytochrome c–catalyzed oxidation of the substrate 2,2'-azino-di-[3-ethyl-benzothiazoline-(6)-sulfonic acid (ABTS) by various concentrations of hydrogen peroxide (Scheme 4.6) was measured according to the method of Radi *et al.* (Radi *et al.*, 1991). Aliquots of dH_2O (~1 mL) were added to various lyophilized FPLC fractions (collected as outlined above) of cytochome c exposed to **1** and **2** and subjected to the *in vacuo* cross-linking procedure (outlined above). The absorbance of each sample was read at 280 nm and compared to a standard calibration curve (comprised of six cytochrome c standards ranging from 6 μg/mL – 760 μg/mL) to determine the cytochrome c content. Based on the absorbance readings, dilutions were made as required such that the concentration of cytochrome c in each sample fell somewhere within a range of between 15 μg/mL and 60 μg/mL. Aliquots of 100 μL of ABTS (20 mg/mL of 0.067 M phosphate buffer pH 7.0) and

aliquots of H_2O_2 (6% wt/wt solution) ranging from 3 µL to 80 µL were added to each of 8 wells in a microplate. The volume of each well was adjusted to 200 µL with 0.067 M phosphate buffer pH 7.0 so that the concentrations of H_2O_2 were 24, 40, 64, 88, 104, 120 and 136 mM after addition of enzyme solution. An aliquot of cytochrome c sample enzyme solution (20 µL) was added to each well immediately prior to analysis by a microplate reader whereupon the increase in absorbance of the oxidized ABTS product, a green-coloured radical cation, was monitored at a wavelength of 405 nm. For comparison purposes, the absorbances were normalized according to a standard cytochrome c solution accurately prepared from the supplier bottle (0.18 µM) and oxidized ABTS product concentrations were determined using a molar extinction coefficient of 31.1 $mM^{-1} \cdot cm^{-1}$. The substrate ABTS is oxidized to a by hydrogen peroxide in the presence of cytochrome c. Lineweaver-Burk plots were prepared from each sample set of normalized curves to determine kinetic parameters.



Scheme 4.6: Cytochrome c activity assay.

4.2.17 Immobilization of Protein to Glass Beads

Small glass beads were activated by amination using the method of Stark and Holmberg (Stark *et al.*, 1989). Glass beads (5 µm) were transferred to a 100 mL round bottom flask to which a 10% solution of 3-aminopropyltrimethoxysilane in toluene (75 mL) was added. The mixture was left to reflux overnight and was then filtered by suction. The filtrate was discarded and the glass beads were washed thoroughly with toluene, then acetone

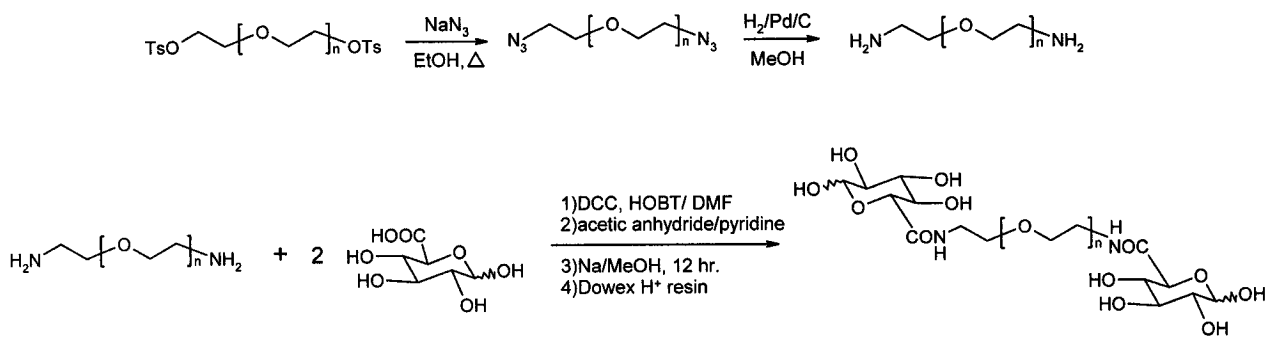
and finally dH_2O. After air-drying the aminated glass beads were stored at 4°C. Amination was confirmed by checking for fluorescence after exposure to dansylchloride for 10 minutes. To one of two Eppendorf tubes containing 27 mg of aminated glass beads each, a 200 µL aliquot of a solution containing ~80 µg of the dialyzed monomer fraction of cytochrome c exposed to **1** (mole ratio of 1:10) under *in vacuo* conditions in dH_2O, was added. The same amount of a solution of the dialyzed monomer fraction of cytochrome c exposed to no cross-linker but treated by the same *in vacuo* conditions was added to the second Eppendorf tube. Foil punctured with small holes was secured on the mouth of the tubes and the protein and beads were lyophilized to dryness in the tubes. The Eppendorf tubes were then placed in a glass beaker in a vacuum oven set at 46°C for 15 hours. To the beads in the Eppendorf, a 500 µL of a solution of $NaBH_3CN$ (1.5 mg/mL phosphate buffer pH 8) was added, swirled and left for 30 minutes. The beads were then filtered by suction over a glass fibre filter mat (2.4 cm) and rinsed several times with ~2 mL of phosphate buffer pH 7.0 containing 0.15 M NaCl, followed by several rinsings of ~50 mL dH_2O. The filter was allowed to air dry and the beads were then transferred to clean Eppendorf tubes. The cytochrome c activity of the beads was tested by adding 100 µL of ABTS (25 mg/mL) to the tubes along with 880 µL of 0.067 M phosphate buffer pH 7.0 and 10 µL of H_2O_2. The absorbance of the resulting solution was measured at 420 nm after 40 minutes.

4.3 Results and Discussion

4.3.1 Synthesis of Reducing Sugar Cross-Linkers:

Many different strategies were attempted in order to synthesize a bifunctional reducing sugar cross-linker by coupling the reducing sugar derivative, glucuronic acid, to an

oxygen-rich alkyl diamine spacer. Some interesting challenges worthy of note were encountered along the way. The first attempt involved a common amide coupling procedure with dicyclohexylcarbodiimide (DCC) or EDC, common carbodiimide coupling agents, in the presence of the auxiliary catalyst N-hydroxybenzotriazole (HOBt) in organic solvent (DMF) according to Scheme 4.7 below.



Scheme 4.7: Synthesis of a divalent glucuronic acid-based cross-linker

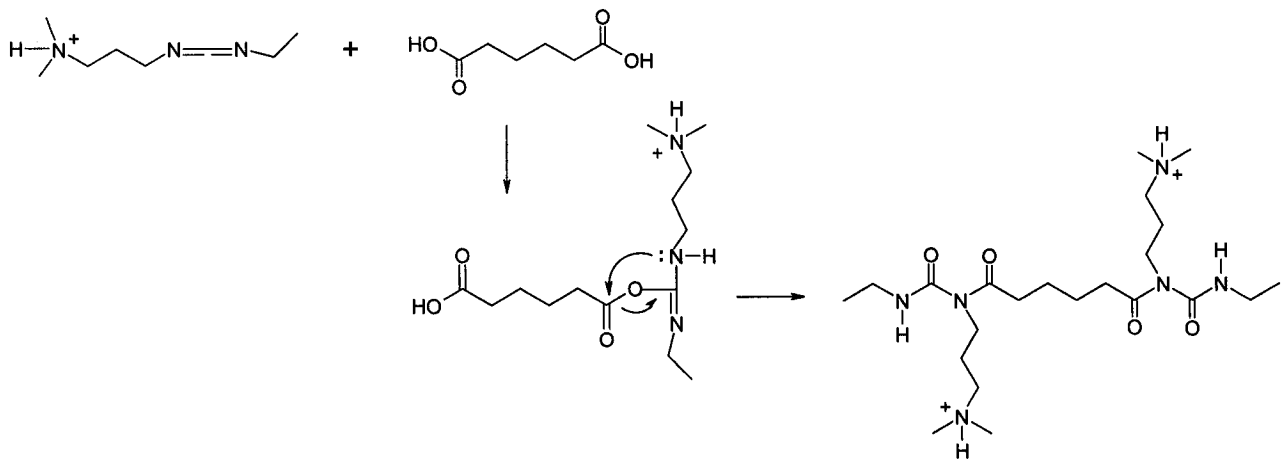
When the reaction was deemed complete by thin layer chromatography, the product was protected by acetylation of the hydroxyls (using acetic anhydride and pyridine) for purification by silica gel and then deprotected under Zemplen conditions to yield the desired product. However, the yield of >5% was surprisingly low. A search through the literature revealed that the probable cause for the poor yield was β-elimination of the labile hydrogen (attached to the tertiary carbon next to the carbonyl group) and the adjacent hydroxyl group which was induced by the presence of pyridine. In his investigation of the degradation of galactopyranuronic acid in the presence of acetic anhydride and various hindered, bulky bases, Tajima found high yields of three β–elimination products (Tajima, 1985).

Since a preliminary test to determine the cross-linking ability of the product showed promise, a simpler, more efficient method was sought by which to prepare the cross-linker. Under aqueous conditions, EDC proved ineffective, possibly due to unfavorable O $\rightarrow$ N migration from the unstable O-acylisourea intermediate to the more stable N-acylurea. In a similar EDC-mediated coupling attempt to form an amide product with 1,6-diaminohexane and hyaluronic acid, a linear polysaccharide containing repeating glucuronic acid units, Kuo *et al.* were also unsuccessful (Kuo *et al.*, 1991). In spite of using a large excess of diamine, they found evidence of an N-acylurea product that they attributed to the formation of an O-acylisourea intermediate possessing a structural conformation unfavourable to attack by the amine. The use of an excess of diamine was avoided in the reaction with glucuronic acid here in order to prevent competitive reaction with the aldehyde of glucuronic acid (acyclic form).

The coupling reagent HATU yielded the desired product **2**, which was easily purified by size exclusion chromatography. Again the yield was unusually low (~10-20%), but this was due in part by the intentional exclusion of a tertiary, hindered base, which is considered essential in preventing racemization and increasing the efficiency of the coupling by uronium-based reagents (Carpino *et al.*, 1994). For the purposes of this investigation, racemization was immaterial and it was considered important to prevent β–elimination for the sake of product purity since co-elution of the desired product and the β -eliminated product during purification would likely be unavoidable.

Another interesting problem arose during the purification of **1** by size exclusion chromatography when an impurity was detected by the presence of a terminal methyl group (δ = 1.1) in the ^{1}H-NMR spectrum of the purified, lyophilized glucosamine-based product.

At first, the presence of this compound was perplexing since a terminal methyl group could only come from EDC or the urea adduct thereof, and both of those compounds were easily separable from **1** on the size exclusion column. It is possible that formation of a bis-urea type of compound according to the mechanism proposed in Scheme 4.8 may have occurred under the conditions used here. With a molecular weight of 458 g/mole, such a compound would be expected to co-elute with **1**, which has a similar molecular weight of 468 g/mole.



Scheme 4.8: Mechanism for bis-urea adduct formation

Since any derivative of EDC bearing a terminal methyl group would be protonated under the neutral dH_2O eluant conditions used here (pH ~5.6), treatment of the product fraction with a cationic resin prior to lyophilization successfully eliminated the co-eluted contaminant.

4.3.2 Cross-Linking Cytochrome c:

Analyses by both size exclusion chromatography and SDS-PAGE showed that cross-linking of cytochrome c occurred with both compounds **1** and **2** and that the formation of

higher molecular weight oligomers was dependent on the cross-linker concentration. Under the reaction conditions used here, cross-linking appears to have been somewhat more efficient with cross-linker **2**.

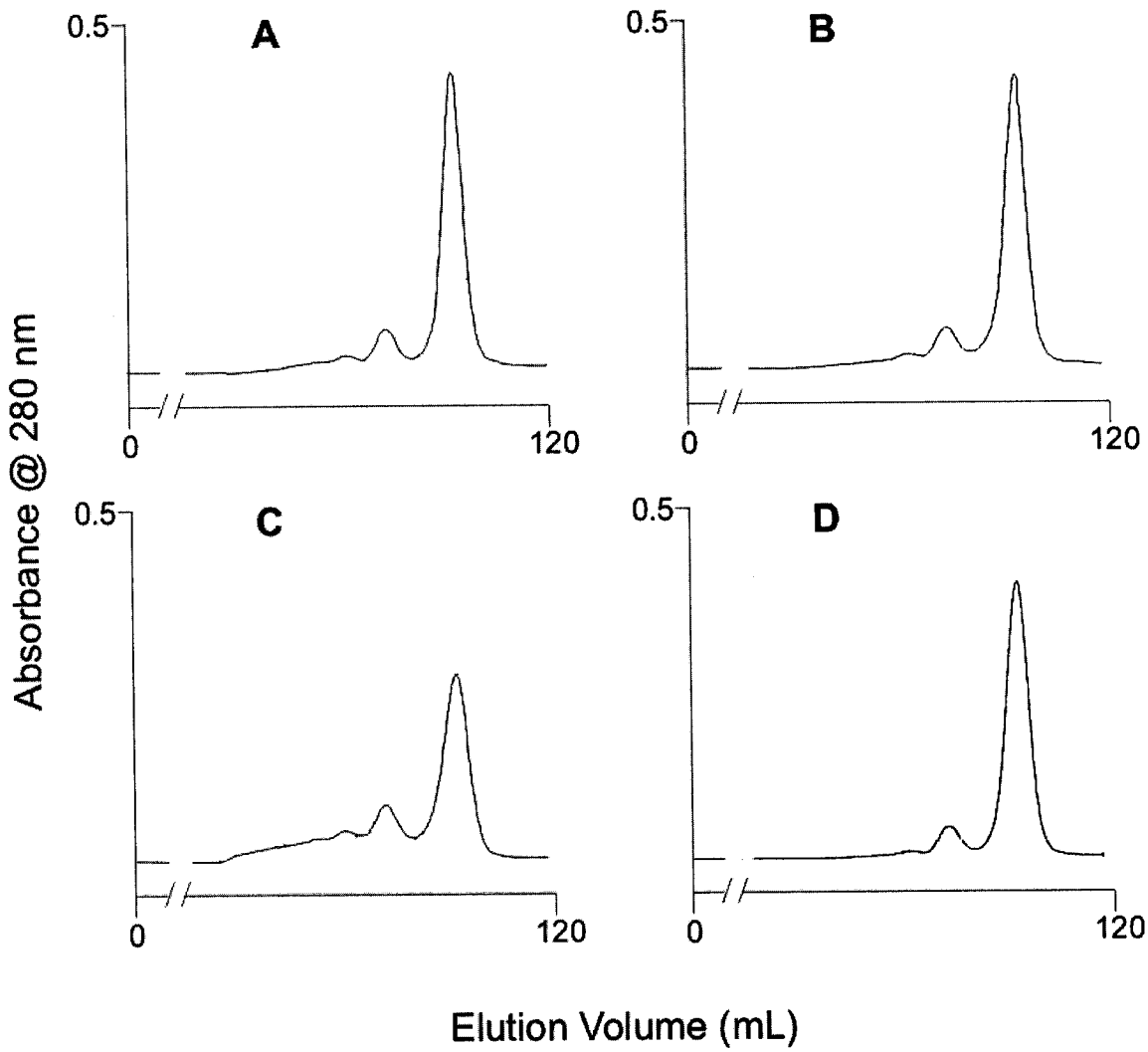


Figure 4.6: SEC traces showing the effect of increasing concentration of **1** on cytochrome c heated at 65°C for 18 hrs under reduced pressure.
Mole ratios of **1** to cytochrome c are as follows:
A – 0.5:1; **B** – 1:1; **C** – 5:1; **D** – 10:1.

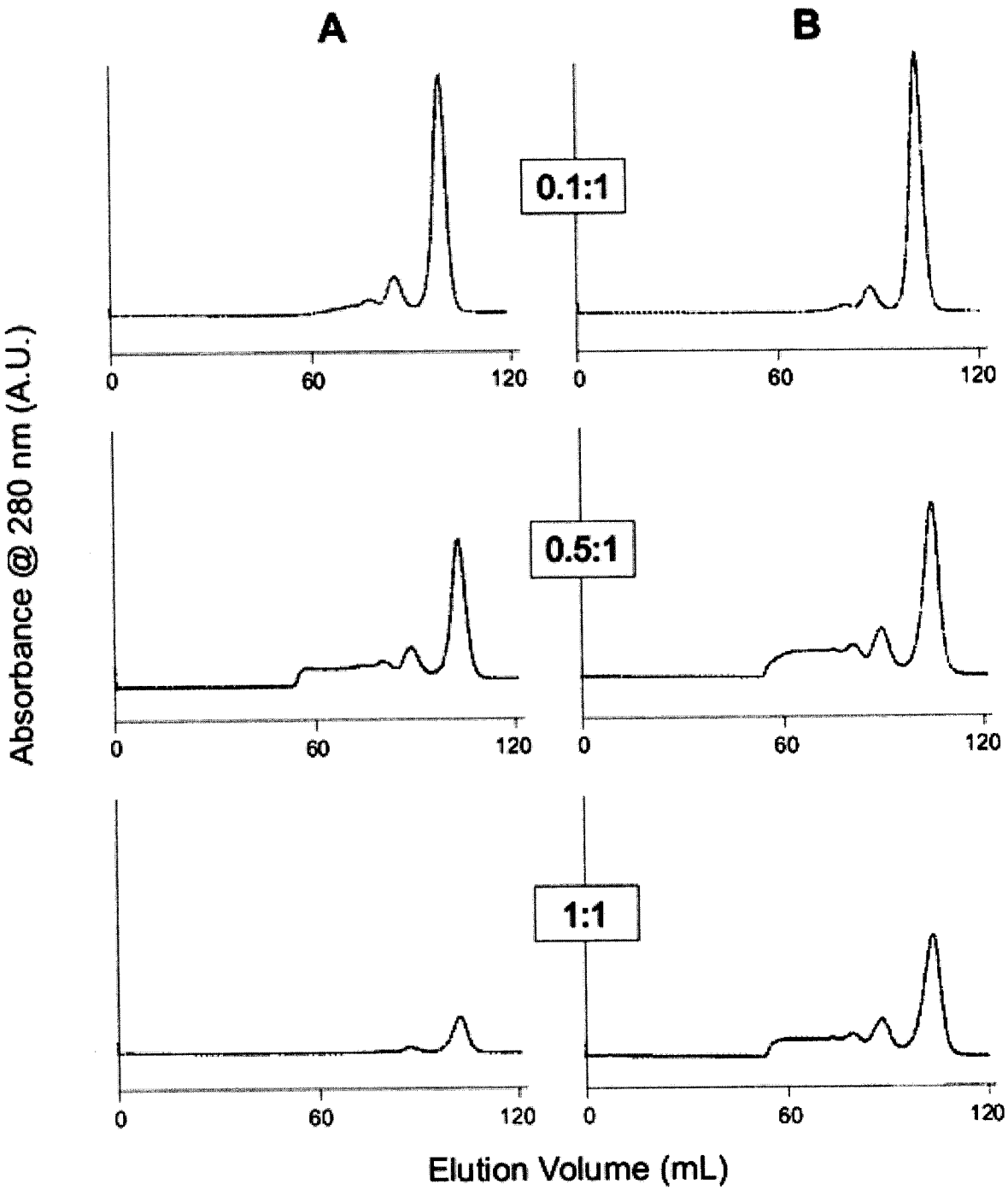


Figure 4.7: SEC traces showing effect of increasing concentration of **2** on cytochrome c heated at 65°C for 18 hrs under reduced pressure in the absence (**A**) and presence (**B**) of trehalose (mole ratio of **2** to cytochrome c is shown in the box between traces).

A comparison of the SEC traces in Figure 4.6 **C** and Figure 4.7 **A** shows that the concentration of cross-linker required to effect a similar yield of cross-linked product was lower for **2** by an order of magnitude – the appearance of a significant proportion of higher molecular weight oligomeric material relative to monomer occurred at a protein to cross-linker ratio of 0.5:1 for **2** and 5:1 for **1**. Figure 4.7 **A** shows that the extent of cross-linking with **2** increased to the point where there was very little soluble protein left in solution. A plot of the amount of soluble protein remaining in solution as a function of cross-linker (Figure 4.8) shows that the amount of soluble protein decreased linearly with increasing ratio of cross-linker to cytochrome c.

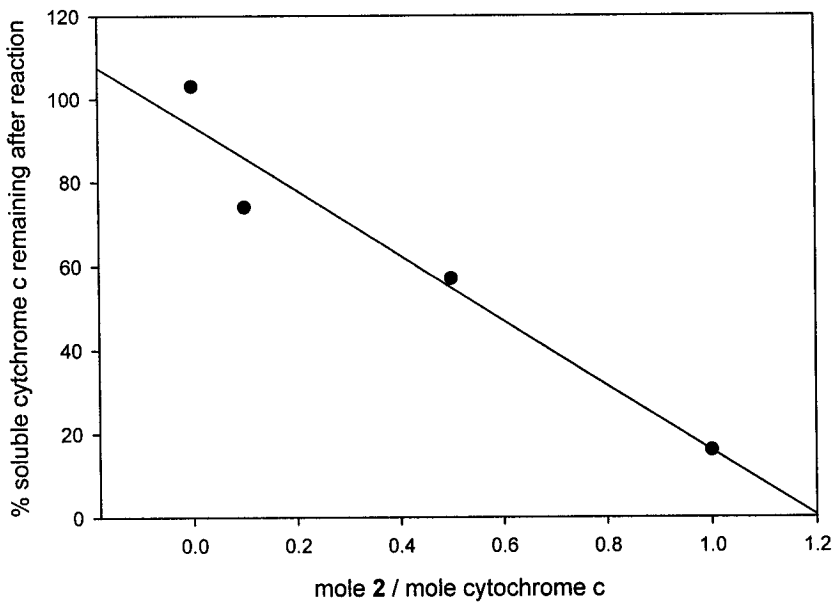


Figure 4.8: Plot showing decreasing solubility in cytochrome c after *in vacuo* reaction with linear cross-linker **2**
at 60°C for 20 hours and reconstitution in 500 μL of dH_2O, as a function of increasing amounts of cross-linker. Solubility was determined by measuring cytochrome c concentrations (interpolated from a standard plot) *via* absorbance readings at 280 nm.

A comparison of the soluble protein oligomers in the SEC traces of cytochrome c co-lyophilized with **2** in the presence and absence of trehalose in Figure 4.7 **A** and **B** shows that more cross-linking occurred with less cross-linker in the absence of trehalose. Addition of the excipient trehalose had a considerable effect on the extent of cross-linking. Trehalose appears to have acted as a solid phase diluent, limiting the interactions between reactive functional groups in the protein and sugars, resulting in an increased amount of smaller, soluble, lower molecular weight oligomers. There appeared to be a threshold ratio whereby trehalose was no longer effective at limiting the formation of insoluble, higher molecular weight cross-linked protein. The ratio at which no protein remained in solution was ~1.2:1 cross-linker **2** to cytochrome c, and in the presence of trehalose that ratio rose to ~5:1. Interestingly, the precipitated protein, which was formed in the presence of trehalose had more of a mesh-like consistency and appearance as compared to that formed in the absence of trehalose, which was more agglomerated and gel-like (Figure 4.9).

Figure 4.6 **D** shows a rather unexpected result in which very little cross-linking occurred when a concentration of cross-linker **1** ten times that of cytochrome c was used. It appears that an upper limit to cross-linking had been reached with cross-linker **1** that was either not reached or not apparent with cross-linker **2**. The decreased retention time and the comparatively broad monomer peak in this SEC trace were suggestive of either intramolecular cross-linking or attachment of the cross-linker by only one sugar, with some sort of 'auto-excipient' effect preventing cross-linking from occurring at the other sugar end. The results of a reducing sugar test of some of the collected SEC fractions shown in Table 4.2 confirmed that the latter had most likely occurred.

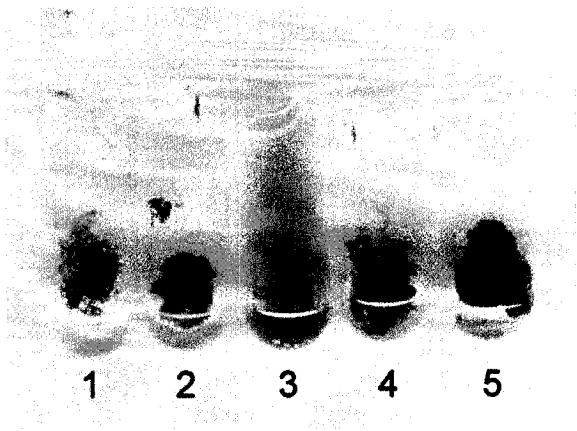


Figure 4.9: Cytochrome c co-lyophilized with varying amounts of linear cross-linker **2** in the presence and absence of trehalose, and heated under vacuum at 60°C for 20 hours.
Mole ratios of cytochrome c to cross-linker **2** are as follows: **1**) 1:10 with trehalose (0.4 mg/mg Cyt c); **2**) 1:5 with trehalose (0.4 mg/mg Cyt c); **3**) 1:0.1; **4**) 1:10; and **5**) 1:5.

While all of the other monomer fractions that were collected from samples having different mole ratios of cross-linker to cytochrome c had reducing sugar contents of between 0 and 18 μg/mg cytochrome c, that of the monomer fraction collected from the 10:1 mole ratio sample was significantly higher at 57 μg/mg cytochrome c.

Table 4.2: Reducing sugar content of cross-linked oligomers of cytochrome c

Cross-linker	Moles cross-linker/ mole cytochrome c	FPLC Fraction	μg Reducing sugar/ mg cytochrome c*
1	1	monomer	17
1	1	dimer	105
1	10	monomer	57
1	10	dimer	15
1	10	trimer	18
2	0.1	monomer	15
2	0.5	monomer	17
2	0.5	dimer	435
2	1	monomer	0

*- results are ~ ±15 μg/mg cytochrome c (determined by Park – Johnson assay)

The presence of tethered, free, antennary reducing sugars on the glycation-modified cytochrome c monomer pointed to the possibility of immobilizing the protein to aminated glass beads by further *in vacuo* glycation reaction. Indeed, a strongly positive test for activity with an absorbance of 0.397 indicated the presence of immobilized glycation-modified cytochrome c on aminated glass beads that had been subjected to such an immobilization attempt. However, since the non-glycated control monomer also showed high activity with an absorbance of 0.387, it was inconclusive as to the extent to which immobilization had occurred by the *in vacuo* reaction between the free reducing sugar termini and the amine groups on the glass beads. The fact that the non-glycated control

monomer had also been immobilized with retention of activity was in itself a significant result, demonstrating that immobilization may be a potential application of the dehydrothermal *in vacuo* reaction previously reported by Simons *et al.* (Simons *et al.*, 2002).

A quantitative assay was carried out to investigate the effects of cross-linking cytochrome c with **1** by the *in vacuo* glycation method on enzymatic activity. A comparison of the activities of one dimer fraction and various monomer fractions from selected samples of the enzyme that had been subjected to *in vacuo* cross-linking experiments is shown in the Lineweaver-Burk plots of Figure 4.10. Calculated activity parameters based on these plots, which were normalized against the standard control plot for comparison purposes, are tabulated in Table 4.3. The linearity of the plots show that all of the cytochrome c species tested followed Michaelis-Menten kinetics for H_2O_2, and the Michaelis constant, K_m, for the standard control was determined to be 75 mM, which is in good agreement with the literature value of 64 mM reported by Radi *et al.* (Radi *et al.*, 1991).

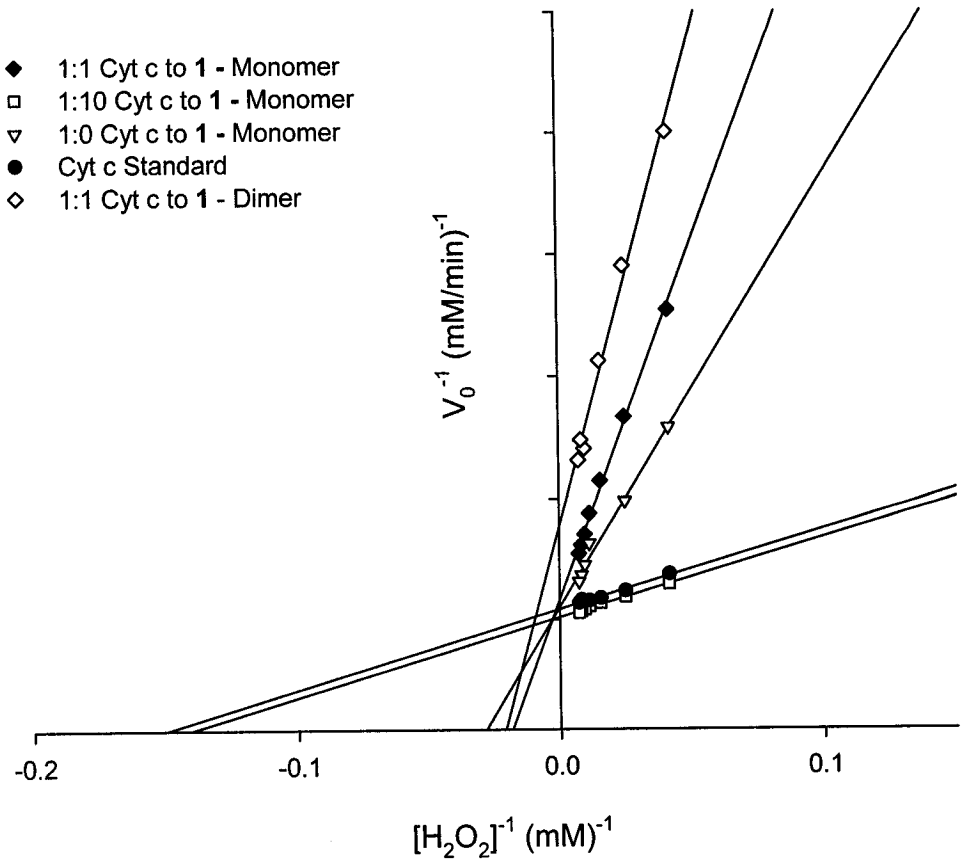


Figure 4.10: Lineweaver-Burk plots of FPLC collected fractions of cytochrome c co-lyophilized at LpH 10 and heated with **1** under *in vacuo* conditions.

Since the K_m is a constant for each enzyme, the higher K_m values for the monomers from cytochrome c samples that had undergone *in vacuo* glycation indicates that the enzyme suffered some damage to the tertiary structure resulting in altered substrate binding ability. This is also reflected in the V_{MAX}/K_m values that, with the exception of the 1:10 ratio (cytochrome c to **1**) monomer, were all lower than the standard control.

It is possible that the pH of 10 that was used in the cross-linking experiments caused denaturation of the enzyme to some extent. Indeed, a reduction in the V_{MAX}/K_m by an order of magnitude observed with the dimer is not surprising since further restricting

conformational movement by the introduction of a cross-link would have the expected effect of impairing the activity of the denatured enzyme even further.

Table 4.3: Comparison of enzyme kinetics of cytochrome c (LpH 10.0) after *in vacuo* cross-linking with **1** calculated from Lineweaver-Burk plots

Sample (Mole ratio Cyt c: 1)	V_{MAX} (mM/min)	K_m (mM)	V_{MAX}/K_m (min^{-1})
Cytochrome c (standard)	5.7×10^{-3}	75	7.6×10^{-5}
1:0 monomer	4.7×10^{-3}	328	1.4×10^{-5}
1:1 monomer	5.5×10^{-3}	342	3.0×10^{-5}
1:10 monomer	2.3×10^{-3}	300	7.7×10^{-5}
1:1 dimer	6.4×10^{-4}	102	6.3×10^{-6}

The reason for the increased V_{max} with the 1:10 monomer that resulted in an increased specificity constant comparable to the standard control sample is unclear. The presence of the covalently bound hydroxyl-rich compound **1** apparently affected the V_{MAX}/K_m of cytochrome c but did not significantly affect the K_m compared to the other monomers. Many studies have been reported in the literature showing that glycated proteins exhibit increased thermal stability with a concomitant slight decrease in activity (Venkatesh *et al.*, 1998; Aoki *et al.*, 1999; Kazan *et al.*, 1997; Sundaram *et al.*, 1998). Baek *et al.* suggest that the reason for the decrease in activity is caused by the conformational change induced by the chemical modification on surface residues (Baek *et al.*, 1997). By the same logic, one can argue that chemical modification done after denaturation has occurred may bring about beneficial conformational changes through hydrogen bonding networks by a compound such as **1** that is

rich in hydroxyl groups, resulting in a compensatory improvement in the V_{MAX}/K_m. However, it is expected that the binding site would be somewhat hindered by the presence of the bulky sugar modifier, and the larger K_m calculated for the glycation-modified monomer of cytochrome c may be evidence of this.

4.3.3 Cross-linking Hemoglobin

All three cross-linkers, **1**, **2** and **3**, were effective in cross-linking hemoglobin under the *in vacuo* conditions used here and the reactivity of the two linear bifunctional cross-linkers, **1** and **2**, toward hemoglobin appeared to be comparable to cytochrome c. Since sample preparation conditions for SDS-PAGE analysis interferes with the non-covalent interactions that bind hemoglobin as αβ-dimers in solution (see Chapter 1), hemoglobin dimers appear as monomers while cross-linked tetramers appear as dimers in the following gels. In the absence of trehalose, cross-linking of hemoglobin with **1** was very extensive at the lower concentrations used; Lanes 2 and 3 of the gel in Figure 4.11 **D** show that very little monomer or soluble oligomers was present. However, as the concentration of **1** was increased, an 'auto-excipient' effect similar to that observed with cytochrome c (10:1 ratio of **1** to cytochrome c) could be seen with hemoglobin.

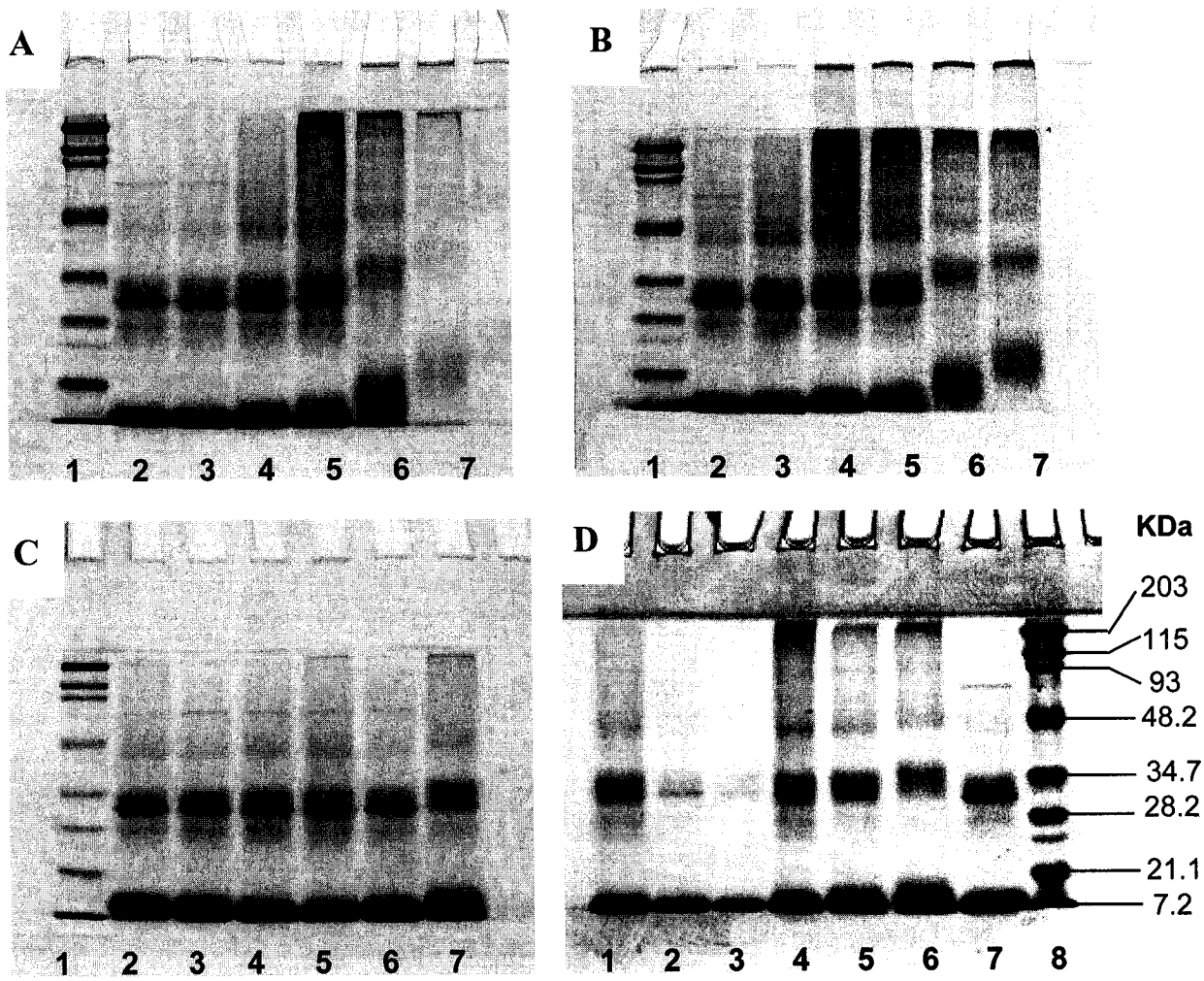


Figure 4.11: SDS-PAGE of human hemoglobin (Hb) co-lyophilized with increasing concentrations of linear homobifunctional cross-linkers at pH 10.0: **A** - **2** with trehalose; **B** - **2** without trehalose (0.6 mg/mg Hb); **C** - **1** with trehalose **D** - **1** without trehalose; all were heated at 60°C for 20 hours under vacuum. Concentrations in each lane expressed as moles cross-linker/mole Hb: For gels **A**, **B**, and **C**: Lane 1- MW markers; Lane 2 – 0; Lane 3 – 0.2; Lane 4 – 1; Lane 5 – 2; Lane 6 – 10; Lane 7 – 20; For gel **D**: Lane 1 – 0.2; Lane 2 - 1; Lane 4 - 2; Lane 5 – 10; Lane 6 – 20; Lane 7 – 10 lyophilized but not heated; Lane 8: - MW markers

Lanes 4 to 6 in Figure 4.11 **D** show an increase in the amount of monomer, and the smeared, wider monomer band indicates modification of hemoglobin by the cross-linker also occurred under the reaction conditions used here. The solubility of hemoglobin also increased with increasing mole ratios of **1** to hemoglobin greater than 2:1. Interestingly, Figure 4.11 **C** shows that in the presence of trehalose, reaction with **1** yielded mainly cross-linked tetramer (appearing as dimer in the gel) with some evidence of modified monomer.

Higher molecular weight oligomers of hemoglobin were observed with reaction by increasing concentration of linear bifunctional cross-linker **2**. Figure 4.11 **B** shows that mole ratios exceeding 1:1 of cross-linker **2** to hemoglobin resulted in extensive cross-linking, producing insoluble aggregates as well as soluble oligomers whose migration through the gel was impeded by molecular weights in excess of 200 kDa, and can be seen lining the wells of the gel in Lanes 4 to 7. Lanes 4 and 5 in Figure 4.11 **B** show that compared to **1** (Figure 4.11 **D**), *in vacuo* reaction with **2** in the absence of trehalose, gave rise to a greater distribution of cross-linked and modified products, including tetramers, oligomers and higher molecular weight polymers of hemoglobin. Reaction of hemoglobin with **2** in the presence of trehalose resulted in less highly polymerized hemoglobin, more soluble oligomers, and more modified monomers and tetramers (Figure 4.11 **A**). Upon reconstitution with water, the viscosity of this set of sample solutions increased with increasing cross-linker concentration to an actual gel at the highest concentration used (i.e., 20:1 mole ratio of **2** to hemoglobin).

Lane 2 of the gel in Figure 4.12 shows that cross-linking by the tetrafunctional branched cross-linker **3** at a ratio of 0.2 moles per mole of hemoglobin produced a significant amount of soluble cross-linked tetramer and interconnected tetramers which were also modified along with hemoglobin monomer. Samples with cross-linker concentrations greater

than this resulted in the formation of higher molecular weight polymers that remained in the wells (Figure 4.12 - Lanes 3 to 7).

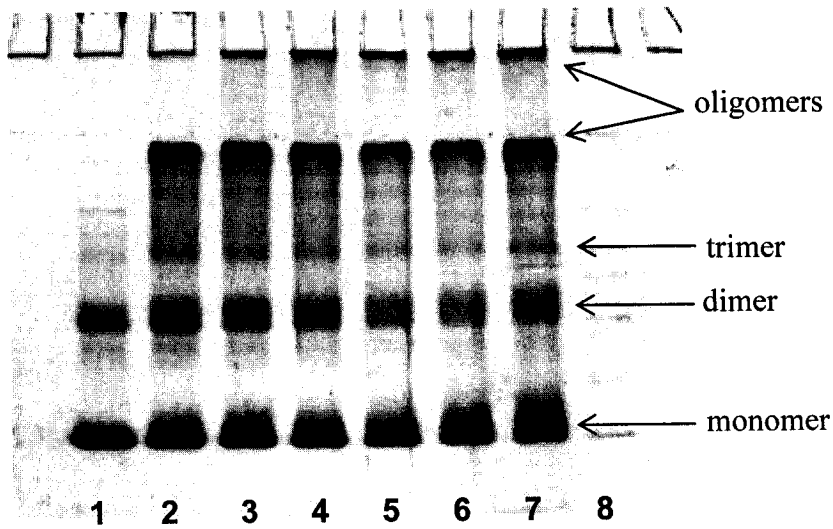


Figure 4.12: SDS- PAGE showing cross-linking between human hemoglobin and the branched cross-linker **3** co-lyophilized at LpH 5.8 (unadjusted) and heated under vacuum at 65°C for 19 hours.
Concentrations in each lane expressed as moles **3**/mole Hb: Lane 1 - 0; Lane 2 – 0.2; Lane 3 – 0.5; Lane 4 – 0.7; Lane 5 – 1.1; Lane 6 – 1.2; Lane 7 – 1.5; Lane 8 – MW markers.

Further analysis of the hemoglobin products by reversed-phase HPLC was carried out to determine the nature of cross-linking. The elution conditions used here (Table 4.1) cause the heme to separate and the globins to dissociate. Analysis of these components was performed by means of a lipophilic C_4 column in which the hemoglobin components elute on the basis of hydrophobicity, whereby the least hydrophobic heme elutes first followed by the β-chains and then the α-chains. The chromatograms typically showed that a decrease in the size of the β-chain peaks at ~28 min. was accompanied by a concomitant increase in a broad peak at a

time of ~70 min as the concentration of cross-linker used was increased, indicating that cross-linking with **3** predominantly involved β-chains (Figure 4.13 **D**).

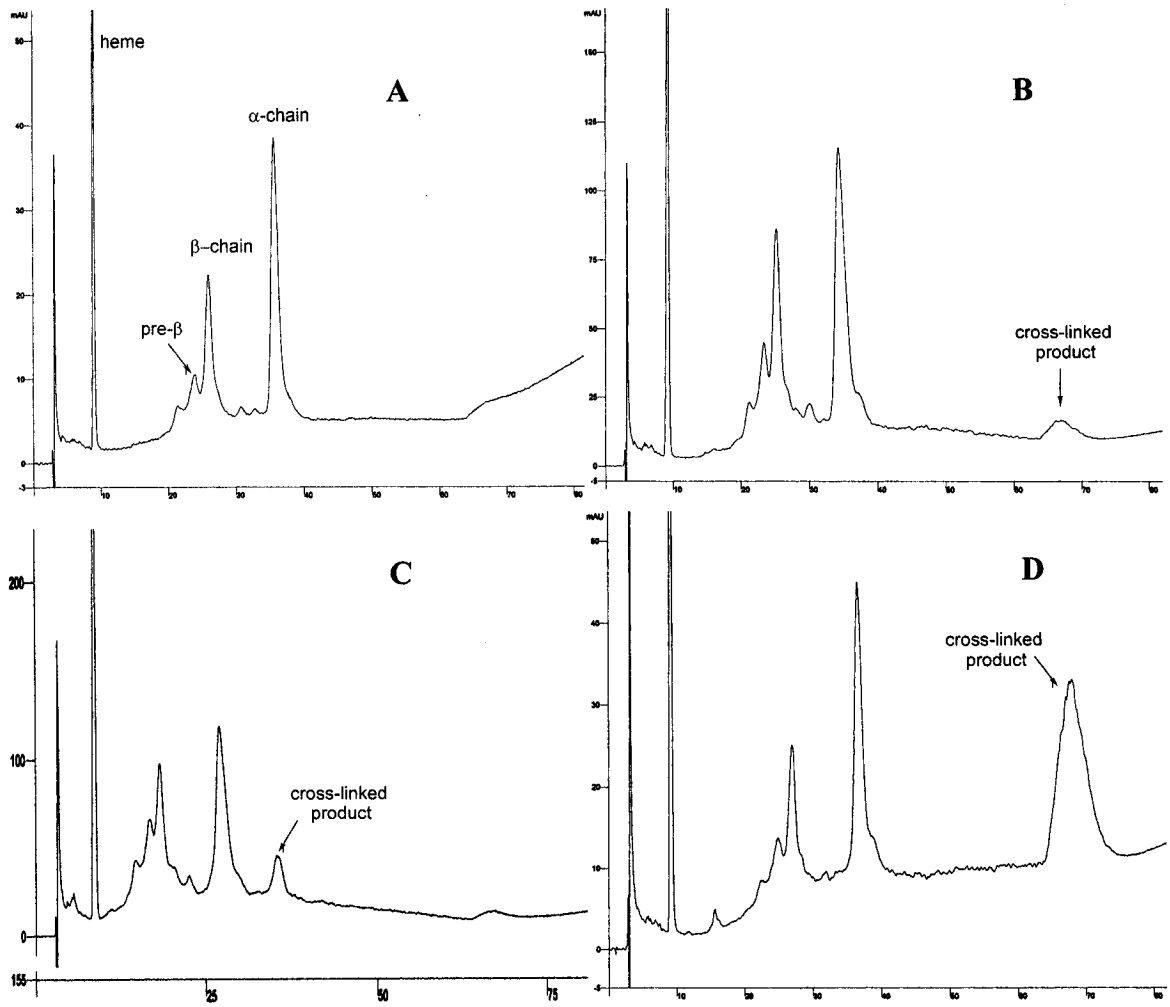


Figure 4.13: Separation of globin chains by HPLC.
Traces show the effects of heating human hemoglobin (LpH 10.0) under reduced pressure in the presence of trehalose and branched cross-linker **3**. **A** – hemoglobin unheated; **B** – hemoglobin heated at 65°C for 18 hrs; **C** – hemoglobin with trehalose (2:1 wt/wt) heated at 65°C for 18 hrs; **D** – hemoglobin with **3** (40:1 wt/wt) heated at 65°C for 18 hrs

In fact, the peaks in the chromatograms typical of the HPLC analyses done on cross-linked hemoglobin here were consistent with (almost identical to) one published recently by Kluger *et al.* in a similar analysis of higher molecular weight oligomers produced by their DBIT cross-linker (Figure 4.2 **B**) (Kluger *et al.*, 1999). They identified the product responsible for the new peak at ~70 min. as a bis-tetramer cross-linked through the α-amino group (N- terminus) of β_1-Val-1 and the ε-amino group of β_2-Lys-82. Although the exact cross-links in the products obtained here have yet to be confirmed by digestion followed by reversed-phase C_{18} HPLC analysis, the evidence obtained to date suggests that the cross-linked product is very similar. Based on Kluger's model of cross-linked hemoglobin bis-tetramers, the schematic representation is proposed in Figure 4.14 to depict the products obtained by *in vacuo* reaction of hemoglobin with the branched, symmetrical, tetrafunctional reducing sugar-based compound **3** designed and synthesized here.

The chromatogram in Figure 4.13 **B** shows that a small amount of cross-linked product having a similar retention time to the cross-linked product observed with **3.** Since no cross-linker was present, this product was formed as a result of a dehydrothermal *in vacuo* proton transfer reaction between ammonium and carboxylate groups (Simons *et al.*, 2002). Interestingly, in the presence of trehalose under the same reaction conditions, a different, less hydrophobic cross-linked product is observed at ~35 minutes suggesting that hydrogen bonding of the non-reducing disaccharide affects the conformational structure of hemoglobin resulting in a favorable interaction between different ammonium and carboxylate groups. It is unclear as to which chains are involved in either of these cross-links without further testing.

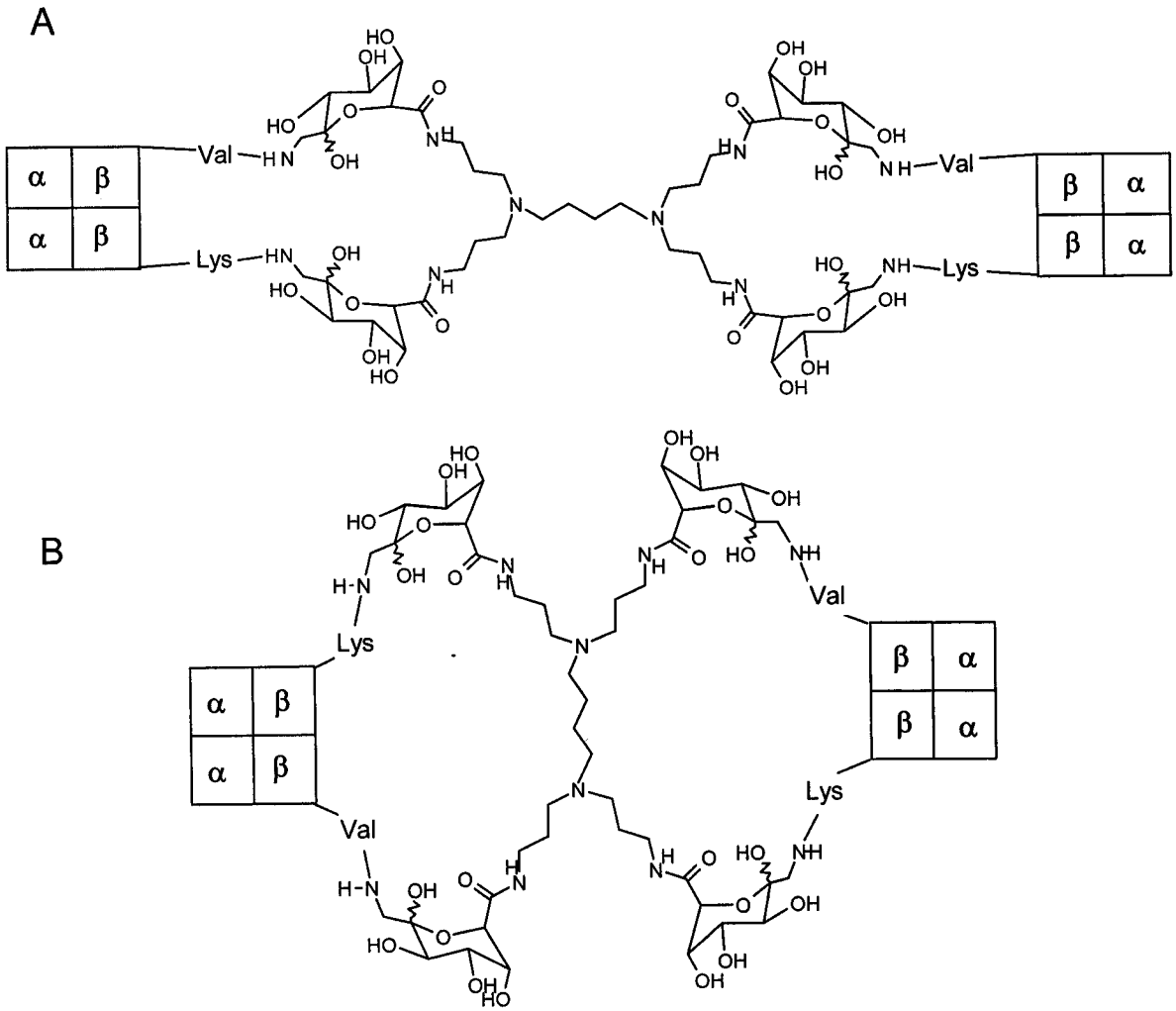


Figure 4.14: Schematic representations of possible cross-linked bis-tetramer configurations formed by *in vacuo* reaction of hemoglobin with **3**.

At a temperature of 82°C, the dehydrothermal reaction was highly favoured and resulted in the formation of an extensively cross-linked high molecular weight hemoglobin product that was insoluble even with exposure to chaotropic agents such as 1M $MgCl_2$ and 8M urea. It is obvious from these results that there is an upper temperature limit in which the *in vacuo* reaction with the reducing sugar cross-linkers is practical.

SDS-PAGE analysis to assess the necessity for reducing the ketoamine product by sodium borohydride (not shown) revealed no difference between the products exposed to the reducing agent and those not exposed, aside from the expected small amount of protein degradation seen with the exposed products (Acharya *et al.*, 1988; Means *et al.*, 1971, p151). The cross-linked products were very stable and did not break down after several days in aqueous solution. This observation suggests that the equilibrium between the acyclic and cyclic ketoamine in solution heavily favours the cyclic form in solution and the kinetics of reversibility are slow. This may be due to the stability introduced by the formation of an intricate network of hydrogen bonds from interaction of nearby side chains with the hydroxyls and the ring oxygen of the sugar. A similar type of network is responsible for the agglutination of multivalent carbohydrate compounds and carbohydrate-specific proteins known as lectins and is illustrated below in Figure 4.15.

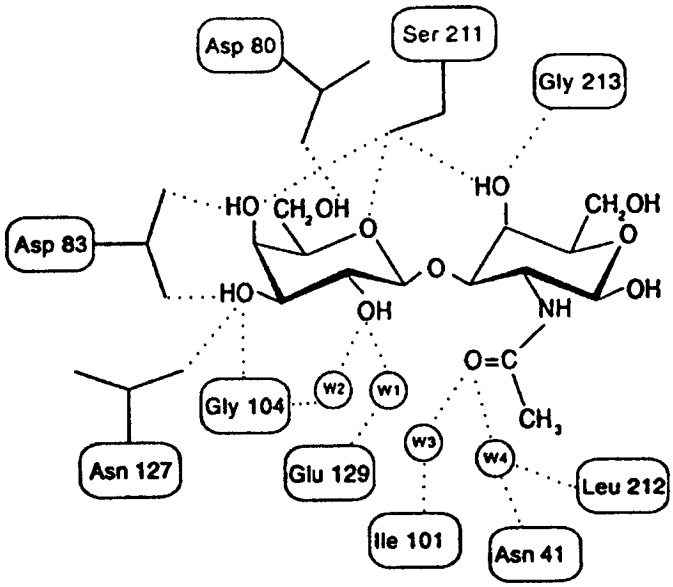


Figure 4.15: Schematic representation of the binding of Gal(β1-3)Gal NAc to peanut lectin *via* a network of hydrogen bonds between the sugars and the lectin residues (Lis *et al.*, 1998).

4.3.4 Mechanistic Considerations

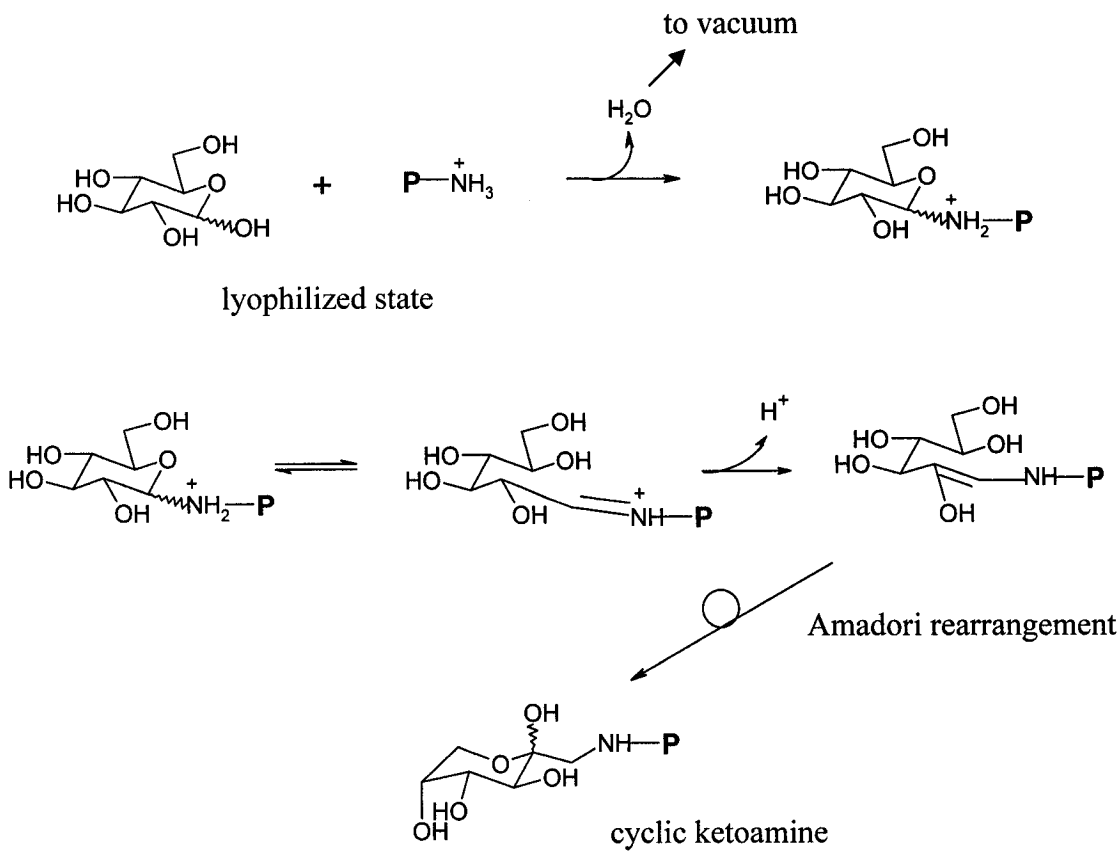
There are three differences between the two linear bifunctional cross-linkers that may influence cross-linking propensity to varying degrees: length of spacer, type of spacer (hydrophobic in **1** *vs.* hydrophilic in **2**) and spatial orientation of the reactive functional group within each of the sugar moieties (glucosamine in **1** and glucuronic acid in **2**). Both optimum spacer length and hydrophobicity are dependant upon the conformation of the protein, while the reactivity of the cross-linker toward the protein is determined by the aldehyde functionality of the sugar moiety. Intramolecular hydrogen bonding between the anomeric hydroxyl and the amide group of the glucosamine derivative **1** is favoured by the geometry of the cross-linker and may influence its reactivity towards amino groups in the protein.

Conversely, the orientation of the anomeric hydroxyl of cross-linker **2** is not conducive to any intramolecular interactions and as such, is more available for intermolecular interactions.

In solution, the acyclic form of the reducing sugar reacts reversibly with an amine group and proceeds very slowly in the absence of a reducing agent. Since the kinetics of the reaction are determined by the equilibrium between the dominant cyclic form and the reactive acyclic form, it is unlikely that the spatial orientation of the anomeric hydroxyl would influence the rate and therefore the extent of the reaction. In the more restrictive environment of the lyophilized state, there is little conformational flexibility and no dynamic equilibrium thus rendering and the spatial orientation of the functional groups and ionization state of both reactants fixed. Initially, it was assumed that Schiff base formation between the protein and cross-linker occurred in solution prior to lyophilization and that more reactive acyclic sugar was made available according to Le Chatelier's principle as the product water was drawn off under vacuum. If this were indeed the case, the presence of excipients would not be expected to influence the extent of cross-linking since the reaction would occur predominantly as a result of solution dynamics. However, the effect of the excipient trehalose on the extent of cross-linking with either bifunctional cross-linker was quite significant and can be seen with both cytochrome c and hemoglobin in Figure 4.7 and Figure 4.11, respectively. Furthermore, in a product analysis as part of a study on *in vacuo* protein glycation with ^{13}C-enriched glucose and ribonuclease, Nicolas Stewart (Stewart, 2002) found a single ketoamine product only after the heating stage of the glycation procedure; there was no product detected after lyophilization. These results suggest that the glycation reaction takes place in the dry state and not in solution, as was initially thought and the mechanism is clearly different under *in vacuo* conditions. Another peculiarity of the *in vacuo* glycation

reaction is that glycation is extensive at LpH values where most of the amino groups are protonated (i.e., pH 5 to pH 9.0) (Stewart, 2002). Indeed, extensive cross-linking at LpH 5.8 occurred with hemoglobin in the presence of the tetrafunctional cross-linker **3** and is shown in Figure 4.12.

The general mechanism depicted in Scheme 4.9 is proposed here to explain these results. It is possible that during lyophilization, the amino groups of the protein and the anomeric hydroxyl group of the sugar moiety orient themselves through hydrogen bonding between the hydrogens of the protonated amino group of the protein and the sugar ring oxygen and anomeric hydroxyl group. The next stage involving heating the lyophilized mixture while under reduced pressure would provide the energy required to facilitate protonation of the anomeric hydroxyl by the proximal protein ammonium group thus generating water, a good leaving group which would be removed under vacuum, as well as a nucleophilic amine group. Alternatively, formation of the cyclic carboxonium cation upon the release of water and just prior to attack by the amine group, would require conversion of the sugar ring to a half-chair geometry in order to accommodate the sp^2 electron configuration of the anomeric carbon. This is the currently accepted mechanism for the hydrolysis of most glycopyranosides (Collins *et al.*, 1995, p.72).



Scheme 4.9: Proposed mechanism of *in vacuo* glycation of proteins.

4.4 Conclusions

Overall, the results of these preliminary studies show that protein cross-linking can be achieved with bifunctional and tetrafunctional reducing sugar reagents under *in vacuo* reaction conditions. However, many of the experiments that were designed to elucidate the nature of the cross-linked products raised more questions than were answered and point to the need for further work. The promising results obtained, especially with hemoglobin, demonstrate the merit of the method and provide justification for future work towards

optimization of the method parameters, as well as further development and investigation of analogous cross-linkers with different proteins.

Although the focus of this research was to investigate cross-linking as an application of the *in vacuo* glycation reaction previously developed by this research group, the use of the reducing sugar cross-linkers designed in this research endeavor is not limited to these reaction conditions. Such reagents can conceivably be used to effect the conjugation of proteins (or other amine-bearing compounds) under reductive amination conditions in solution (Roy *et al.*, 1984), if necessary. However there are several advantages to the *in vacuo* reaction method; 1) less reagent and protein are required; 2) proteins cross-link in their most natural conformation thus reducing conformational distortion and strain from the cross-link; 3) the cross-linker is not expected to be toxic; 4) no solvents or other reagents are required; 5) the protein can be lyophilized with the cross-linker at a pH value where it retains its native structure and biological activity; 6) extremely mild reaction conditions are conducive to sensitive proteins; and 7) the extent of cross-linking can be controlled either by adjusting the protein/cross-linker ratio or by the addition of excipients.

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Chapter 5 Preparation of Neoglycoconjugates by Glycation

5.1 Introduction

The many potential roles of carbohydrates covalently attached to proteins are of interest both for practical applications and theoretical structure-function studies. Naturally occurring glycoproteins are involved in a wide variety of interactions of physiological importance including inflammatory and immune responses and cellular communication. A great deal of medicinal chemistry research is dedicated to the development of efficient methods for chemically attaching carbohydrates to proteins for the production of neoglycoproteins suitable for use as vaccines (Wong, 1995; Roy, 1996) and antiviral agents (Mammen *et al.*, 1998; Maeder, 2002). Carbohydrate-protein interactions are known to be weak, with typically high dissociation constants (Mammen *et al.*, 1998). By increasing the number of carbohydrates on a single conjugate molecule, thereby engendering multivalency, the average free energy of the interactions can be amplified. In this way, multivalent neoglycoproteins are immunogenic and as such, are candidates for synthetic vaccines while the high valencies of glycodendrimers render them effective as clustering agents for the prevention of many pathogenic diseases (Roy, 1996).

Covalent attachment of sugars to proteins is commonly achieved by enzymatic glycosylation or by chemical methods such as reductive amination (Roy *et al.*, 1984), (Kamicker *et al.*, 1977) and others reviewed by Stowell and Lee (Stowell *et al.*, 1980). In spite of the fact that the nature of the protein-carbohydrate linkage can be designed with existing available chemical methods, one shortcoming is that these methods often result in

mixtures of products. In an attempt to circumvent this problem, Davis *et al.* developed a method which combines site-directed mutagenesis to introduce a cysteine at a preselected site on the protein scaffold with chemical methods to attach a sugar moiety *via* the thiol thus provided (Davis *et al.*, 1998).

Protein glycation, which is the least efficient chemical method, is a direct, reversible chemical reaction between a reducing sugar and a protein and is often carried out under aqueous conditions where the reaction is unfavorable due to the fact that water is a product. Current aqueous glycation methods require relatively large amounts of protein and carbohydrate (Yeboah *et al.*, 1999; Roy *et al.*, 1984), and the extent of glycation is very difficult to control (Wrodnigg *et al.*, 2001; Yaylayan *et al.*, 1994). Another significant drawback is that the desired glycation product, cyclic ketoamine, may be contaminated with advanced glycation products (Wrodnigg *et al.*, 2001; Yaylayan *et al.*, 1994).

An effective method of increasing the efficiency of a reaction is by the removal of a product. The first step in the glycation of an amino group is believed to be the reaction of a deprotonated amine with the aldehyde group of a reducing sugar yielding a Schiff base and water as products (see Scheme 1.3). In aqueous media, formation of the Schiff base is reversible, and *in situ* reductive alkylation of the Schiff base forming a stable derivative has been employed to achieve efficient glycation (Cayot *et al.*, 1999; Wrodnigg *et al.*, 2001). Theoretically, another way of promoting the glycation reaction is by the removal of the water but there is no obvious experimental approach for such a strategy under aqueous conditions.

It has been shown that water can be effectively removed and glycation can be achieved by carrying out the reaction in the dry state under vacuum (Kaplan *et al.*, 2002). Although dry state glycation has been attempted under a variety of conditions (Boratynski *et*

al., 1998; Boratynski, 1998; Boratynski, 2002; Quan *et al.*, 1999; Morgan *et al.*, 1999; Yeboah *et al.*, 1999; French *et al.*, 2002), a common observation is that many advanced glycation products are observed and the mechanism of their formation is unclear. While the influence of reaction conditions such as high humidity and the presence of oxygen have been investigated, none of these studies have used a vacuum to promote the glycation reaction by the removal of water or to prevent the formation of advanced glycation end products.

5.1.1 Neoglycoproteins

The research presented in this chapter deals with the preparation of potential multivalent neoglycoconjugates using the *in vacuo* glycation methodology with proteins and dendrimers as scaffolds. In this preliminary investigation, bovine serum albumin (BSA) and lactose were selected as the protein scaffold and disaccharide respectively, since multivalent BSA conjugates containing reductively aminated disaccharides such as cellobiose, maltose and lactose are known to induce an immunogenic response in rabbits (Kamicker *et al.*, 1977). Although *in vacuo* glycation has been successfully accomplished using the monosaccharide, glucose, (Kaplan *et al.*, 2002), this is the first time the method has been used to achieve glycation with a disaccharide.

In order to be antigenic, a threshold of sugar residues on the neoglycoproteins is required (Kamicker *et al.*, 1977). The assay that was used here to determine whether the BSA-lactose glycoconjugate product of *in vacuo* glycation was sufficiently multivalent is an immunoprecipitation assay, which tests the ability of the conjugate to bind to a model lectin. Lectins are a class of proteins that contain two or more carbohydrate-binding sites and their binding activity is highly specific. A lectin possessing more than two binding sites will cross-link divalent or multivalent oligosaccharides to form a three-dimensional lattice

resulting in a precipitation of the complex (Lis *et al.*, 1998). In the BSA-lactose neoglycoconjugate prepared here, galactose was the antennary sugar, hence *Ricinus communis*, a galactose-specific lectin isolated from castor beans (Nicolson *et al.*, 1974), was used to determine the presence and extent of multivalency on the conjugate by an immunoprecipitation assay.

5.1.2 Glycodendrimers

The success of protein glycation using the *in vacuo* methodology inspired efforts to investigate the feasibility of extending the methodology to macromolecular scaffolds other than proteins. Dendrimer-based glycoconjugates have shown promise for biological applications that require multivalency (Roy *et al.*, 2001) or clustering (Roy, 1996; Roy, 2001), and since dendrimers are equipped with the requisite terminal, reactive amine groups, they were obvious candidates for such an investigation. Current methods of preparing glycodendrimers involve a variety of multi-step synthetic strategies involving either a convergent approach (Ashton *et al.*, 1997) or a divergent modification in which sugar moieties are attached to a dendrimer core by way of spacers which are directly coupled to the dendrimer periphery (Andre *et al.*, 1999). Labeled starting monosaccharides were used to generate glycodendrimers by divergent modification of existing DAB dendrimers (G1.0 and G2.0) since the objective of the work presented in this chapter using the *in vacuo* glycation methodology to prepare glycodendrimers was intended to be a feasibility study.

$^{13}C_1$-enriched glucose and $^{13}C_1$-enriched mannose were used as the starting material for i*n vacuo* glycation of dendrimers to facilitate product analysis using ^{13}C NMR. Routine ^{13}C NMR spectra are usually acquired using broad band proton decoupling parameters in order to take advantage of the sensitivity enhancement provided by the polarization transfer

from ^{1}H to ^{13}C via the nuclear Overhauser effect (NOE). Since ^{13}C spin-lattice relaxation times (T1) tend to be quite long and the NOEs between ^{1}H and ^{13}C nuclei are uneven, intensities of ^{13}C signals are not indicative of the number of carbons in the molecule as integration is not possible. However, the NOE can be suppressed by utilizing a technique known as inverse gated decoupling where the decoupler is turned on during acquisition and is turned off during the during the relaxation delay, i.e., the time delay between successive rf pulses. Provided the relaxation delay time is sufficient for magnetization to achieve equilibrium (usually about five times the T1 of the carbon with the longest T1), the acquired spectrum can be integrated and quantified. While the products resulting from the *in vacuo* glycation of dendrimers could be readily identified by ^{13}C NMR spectra acquired using continuous proton decoupling parameters, product stability was monitored using the inverse gated decoupling technique in order to observe any changes in the ratio of products to starting material over time.

5.2 Materials and Methods

Materials:

Polypropyleneimine dendrimers, G1.0 and G2.0, were a kind gift from Bas de Waal, University of Eindhoven, Netherlands. D-$^{13}C_1$-glucose and D-$^{13}C_1$-mannose (both 99 atom % enriched) were purchased from Sigma. D_2O (99.9 atom % ^{2}H, Cambridge Isotope Laboratories) and DMSO-d_6 were purchased from Aldrich Chemical Co. Bovine serum albumin, lactose and *Ricinus communis* agglutinin (RCA_1 – MW= 120,000, 1 mg/mL in PBS) were purchased from Sigma and were used as received.

pH Adjustment/Measurement:

The pH of aqueous solutions were measured using a Radiometer Copenhagen type PHM26 pH meter fitted with an ORION semi-micro gel-filled combination electrode model 91-15.

Lyophilization:

Lyophilization, when required in experiments involving both proteins and dendrimers, was carried out either by a VIRTIS-24 commercial freeze dryer or a home-made freeze dryer equipped with an ethanol/dry ice trap and a 70 mTorr vacuum pump.

5.2.1 Preparation of BSA-Lactose Glycoconjugates

A stock solution of BSA (4 mg/mL in dH_2O) was prepared and the pH was adjusted to 5.0, 7.2, and 9.1 with either 1N NaOH or 1N HCl. Aliquots of the pH-adjusted protein stock solution (450 μL) were transferred to borosilicate glass disposable culture tubes (13 x 100 mm) such that each tube contained 2.0 mg of protein. Another stock solution of lactose (3 mg/mL) in dH_2O was prepared and to each set of tubes containing the pH-adjusted BSA, aliquots of the lactose stock solution were added such that the tubes contained the following amounts of lactose: 0 μg, 16 μg, 78 μg, 155 μg, 310 μg, 775 μg. The volumes in the tubes were adjusted with dH_2O to a total volume of 600 mL and then the tubes were briefly vortexed to mix. Each tube was then flash frozen, lyophilized to dryness overnight and placed in a vacuum oven set at 60°C for 18 hrs. An aliquot of dH_2O (500 μL) was added to each tube and mixed well prior to analysis by SDS-PAGE, FPLC and HPLC.

5.2.2 SDS-PAGE Analysis of Cross-linked Proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli (Laemmli, 1970) using a Bio-Rad Mini-PROTEAN II dual slab cell apparatus. All reagents were electrophoresis grade quality purchased from Sigma or BioRad (buffers were prepared as required using dH_2O). All of the SDS-PAGE analyses were conducted under discontinuous gel conditions with the stacking gel and the samples containing Tris•Cl at pH 6.8 and the resolving gel containing Tris•Cl at pH 8.8. The running buffer was prepared from Tris and glycine with a pH of 8.3 and reducing conditions were provided by β-mercaptoethanol, which was added to the sample buffer just prior to heating. The sample buffer, gels, and running buffer all contained 2% SDS. The molecular weight marker was purchased from BioRad, a broad range of SDS-PAGE standards consisting of aprotinin (MW = 6.5 kDa), lysozyme (MW = 14.4 kDa), soybean trypsin inhibitor (MW=21.5 kDa), carbonic anhydrase (MW = 31 kDa), ovalbumin (MW = 45 kDa), bovine serum albumin (MW = 66.2 kDa), phosphorylase B (MW = 97.4 kDa), β-galactosidase (MW = 116.3 kDa) and myosin (MW = 200 kDa). Approximately 5 - 20 μg (10 μL volume) was loaded into each of the wells of a 1.0 mm thick, 12% polyacrylamide gel and electrophoresis was carried out at a constant current of 20 - 25 mA/gel (with a voltage gradient of 70 - 150 mV). Protein bands were visualized by staining with Coomassie Brilliant Blue R-250.

5.2.3 FPLC Separation of BSA-Lactose Glycoconjugates

A Pharmacia FPLC gel filtration system consisting of a Pharmacia HiLoad 16/10 Superdex 75 preparative grade column connected to a high precision pump P-500 fitted with

a 200 μL sample injection loop controlled by a LCC-500 Plus liquid chromatography controller was used to separate and isolate protein fractions. Peaks were detected by a Pharmacia Single Path Monitor UV-1 Optical Unit with a wavelength of 280 nm and were recorded on a Dual Channel Chart Recorder REC-482. The eluant phosphate buffer pH 7.0 prepared with 0.15 M NaCl in dH_2O was filtered through 0.45 micron cellulose acetate by suction and degassed prior to use. A flow rate of 1 mL/min. was used. The first (higher MW oligomer) and second fractions (monomer) were manually collected and dialyzed (using 3,500 MW cut-off dialysis tubing) against distilled water at 4°C for 48 hours with six changes of dialysate. The samples were then transferred to plastic centrifuge vials, flash frozen and lyophilized overnight. The dialyzed, lyophilized samples were then reconstituted with 300 μL of dH_2O in the plastic vials which were then placed in a -80°C freezer until required for further analyses.

5.2.4 Protein Assay for BSA-Lactose Glycoconjugates

The amount of protein in the glycoconjugate samples was determined by the Bradford assay (Bradford, 1976) whereby the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 (Bio-Rad Protein Assay Dye Reagent Concentrate) shifts from 465 nm to 595 nm upon protein binding. Bio-Rad protein assay dye reagent concentrate was diluted 1 part reagent with 4 parts dH_2O and then filtered through Whatman #1 filter to remove particulates just prior to use. An aliquot of each sample (20 μL) was withdrawn from the reconstituted FPLC fractions and transferred to the wells of a microplate to which 400 μL of the diluted dye reagent concentrate was also added. Aliquots of a set of five standards ranging from 40 to 400 μg/μL (20 μL) were added to the same microplate with 400 μL of the diluted dye reagent concentrate added to each of the wells. After the

microplate was covered and shaken on a rotary plate shaker for 5 minutes at r.t., the absorbance of the standards and samples were measured at a wavelength of 595 nm by a microplate reader. The protein content was determined by interpolation of a standard curve.

5.2.5 Neutral Sugar Assay for BSA-Lactose Glycoconjugates

The neutral sugar content for the BSA-lactose glycoconjugate samples was determined according to the method of Monsigny *et al.* (Monsigny *et al.*, 1988). A lactose standard stock solution was prepared (0.340 mg/200 mL dH_2O) and was added to 7 wells in a 96-well round bottom Corning brand microplate along with dH_2O such that a range of standards from 0 μg to 13.6 μg of lactose in a total volume of 20 μL was prepared in the wells. Sample aliquots (20 μL) of two fractions collected for each LpH set (i.e., 5.0, 7.0, and 9.0) were added individually to the remaining wells. An aliquot of resorcinol solution (20 μL, 0.6% in dH_2O) was added to all of the wells followed by the addition of sulfuric acid (100 μL, 75% in dH_2O). The microplate was covered and shaken on a rotary plate shaker for two minutes and then incubated for 30 minutes on the top shelf of an oven with the temperature set to 90°C. The microplate was then placed in a dark cupboard at room temperature where it remained for 30 minutes. The absorbance of each well was read at a wavelength of 490 nm by a microplate reader and the sugar content was determined by interpolation from a standard curve.

5.2.6 Gel Filtration Analysis of BSA-Lactose Glycoconjugates by HPLC

Analytical reverse phase high performance liquid chromatography was carried out using a Varian Prostar quaternary pump HPLC system equipped with a 50 μL sample injection loop and a TosoHaas TSK-GEL G2500PWXL column (30 cm x 7.8 mm ID). Peaks

were detected by a sequential UV-vis detector set at a wavelength of 280 nm. The instrument was controlled and data was recorded by Varian Star Chromatography Workstation software version 5.3. Samples were manually injected and analyzed under ambient, isobaric, non-gradient, elution conditions with a flow rate of 1 mL/min using 0.15 M sodium phosphate buffer pH 7.0 as the eluant.

5.2.7 Double Radial Immunodiffusion Assay for BSA-Lactose Glycoconjugates

Agarose (200mg) was dissolved in phosphate buffer (1.0 M, pH 7.0) containing 0.15 M NaCl (16 mL) by heating in a microwave oven to boiling in a 125 mL conical flask. A solution of 10% polyethylene glycol (PEG) 8000 (4 mL) was immediately added to this solution and swirled to mix. While the agarose - PEG solution was still hot, a 2.8 mL aliquot was transferred to glass Petri dishes (1.5 inch diameter). When the solution had solidified to a gel (after about 30 minutes), one small hole surrounded by 4 small holes to make wells with a volume capacity of 12 - 15 μL were bored out of the gel. The center well was carefully filled with a galactose-specific lectin solution, *viz.*, *Ricinus communis* agglutinin, (1 mg/mL in PBS), using a syringe (12 μL). A multivalent galactose polymer synthesized by Frank Tropper was prepared in a concentration of 1 mg/mL PBS for use as a positive control and was added to one of the surrounding wells (12 μL). A solution of BSA (12 μL, 1 mg/mL in PBS) served as a negative control which was added to a second well. The reconstituted first collected FPLC fraction of the separated BSA-lactose glycoconjugate (12 - 15 μL) was added to a third well, while the reconstituted second collected fraction was added to the remaining fourth well (12 - 15 μL). The agar dish was covered and placed in a sealed, humid chamber for 12 - 24 hours. Precipitation with the lectin (positive result) was indicated by the presence

of a white band in the clear agarose gel between the centre well and that of the interacting sample.

5.2.8 Reaction of DAB Dendrimers with Reducing Monosaccharides

Effect of LpH

Experiment A – Reaction of G1.0 Dendrimers with Glucose and Mannose:

The effect of pH on the products observed as a result of *in vacuo* glycation of G1.0 DAB dendrimers with ^{13}C-enriched in a 1:1 mole equivalent ratio was quantified by inverse gated decoupled ^{13}C-NMR analysis. A stock solution of G1.0 DAB dendrimer (6.9 mg/mL in dH_2O) was prepared and aliquots (360 μL) of the pH-unadjusted stock solution, at pH 10.6 were added to two test tubes. The pH of the remaining dendrimer stock solution was then adjusted to pH 9.5 with concentrated HCl and aliquots were transferred to two test tubes. The pH of the remaining dendrimer solution was adjusted sequentially and aliquots were transferred in this way for the following pH values: 8.3, 7.0, 5.5, and 4.0, such that two sets of 6 test tubes containing 2.5 mg of dendrimer in solutions of varying pH were prepared. Stock solutions of $^{13}C_1$-enriched glucose and $^{13}C_1$-enriched mannose (5.7 mg/mL in dH_2O) were also prepared, and to one set of test tubes containing dendrimer, aliquots of $^{13}C_1$-enriched glucose solution (440 μL) were added while aliquots of $^{13}C_1$-enriched mannose (440 μL) were added to the other set. Each of the tubes was vortexed briefly to mix, and the solutions were flash frozen and lyophilized overnight to dryness. The tubes were then placed in a glass beaker in a vacuum oven set at 40°C for 12 hrs. The residue remaining in the each tube was dissolved in 540 μL of dH_2O to which 60 μL of D_2O was added, mixed by brief vortexing, and transferred to an NMR tube immediately prior to analyzing by inverse gated decoupled ^{13}C-NMR.

Experiment B - Reaction of G2.0 Dendrimers with Glucose:

The effect of pH on the products of *in vacuo* glycation of G2.0 DAB dendrimers with ^{13}C-enriched in two sets of samples, 1:8 and 2:8 ratios of glucose to dendrimer amine group, was observed using continuously decoupled ^{13}C-NMR. A stock solution of G2.0 DAB dendrimer (10.1 mg/mL in dH_2O) was prepared and the pH was adjusted in the same manner as described in **Experiment A** above. Aliquots of the pH-adjusted dendrimer solutions (500 μL) were transferred to test tubes (16 x 150 mm), such that the pH of the dendrimer solutions in each set of 6 tubes was as follows: 10.7, 9.0, 6.7, 5.4, 4.1, and 2.7. To each of the tubes in the first set was added 235 μL of $^{13}C_1$-glucose stock solution (5.6 mg/mL in dH_2O) and 235 μL of dH_2O, and to each of those in the second set, 470 μL of the same $^{13}C_1$-glucose stock solution was added such that each tube in the first and second set contained 5 mg of G2.0 dendrimer and 1.2 mg and 2.7 mg of $^{13}C_1$-glucose, respectively. Each of the tubes was vortexed briefly to mix, and the solutions were flash frozen and lyophilized overnight to dryness. The tubes were then placed in a glass beaker in a vacuum oven set at 60°C for 2.5 hrs and then the temperature was reduced to 40°C for 13.5 hrs. The residue remaining in the each tube was dissolved in 700 μL of dH_2O, mixed by brief vortexing, and transferred to an NMR tube immediately prior to analyzing by continously decoupled ^{13}C-NMR.

Effect of Temperature

The effect of increasing the temperature while heating under vacuum was assessed with respect to the products. In one experiment, a set of $^{13}C_1$-glucose/G1.0 dendrimers and $^{13}C_1$-mannose/G1.0 dendrimers samples was prepared as described above (in ***Effect of LpH***) with the exception that, after lyophilization the samples were placed in a vacuum oven set at 50°C for 12 hrs. The residue remaining in the each tube was dissolved in 700 μL of dH_2O,

mixed by brief vortexing, and transferred to an NMR tube immediately prior to analysis by inverse gated decoupled ^{13}C-NMR.

Effect of Glucose Concentration

A stock solution was prepared with G1.0 DAB dendrimer, which had been vacuum dried for 48 hrs at 25 °C just prior to weighing, in dH_2O (7.7 mg/mL), and the unadjusted pH of the solution was measured and found to be 10.5. Aliquots of the dendrimer stock solution (330 μL) at pH 10.5 (unadjusted) and pH 7.3 (adjusted with concentrated HCl) were added to two sets of 4 test tubes (10 x 75 mm) such that each tube contained 2.5 mg dendrimer. A second stock solution of $^{13}C_1$-glucose was prepared (10.6 mg/mL in dH_2O) and the following aliquots were added in succession to both sets of 4 tubes containing the dendrimer solutions: 140 μL, 290 μL, 575 μL, and 1,145 μL such that the tubes contained the following mole equivalents of glucose per dendrimer amine: 0.25, 0.5, 1.0, and 2.0, respectively. The volume in each tube was adjusted to a total of 1,475 mL with dH_2O where necessary and then mixed by vortex. The solutions in the tubes were lyophilized to dryness overnight and the tubes were then transferred to a vacuum oven set at 40°C for 18 hrs. The residue in the tubes was dissolved in an aliquot of 700 μL of D_2O and the resulting solution was then transferred to an NMR tube just prior to ^{13}C NMR analysis.

5.2.9 NMR of Glycated Dendrimers

^{13}C-NMR spectra were collected on a Bruker Avance 300 NMR, 300 MHz spectrometer operating at 75.48 MHz or a Bruker Avance 700 NMR, 700 MHz spectrometer operating at 176.04 MHz. Spectra were routinely acquired using continuous proton decoupling and when inverse gated proton decoupling was required for integration, a relaxation delay time of 300 seconds was used.

5.2.10 IR Analysis of Glycated Dendrimers

Infrared spectra were recorded using a Bomem Michelson series FT-IR instrument using oven-dried FT-IR grade potassium bromide for solid pressed-disk sample preparation.

5.3 Results and Discussion

5.3.1 BSA-Lactose Neoglycoproteins

Bovine serum albumin with varying concentrations of lactose lyophilized at three different LpH values (*viz.*, 5, 7 and 9) and reacted under *in vacuo* glycation conditions, resulted in a mixture of monomeric glycated protein conjugates and oligomeric glycated protein conjugates that can be seen in the representative gel of Figure 5.1. With increasing initial lactose concentration samples, the monomeric BSA bands (dark bands in the middle of the gel) appear broader and higher (slower moving), indicative of an increase in the molecular weight distribution due to increasing incorporation of lactose. Higher molecular weight oligomers of cross-linked BSA and cross-linked BSA glycoconjugates, due to dehydrothermal cross-linking (Simons *et al.*, 2002), were also formed as indicated by the presence of bands closer to the top of the gel. Oligomers too large to enter the gel can be seen occupying the wells of those samples that were prepared with lower initial lactose concentration, i.e., Lanes 1 and 5, having 16 and 78 μg lactose per 2 mg protein, respectively. The absence of oligomers in the wells of the other glycated samples appears to be another example of the excipient effect induced by the presence of a threshold concentration of sugar (Chapter 4, Results and Discussion) which impedes intermolecular interaction, thereby preventing the formation of higher molecular weight oligomers.

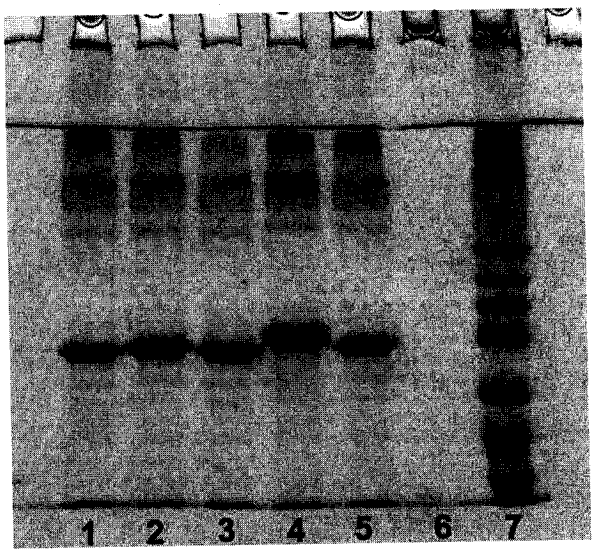


Figure 5.1: SDS-PAGE 10% polyacrylamide gel showing BSA-lactose glycoconjugate prepared with 2.0 mg BSA and various amounts of lactose under *in vacuo* conditions at LpH 9.0:
Lane 1: 16 μg lactose; Lane 2: 155 μg lactose; Lane 3: BSA control – no lactose; Lane 4: 775 μg lactose; Lane 5: 78 μg lactose; Lane 6: blank; Lane 7: MW markers.

This effect is also evident in the HPLC traces in Figure 5.2, particularly in the D row of traces, where the first peaks, corresponding to the fastest eluting, higher molecular weight compounds are somewhat diminished compared to those of row B, where comparatively lower amounts of lactose was present during lyophilization of these samples. The traces in the columns for LpH 5 and LpH 9 indicate the presence of a greater amount of oligomeric products for these samples compared to those of neutral pH.

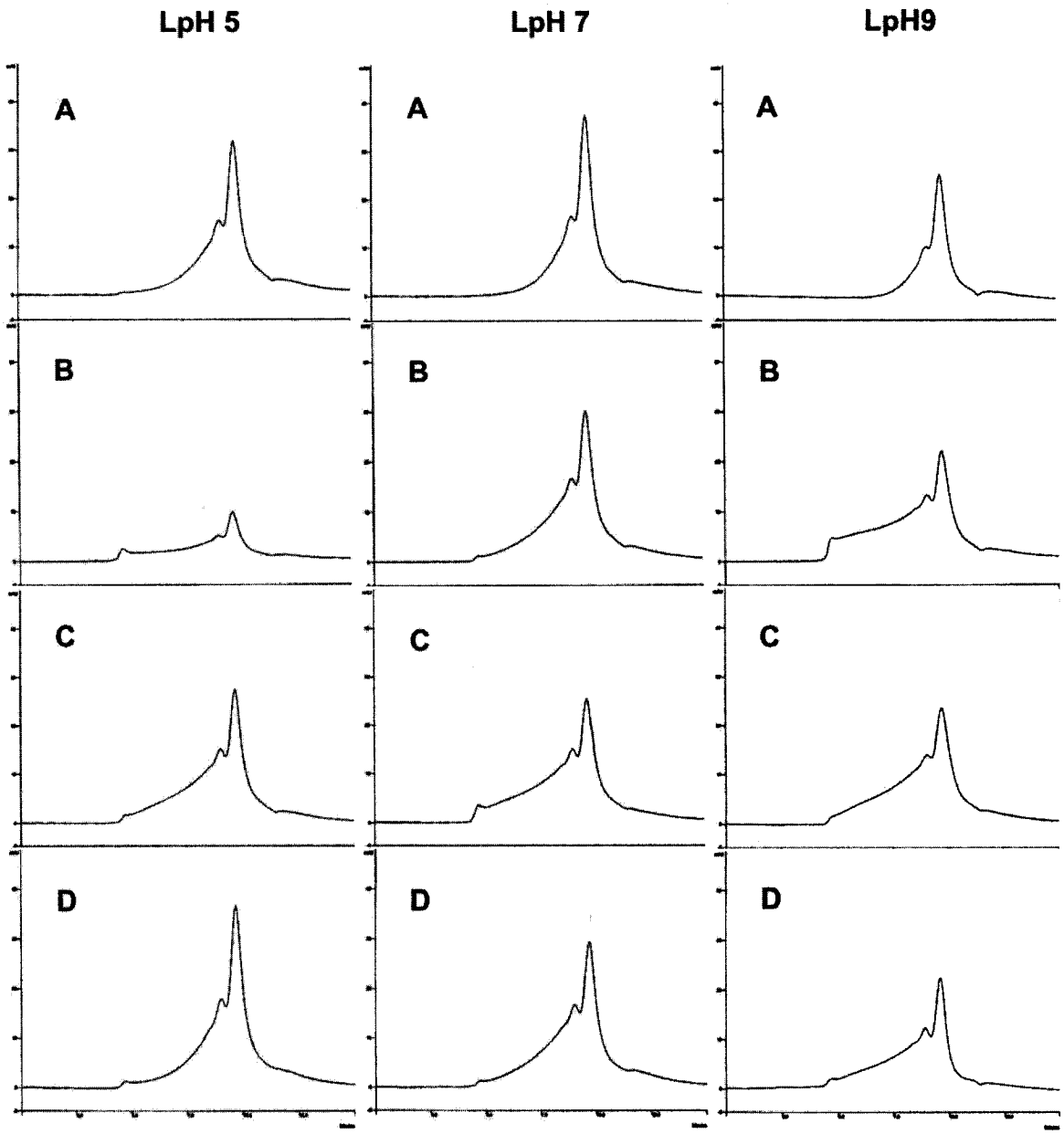


Figure 5.2: Comparison of HPLC chromatograms of BSA (2 mg) co-lyophilized with varying amounts of lactose
(**A** – 0 μg; **B** – 78 μg; **C** - 155 μg; **D** – 775 μg) at three different LpH values (columns), and heated at 60°C under vacuum for 16 hrs.

The presence of a wide distribution of higher molecular weight products in the samples is evident by the relatively large area under the traces as they slope towards the more

sharply defined monomer peak (last peak), particularly noticeable in the traces of the samples of LpH 9. The more acidic samples at LpH 5 also contained aggregated material, especially in the presence of 78 μg of lactose (LpH 5 **B**), which was not observed with any of the other samples prepared under neutral or basic conditions.

Lactose, 4-*O*-*β*-D-galactopyranosyl-D-glucose, is a reducing heterodisaccharide composed of galactose and glucose and since glucose is the reducing moiety, its structure is altered by the formation of the conjugate linkage under the *in vacuo* reaction conditions; however the structure of galactose remains intact and thus is capable of binding with galactose-specific lectins. Efforts to quantify the amount of neutral sugar present in the BSA-lactose conjugates by way of chemical assay in order to determine the extent of glycation were inconclusive, despite repeated attempts. In reviewing the data, it is possible that some of the samples were incompletely dialysed, and the assay should be repeated after more extensive dialysis (the assay was included in the Methods and Materials of this section as a matter of record for future work). Nonetheless, the results of the precipitation assay with the galactose-specific lectin, *Ricinus communis,* in Figure 5.3 show conclusively that both monomeric and oligomeric BSA-lactose conjugate products resulting from the *in vacuo* glycation procedure were indeed sufficiently multivalent at lactose concentrations as low as 0.155 mg lactose per 2 mg of BSA, regardless of LpH. Only those gels of samples where precipitation bands were evident were included in Figure 5.3.

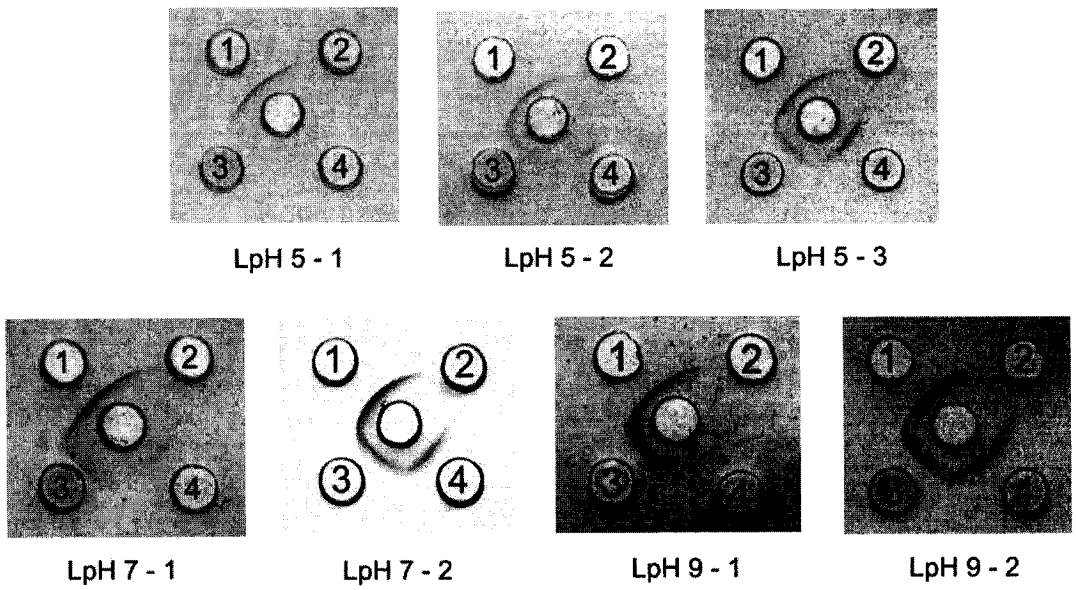


Figure 5.3: Precipitation of BSA-lactose conjugates with *Ricinus communis* lectin in agarose gel (1%).
The centre well was loaded with lectin (1 mg/mL, 12 μL), Wells 1 and 2 were loaded with the positive control galactose polymer (1 mg/mL, 12 μL) and the negative control BSA (1 mg/mL, 12 μL) respectively, and Wells 3 and 4 were loaded with BSA-lactose monomer (second FPLC fraction, ~2 mg/mL) and BSA-lactose conjugate oligomers (first FPLC fraction, ~2 mg/mL), respectively. The amount of lactose initially reacted with BSA, top row from left to right, LpH 5-1: 155 μg; LpH 5-2: 310 μg; LpH 5-3: 755 μg. Amount of lactose initially reacted with BSA, bottom row from left to right, LpH 7-1: 310 μg; LpH 7-2: 755 μg, LpH 9-1: 310 μg; LpH 9-2: 755 μg.

In their attempts to generate similar conjugates simply by heating dry mixtures of lactose and BSA, Boratynski *et al.* used much higher amounts of lactose and heated the samples at a much higher temperature (120°C) under conditions of atmospheric pressure and oxygen (Boratynski *et al.*, 1999). While they were successful in generating multivalent glycoconjugates, it has been shown that oxygen, at levels found in air, catalyzes

autooxidation of Amadori rearrangement products resulting in protein cross-linking and degradation (Yeboah *et al.*, 1999; Yaylayan *et al.*, 1994). Thus the long-term stability of neoglycoconjugates prepared in this way, is questionable. By comparison, the conditions of the *in vacuo* method used here are much less harsh and, although this is yet to be evaluated, the products are more likely to be less complex. While both glycated oligomeric and monomeric fractions were found to be multivalent in nature, the sharper monomeric peaks seen in the gel filtration traces of Figure 5.2 indicate the presence of a relatively narrow distribution of glycated monomer products that exhibit a high degree of multivalency (as seen by the intense precipitation bands in Well 3 of many of the gels in Figure 5.3) easily separated from the oligomers by FPLC. In this way, more homogeneous, multivalent neoglycoproteins can be generated.

5.3.2 Glycated and Mannosylated Dendrimers

The extent of dendrimer glycation under the reaction conditions of the *in vacuo* glycation procedure was determined by the integration of peaks present in the ^{13}C NMR spectra of the products relative to that of the sugar starting material. The products of glycation with glucose and mannose, identified by the position of the peaks in Figures 5.4 and 5.5, respectively, were not unexpected and were typical of those identified previously in solution protein glycation experiments (Neglia *et al.*, 1983; Neglia *et al.*, 1985). However, unlike proteins, which exist mainly in the protonated state in the pH range studied, the ionization state of the primary amines of dendrimers is more responsive to changes in the pH of the initial solution from which they were lyophilized.

Figure 5.4 shows that at the basic, unadjusted pH of 10.6, glycation with glucose was extensive, and the main product, appearing at a chemical shift of 89.7 ppm, was identified as

the β-glycosylamine with the α-glycosylamine present as a minor product at 86.7 ppm. At the other pH extreme, i.e., LpH 4.0, no product was observed and only two peaks, identified as the α- and β-glucopyranose anomers of the glucose starting material at 92.4 ppm and 96.2 ppm respectively, can be seen in Figure 5.4. As the initial pH of the solution from which the mixture was lyophilized decreased, the appearance of another product, identified as the ketoamine at 53.4 ppm, is evident among the starting material and glycosylamine product peaks in the spectra. The pH dependency is not exclusive to glucose and is also apparent in the spectra of the mannosylated dendrimers in Figure 5.5; as expected, the chemical shifts of the peaks are different from the glycated dendrimers (peak assignments for mannose anomers, mannosylamine and ketoamine products are given in the legend of the Figure), but the product trend appears to be very similar to that of glucose.

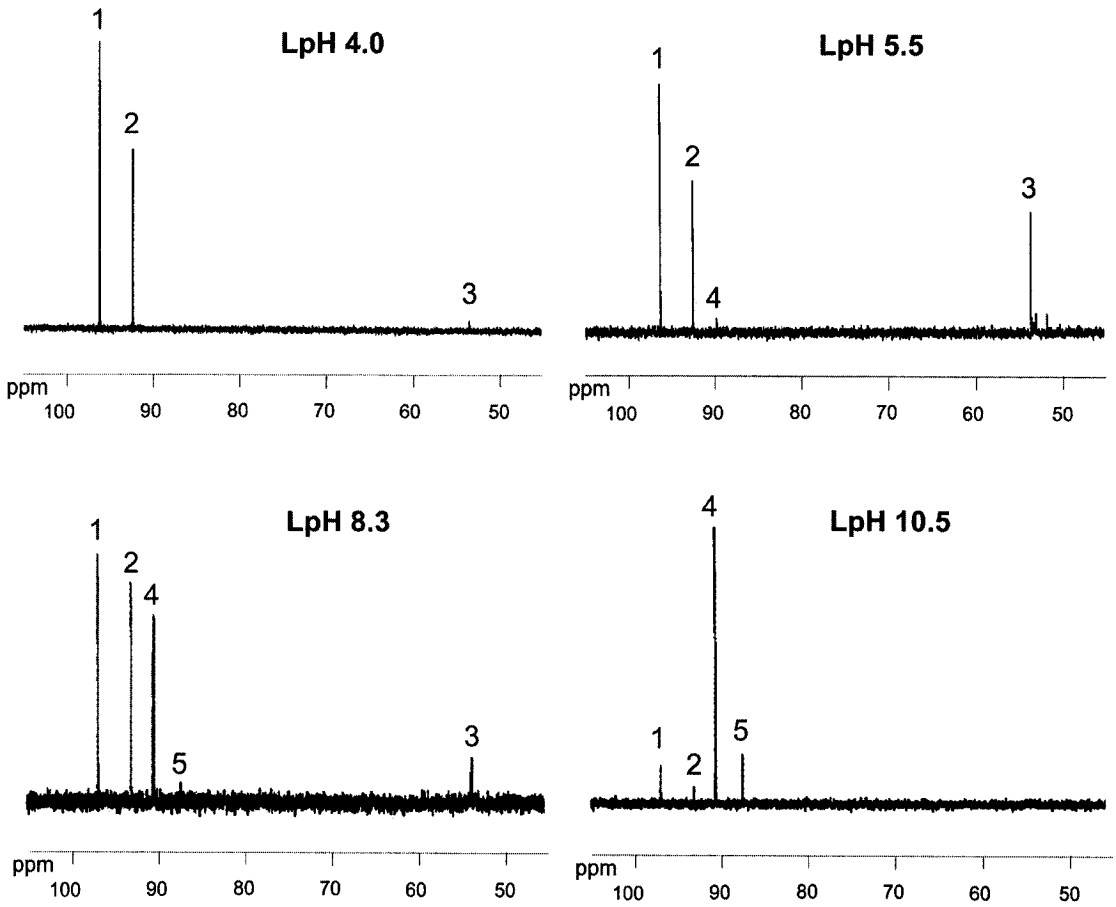


Figure 5.4: ^{13}C NMR spectra (700 MHz, inverse gated ^{1}H decoupled, 10%D_2O in dH_2O) of G1.0 DAB dendrimers co-lyophilized with $^{13}C_1$-enriched glucose under conditions of varying pH and heated at 40°C under vacuum for 24 hrs. Numbered peaks are assigned as follows: **1** – C_1 β-glucopyranose (96.2 ppm); **2** - α-glycosylamine (86.7 ppm); **3** – ketoamine (53.4 ppm); **4** – β-glycosylamine (89.7 ppm); **5** C_1 α-glucopyranose (92.4 ppm).

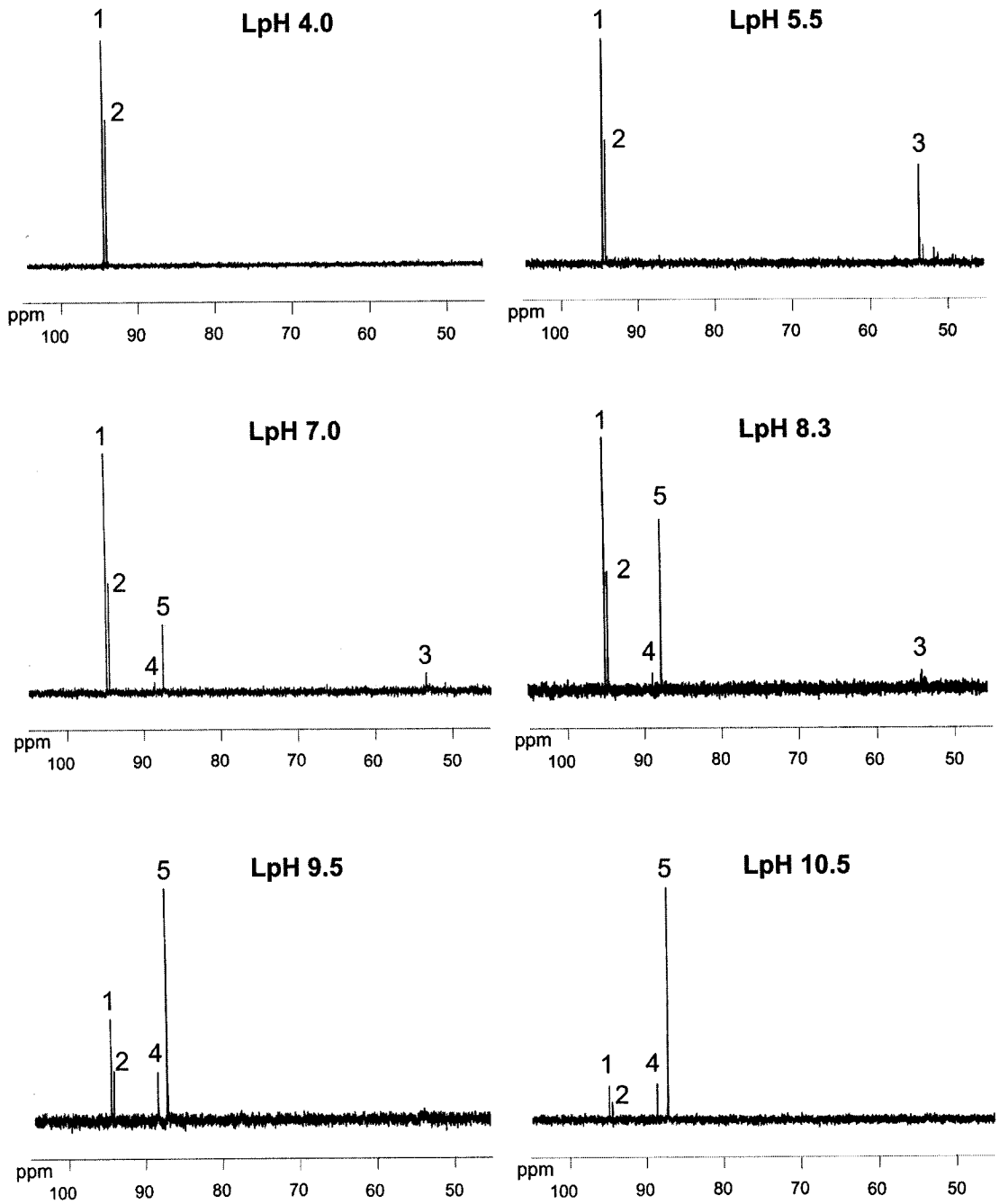


Figure 5.5: ^{13}C NMR spectra (700 MHz, inverse gated ^{1}H decoupled, 60% D_2O in dH_2O) of G1.0 DAB dendrimers co-lyophilized with $^{13}C_1$-enriched mannose at various LpH conditions and heated at 40°C under vacuum for 24 hrs. Numbered peaks are assigned as follows: **1** – C_1 α-mannopyranose (94.4 ppm); **2** - C_1 β-mannopyranose (94.0 ppm); **3** – ketoamine (53.4 ppm); **4** – α-mannosylamine (87.1 ppm); **5** – β-mannosylamine (88.2 ppm).

Both Figures 5.4 and 5.5 show that while the intensity of the ketoamine peak rises with decreasing pH to a maximum at LpH 5.5, the peaks of the glucose anomers rise continuously with decreasing LpH, concomitant with a decrease in the intensity of the glycosylamine anomers peaks. This pH trend is depicted in the plot in Figure 5.6 below:

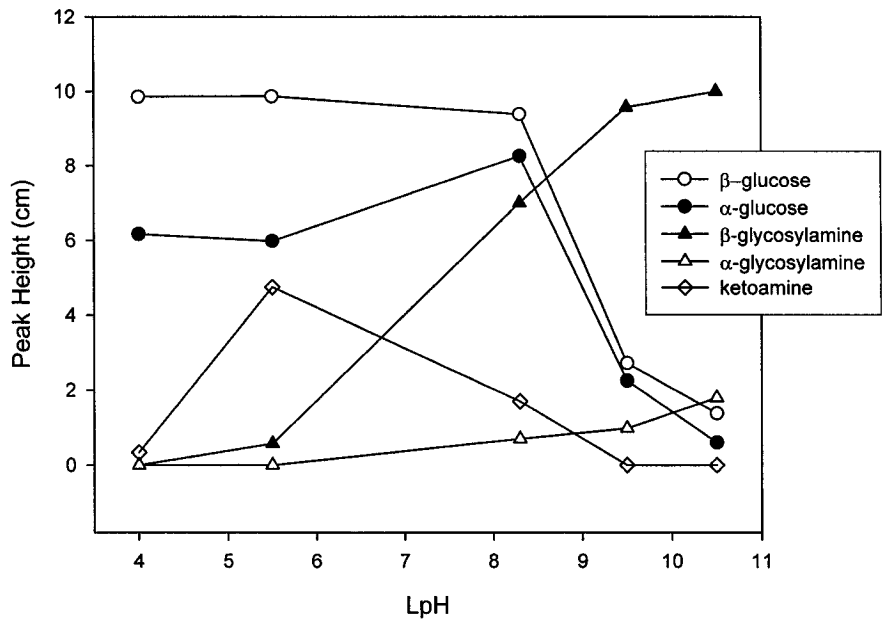


Figure 5.6: Effect of LpH on product formation and glucose content for *in vacuo* glycated G1.0 dendrimers (prepared using 1:1 stoichiometric equivalents of glucose to dendrimer).
Determined by peak height integration of inverse gated ^{1}H decoupled ^{13}C NMR (700 MHz, 1 scan acquired per sample, samples dissolved 10% D_2O in dH_2O).

The spectra in Figure 5.7 show an almost identical pH dependency for the products of *in vacuo* glycation of the higher generation G2.0 dendrimers. The main difference is the observation that the ketoamine product rises to a maximum at the LpH of 4.1 where no product is observed for either the glycated or mannosylated G1.0 glycated dendrimers.

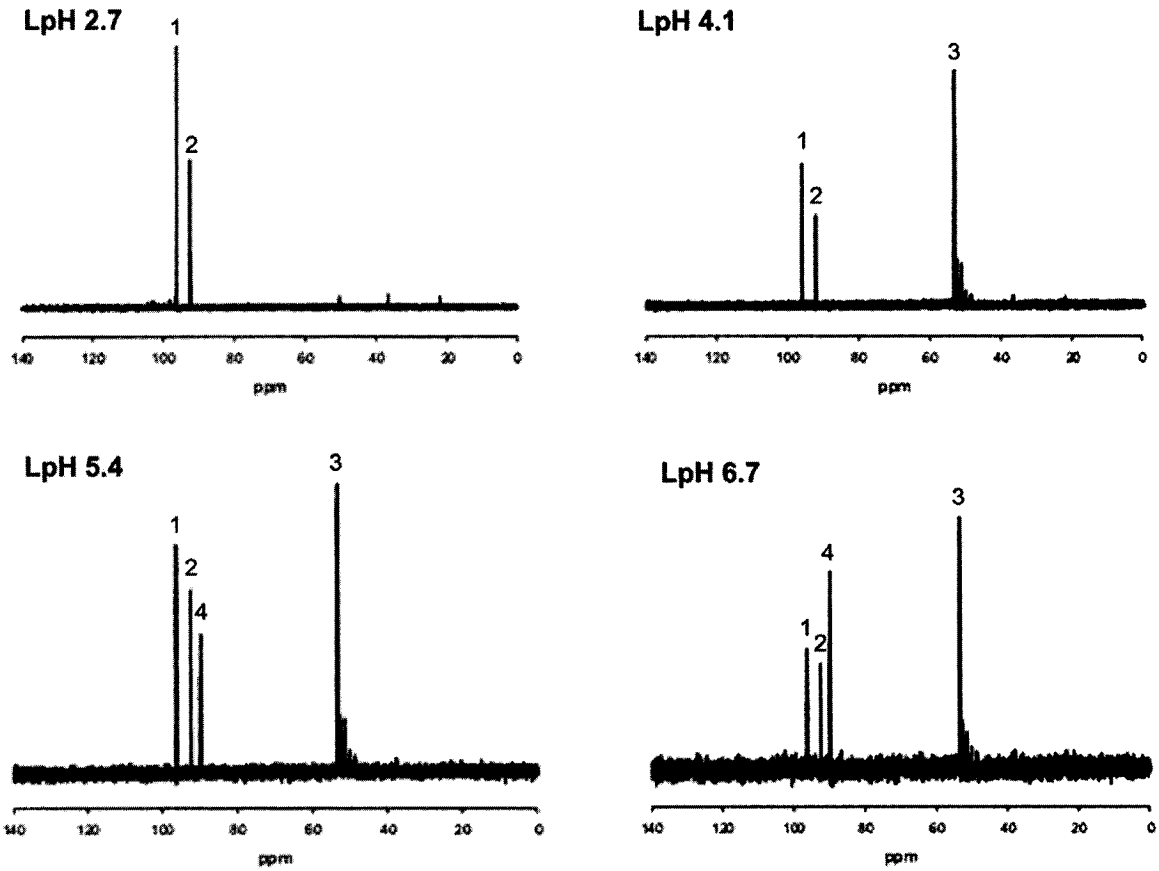


Figure 5.7: ^{13}C NMR spectra (300 MHz, continuously ^{1}H decoupled, D_2O) of G2.0 DAB dendrimers co-lyophilized with $^{13}C_1$-enriched glucose (2:4 mole equivalence ratio) at various LpH conditions and heated at 40°C under vacuum for 24 hrs. Numbered peaks are assigned as follows: **1** – C_1 β-glucopyranose (96.2 ppm); **2** - C_1 α-glucopyranose (92.4 ppm); **3** – ketoamine (53.4 ppm); **4** – β-glycosylamine (89.7 ppm); **5** – α-glycosylamine (86.7 ppm).

Although they are relatively stable, both cyclic and acyclic forms of the ketoamine product possess reactive sites, *viz.*, secondary amine and carbonyl, respectively, and are capable of further reaction. In order to test whether the secondary amine would participate in further glycation under *in vacuo* conditions to yield a branched product, the effect of increasing glucose concentration on G1.0 dendrimer glycation products was investigated. In

Figure 5.8, the peak at 64.1 ppm in the spectrum corresponding to the 2:1 mole equivalence ratio of sugar to dendrimer is interesting, and may indeed be indicative of a branched product.

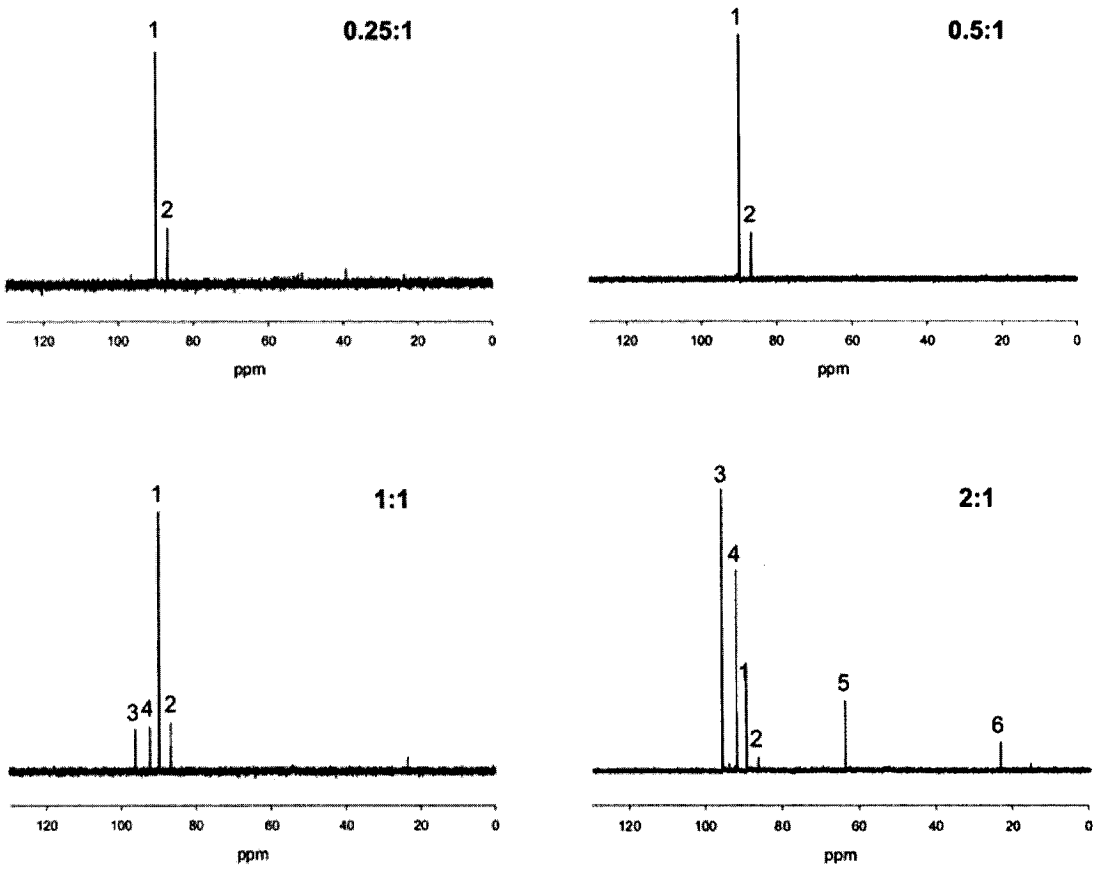


Figure 5.8: ^{13}C NMR spectra (300 MHz, continuously ^{1}H decoupled, D_2O) of different ratios of $^{13}C_1$-enriched glucose to G1.0 DAB dendrimers
Dendrimers co-lyophilized with varying amounts of glucose and heated at 40°C under vacuum for 24 hrs (0.25:1; 0.5:1; 1:1; and 2:1 mole equivalents glucose to dendrimer). Numbered peaks are assigned as follows: **1** – β-glycosylamine (89.7 ppm); **2** - α-glycosylamine (86.7 ppm); **3** – C_1 β-glucopyranose (96.2 ppm); **4** – C_1 α-glucopyranose (92.4 ppm); **5** and **6** – unassigned (64.1 ppm).

Another interesting observation in Figure 5.8 is that the extent of glycation in the presence of twice the stoichiometric amount of sugar (2:1) appears to be lower than that seen in the spectrum obtained for the 1:1 sample. Once again, similar to that observed with the *in vacuo* glycation of proteins, there appears to be threshold limit to the extent of glycation that can be achieved; in this case, a 1:1 mole equivalence of sugar to dendrimer. Concentrations in excess of this threshold limit appear to induce an excipient effect.

Another significant difference between proteins and dendrimers with respect to *in vacuo* glycation is that dendrimers cannot withstand the elevated temperatures used for protein glycation during the heating stage of the procedure. At 50°C, both G1.0 and G2.0 dendrimers started to turn brown after just 2 hours during glycation with either glucose or mannose. Moreover, product analysis by ^{13}C NMR (not shown) showed an entirely different set of peaks, and neither the glycosylamine nor the mannosylamine peaks was among them in their respective spectra. These observations suggest that at temperatures above 40°C, the Maillard reaction progresses more rapidly, giving rise to advanced glycation products.

5.3.3 Glycated Dendrimer Product Stability

Integration of inverse gated spin decoupled ^{13}C NMR spectra of dendrimer glycated with $^{13}C_1$-enriched glucose by the *in vacuo* method that was recorded at different time intervals showed that while initially there is almost complete incorporation of glucose, the neoglycoconjugate is unstable and degrades back to the starting material over time. The rate of degradation is illustrated in the comparison plots of peak height *vs.* time for product and starting material (Figure 5.9), where the plots show that the α- and β- anomeric forms of the glycosylamine product began to decrease as the α- and β- forms of the glucose starting

material increased significantly in a matter of a few hours; almost complete reversion back to the starting α- and β- glucose anomers occured after about 48 hours. The plots in Figure 5.10 show that the stability of the products prepared *in vacuo* at LpH 10.5 is somewhat greater in the short term, where the products appear to be stable in solution for up to 12 hours.

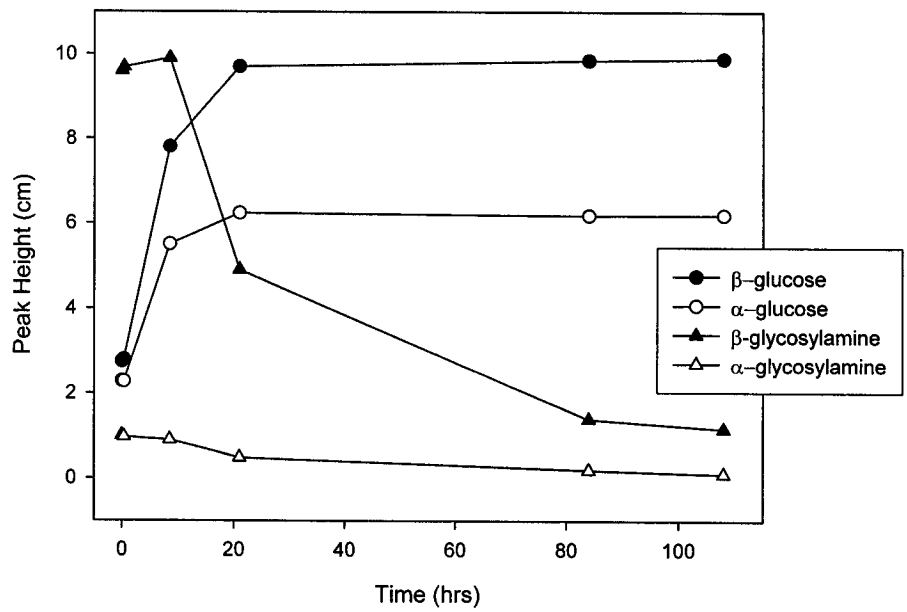


Figure 5.9: Plot showing long-term degradation rate of G1.0 DAB dendrimer glycated with $^{13}C_1$-enriched glucose by *in vacuo* glycation procedure at LpH 9.5. As determined by peak height integration of ^{13}C NMR spectra (700 MHz, inverse gated ^{1}H decoupled, 8 scans acquired per sample, 10% D_2O in dH_2O) as a function of time.

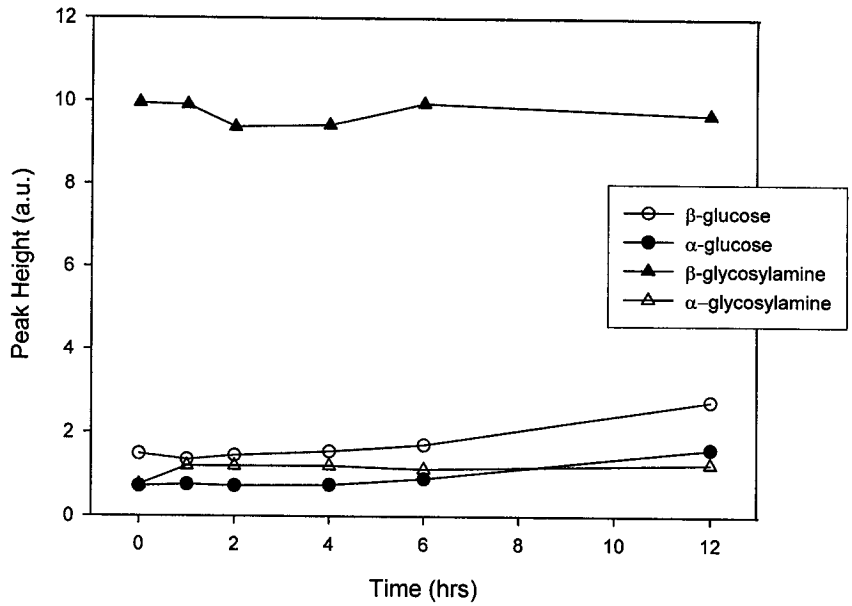


Figure 5.10: Plot showing short-term degradation rate of G1.0 DAB dendrimer glycated with $^{13}C_1$-enriched glucose by *in vacuo* glycation procedure at LpH 10.5. As determined by peak height integration of ^{13}C NMR spectra (700 MHz, inverse gated ^1H decoupled, 8 scans acquired per sample, 10% D_2O in dH_2O) as a function of time.

Attempts to introduce stability by post-glycation reduction of the dendrimer neoglycoconjugate with both sodium borohydride and sodium cyanoborohydride resulted in an acceleration of product degradation back to the starting materials, as detected by the almost instantaneous appearance of ^{13}C-enriched glucose peaks by inverse gated decoupled ^{13}C NMR analysis (not shown).

5.3.4 Mechanistic Considerations

In his work on *in vacuo* glycation of RNase A and hemoglobin with glucose, Nicolas Stewart (Stewart, 2002) found no evidence of a reaction product until the lyophilized samples had been heated under vacuum. Surprisingly, results of ^{13}C NMR analysis of dendrimers that

had been co-lyophilized with $^{13}C_1$-enriched glucose at the unadjusted pH of 10.5 but had not yet been heated under vacuum, showed the presence of a glycosylamine product, suggesting that the mechanism for *in vacuo* glycation of dendrimers differs somewhat from that of proteins, and may involve initial rapid nucleophilic attack of the open chain sugar as it occurs in solution. The spherical symmetry and the relative molecular simplicity of dendrimers compared to proteins may, to a much greater extent, facilitate alignment or favorable orientation of the reactive functional groups due to weak interactions in solution which become stronger as the solvent water is drawn off during lyophilization. As removal of water drives the reaction forward, the energy required for molecular mobility in order for functional group interaction in the absence of solvent would be expected to be much less than for the more complex proteins. This might explain the much higher extent of reaction observed with dendrimers, where at LpH 10.5, reaction with the nucleophilic terminal amine groups of the dendrimers is almost complete (see Figures 5.5 and 5.4).

The characteristic absorption band of the acyclic ketoamine carbonyl between 1715 and 1725 cm^{-1} (Yaylayan *et al.*, 1994) is not apparent in any of the solid state infrared spectra of glycated dendrimers that were acquired after lyophilizing and heating (Figure 5.11). In the samples reacted at LpH 7.2 and lower, where evidence of the presence of ketoamine was detected in ^{13}C NMR spectra (*vide supra*), it is quite probable that a distribution of ketoamine products with up to four ketoamine moieties on the glycated G1.0 dendrimer would be present in the samples. Thus amplification of the carbonyl stretching in the infrared spectra would be expected, were it present at all, giving rise to a significant, identifiable peak. In their work on solution glycation of polypeptides with polysaccharides, Berry *et al.* (Berry *et al.*, 1998) show IR evidence of a very moderate ketone stretching vibration, which is typical

for the products of solution glycation since a very small proportion of the ketoamine exists in equilibrium in solution following rearrangement (Figure 1.3).

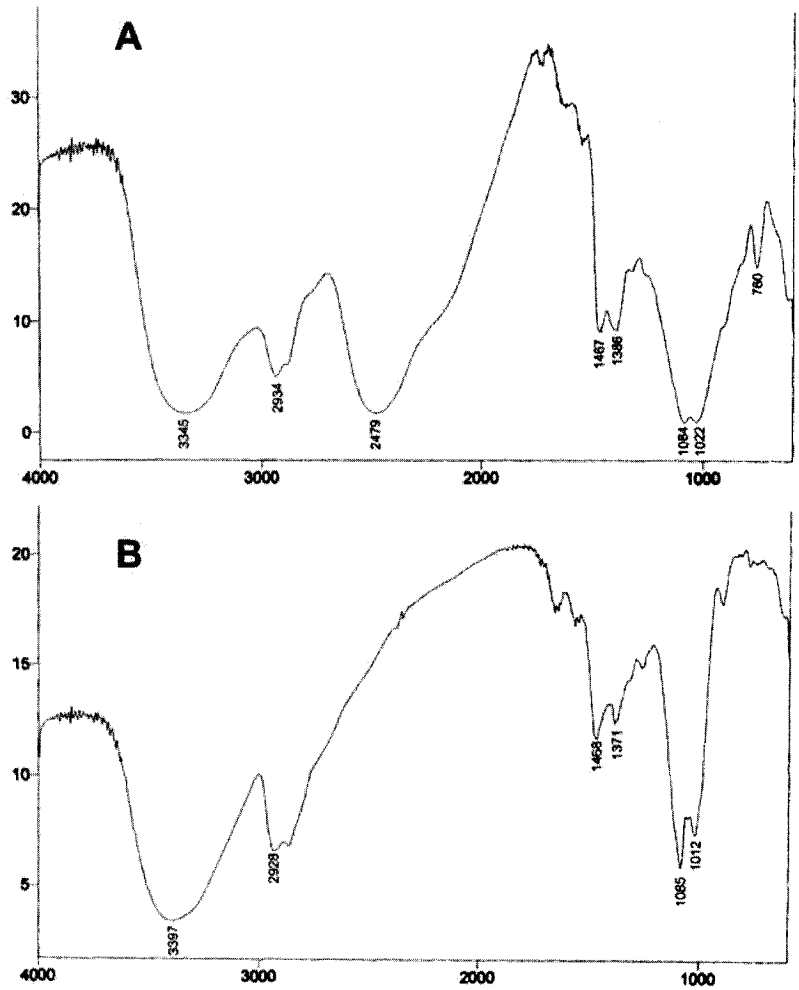
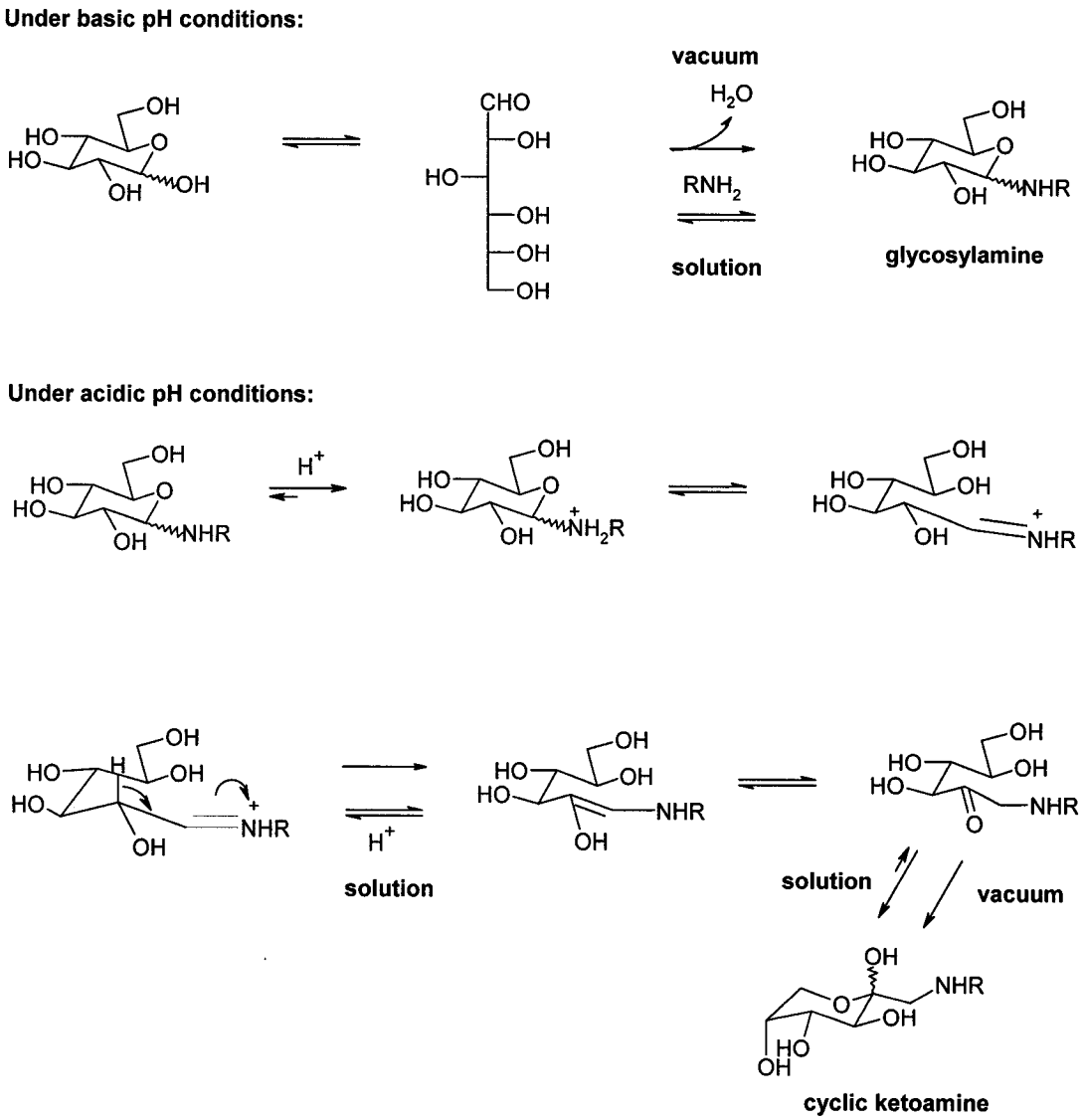


Figure 5.11: Infrared spectra of polypropyleneimine dendrimer G1.0 co-lyophilized with glucose (1:1 equivalents) and heated at 40°C for 12 hours
A) LpH 7.2 (pH adjusted with concentrated HCl); B) LpH 10.4 (pH unadjusted).

In the case of the dendrimers glycated in the absence of water here, no equilibrium of the products exists, and the absence of this absorption band in the IR spectra shown in Figure 5.11 **A** suggests that the dendrimer glycation products exist predominantly in the ring form as opposed to the open chain. In fact, the only difference between the IR spectra of the LpH 7.2 samples compared to that of the LpH 10.5 samples, where the sole product of *in vacuo* glycation is glycosylamine (*vide supra*), is the presence of the large, broad protonated amine peak at 2479 cm^{-1}.

Likewise, the absence of a peak in the azomethine (R_2-C=N-) region at 145-165 ppm in the ^{13}C spectra of the glycated dendrimers (not shown) indicates that there is no measurable open-chain Schiff base glycosylamine; an observation also made by Stewart (Stewart, 2002) in his investigation of *in vacuo* glycation of RNase A protein with glucose.

Based on these results and postulates presented, a reaction scheme depicting the glycation of dendrimers under *in vacuo* conditions compared to that in solution is proposed in Scheme 5.1 below.



Scheme 5.1: Proposed mechanism for glycation of dendrimers under vacuum and in solution.

5.4 Conclusions

The protein neoglycoconjugates generated by reaction between lactose and BSA using the *in vacuo* glycation method proved to be extremely stable even after lengthy exposure to PBS during size exclusion chromatographic analysis, lengthy dialysis,

lyophilization and reconstitution followed by the time-consuming double radial immunodiffusion assay. This stability may be due to a three-dimensional network of hydrogen bonding arising from the spatial arrangement of the hydroxyl groups in the cyclic ketoamine and their proximity to adjacent residues on the protein (see Chapter 4, Results and Discussion).

The contrasting lack of stability observed with dendrimeric glycoconjugates prepared using the *in vacuo* methodology further reinforces the hypothesis that a hydrogen bonding network, similar to that depicted in Figure 4.15, is responsible for the stability observed with protein glycoconjugates. Since dendrimers are highly symmetrical and lack the multifunctionality of proteins, no such hydrogen bonding network would be facilitated in the resulting dendrimer neoglycoconjugate product even though the ketoamines and glycosylamine moieties are also cyclic. Furthermore, there are no analogous proximal pendant groups spatially oriented to favour such interconnected, intramolecular stabilizing interactions with dendrimeric glycoconjugates. However, it is conceivable that dendrimeric glycoconjugates prepared from dendrimers of generations higher than those used here, i.e., Generations 3.0 and greater, may support a hydrogen bonding network between the ring oxygen and the hydroxyls of nearby sugars since their peripheries would be expected to be more sterically congested.

From the results presented here, it would appear that while the *in vacuo* glycation method is very amenable to the preparation of stable protein neoglycoconjugates, it may not be suitable for the synthesis of stable dendrimer neoglycoconjugate end-products. However, since they can be easily prepared in fairly high yield under optimal conditions of pH and temperature, and maintain reactive secondary amine functionality, dendrimer glycoconjugate

products prepared in this way may be useful as starting materials for divergent dendrimeric modification. Further investigation into this and the stability of higher generation dendrimeric glycoconjugates prepared using the *in vacuo* glycation technique is warranted since there are many potential therapeutic applications.

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Yeboah, F. K., Alli, I. and Yaylayan, V. A. (1999), *J. Agric. Food Chem.*, **47**: 3164-3172.

Chapter 6 Conclusions

The discovery that proteins can readily undergo modification in the lyophilized state has many practical benefits. Since this research is still in the early stages, the work presented in this thesis barely scratches the surface of that which needs to be done, particularly towards characterization of cross-linking sites and determination of optimal reaction conditions, in order to fully exploit the potential of the methodologies arising from this discovery. Nonetheless, some interesting theoretical insights and practical results have been gained by the progress made thus far.

6.1.1 Theoretical Aspects

One of the most interesting theoretical aspects arising from the results of this and related work currently ongoing in the Kaplan laboratory is the evidence for proton transfer that occurs among ionizable functional groups of compounds in the lyophilized state under conditions of elevated temperature and reduced pressure. Reactions involving protonated amine groups, which are not observed in a solvated environment, occur readily under *in vacuo* conditions giving rise to covalently bonded products. Evidence for this was observed in the protein modification and cross-linking experiments using *p*-nitrophenylchloroformate as well as in the dendrimer and protein glycation and reducing sugar-based cross-linking experiments.

The accepted mechanism for the glycation reaction in aqueous solution involves nucleophilic attack of the amino group of a protein on the carbonyl group of the open chain form of the reducing sugar. The resulting Schiff base undergoes Amadori rearrangement to form the ketoamine derivative. Since lysine ε-amino groups in proteins normally have

ionization constants of 10.5 to 11, very little glycation would be expected below pH 9.5 where the protonated form of these groups is dominant. However, the results of experiments with proteins and protonated dendrimers reported in this thesis and from work done previously by the Kaplan research group (Kaplan *et al.*, 2002) have shown that the protonated form of the amino group is readily glycated in the dry state and the kinetics of the reaction can be dramatically accelerated by first lyophilizing and heating under reduced pressure. The mechanism by which *in vacuo* protein glycation occurs has not been definitively established but it is clearly different from that postulated for glycation in solution.

The results obtained to date indicate that, unlike many of the available solution methods, protein cross-linking *via* the *in vacuo* method does not result in uncontrolled random cross-linking. In solution, the conformational mobility of proteins is unrestricted and the intramolecular and intermolecular interactions between side chains and any other molecules present are usually weak. During lyophilization protein mobility becomes more restricted and as water is drawn off, the interactions become significantly stronger, steering the protein towards a 'fixed' lowest energy conformation. Incubation of the lyophilized material at elevated temperatures provides the energy necessary to drive proton and/or electron transfer reactions between the proximally oriented reactive functional groups, resulting in covalent bond formation. Any gaseous products will be drawn off in the evacuated environment thus driving forward otherwise unfavorable reactions. Since this is very much an entropically driven process, the cross-linked product should retain conformational order and in the case of enzymes, catalytic integrity.

6.1.2 Practical Aspects

Indeed, this is exemplified by the retention of full catalytic activity observed with the covalently linked dimer of ribonuclease, A which was a product of *p*-nitrophenylchloroformate activation under *in vacuo* conditions. In contrast, chemical modification or cross-linking by conventional solution chemistry often involves exposing the protein to highly reactive reagents which may induce conformational changes resulting in non-specific, random cross-linked molecular orientations and decreased catalytic efficiency with enzymes. The fact that a protein can be lyophilized at a pH value at which it retains its native structure and biological activity prior to modification, is a big advantage of the *in vacuo* procedure in terms of retention of activity.

Some of the other practical advantages of the *in vacuo* reaction method in terms of glycation, can be summed up by the following points: 1) a wide range of protein and/or carbohydrate quantities, *viz.* gram to picogram quantities can be used, thus making it applicable to both microscale and larger scale reactions; 2) elevated temperatures can be used to increase the rate of glycation without structural damage to the protein or carbohydrate; and 3) the extent of glycation can be controlled either by adjusting the protein/carbohydrate ratio or by the addition of excipients. Temperature and duration of heating may also play a role in product outcome, but this has yet to be determined.

The success of the *in vacuo* glycation methodology for the facile preparation of a multivalent neoglycoprotein was demonstrated by the results obtained using BSA and the disaccharide, lactose. Results from reactions with the reducing sugar-based cross-linkers developed in conjunction with this methodology show that antennary sugars can also be tethered to a protein by way of two or more reducing sugars separated by a spacer. While

such neoglycoproteins prepared in this way appear to be stable *in vitro*, the potential for use as therapeutic agents in terms of stability has yet to be assessed under *in vivo* conditions. In order for these neoglycoproteins to be effective and considered worthy candidates for synthetic vaccines, they must be sufficiently stable to elicit an immune response *in vivo*, yet not so stable as to thwart eventual necessary biodegradation by catabolism. Unlike many other therapeutic protein or polymer-based neoglycoconjugates and implantable biomaterials prepared using chemical methods, the products of degradation of the neoglycoproteins prepared using the *in vacuo* methodology are not be expected to be toxic since they would simply return to their native proteins and sugars. This, coupled with the fact that no costly reagents or toxic solvents are required for the synthesis and purification of such neoglycoproteins, justifies further research into the preparation of neoglycoproteins using the *in vacuo* glycation methodology for therapeutic applications.

6.1.3 Future Work

The results reported in this thesis establish *in vacuo* protein modification as a new platform technology. Many new avenues of research for the development of novel protein products now present themselves. Modification and cross-linking of biocompatible naturally occurring polymers such as collagen, chitosan and hyaluronic acid for the preparation of biomaterials is the subject of much ongoing biomedical engineering research. Many of these natural macromolecules possess functional groups that are amenable to modification by the *in vacuo* procedures described in this thesis. For example, there is a good possibility that modification of the glucuronic acid-based polysaccharide, hyaluronic acid, may be achieved through activation of its repeating carboxylic acid groups by *in vacuo* reaction with *p*-nitrophenylchloroformate. Collagen, a protein rich in lysines, could be easily cross-linked

using reducing sugar-based cross-linkers such as those developed here under *in vacuo* glycation conditions. Similarly, the amino groups of repeating glucosamine units found throughout the chitosan polysaccharide backbone are available for reaction. Since cross-linking using this *in vacuo* glycation technique can be achieved under acidic conditions, it is very suitable for chitosan, which prefers an acidic environment to maintain solubility in solution. Through judicious selection of spacers in designing an appropriate reducing sugar-based cross-linker molecule, physical properties, such as solubility, tensile strength, and elasticity, which may be desirable or necessary for particular applications, could be imparted to biomaterials prepared from either of these naturally occurring polymers.

Since one of the goals of this work was to design and develop reducing sugar cross-linkers to be used under *in vacuo* conditions, optimizing the synthetic methods to increase yields was deemed unimportant at this stage. However, in order for the cross-linkers to have practical value, this is an important issue that should be addressed in future work. Recent research has lead to the development of an improved coupling agent, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride, which efficiently mediates amide coupling in a one-pot reaction between a carboxylic acid and amine in THF for a few hours with reportedly good yields (Kunishima *et al.*, 1999). The appeal of this reagent is that it does not require the presence of a base and thus may be a good alternative to HATU and carbodiimide to improve yields by minimizing problematic β-elimination. Since cross-linking of hemoglobin using sugar-based cross-linkers was successful, and the applications of such cross-linked products have therapeutic and economic value, efforts towards the improving the efficiency of the synthetic procedures warrant further development.

Another advantage of the *in vacuo* methodology for protein modification and glycation is the control over the extent of reaction afforded by the use of excipients. In this and prior work done using this methodology, most of the excpients employed were small molecules, such as trehalose and sorbitol. The interesting results obtained in the *p*-nitrophenylchloroformate experiments with the use of cyclodextrin suggest that larger, more complex, higher molecular weight, non-reducing macromolecules, such as polysaccharides, dextran, and polyethylene glycol may be of merit as excipients. For example, Wehtje *et al.* saw an improvement in the activity of enzymes that had been immobilized onto glass beads by adsorption when polyethylene glycol or innocuous proteins, such as gelatin, had been included as additives in the immobilization preparation (Wehtje *et al.*, 1993). The stabilization and thermal protection that had been provided in those experiments could be equally advantageous in cases where more sensitive or heat-labile proteins are to be modified by the *in vacuo* methodology.

In closing, one of the most satisfying aspects of this research was that each success gave rise to many new avenues of investigation. On the other hand, it was impossible to investigate within a reasonable timeframe many of the potential possibilities for the methodology, let alone investigate in sufficient depth those that were studied. While it started out as a quest for a means to couple a hydrophilic protein to a lipophilic scaffold without damaging the catalytic properties of the enzyme, this work has given rise to novel methodologies for the facile preparation of macromolecular conjugates that is otherwise difficult or impossible to accomplish by current chemical methods.

References

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CLAIMS TO ORIGINAL RESEARCH

1. The reagent *p*-nitrophenylchloroformate was used to activate carboxyl groups of a protein in the lyophilized state under conditions of reduced pressure and elevated temperature in order to achieve homomolecular and heteromolecular cross-linking. Dimers of ribonuclease generated by this technique exhibited twice the activity of the monomeric ribonuclease both in the presence and ablsence of a cytosolic ribnuclease inhibitor.

2. Branched homotetrafunctional and reducing sugar-based cross-linkers were designed and synthesized by coupling glucuronic acid to a G1.0 dendrimer and linear homobifunctional analogues were designed and synthesized by coupling glucuronic acid to a diamine and glucosamine to a diacid.

3. Cross-linked protein products were generated by co-lyophilization of proteins with the reducing sugar-based cross-linkers followed by heating under conditions of reduced pressure. These cross-linkers constitute a novel class of protein cross-linkers.

4. Co-lyophilization of bovine serum albumin and low levels of lactose from neutral, acidic and basic solutions followed by heating under conditions of reduced pressure resulted in the generation of stable, multivalent glycated protein monomers and higher molecular weight oligomers, both capable of binding with a galactose-specific lectin.

5. Lipophilic modified poly(propyleneimine) dendrimers partitioned various analgesics demonstrating potential as a novel detoxication construct design in an artificial liver.

PUBLICATIONS AND CONFERENCE PROCEEDINGS

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2. Kaplan, H., King, M.C., Stewart, N.A.S., *In Vacuo* Glycation of Proteins, Canadian Patent Application, Filing Date, August 4, 2002

3. Kaplan, H., Hefford, M.A., King, M.C., Simons, B.L., Stewart, N.A.S., "Covalent Cross-linking of Proteins Without Chemical Reagents", 5th European Symposium of the Protein Society, Florence, Italy. Mar.29-Apr.2, 2003. Abs. 249

4. King, M.C., Stewart, N.A.S., Kaplan, H., "*In Vacuo* Glycation as an Approach to Covalent Cross-linking of Proteins", 16th Symposium of the Protein Society, San Diego, California, USA. Aug.17-21, 2002. Abs. 601

5. King, M., Roy, R., Sheardown, H., "Modified Dendrimers for Partitioning of Lipophilic Drugs and Applicability in Removal of Toxins from Blood", 6th World Biomaterials Congress, Kamuela (Big Island), Hawaii, USA. May 15-20, 2000. Abs. P 1448