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DEVELOPMENT OF GLYCOPEPTIDE CARRIERS FOR RECEPTOR MEDIATED GENE DELIVERY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By

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* * * * *

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DEDICATION

To
My Parents

Inderjeet Kaur Wadhwa
&
Jaimal Singh Wadhwa
ACKNOWLEDGEMENTS

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INTRODUCTION

Receptor Mediated Gene Delivery and the Asialoglycoprotein Receptor

Gene delivery into cells has been an important research tool for many years to study the biology of living organisms. Over the last decade however, increasing attention has been focussed on gene delivery methods due to the emerging science of gene therapy. This is because genes are attractive candidates for use in a variety of disease states due to the ability to produce therapeutic biomolecules using the biosynthetic machinery provided by host cells. Methods for transfecting genes into cells in culture include calcium phosphate or DEAE dextran coprecipitation, electroporation, particle bombardment, scrape loading, sonication, direct injection, liposomal delivery, gene transfer by viral vectors, and receptor mediated gene delivery. However, transfection of cells in vivo for gene therapy poses special problems, thus restricting the use of most of these methods to ex vivo protocols.

Engineered viruses, cationic liposomes, receptor targeted DNA carriers, and naked DNA plasmids have emerged as promising vectors for the introduction of therapeutic genes into cells both ex vivo and in vivo. Of these, viral vectors are the most efficient, and are being used currently in many gene therapy clinical trials.
However, due to long term safety concerns, non-viral methods may eventually find more acceptance for routine clinical use. Among non-viral methods, receptor mediated gene delivery has the greatest potential for targeting specific tissue or cell types based on the specific recognition of ligands by unique receptors on these cells.

This approach, schematically outlined in Figure 1, was pioneered by Wu and Wu\textsuperscript{13,14}, and employs a receptor ligand covalently attached to a polycationic anchor that binds DNA by non-covalent, but strong ionic interactions. The choice of the ligand is based on the binding characteristics of the receptor system to be targeted, which must be capable of internalizing the bound ligand by endocytosis. DNA carriers have been designed to transfect hepatocytes via the asialoglycoprotein receptor (ASGP-R)\textsuperscript{13-21} or the insulin receptor\textsuperscript{22,23}; lung cells have been targeted via thrombomodulin\textsuperscript{24}, surfactant protein A\textsuperscript{25}, or lectins\textsuperscript{26}; and leukaemic T cells targeted by means of a mucin antigen\textsuperscript{27}. Alternatively, diverse cell types derived from both normal and tumor tissues have been transfected by means of the transferrin receptor\textsuperscript{28-30}.

Of all these receptor systems, the ASGP-R has been studied in greatest detail, and is the first discovered mammalian lectin\textsuperscript{31,32}. This receptor is found exclusively on hepatocytes, and is believed to function in part by maintaining proper serum concentrations of circulating glycoproteins, by selectively removing those that have been de-sialylated and possess exposed galactose residues\textsuperscript{32-34}. In addition to asialoglycoproteins, the receptor binds a broad range of artificial molecules that
Figure 1. Receptor Mediated Gene Delivery. A. Plasmid DNA encoding the desired gene(s) binds with carrier molecules, leading to condensed carrier-DNA complexes with receptor ligands anchored on the surface. B. Carrier-DNA complexes bind to cell surface receptors, and are internalized into endosomes following receptor mediated endocytosis. However, internalized DNA must escape from the endosomal compartment to the nucleus if gene expression of the therapeutic protein(s) is to occur.
possess terminal galactose or N-acetyl-galactosamine residues, and internalizes them by endocytosis in a manner relatively uninfluenced by the size or composition of the aglycone. Further, the ASGP-R is also well conserved in structure and ligand specificity across many mammalian species, allowing its study in various animal models.

These aspects of the ASGP-R have led to the design and testing of numerous hepatocyte targeted drug carriers, with potential applications in antiviral, immunoactivation, enzyme replacement, anticancer and gene therapies. With respect to the latter, selective targeting of the ASGP-R has been accomplished with ligands possessing terminal galactose residues such as asialo-orosomucoid, galactosylated proteins or polymers, and galactosylated synthetic ligands. The anchor utilized most often is poly-L-lysine in the molecular weight range of 20-60 kDa. An important property of polylysine is its ability to condense DNA into compact structures which may be small enough for internalization into cells by endocytosis.

However, internalization of condensed DNA into cells is not sufficient for successful gene delivery and expression, since the DNA must reach the nucleus in order to be expressed. It is known that after endocytosis, the internalized receptor and ligand may be sorted into various pathways. In the case of the ASGP-R, the receptor bound ligand is sorted into endosomes, which mature into (or fuse with) lysosomes while the receptor shuttles back to the surface to accept more ligand. In the
lysosomes, the internalized ligand is ultimately degraded by the action of various enzymes. This fate represents a serious obstacle for receptor-mediated gene delivery, and efficient transfection is obtained only when a substantial proportion of the delivered genes is allowed to escape from the lysosomes. Endosomal escape of internalized complexes has been achieved by the use of various lysosomotropic agents such as chloroquine\textsuperscript{29,44}, fusogenic peptides\textsuperscript{17,45-48}, or even cotransfected virus particles\textsuperscript{16,49-54}.

It is not known how DNA complexes reach the nucleus once delivered to the cytoplasm. It may be speculated that the cationic nature of the complexes is a determinant for nuclear transport, since nuclear proteins such as histones are predominantly cationic. However, intracellular nuclear transport probably represents another significant barrier to effective gene delivery, one that may be potentially overcome by the use of nuclear targeting signals\textsuperscript{55-57}.

STATEMENT OF THE PROBLEM

Receptor mediated gene delivery is a promising non-viral approach for the selective transfer of DNA into specific cell types, for the purpose of safe and controlled gene therapy. However, this approach has also remained the most undeveloped of the non-viral methods, due to several reasons. Although it has been shown to work in principle by several groups, gene transfer efficiencies remain low, in part due to the several biological barriers that must be navigated, especially for \textit{in}
vivo gene delivery. Further, the DNA carriers that have been described are structurally
heterogeneous collections of crosslinked macromolecules which are difficult to
characterize and study with precision. It follows therefore, that complexes between
these carriers and plasmid DNA present even greater difficulties in characterization,
formulation, and biological testing.

Carriers for efficacious receptor mediated gene delivery need to satisfy several
requirements. They must form stable and well characterized complexes with plasmid
DNA encoding therapeutic gene(s) and controlling elements. They must offer
protection to the DNA from enzymatic or biochemical degradation until it reaches its
site of action. They must cause targeting of the DNA to specific cells types, tissues
or organs to allow small doses to be effectively concentrated at desired sites. They
must allow transport of the DNA through various extracellular and intracellular
barriers to reach the nucleus. And as with any drug carrier to be used in vivo, they
must be non-toxic, and non-antigenic. Given the multitude and complexity of these
criteria, it is clear that along with rationally designed therapeutic plasmids, receptor
mediated gene delivery for clinical use requires the careful, stepwise, and rational
design of DNA carriers.

Fortunately, due to the seminal work done by several researchers in the field,
many steps towards constructing targeted DNA carriers have already been taken, and
receptor mediated gene delivery has been demonstrated both in vitro and in vivo[4,41,58-]
62. Various carrier molecules targeted to the ASGP-R have been described in the
literature, that are glycoprotein$^{13,14,16}$, glycosylated polymer$^{15,17,18}$ or neoglycoprotein conjugates$^{20,21}$, in the approximate molecular weight range of 50 kDa to 500 kDa. However, the heterogeneous components of these high molecular weight conjugates and the consequent carrier design utilizing random chemical coupling makes it difficult to exert fine control over structure and function.

In order to overcome these problems, this thesis project is focussed towards the development of low molecular weight glycopeptide carriers. The overall objective is to initiate the design of well defined DNA carriers, whose structure and function can be controlled and modulated in order to study and optimize receptor mediated gene delivery. It is hoped that this approach will not only result in efficient and well characterized DNA carriers, but will also lead to insights into the physico-chemical properties of carrier-DNA complexes, and further allow an increased understanding of how these properties impact their biological function.

The asialoglycoprotein receptor was selected in order to achieve gene delivery into hepatocytes. Therefore the first major goal of this thesis project was to prepare an oligosaccharide ligand capable of binding the ASGP-R with high affinity. The second major goal was to use this oligosaccharide ligand to prepare a prototype glycopeptide carrier and demonstrate the feasibility of using such low molecular weight carriers for receptor mediated gene delivery. And the third major goal was to improve upon the prototype design in order to allow stepwise optimization of structure and function.
The function of DNA carriers cannot be studied using the carriers in isolation; it can only be determined in complex with the DNA to be delivered into cells. Further, the necessity of making structurally defined carriers is related not only to the production of homogeneous and well characterized carrier molecules, but even more so to the preparation of well characterized DNA complexes with predictable and controllable properties. Accordingly, a fourth major goal of this thesis project was to develop analytical methods to probe and monitor DNA-carrier complexes, with the hope that such techniques would allow insights into the complexation process and aid in the optimization, formulation, and quality control of the complexes.
CHAPTER I

Isolation and Purification of N-Linked Oligosaccharides from Bovine Fetuin.

INTRODUCTION

Receptor binding affinity is one of the most important criteria for targeted drug (or gene) delivery, since carrier molecules must compete for receptor binding with all endogenous ligands present in vivo. Receptor binding in vivo must also compete with any other elimination processes occurring at the same time, and further competition may occur due to non-specific binding to plasma proteins. Although the ASGP-R binds and internalizes most galactose terminated ligands, it shows markedly different affinities for these depending on structure\textsuperscript{63,64}. Monovalent ligands such as galactose, lactose, and monoantennary galactosides bind with a millimolar dissociation constant, which is far below that needed to achieve targeting under physiological conditions. In comparison, divalent galactose terminated oligosaccharides bind with affinities that are three orders of magnitude higher but still fail to target the receptor appreciably in vivo. However, oligosaccharide ligands containing a cluster of three galactose residues bind to the receptor with a nanomolar dissociation constant which is sufficient to achieve significant targeting under physiological conditions\textsuperscript{65}. 

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This relationship between sugar clustering and enhanced binding affinity is related to the structure of the ASGP-R receptor which is composed of three subunits, arranged on the cell surface in a geometrically defined manner, with a sugar binding site on each subunit\(^{66,67}\). Accordingly, the best ligands for the ASGP-R possess the proper conformation (and sufficient flexibility) to present a multivalent cluster of terminal galactose residues in a suitable spatial arrangement, as shown schematically in Figure 2. An even further increase in binding affinity is obtained by substituting the galactose residues with N-acetylgalactosamine residues, which display tighter binding to the individual subunits\(^{65,68}\).

Due to these considerations, multivalent N-linked oligosaccharides with terminal galactose or N-acetylgalactosamine residues are the best known ligands for the ASGP-R. Glycoproteins with appropriate oligosaccharides, and neoglycoproteins prepared by chemically coupling galactose or N-acetylgalactosamine to proteins at a high density (to obtain clustered residues) may also provide high affinity ligands and ASGP-R targeting; however, these species often cannot be obtained and characterized as homogeneous structures, and may possess additional disadvantages such as antigenicity. On the other hand, low molecular weight oligosaccharides can be purified to homogeneity and fully characterized. They are hydrophilic and fully biodegradable, and since these are endogenous structures, no toxicity or antigenicity is expected. Furthermore, similar to the case of ASGP-R, there is evidence that many
Figure 2. Binding of Complex N-Linked Oligosaccharides to the Asialoglycoprotein Receptor. The ASGP-R presents its extracellular carbohydrate binding domains (CRDs) in a trimeric arrangement that is spatially defined. Therefore, binding affinity is influenced not only by the specificity of the individual CRDs for galactose and N-acetylglactosamine residues, but also by the clustered arrangement of these residues as determined by oligosaccharide conformation and flexibility.
other lectins found in mammalian cells have evolved to bind endogenous oligosaccharide ligands with high affinity and exquisite specificity\textsuperscript{69-72}. This raises the intriguing possibility of using oligosaccharides as highly specific "glycotargeting" agents for cell types that possess unique endocytosing lectins\textsuperscript{38}.

In order to use N-linked oligosaccharides as targeting ligands for site-specific gene delivery, purified and well characterized oligosaccharide structures are required. However, synthetic pathways for producing complex oligosaccharides have not been yet fully developed. Therefore, at present, isolation of oligosaccharides from naturally occurring glycoproteins followed by their purification is the only option to obtain milligram quantities of complex N-linked oligosaccharides.

Release and subsequent isolation of N-linked oligosaccharides from glycoproteins can be accomplished by two general approaches, chemical and enzymatic\textsuperscript{73,74}. Enzymatic methods rely upon either the enzyme N-Glycopeptidase F (GPF), or Endo-β-N-acetylglucosaminidases. GPF has broad specificity, and can release most N-linked oligosaccharides in their unreduced form by cleavage at the N-glycosidic bond. Various N-acetylglucosaminidases are known, and these cleave within the chitobiose core found in all N-linked oligosaccharides. However they have certain glycon specificities, and result in truncated core structures. Chemical methods for N-linked oligosaccharide release include the use of alkali, lithium aluminum hydride, and hydrazine; and these methods also allow the simultaneous release of O-linked oligosaccharides. However, only hydrazinolysis allows the oligosaccharides
to be obtained in their unreduced form (which allows further derivatization and labelling), although substituents on sialic acids may be lost. On the other hand, hydrazinolysis also requires the use of potentially explosive chemicals at elevated temperatures, and is therefore best performed using specialized equipment. Based on these considerations, enzymatic release by GPF was selected for this study.

Unfortunately, the purification of oligosaccharides isolated from glycoproteins is difficult, especially in large quantities. This is due to the fact that most glycoproteins possess a variety of oligosaccharide structures, many of which are very closely related and difficult to separate. Oligosaccharides do not have any chromophore for facile detection, nor do they possess hydrophobic groups for manipulation by high resolution techniques like reverse phase - high performance liquid chromatography (RP-HPLC). Accordingly, most approaches for separation of oligosaccharides rely upon the derivatization of the reducing end with a hydrophobic chromophore or fluorophore. These procedures have been proven to be very sensitive and useful for analytical purposes. However, the derivatized oligosaccharides produced may not be optimal for probing biological activity and binding since the natural closed ring form of the reducing end N-acetylglucosamine (GlcNAc) residue is permanently altered and this may influence biological recognition by lectins. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been used with great success to separate closely related oligosaccharides without any derivatization. However at
present this technique is useful for analytical purposes only due to the low capacity of pellicular anion exchange resins used in HPAEC columns\textsuperscript{83}.

Recently, new coupling schemes have been developed for the reducing end modification of oligosaccharides via glycosylamine formation\textsuperscript{80,84-86}. Oligosaccharide glycosylamines can be derivatized without altering the closed ring form of the reducing terminal, and the bond formed with the derivatizing moiety closely resembles the natural linkage of N-linked oligosaccharides to asparagine\textsuperscript{87}. Using a similar strategy, a procedure was developed for the derivatization of the reducing end of oligosaccharides with boc protected tyrosine\textsuperscript{88}. The tyrosinamide derivatives can be sensitively detected and quantitated by both absorbance and fluorescence, have excellent resolution characteristics on reverse phase HPLC, and may be radioiodinated for biological studies\textsuperscript{89}. In addition, further derivatization of the amine terminus of tyrosine may be carried out after the facile removal of boc, and the derivatization can also be reversed to produce purified reducing oligosaccharides\textsuperscript{90}.

In this chapter, the preparation and characterization of the oligosaccharides from bovine fetuin will be described, with emphasis on a galactose terminated triantennary oligosaccharide which has been utilized throughout in this study. Fetuin was selected due to its high level of glycosylation, and due to the presence of the aforementioned triantennary as the major oligosaccharide species. However, this approach for the preparation of tyrosinamide oligosaccharides has subsequently also been applied to porcine fibrinogen\textsuperscript{91}, ovotransferrin\textsuperscript{92} and ovalbumin\textsuperscript{93}, and the overall
procedure has been further optimized.

MATERIALS AND METHODS

Bovine fetuin, iodoacetamide, dithiothreitol, boc-tyr-NHS ester, Sephadex G-25, and α-monothioglycerol were purchased from Sigma Chemical Co., St. Louis, MO. TPCK treated trypsin was from Worthington Enzymes, Freehold, NJ. Reverse phase "Spherisorb" HPLC columns were purchased from Phase Separations, Norwalk, CT. Carbopac PA1 HPAEC column was from Dionex Corp., Sunnyvale, CA. Anion and cation exchange resins AG1X2 and AG50WX2 were from Bio-Rad, Richmond, CA. N-glycosidase F (EC 3.2.2.18) was obtained from New England Biolabs, Beverly, MA. Neuraminidase from Clostridium perfringens (EC 3.2.1.18) was from Boehringer Mannheim, Indianapolis, IN. Dialysis tubing was purchased from Spectrum, Houston, TX. Only distilled and deionized water was used. All other chemicals were of reagent grade or better.

Isolation of Oligosaccharides from Fetuin.

Bovine fetuin (5 g) was dissolved in 40 ml of 8 M guanidine hydrochloride buffered with 0.2 M tris (pH 8.2), and treated with 1.1 g of dithiothreitol for 1 h at room temperature. The reduced disulfide bonds were alkylated by treatment with 5.1 g of iodoacetamide for 30 min at room temperature. Excess reagents were removed by dialysis in 12,000 MWCO tubing against distilled running water for 24 h, followed
by dialysis against 4 L of 50 mM ammonium bicarbonate pH 8.0. The retentate was
digested with 50 mg of trypsin for 24 h at 37°C, followed by dialysis against water
for 24 h in 12,000 MWCO tubing to remove low molecular weight peptides and
buffer salts.

The retentate was lyophilized, and then dissolved in 100 ml of 50 mM
ammonium acetate, and pH adjusted to 7.5 by adding 1 M ammonium hydroxide. The
N-linked oligosaccharides were released by treatment with 100μl (0.2 IU) of GPF for
72 h at 37°C. The time course of the reaction was monitored by HPAEC-PAD using
a PA1 column (eluted at 1 ml/min with 100mM sodium hydroxide, with a gradient of
0 to 250 mM sodium acetate applied over 30 min), while detecting eluting sialylated
oligosaccharides. After complete release of oligosaccharides, the pH was adjusted to
5.0 with glacial acetic acid and 3.5 units of neuraminidase from clostridium
perfringens added for desialylation of oligosaccharides at 37°C for 24 h.
Desialylation was monitored by HPAEC-PAD using the same conditions as described
above and was evidenced by the appearance of sialic acid in the chromatogram, as
well as by decreased retention time of the asialo-oligosaccharides.

Deglycosylation and desialylation was accompanied with precipitation of some
peptides, and these were separated by centrifugation at 13,000 g. The released
oligosaccharides remained in the supernatant and were lyophilized, reconstituted in
water, and chromatographed in two equal parts on a mixed bed ion exchange column
(2.5 cm x 40 cm; upper resin: AG50WX2 hydrogen form; lower resin: AG1X2 acetate
form) eluted with water with detection at 214 nm. The asialo oligosaccharides eluted as a mixture in the first UV absorbing peak in the void volume (125 ml). On eluting the ion-exchange column with 1 M acetic acid, sialyl oligosaccharides were obtained between 130 and 240 ml, slightly separated from sialic acid. The presence of carbohydrate was determined by analyzing each fraction by the phenol-sulfuric acid assay\textsuperscript{24}, and the appropriate fractions were pooled and freeze dried. Yield data was obtained by quantitative glucosamine analysis using HPAEC-PAD\textsuperscript{95}.

**Reducing End Modification of Oligosaccharides with Tyrosine.**

Lyophilized asialo-oligosaccharides (10 μmol) were dissolved in 1 ml water and 1 g of ammonium bicarbonate was added, followed by heating at 50°C for 24 h. HPAEC-PAD was used to monitor the formation of oligosaccharide-carbonate and oligosaccharide-glycosylamines (using a PA1 column eluted at 1 ml/min with 100 mM sodium hydroxide, with a gradient of 0 to 250 mM sodium acetate applied over 30 min). Excess ammonium bicarbonate was removed by repeated freeze drying (5-6 times) during which time the oligosaccharide carbonate converted into the glycosylamine form as monitored by HPAEC. Tyrosine derivatization of the glycosylamine was performed by adding 100 molar excess of boc-tyr-NHS ester in 1.2 ml of DMF, and incubating at 50°C for 3 h, while monitoring the formation of the derivative by HPAEC as described above. The reaction also resulted in the esterification of the hydroxyl groups of the oligosaccharides, and the esters were
lysed by treatment with 4 ml of 1 M sodium hydroxide. The precipitate that formed was removed by centrifugation and extracted twice with sodium hydroxide. The pooled extracts were directly gel-filtered on a Sephadex G-25 column (2.5 x 50 cm) eluted with 1% v/v pyridine-acetic acid (1:1) buffer while monitoring absorbance at 280 nm. Tyrosinamide oligosaccharides as well as underivatized oligosaccharides eluted as a single peak between 75-125 ml, separated from the excess tyrosine reagent which eluted later, as judged by phenol sulfuric acid assay of the fractions. The quantity of recovered oligosaccharides was estimated by glucosamine analysis as well as by absorbance at 280 nm (ɛ = 1330 M⁻¹ cm⁻¹).

Mono-sialylated oligosaccharides were derivatized with some changes in the procedure due to low solubility in DMF. Ethylene glycol (0.5 ml) was added to dissolve the oligosaccharide-glycosylamine (10 μmol), warmed to 50°C, followed by the addition of 150 molar excess of boc-tyr-NHS ester in 2 ml of DMF. The reaction was allowed to proceed for 2 h at 50°C, and the same extraction and gel filtration procedures were then performed as described above.

**HPLC Purification of Derivatized Oligosaccharides.**

A computer interfaced HPLC (ISCO Inc. Lincoln NE) was used, consisting of two pumps (Model 2350), a variable UV/Vis detector (V4), and an automated fraction collector (Foxy 200). The tyrosinamide oligosaccharides were dissolved in water (10 μmol/ml) and 2 μmol portions were chromatographed on a 2 x 25 cm semi-
preparative C8 reverse phase column eluted isocratically at 10 ml/min with 35 mM acetic acid and 8% acetonitrile, with detection at 280 nm. Each major peak was collected, lyophilized, and found to rechromatograph as a single peak on analytical reverse phase HPLC as well as on HPAEC.

In the case of sialyl oligosaccharides, removal of boc was required to enhance the separation in order to remove minor contaminants. Boc was removed by treatment of the derivatized oligosaccharide (5 μmol) with 500 μl of TFA at room temperature for 10 min, and the acid was removed by repeated freeze drying. The deprotected oligosaccharides were dissolved in 500 μl 0.1% TFA, and 2 μmol portions chromatographed on a reverse phase semi-preparative HPLC column as described above, eluted isocratically with 1.25 % acetonitrile in 0.1% TFA. The major peak was collected, lyophilized, and rechromatographed to assess purity.

Structural Characterization of Tyrosinamide Oligosaccharides.

Purified tyrosinamide oligosaccharides were characterized by quantitative monosaccharide analysis, 500 MHz proton NMR, and by FAB-MS. Monosaccharide analysis was carried out according to published methods95, utilizing HPAEC-PAD. For NMR, 1 μmol samples were freeze dried repeatedly in D₂O and prepared in 0.5 ml 99.98% D₂O containing 0.01% acetone as internal standard. Spectra were acquired on a Bruker Aspect spectrometer and processed offline with resolution enhancement using the software package Felix™ (Hare Research, Eugene OR).
For mass spectrometry, each oligosaccharide sample (20 nmol) was prepared in 10 µl water containing 1 µl α-monothioglycerol. The water was removed by speed vacuum and the 1 µl sample was applied to the probe of the spectrometer (Finnigan Matt 900) operated in the positive ion mode.

RESULTS AND DISCUSSION

The steps performed in isolation of reducing oligosaccharides from fetuin are shown in the flowchart in Figure 3, and are briefly summarized here. Fetuin was initially reduced and alkylated in order to allow efficient proteolysis by trypsin. Proteolysis by trypsin is necessary to produce moderate length glycopeptides so that GPF can release oligosaccharides efficiently. This allowed the use of analytical amounts of GPF to deglycosylate gram quantities of glycopeptides. Neuraminidase was used to remove terminal sialic acid residues from the oligosaccharides released by GPF. This is necessary to remove heterogeneity which arises due to incomplete sialylation on some of the released oligosaccharides. Further, in order to target the asialoglycoprotein receptor, subterminal galactose residues on oligosaccharides need to be exposed.

It was found that neuraminidase from Clostridium perfringens was unable to desialylate all the N-linked oligosaccharides obtained from fetuin\textsuperscript{88,89}. This selective desialylation allowed the facile separation of a monosialyl triantennary
Bovine Fetuin

1. Alkylation and Reduction
2. Dialysis and lyophilization

Denatured Protein

3. Trypsin Digestion
4. Dialysis and lyophilization

Peptides and Glycopeptides

5. N-Glycosidase F Digestion
6. Sialidase Digestion
7. Lyophilization

Peptides, Oligosaccharides, and Sialic Acid

8. Ion Exchange Chromatography
9. Lyophilization

Reducing Oligosaccharides (heterogeneous)

Figure 3. Isolation of N-Linked Oligosaccharides from Bovine Fetuin. The oligosaccharides were isolated by a combination of chemical, enzymatic, and chromatographic treatments.
oligosaccharide from the other asialo-oligosaccharides since the former was retained on an ion exchange column upon elution with water while the latter were not. The monosialylated oligosaccharide was subsequently eluted with 1M acetic acid. It is interesting to note that neuraminidase from another source, *Arthrobacter ureafaciens* does not show this selectivity in the desialylation of fetuin oligosaccharides.

The chemical pathway to obtain tyrosinamide oligosaccharides is shown in Figure 4. Reducing oligosaccharides were converted into their glycosylamines by incubation with excess ammonium bicarbonate. The rate of glycosylamine formation could be accelerated, from 5 days at room temperature to 24 h, by carrying out the incubation at 50°C (with a 90% decrease in starting material). Two species were formed - oligosaccharide glycosylamines and glycosylamine carbonates. The latter could be reversed into their glycosylamines by repeated freeze drying, which results in removal of carbonate in the form of carbon dioxide. The reaction progress was monitored (by noting the formation of product and disappearance of reactant) using HPAEC-PAD as shown in Figure 5.

Derivatization of oligosaccharide with boc-tyr was performed in dimethyl formamide (DMF). Any water in the reaction mix caused problems due to a) low solubility of boc-tyr-NHS ester in water and b) reversal of the glycosylamine back to the reducing oligosaccharide. On the other hand, exclusion of water from the reaction promoted a side reaction where hydroxyl groups of the oligosaccharides were esterified with the boc-tyrosine reagent. The presence of esters could not be
Figure 4. Reducing-End Modification of N-Linked Oligosaccharides with Tyrosine. Reducing oligosaccharides (only the reducing end disaccharide shown, "R" represents the rest of the structure) were converted into their glycosylamines and glycosylamine-carbonates by treatment with ammonium bicarbonate. After reversal of the carbonates, the glycosylamines were reacted with the NHS ester of boc-tyrosine to form tyrosinamide oligosaccharides.
Figure 5. HPAEC-PAD Analysis of the Formation of Glycosylamine and Tyrosinamide Oligosaccharides. A. HPAEC-PAD elution profile of asialo-reducing oligosaccharides. B. Upon treatment with excess ammonium bicarbonate, the formation of glycosylamines and glycosylamine-carbonates was observed. C. The glycosylamine-carbonates were reversed upon freeze drying. D. The glycosylamines were converted to tyrosinamide oligosaccharides by conjugation with the NHS ester of boc-tyrosine. Identical elution conditions (described in text) were used for the chromatograms shown in all four panels.
monitored by HPAEC due to rapid de-esterification under the basic conditions used (100mM NaOH) to elute the PA1 column; however the low solubility of derivatized oligosaccharides in water and the low recovery of tyrosinamide oligosaccharides from gel filtration indicated the same. The problem was resolved by hydrolysis of esters by treatment with sodium hydroxide prior to gel filtration, resulting in good recoveries of the desired product (70-80%). It is felt that previous published reports showing low coupling yields via glycosylamine formation were probably due to incomplete removal of carbonate and due to glycosylamine reversal in aqueous conditions.

The glycosylamine of the monosialylated oligosaccharide was very poorly soluble in DMF, and this problem was partially overcome by including ethylene glycol in the reaction mix, resulting in reasonable yields of the coupled product (50%). Further, boc-tyr coupled monosialyl-oligosaccharide could not be purified to homogeneity on reverse phase HPLC under the conditions used for boc-tyr coupled asialo oligosaccharides. Removal of boc from monosialyl-oligosaccharide resulted in an enhanced separation under ion-pairing conditions that allowed purification to homogeneity. Boc was removed by exposure of the lyophilized oligosaccharide to neat TFA at room temperature for 10 min. Even though sialic acids are ordinarily labile under acidic conditions, in absence of any water no desialylation or degradation of glycosidic linkages was observed, when exposure to TFA was limited to 10 min.

Removal of boc from asialo-oligosaccharides was not necessary for their purification. However this procedure can be applied whenever the amine on the
tyrosine needs to be exposed to allow further derivatization. The tyrosine ring itself can be radioiodinated with or without the presence of boc whenever required. This is a very good advantage of these derivatives since previous methods of radioiodinating oligosaccharides required tyramine derivatization\(^9\) by reductive amination which permanently alters the closed ring structure of the reducing end.

The HPLC separations of tyrosine derivatized oligosaccharides showed very good resolution and capacity, and representative chromatograms are shown in Figure 6. The separations were primarily based on hydrophobicity. Triantennary oligosaccharide, having two more sugar residues than bi-antennary, is more hydrophilic and eluted 10 minutes earlier under the conditions described. Three major tyrosinamide oligosaccharides were recovered from fetuin by HPLC purification: triantennary, bi-antennary, and sialyl-triantennary. The structures of these three oligosaccharides are shown in Figure 7, and their yields are detailed in Table 1. The relatively high yield of triantennary was an important factor in allowing the use of this oligosaccharide for the studies described in subsequent chapters.

The structures of the purified oligosaccharides were confirmed using quantitative monosaccharide analysis, high field proton NMR and further by FAB-MS. Monosaccharide analysis provided the identity and molar ratios of the individual sugar residues, and the analysis for the triantennary oligosaccharide is shown in Figure 8. NMR analysis was performed by comparing the chemical shifts of the
Figure 6. Semipreparative RP-HPLC Purification of Tyrosinamide Oligosaccharides from Fetuin. A. Purification of boc-tyrosinamide-asialo-oligosaccharides. Peaks 1 and 2 are the triantennary and bi-antennary oligosaccharides, respectively. B. Purification of tyrosinamide-sialyl-triantennary oligosaccharide (Peak 3). Structures of the numbered peaks are shown in Figure 7.
Figure 7. Structures of the Purified Tyrosinamide Oligosaccharides from Bovine Fetuin. The structures of oligosaccharides designated as boc-tyrosinamide-triantennary (Fig. 6A, Peak 1), boc-tyrosinamide-biantennary (Fig. 6A, Peak 2), and tyrosinamide-sialyl-triantennary (Fig. 6B, Peak 3).
Table 1. Yield Analysis of N-linked Oligosaccharides Obtained from Bovine Fetuin.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Amount Recovered</th>
<th>Yield</th>
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<tbody>
<tr>
<td>Ion Exchangee</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asialo-oligosaccharides</td>
<td>78</td>
<td>52%</td>
</tr>
<tr>
<td>Sialyl-oligosaccharides</td>
<td>8</td>
<td>5%</td>
</tr>
<tr>
<td>Tyrosine Conjugationd</td>
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<tr>
<td>Asialo-oligosaccharides</td>
<td>70</td>
<td>47%</td>
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<td>Sialyl-oligosaccharides</td>
<td>4</td>
<td>3%</td>
</tr>
<tr>
<td>Reverse Phase HPLCc</td>
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<td></td>
</tr>
<tr>
<td>Triantennary</td>
<td>35</td>
<td>23%</td>
</tr>
<tr>
<td>Biantennary</td>
<td>5</td>
<td>3%</td>
</tr>
<tr>
<td>Sialyl-Triantennary</td>
<td>2</td>
<td>1%</td>
</tr>
</tbody>
</table>

a The μmols of N-linked oligosaccharides recovered from 5g of bovine fetuin as measured by quantitative glucosamine analysis.

b The percent of oligosaccharide recovered at each step starting with 5g of bovine fetuin containing 149 μmol of N-linked oligosaccharides.

c The oligosaccharides recovered from mixed bed ion exchange chromatography.

d The tyrosinamide oligosaccharides recovered from gel filtration chromatography.

e The final yield of purified tyrosinamide oligosaccharides recovered from RP-HPLC.
Figure 8. Quantitative Monosaccharide Analysis of Boc-Tyrosinamide-Triantennary Oligosaccharide. Monosaccharide analysis was conducted using HPAEC-PAD. A. Elution profile of monosaccharide standards (1 nmol each of fucose [Fuc], N-acetylgalactosamine [GalNAc], N-acetylglucosamine [GlcNAc], galactose [Gal], glucose [Glc], and mannose [Man]). B. Elution profile of the monosaccharides obtained after acid hydrolysis (2M TFA) of 0.33 nmol of boc-tyrosinamide-triantennary. Integration of the monosaccharide peaks yielded the correct amounts of N-acetylglucosamine, galactose and mannose residues.
purified oligosaccharides against characteristic chemical shifts for oligosaccharides and glycopeptides from fetuin reported in the literature. The NMR resonances resulting from the anomeric and acetyl protons of the triantennary oligosaccharide are shown in Figure 9, and the chemical shifts detailed in Table 2. It was found that coupling boc-tyr in a β-glycosylamide linkage preserves the ring closed structure of GlcNAc1 and the linkage closely resembles the natural β amide linkage found between N-linked oligosaccharides and asparagine since the chemical shifts of the anomeric protons in coupled oligosaccharides were found to be in close agreement to chemical shifts reported previously for triantennary and biantennary containing glycopeptides from fetuin. However, the presence of boc caused a significant broadening in the signals from the N-Acetyl part of GlcNAc1, which reverted to normal upon the removal of the boc group.

The molecular mass values of the purified tyrosinamide oligosaccharides were calculated using the computer program "OligoMass" (Appendix), and were found to be in close agreement with the values obtained from FAB-MS, confirming the structural analysis. The major mass ion (M+Na') for the triantennary oligosaccharide was observed to be 2290.9 amu as compared to a calculated exact mass of 2290.8 amu.
Figure 9. Proton NMR Structural Reporter Group Signals for Boc-Tyrosinamide-Triantennary Oligosaccharide. A. The structure of the oligosaccharide, with the individual monosaccharide residues numbered for identification of NMR signals. B. The structures of the individual monosaccharides (in their reducing forms), with the carbon atoms numbered for identification of linkages. C. The resonances from the anomeric protons of the numbered monosaccharides are shown in the left and middle panels. The resonances from the acetyl protons (scaled down in height) are shown in the right panel.
Table 2. Chemical Shifts of the Structural Reporter Group Signals Identified for Boc Tyrosinamide Triantennary Oligosaccharide.

<table>
<thead>
<tr>
<th>Proton</th>
<th>Chemical Shift*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anomeric Proton (H-1) of</strong></td>
<td></td>
</tr>
<tr>
<td>GlcNAc 1</td>
<td>5.017</td>
</tr>
<tr>
<td>GlcNAc 2</td>
<td>4.617</td>
</tr>
<tr>
<td>Man 4</td>
<td>5.119</td>
</tr>
<tr>
<td>Man 4'</td>
<td>4.923</td>
</tr>
<tr>
<td>GlcNAc 5</td>
<td>4.568</td>
</tr>
<tr>
<td>GlcNAc 5'</td>
<td>4.583</td>
</tr>
<tr>
<td>Gal 6</td>
<td>4.463</td>
</tr>
<tr>
<td>Gal 6'</td>
<td>4.475</td>
</tr>
<tr>
<td>GlcNAc 7</td>
<td>4.547</td>
</tr>
<tr>
<td>Gal 8</td>
<td>4.469</td>
</tr>
<tr>
<td><strong>Acetyl protons of</strong></td>
<td></td>
</tr>
<tr>
<td>GlcNAc 1</td>
<td>1.979</td>
</tr>
<tr>
<td>GlcNAc 2</td>
<td>2.082</td>
</tr>
<tr>
<td>GlcNAc 5</td>
<td>2.049</td>
</tr>
<tr>
<td>GlcNAc 5'</td>
<td>2.046</td>
</tr>
<tr>
<td>GlcNAc 7</td>
<td>2.076</td>
</tr>
</tbody>
</table>

* Chemical shift values (ppm) were recorded relative to an internal standard of acetone (2.225 ppm) at 23°C.
Figure 10. Absorbance and Fluorescence Spectra for Boc-Tyrosinamide-Triantennary Oligosaccharide. A. The UV absorbance spectrum, with a maximum at 274 nm. B. The fluorescence spectra with excitation and emission maxima at 275 nm and 305 nm respectively.
An important feature of tyrosinamide oligosaccharides is their absorbance and fluorescence properties (Figure 10) due to the presence of the tyrosine residue. This chromophore allows fast, sensitive and convenient detection and quantitation. The molar absorbance of tyrosine ($\varepsilon_{274} : 1450$ or $\varepsilon_{280} : 1330$ M$^{-1}$cm$^{-1}$) was unchanged upon conjugation to oligosaccharides, as determined by quantitative monosaccharide analysis of the oligosaccharides whose concentration was predetermined by absorbance measurement. UV absorbance was used extensively throughout this project to quantitate tyrosinamide oligosaccharides and their conjugates, with a limit of detection of about 5 nmol in a cuvette, and about 0.5 nmol in a HPLC flow cell. These detection limits can be improved dramatically, to about 5 pmol, by the use of fluorescence.

CONCLUSION

The high affinity binding of galactose terminated oligosaccharides with the asialoglycoprotein receptor may serve to accomplish targeted gene delivery to hepatocytes. Based on this assumption, multi-milligram quantities of a triantennary oligosaccharide were isolated from bovine fetuin, and purified as a tyrosinamide conjugate using newly developed methodology. The oligosaccharide was purified to homogeneity and was structurally characterized, in a first step towards the rational design of receptor targeted glycopeptide carriers for gene delivery.
CHAPTER II

Whole Body Biodistribution and Pharmacokinetics of Triantennary Oligosaccharide In Vivo

INTRODUCTION

As detailed in the previous chapter, milligram quantities of galactose terminated triantennary oligosaccharide (Gal-Tri) were purified from fetuin after tyrosinamide derivatization (Boc-Tri), with the objective of using it as a targeting ligand for the asialoglycoprotein receptor (ASGP-R). Although it is well known that Gal-Tri linked covalently to endogenous glycoproteins binds with high affinity to the ASGP-R\textsuperscript{32,33}, the \textit{in vivo} behavior of the purified oligosaccharide had not been reported in the literature prior to this study. Furthermore, it was not known if the tyrosinamide derivatization of Gal-Tri would compromise the targeting activity.

To address these concerns, the whole body biodistribution and pharmacokinetics of Boc-Tri were studied in mice, after radiolabelling the tyrosine ring with \textsuperscript{125}I. Although the study\textsuperscript{65} also included other purified oligosaccharides and enzymatically remodelled N-acetylgalactosamine terminated oligosaccharides, only the results obtained with Boc-Tri will be detailed in this chapter.
MATERIALS AND METHODS

Sodium $^{125}$Iodide was purchased in 1 mCi amounts (in 100 µl 0.1 M sodium hydroxide) from DuPont, Boston MA. Silica gel TLC plates (60F254), were from Alltech, Deerfield, IL. Sephadex G-10 and carboxymethylcellulose (CMC) were obtained from Sigma Chemical Co., St. Louis MO. Ketamine hydrochloride was from Parke-Davis; silastic catheters (0.012" i.d., 0.025" o.d.) were purchased from Baxter, McGaw Park, IL.

Preparation and Radioiodination of N-linked Oligosaccharides.

The isolation of Boc-Tri from fetuin, and its purification by reverse phase HPLC after tyrosine derivatization\cite{8} has been described in detail in Chapter I. Radioiodination was performed using a modification of the Chloramine T method\cite{102}. 2 nmol of Boc-Tri was prepared in 60 µl of phosphate buffer (0.5 M, pH 7.0) and added to 50 µl of Na$^{125}$I (0.5 mCi) in 0.1 M sodium hydroxide. The iodination was initiated by the addition of 20 µl of 10 mM chloramine T prepared in phosphate buffer; and the reaction quenched 3 minutes later by the addition of 80 µl of 10 mM sodium metabisulfite in phosphate buffer. The radiolabelled oligosaccharide was immediately purified by gel filtration on a disposable Sephadex G-10 column (0.8 x 25 cm) eluted with sterile saline while collecting 0.5 ml fractions. The radioactive peak eluting between 3 - 4 ml consisted of labelled oligosaccharide, and had a specific activity of 125 µCi/nmol, assuming quantitative recovery.
The purity of the radiolabelled oligosaccharide was assessed by thin layer chromatography (TLC). The labelled oligosaccharide (1 μl; 2 nCi) was spotted onto a TLC plate and developed by a solvent system consisting of ethyl acetate, acetic acid, pyridine and water (4:3:1.5:2.5 volume ratio). After development and drying, the plates were exposed to a phosphor storage screen for 12 h at room temperature. The screen was scanned on a PhosphorImager™ system (Molecular Dynamics, Sunnyvale CA), and the data analyzed by quantitative densitometry using ImageQuant™ software. Purity of the oligosaccharide spot was established by obtaining an integration intensity of greater than 95% of the total counts on the lane.

**Pharmacokinetics of Boc-Tyrosinamide Triantennary Oligosaccharide**

Prior to obtaining pharmacokinetic data, the *in vitro* stability of the radiolabelled oligosaccharide was determined in mouse whole blood. The oligosaccharide (1 μl; 50 nCi) was incubated with 100 μl of heparinized blood at 37°C. A 10 μl aliquot was extracted (described on Pg. 39) at time points ranging from 1 to 6 h, and analyzed using TLC and quantitative densitometry as described above.

Adult ICR albino mice (Harlan Sprague-Dawley, Indianapolis IN) weighing 28-32 g were used in the study. They were housed in mice cages located in a limited access area with controlled temperature (26-28°C) and 12 h light-dark cycle; and fed with standard laboratory mice food *ad libitum* for at least 3 days before use. Following general anaesthesia administered by intraperitoneal injection of ketamine
hydrochloride (100 mg/kg), surgery was performed on each mouse to place silastic catheters in both left and right jugular veins, secured in place by silk sutures. Radiolabelled oligosaccharide (1.8 μCi) prepared in sterile saline was administered by 10 μl bolus i.v. dose in the right side catheter, and washed with 20 μl saline. Blood samples (10 μl) were withdrawn by microsyringe from the other catheter at 1, 3, 6, 10, 15, 20, 30, 40, and 60 min after dosing. Each blood sample withdrawn was replaced by an equal amount of sterile saline in the same catheter. Mice were sacrificed by cervical dislocation at the end of each study.

Blood samples were added to 60 μl water placed in microtubes, and the total radioactivity in the samples quantitated by a gamma counter (Hewlett Packard, Palo Alto CA) to obtain blood levels of the oligosaccharide at each time point. To unambiguously determine the amount of unmetabolized radiolabelled oligosaccharide in each sample, the following procedure was used: 200 μl acetonitrile was mixed with vortexing to precipitate serum proteins and the supernatant collected after centrifuging samples for 10 min at 10,000 g. The pelleted protein was washed twice with 50 μl of 80% acetonitrile and the combined supernatants were dried by speed-vacuum evaporation. Sample residues were reconstituted in 10 μl water and 1.5 μl of each sample was spotted onto adjacent lanes of a TLC plate. The plate was developed, autoradiographed for 48 h at room temperature, and analyzed by densitometry. Quantitative estimates of radioactivity in the oligosaccharide spots in each lane were obtained from a calibration curve prepared by spotting known amounts of labelled
oligosaccharide onto a TLC plate and performing autoradiography and data analysis as before.

Pharmacokinetic analysis was performed on a triplicated data set with the aid of PCNONLIN™ (SCI Software, Lexington KY) by obtaining iterative least-squares nonlinear fits to the direct count data, using a two compartment open model described by the integrated equation:

\[ C_b = Ae^{-\alpha t} + Be^{-\beta t} \]

where \( C_b \) is the concentration of the labelled oligosaccharide in blood while \( A \), \( B \) are the y intercepts, and \( \alpha \), \( \beta \) are the slopes of the alpha and beta phases of decline in a log-linear plot of concentration vs. time. Pharmacokinetic parameters were calculated using the fitted values of \( A \), \( B \), \( \alpha \), and \( \beta \) obtained from each mouse.

Biodistribution of Radiolabelled Oligosaccharide

A silastic catheter was surgically inserted into the right jugular vein of an anaesthetized mouse. The radiolabelled oligosaccharide prepared in sterile saline (10 \( \mu l \); 15 x \( 10^6 \) dpm) was administered by i.v. bolus dose into the catheter and washed with 20 \( \mu l \) saline. The catheter was removed and the vein was immediately ligated with a silk suture. After 30 min, the deeply anaesthetized mouse was prepared for whole body cryosectioning by immersing in a hexene dry-ice bath (-70°C) for 5 min, and mounting in 4% (w/v) carboxymethylcellulose cuboid block, which was then frozen at -20°C. Eight longitudinal whole body sections 25 \( \mu m \) thick were obtained.
on a cryo-microtome (LKB 2250, Sweden) at different depths to reveal all major organs. The sections, collected on transparent adhesive tape (Scotch 810, 3M Co. Minneapolis, MN), were allowed to dry at -15°C for 24 h, and then exposed to a PhosphorImager™ screen for 48 h at room temperature. Images were scanned and analyzed utilizing ImageQuant™ software as with TLC autoradiograms.

Quantitative tissue distribution was obtained by direct counting of major organs of intravenously dosed mice. Briefly, mice were dosed as described above with 1.8 μCi radiolabelled oligosaccharide and sacrificed 30 min later by cervical dislocation. Major organs (liver, heart, lungs, spleen, kidney, stomach, small intestine and large intestine) were quickly dissected out, washed with saline, weighed, placed in dry scintillation vials, and the radioactivity in each vial was obtained by direct gamma ray counting.

RESULTS AND DISCUSSION

The aim of the study was to ascertain the biodistribution and pharmacokinetics of tyrosine modified, galactose terminated triantennary oligosaccharide in vivo in mice. The oligosaccharide has a tyrosine ring attached covalently to the reducing end via a β-glycosylamide linkage which resembles the natural linkage of N-linked oligosaccharides to asparagine. The presence of this tyrosine allowed iodination for the radiotracer monitoring of the oligosaccharide.

The biodistribution study was preceded by a stability and pharmacokinetic
analysis of the radiolabelled oligosaccharide in blood. Boc-tyrosinamide triantennary
oligosaccharide was found stable in whole mouse blood both *in vitro* and *in vivo*
within the time frame studied, a useful advantage for its use as a targeting ligand.
Further, its stability allowed the use of direct gamma counts in blood samples for
pharmacokinetic analyses. This was very advantageous since the direct counting
protocol allowed for elimination of time and labor involved in carrying out TLC
analyses of extracted samples; reduced the handling of radioactive materials, and
eliminated experimental errors introduced in extraction procedures.

Pharmacokinetic analysis utilizing a two compartment open model with
elimination from the central compartment was found adequate to describe the kinetics
of the radiolabelled oligosaccharide in blood within the time frame studied (Figure
11 and Table 3). A very fast clearance rate from blood was observed, resulting in a
overall mean residence time of approximately 45 min. The alpha half life was less
than 1 minute, and is a reflection of the extremely rapid initial uptake by the liver.
This should be an advantage for gene delivery since fast clearance from the
bloodstream may provide fewer opportunities for DNA complexed carriers to be
phagocytosed or marked for destruction by circulating opsonins.

A high level of targeting or binding to a peripheral compartment is also
indicated by the values obtained for the volumes of distribution. The volume of the
Figure 11. Pharmacokinetic Analysis of $^{125}$I-Labelled Boc-Tyrosinamide-Triantennary Oligosaccharide. A. The oligosaccharide was extracted from blood samples at various time points, and analyzed by densitometry after TLC separation and autoradiography. B. Both the TLC separated and whole blood concentrations were fitted by least squares regression and yielded similar results.
Table 3. Pharmacokinetic Parameters for Boc-Tyrosinamide-Triantennary Oligosaccharide in Mice^a

<table>
<thead>
<tr>
<th>Tri (n=3)</th>
<th>^b(t_{1/2,\alpha}) (min)</th>
<th>^c(t_{1/2,\beta}) (min)</th>
<th>^dMRT (min)</th>
<th>^eCL_{lb} (ml/min)</th>
<th>^fV_e (ml)</th>
<th>^gV_{ss} (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.8</td>
<td>34.4</td>
<td>43.0</td>
<td>0.7</td>
<td>4.3</td>
<td>24.4</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.3</td>
<td>18.6</td>
<td>26.3</td>
<td>0.3</td>
<td>1.7</td>
<td>10.2</td>
</tr>
</tbody>
</table>

^a Derived from nonlinear least squares curve fitting of Equation 1 (Pg. 40) to the dataset, from which values of A, B, \(\alpha\) and \(\beta\) were obtained. Pharmacokinetic parameters were calculated for each dataset individually, and the average and standard deviation calculated from the triplicated parameters.

^b Alpha half life : \(t_{1/2,\alpha} = \ln \frac{2}{\alpha}\)

^c Beta half life : \(t_{1/2,\beta} = \ln \frac{2}{\beta}\)

^d Mean residence time : MRT = \(\frac{A/\alpha^2 + B/\beta^2}{A/\alpha + B/\beta}\)

^e Total body clearance : Cl_{lb} = \frac{Dose}{A/\alpha + B/\beta})

^f Volume of distribution, central compartment : V_e = \frac{Dose}{A + B})

^g Volume of distribution, steady state : V_{ds} = Cl_{lb} \times MRT
"central compartment" (4.3 ml, Table 3) is more than twice the total volume of blood expected for mice of the size used, indicating that significant extraction of the oligosaccharide from blood occurs even during the alpha phase of 1 minute. The volume of distribution at steady state (~24 ml, Table 3), is three fold higher than that obtained for oligosaccharides that are not targeted to any site\textsuperscript{65}.

Pharmacokinetic analysis was found to be useful to arrive at a rational time for taking sections for biodistribution studies: approximately one beta phase half life or 30 min. It was found that taking sections at too early a time point resulted in high background due to the presence of substantial amount of radioactivity in the bloodstream, while taking sections at a much later time resulted in low definition of organ boundaries, making identification difficult.

Whole body autoradiographic exposures (Figure 12A) of cryosectioned mice dosed with (\textsuperscript{125}I) Boc-Tri clearly showed high level of binding to liver, as early as 5 minutes after dosing. Some radiolabel was seen in the kidney, where it underwent renal excretion. Quantitative tissue distribution data based on direct organ counting (Figure 12B) served to confirm the whole body imaging results. At 30 min post administration, about 60% of the total radioactivity was found associated with the liver and comparatively, very little binding was seen in other organs. This level of targeting is very significant, since the oligosaccharide is very hydrophilic, and undergoes rapid renal filtration. Therefore the liver targeting is in competition with renal clearance. In the case of DNA-carrier complexes, where the oligosaccharide is
Figure 12. Biodistribution Analysis of $^{125}$I-Labelled Boc-Tyrosinamide-Triantennary Oligosaccharide. A. Whole body autoradiography of cryosectioned mice demonstrated targeting of the oligosaccharide to the liver. B. Quantitative analysis to determine the targeting efficiency at 30 min post-administration (average and standard deviation from five mice) demonstrated that ~60% of the dose was targeted to the liver.
anchored onto the surface of DNA, renal filtration is expected to be greatly reduced.

CONCLUSION

The results obtained indicate that N-linked oligosaccharides modified with tyrosine are able to retain the proper conformation to be able to bind to their natural receptors. The tyrosinamide triantennary oligosaccharide was a potent ligand for liver targeting in vivo, and this activity is consistent with the known localization of the asialoglycoprotein receptor on hepatocytes, and its binding specificity. Therefore, the oligosaccharide appears to be a good ligand for hepatocyte targeted gene delivery. This is not only because of its ability to target the liver in vivo, but also due to the fact that it can be obtained from natural sources in relatively high yield and can be conveniently purified by reverse phase HPLC to homogeneity. Further, it can be quantitated by UV absorbance, can be labelled by iodination for radiotracer studies, and seems to possess good stability in vivo.

Pharmacokinetic and biodistribution studies have also been reported for other purified oligosaccharide structures65,106,107. These data demonstrate that several oligosaccharides fail to target the liver in vivo, whereas other oligosaccharides, notably N-acetylgalactosamine terminated structures, are extremely potent ligands for the asialoglycoprotein receptor, and display 80% (of dose) or higher liver targeting in vivo. Further, preliminary evidence indicates that some oligosaccharide structures may be able to target organs such as the kidney108, although at low targeting
efficiencies\textsuperscript{109}. These results clearly demonstrate the influence of oligosaccharide structure on \textit{in vivo} targeting activity, and provide promise that continued research in this area may allow specific cells and tissues to be targeted for drug and gene delivery by the use of purified oligosaccharide structures.
CHAPTER III

Preparation and Characterization of the Glycopeptide Carrier "TriPL"

INTRODUCTION

Carriers for receptor mediated gene delivery typically employ a receptor ligand covalently attached to a polycationic anchor that binds DNA by ionic interactions. Selective transfection of hepatocytes via the ASGP-R has been accomplished with ligands possessing terminal galactose residues such as asialo-orosomucoid, galactosylated proteins or polymers, and galactosylated synthetic ligands. The anchor utilized most often is poly-L-lysine in the molecular weight range of 20 - 60 kDa.

Asialo-orosomucoid (ASOR) is the most commonly used ligand for hepatocyte targeted gene delivery, and has the advantage of very high efficiency targeting to the ASGP-R on hepatocytes. The high level of targeting is mediated by the several galactose terminated N-linked oligosaccharides covalently bound to ASOR. However, the presence of several different types of oligosaccharides is also a source of heterogeneity in ASOR, since the type and extent of glycosylation may vary. The heterogeneity of ASOR-polylysine conjugates is increased by the fact that there are
multiple reactive groups on ASOR, which are linked randomly to amine groups of polydisperse high molecular weight polylysine. Further, ASOR is obtained from pooled human blood, and there is the risk of copurification of infectious viral particles as with all blood protein products.

To alleviate some of these problems, simpler carriers have been designed that are easier to prepare and characterize. Hanson et al. have reported the use of galactosylated polylysine, whereas Midoux et al. have designed carriers consisting of lactosylated polylysine. In these cases, multiple galactose or lactose residues were conjugated randomly to the amine side chains of high molecular weight polylysine. These carriers are attractive with respect to low cost of starting materials, but are still quite heterogeneous. More importantly, although the clustering of multiple galactose or lactose residues on polylysine may be sufficient to cause binding to the ASGP-R, it is unclear how the in vivo binding affinity of these carriers compares with the binding of natural oligosaccharides found on glycoproteins. Previous studies in the literature indicate that the subunits of the ASGP-R are arranged in a specific geometric arrangement, requiring properly positioned galactose residues for optimal binding. Further, it is also known that ligands that show appreciable binding to the ASGP-R in vitro may fail to target hepatocytes in vivo.

Due to the above-mentioned concerns, it was decided to prepare glycopeptide carriers which would not only bind to the ASGP-R and DNA with high affinity, but could also be highly characterized and purified. These properties would then allow
for rational evolution of carrier design in order to study and optimize receptor mediated gene delivery. A first step was taken towards these objectives by selecting the previously prepared triantennary oligosaccharide as a fully characterized, high affinity ligand for the ASGP-R, which was coupled to one of the amines on 2.5 kDa poly-L-lysine to produce a low molecular weight glycopeptide carrier, "TriPL".

MATERIALS AND METHODS

Poly-L-lysine hydrobromide, of average dp 19 (PL), succinic anhydride, 1-ethyl-3-[3-dimethyl-aminopropyl] carbodiimide methiodide (EDC) and chloroquine were obtained from Sigma Chemical Co, St. Louis MO. β-galactosidase (EC 3.2.1.23) from bovine testes was obtained from Boehringer Mannheim, Indianapolis, IN. Gel filtration HPLC column (G2000 SWXL) was purchased from Tosohaas, Montgomeryville, PA; C8 reverse phase columns (MV Microsorb, with 5 µm packing) were from Rainin, Emeryville, CA. Analytical and semi-preparative reverse phase polymer columns (PRP-1, with 10 µm packing) were from Hamilton Co., Reno, NV. HPLC was performed using equipment from ISCO (Lincoln, NE), consisting of computer interfaced pumps, variable wavelength UV detector, and automated fraction collector. High pH anion exchange chromatography (HPAEC) was performed on a carbohydrate analyzer with a CarboPac PA1 column, and pulsed amperometric detector (PAD), all from Dionex, Sunnyvale, CA.
Succinylation of Triantennary Oligosaccharide

Boc protected tyrosinamide triantennary oligosaccharide (Boc-Tri) was prepared from bovine fetuin as described in Chapter I. Boc-Tri (1 μmol) was freeze dried and reacted with 200 μl TFA for 10 min at room temperature. The boc deprotected oligosaccharide (Tri) was then freeze dried three times to obtain a neutral pH.

Tri (500 nmol, prepared in 450 μl 0.2 M sodium bicarbonate buffer, pH 8.0) was reacted with succinic anhydride (12.5 mg in 50 μl DMF) for 15 min at room temperature, along with the addition of 150 μl 1 M sodium hydroxide to maintain the pH between 7.5-8. The reaction was terminated by adjusting the pH to 12 by the addition of 150 μl 1 M sodium hydroxide and incubated at 37°C for 10 min, followed by acidification to pH 3 by adding 100 μl 4 M TFA prior to HPLC purification.

Succinyl-Tri was purified from a semi-preparative polymeric RP-HPLC column (305×7 mm) equilibrated at 3 ml/min with 0.1% TFA and 4% acetonitrile. Following injection of 500 nmol of Succinyl-Tri (900 μl) into a 1 ml loop, an acetonitrile gradient of 4% to 15% was developed over 13 min while monitoring absorbance at 274 nm (0.2 AUFS). The peak eluting at 9 min was collected and freeze dried, and the yield estimated by Abs274nm (ε = 1450 M⁻¹ cm⁻¹).

Boc-Tri, Tri, and Succinyl-Tri were prepared for proton NMR spectroscopy by freeze drying 1 μmol twice in 99.98% deuterium oxide containing 0.01% acetone as an internal standard and analyzed on a Bruker 500 MHz NMR spectrometer.
operating at 23°C. The acquired spectra were processed utilizing resolution enhancement parameters supplied with Felix™ software (Hare Research, Eugene, OR). Samples were prepared for FAB-MS by dissolving 20 nmol in 10 μl water and 1 μl of α-monothioglycerol. The water was removed by speed vacuum and the 1 μl sample was applied to the probe of a Finnigan Matt 900 spectrometer operated in the positive ion mode.

Conjugation of Succinyl-Tri with Polyllysine

Succinyl-Tri (400 nmol in 400 μl water) was added to PL (2.5 μmol in 400 μl water) and the coupling reaction was initiated by adding 800 μl of EDC (500 mM in 20 mM borax / hydrochloride buffer, pH 7.5). After incubation at room temperature for 2 h, the reaction was quenched by the addition of 16 μl of 2 M hydrochloric acid.

The glycopeptide conjugate (TriPL) was purified in 200 nmol (800 μl) portions on an analytical (250 × 4.1 mm) polymeric RP-HPLC column (50°C) equilibrated at 1 ml/min with 0.1% TFA and 1% acetonitrile. The acetonitrile concentration was held at 1% for 20 min followed by a step to 15% acetonitrile over 1 min and elution continued for 10 min while monitoring Abs_{274 nm} (0.1 AUFS). The peak eluting at 23 min was collected and freeze dried and the yield determined by absorbance at 274 nm.
Preparation of Agalatco-Triantennary-Polylysine Conjugate

Agal-TriPL was prepared by incubating 100 nmol of TriPL (in 200 μl 50 mM sodium phosphate citrate buffer, pH 4.3) with 40 mU of β-galactosidase for 24 h at 37°C. The product was purified on an analytical polymer reverse phase column as described for TriPL and characterized by monosaccharide compositional analysis as described below.

Compositional Analysis of TriPL

The amine content of TriPL (determined by Abs$274$) was obtained by fluorescamine assay as described$^{101}$, using a PL standard, with the fluorescence being measured at excitation and emission wavelengths of 390 and 475 nm (5 nm slit widths). Amino acid analysis of the conjugates was performed by Picotag™ analysis$^{112}$, and monosaccharide composition analysis was performed by HPAEC-PAD following TFA and hydrochloric acid hydrolysis$^{95}$.

RESULTS AND DISCUSSION

A hepatocyte targeted gene delivery carrier was prepared by conjugating a natural triantennary oligosaccharide to an amine side chain of dp 19 polylysine. The conjugation results in an amide linkage between the oligosaccharide and polylysine such that the three galactose terminated antennae are accessible for binding with the ASGP-R.
The reaction scheme is outlined in Figure 13. Triantennary oligosaccharide was purified from fetuin after reducing end modification as previously described in Chapter I, and obtained as a boc-protected tyrosinamide oligosaccharide (Boc-Tri, Fig. 13A). Treatment of Boc-Tri with concentrated TFA (~13 M) removed the boc group and exposed the N-terminal amine on tyrosine for further modification (Fig. 13B). Deprotection was carried out for 10 minutes in the absence of water and the product frozen in dry ice immediately to avoid hydrolysis of glycosidic linkages of the oligosaccharide. Exposure to TFA for shorter periods of time resulted in incomplete removal of boc. The side products of the reaction, carbon dioxide and tert-butyl alcohol are volatile and were removed by freeze-drying along with excess TFA. Under these conditions, the conversion to deprotected oligosaccharide was quantitative, as evidenced by elution on RP-HPLC.

A carboxylic group was introduced into the oligosaccharide by succinylation of the exposed amine using 250 molar excess of succinic anhydride (Fig. 13C). The reaction proceeded quickly when the pH was maintained between 7.5 and 8 in order to deprotonate the amine terminus on Tri. This required the addition of base after adding succinic anhydride since some of the excess reagent hydrolyzed in aqueous media and caused a decrease in pH due to the production of succinic acid. In addition to succinylation of the amine, esterification of hydroxyl groups of the oligosaccharide also occurred but was reversed at an elevated pH while maintaining the succinimide linkage. Succinyl-Tri was isolated from a polymeric reverse phase column at greater
Figure 13. Reaction Scheme for Conjugation of Triantennary Oligosaccharide to Polylysine. Boc-Tri (A) was converted to Tri (B) by treatment with TFA. The exposed N-terminus of Tri was reacted with succinic anhydride to introduce a carboxylic group in Succinyl Tri (C). The carboxyl was activated by EDC, and coupled with an amine on polylysine (of average dp 19) to produce TriPL (D). The structure shown for TriPL is meant for illustration only since the linkage was not site specific.
than 90% yield with respect to Boc-Tri.

The elution of Boc-Tri, Tri and Succinyl-Tri on RP-HPLC is shown in Figure 14, A-C. Tri was much less hydrophobic than Boc-Tri and eluted earlier, while Succinyl-Tri had intermediate hydrophobicity under identical conditions. These differences in properties on RP-HPLC allowed the monitoring of Boc-Tri, Tri, and Succinyl-Tri for optimizing reaction conditions and evaluating purity.

Proton NMR analysis of purified Boc-Tri, Tri, and Succinyl-Tri showed characteristic signals from anomeric and acetyl protons on the oligosaccharide. In addition, proton signals at 1.35 ppm from methyl groups on Boc-Tri were absent from Tri and Succinyl-Tri, and the latter showed the presence of methylene protons on the succinyl group between 2.30-2.45 ppm. FAB-MS analysis provided a molecular ion corresponding to M+2Na+ for Succinyl-Tri (2313.3) which was within 0.5 a.m.u. of the calculated molecular mass.

Succinyl-Tri was converted to TriPL by coupling its carboxylic group to an amine side chain of PL after activation with EDC (Fig. 13D). The reaction was monitored by gel filtration HPLC. High molar excess of EDC was found necessary for the reaction to proceed, and varying the oligosaccharide to polylysine ratio affected the composition of reaction products. Increasing the molar ratio (Succinyl-Tri to PL) progressively from 1:6 to 4:3 resulted in higher molecular weight conjugates which were resolved on gel filtration HPLC (Figure 15, A-C). Amino acid analysis of the isolated products indicated that these contained multiple
Figure 14. Analytical RP-HPLC Characterization of Oligosaccharide Conjugates. Purified Boc-Tri (A), Tri (B), and Succinyl-Tri (C) were analyzed on a C8 HPLC column under identical elution conditions. TriPL (D) was chromatographed on the same column using similar conditions, however, with a step gradient. Analytical RP-HPLC allowed monitoring of reactions showed in Figure 13, and established the purity of each intermediate.
Figure 15. Optimization of Reaction Stoichiometry between Succinyl-Tri and Polylysine. Reactions between succinyl-tri and polylysine were analyzed by gel filtration HPLC. As the oligosaccharide ratio increased, higher molecular weight products (dashed line) were obtained in addition to the 1:1 conjugate (A and B). With large excess of polylysine, conjugation was inhibited (D), resulting in unreacted oligosaccharide (dotted line). Optimal stoichiometry was observed at a oligosaccharide to polylysine molar ratio of 1:6 (C), resulting in a 1:1 conjugate (dotted and dashed line).
oligosaccharide units conjugated to each polylysine. Alternatively, decreasing the molar ratio to 1:12 inhibited the reaction (Fig. 15D). A Succinyl-Tri to PL stoichiometry of 1:6 was chosen to allow the reproducible isolation of a 1:1 conjugate as determined by amino acid analysis.

Gel filtration under gravity flow with Sephadex G-25 or G-50 (Pharmacia), and P-6 (BioRad) resins was found to lack the resolution needed to separate TriPL from excess polylysine. Better resolution was obtained using gel filtration HPLC, and although this technique proved to be very useful for analysis of the conjugation reactions (by monitoring tyrosine absorbance at 274 nm), it allowed only partial purification, and limited scale up. These problems were overcome by purification on a polymer RP-HPLC column (PRP-1, Hamilton Co.), and TriPL was isolated with an overall 60% yield with respect to Boc-Tri. The purification removed free polylysine and excess EDC which eluted earlier.

The purity of TriPL was assessed by RP-HPLC and gel filtration HPLC. The chromatographic profile of purified TriPL on a C-8 silica column is shown in Figure 14D, while the gel filtration HPLC profile is shown in Figure 16B. TriPL eluted earlier compared to succinylated oligosaccharide on gel-filtration HPLC due to its increased molecular weight. Further, gel filtration HPLC of purified TriPL showed absence of polylysine upon monitoring absorbance at 220 nm. The tyrosine chromophore in purified TriPL was unchanged from Tri or Succinyl-Tri, permitting routine quantitation by UV absorbance measurement at 274 nm as before. However,
Figure 16. Analytical Gel Filtration Characterization of TriPL. Succinyl Tri (A), and TriPL (B) were chromatographed on a gel filtration HPLC column eluted at 1 ml/min with 50 mM sodium phosphate (pH 4.5) and 300 mM sodium chloride. TriPL (MW: ~4700) eluted 40 sec earlier than Succinyl-Tri (MW: 2269) due to its higher molecular weight.
Figure 17. UV Absorbance Profiles of Succinyl-Tri and TriPL. Absorbance spectra of Succinyl-Tri (solid line) and TriPL (dashed line) showed unchanged maxima of the tyrosine chromophore at 274 nm. However, TriPL had higher absorbance at lower wavelengths due to the presence of multiple lysine residues.
Figure 18. Preparation of a Control Carrier, Agal-TriPL. A control agalacto carrier was prepared by enzymatically removing the terminal galactose residues of TriPL, followed by purification on HPLC.
the absorbance spectrum of TriPL was changed at lower wavelengths due to the presence of multiple lysines in the conjugate (Figure 17).

The monosaccharide composition of TriPL was identical to that of Boc-Tri, and fluorescamine analysis of TriPL using a PL standard indicated 1.03 nmol of polylysine dp 19 for every nmol of tyrosine absorbance. Furthermore, amino acid analysis resulted in a lysine to tyrosine ratio of 20:1, establishing an approximate 1:1 conjugate between polylysine and oligosaccharide.

A control carrier molecule was prepared by trimming terminal galactose residues from TriPL with β-galactosidase (Figure 18), to render the carrier incapable of binding to the ASGP-R. Agal-TriPL was purified in a manner similar to TriPL, and was found to be devoid of galactose residues upon monosaccharide analysis.

CONCLUSION

A low molecular weight glycopeptide carrier was constructed for targeted (receptor-mediated) gene delivery and expression. A galactose terminated triantennary oligosaccharide was purified from bovine fetuin and derivatized at the reducing end with boc-tyrosine. The tyrosinamide-oligosaccharide was treated with TFA to remove boc and the N-terminus of tyrosine was reacted with succinic anhydride to introduce a carboxyl group. This carboxyl group on the succinylated triantennary was activated using a carbodiimide and covalently conjugated to one of the amines on low molecular weight (dp 19) polylysine. The resulting glycopeptide carrier, TriPL, was
purified; and characterized by its tyrosine chromophore, presence of amines, elution on gel filtration HPLC, monosaccharide analysis, and amino acid analysis.

The use of a low molecular weight ligand and anchor creates a distinct advantage in the preparation and characterization of carrier conjugates. Purification procedures employed RP-HPLC separations, which gave reproducible and scalable isolation of desired products. In contrast, carriers made with proteinaceous ligands and high molecular weight polylysine pose many difficulties in characterization and purification\textsuperscript{30,113}. This is in part due to the presence of several reactive groups on both ligand and anchor which makes the conjugation chemistry difficult to control and results in considerable heterogeneity in products. In the case of TriPL, the triantennary oligosaccharide has only one reactive carboxylic group, but it is attached randomly to one of the amines of polylysine. An additional source of heterogeneity is the polydispersity of commercially available polylysine, and the potential to form crosslinks, which is however compounded in the case of carriers prepared with higher molecular weight polylysines.
CHAPTER IV

Receptor-Mediated Gene Delivery with DNA-TriPL Complexes

INTRODUCTION

A. DNA-Carrier complexation.

Polycationic molecules like polylysine strongly interact with the negatively charged phosphate groups of DNA, resulting in complexes held together by electrostatic bonds. This is the basis for the use of "carrier" molecules like polylysine to anchor receptor ligands on the surface of DNA. Although the existence of polylysine-DNA complexes has been known for decades\textsuperscript{114-116}, it was only recently that their utility in receptor mediated gene delivery was described\textsuperscript{13,14}.

It is also striking that in nature, DNA within the chromatin of eukaryotic cells always exists in complex with polycationic nucleoproteins called histones. Five different types of histones are known\textsuperscript{117}, and the interactions involved are highly complex; ultimately giving rise to nucleosomal structures that are further arranged in superstructures\textsuperscript{117,118}. Two important attributes of this arrangement are that an extremely high level of DNA compaction is achieved, and further, the DNA is resistant to enzymatic degradation.
In a similar fashion, polycationic carrier molecules not only serve to anchor receptor ligands to DNA, but also allow a high level of DNA "condensation", and further, protect it from enzymatic digestion\textsuperscript{39,40}. Further considerations include the formulation of uniform and predictable, soluble DNA complexes; and the stability of these complexes. All of these attributes can be expected to be critical to successful receptor mediated gene delivery, and depend upon the proper complexation of the carrier molecule to DNA. Accordingly, part of this thesis project was devoted to developing methods of monitoring DNA complexation in the anticipation that these methods would yield information that could be utilized in the formulation, optimization, and quality control of DNA-carrier complexes.

**B. Evaluation of DNA-TriPL Complexes for Receptor-Mediated Gene Delivery.**

In order to study TriPL mediated gene delivery, it was desirable to use an assay system which could be routinely used for the rapid evaluation of DNA-carrier complexes in a biologically relevant manner. The assay would ideally be sensitive and quantitative, and would also allow the processing of large number of sample treatments economically. Keeping these concerns in mind, it was decided to test the carrier-DNA complexes for receptor mediated gene delivery by the use of human hepatocytes possessing the asialoglycoprotein receptor. Accordingly, HepG2 cells (a well characterized human hepatoma cell line\textsuperscript{119-121}), were selected as a model.
The rapid, sensitive and accurate quantitation of foreign DNA delivered within cells poses many technical difficulties, and can be very tedious. Further, a test of this nature may not indicate if the delivered DNA was degraded or otherwise biologically inactive. Therefore, a functional test of gene delivery was used by measuring the expression of the luciferase gene\(^{122-124}\) (derived from the firefly *Photinus pyralis*) under control of the cytomegalovirus enhancer/promoter\(^{125,126}\). Luciferase is not found endogenously in mammalian cells and is a popular reporter of gene expression. In the presence of ATP, magnesium ions and luciferin, it emits a flash of light which can be detected easily by a luminometer. Under appropriate conditions, the peak height as well as integrated light intensity are directly proportional to luciferase concentration\(^{123,124}\). The assay is extremely sensitive, non-isotopic, easy and rapid to perform, inexpensive relative to most other reporter assays, and provides a linear response over several orders of magnitude. In addition, luciferase does not undergo any post-translational processing and thus is available for detection immediately upon translation.

**MATERIALS AND METHODS**

LB media, LB agar, D-luciferin, and luciferase from *Photinus pyralis* (EC 1.13.12.7) were obtained from Boehringer Mannheim, Indianapolis, IN. HepG2 cells were from American Type Culture Collection, Rockville, MD. Minimum essential media (MEM), media supplements, heat inactivated "qualified" fetal bovine serum
(FBS), and competent E. coli (DH5α) were from Gibco BRL, Grand Island, NY. Bradford reagent was purchased from BioRad, Hercules, CA and thiazole orange was a gift from Beckton Dickinson Immunocytometry Systems, San Jose, CA. The 5.6kbp plasmid pCMVL (Figure 19) encoding the reporter gene luciferase under the control of the cytomegalovirus promoter was a gift from Dr. M.A. Hickman at the University of California, Davis. Fluorescence and light scattering were performed using a computer interfaced fluorimeter (LS50B) from Perkin Elmer, UK. UV spectroscopy was conducted on a Beckman DU640 spectrophotometer, and luciferase light units were recorded on a luminometer (Lumat LB 9501) from Berthold Systems, Germany.

**Large Scale Plasmid Preparation of pCMVL**

Large scale plasmid preparation was carried out using established procedures\textsuperscript{1,2,7,12,8}. Briefly, competent E. coli were transformed with pCMVL containing an ampicillin resistance gene and selected on LB-Agar plates supplemented with 50 μg/ml ampicillin. The bacteria were grown in ampicillin supplemented LB broth in 2 liter flasks, and the harvested cells lysed by alkali. The isopropanol precipitated DNA was separated into linear, circular and supercoiled forms by ultracentrifugation on a cesium chloride/ethidium bromide gradient, and the supercoiled DNA repurified by equilibrium ultracentrifugation. Ethidium bromide was removed by partitioning into isobutanol and cesium chloride was removed by precipitating the DNA twice with ethanol. The ethanol precipitate was recovered in water and used as such.
Figure 19. Gene Sequences of the Plasmid pCMVL. The plasmid pCMVL has a luciferase (Luc) gene, downstream of a cytomegalovirus (CMV) enhancer/promoter. The plasmid also has an ampicillin (Amp) resistance gene and ColE1 origin for selection and maintenance in E. coli; polyadenylation signal from bovine growth hormone (BGH) for RNA transcript stability; and a SV40 origin for episomal replication.
Quantitation was by absorbance at 260 nm (1.0 absorbance unit equivalent to 50 μg/ml of DNA). Purity of the plasmid was confirmed by agarose gel electrophoresis resulting in a predominantly single band corresponding to the supercoiled form, and by an $A_{260}/A_{280}$ ratio of >1.95 (absorbance measurements performed in 10mM Tris.HCl - 1mM EDTA buffer, pH 8.0).

**Preparation of TriPL-pCMVL complexes.**

TriPL-pCMVL complexes were prepared with DNA concentrations ranging from 0.2-40 μg/ml and TriPL-pCMVL ratios (nmol carrier/μg DNA) varying from 0.1 to 1.2. The optimized complex was prepared at a DNA concentration of 20 μg/ml and a carrier-DNA ratio of 0.8 by adding TriPL (16 nmol, in 500 μl solvent) to pCMVL (20 μg, in 500 μl solvent) while vortexing, and allowing the mixture to incubate at room temperature for 30 min. The amounts and volumes of carrier and DNA were linearly scaled for preparing different amounts of complex. Solvents used were 0.15 M sodium chloride (saline), 20 mM hepes (pH 7.4) with 0.15 M sodium chloride (HBS), and 0.72 M mannitol. For studying the effect of salt concentrations upon solubility, complexes were prepared at the optimized carrier-DNA ratio of 0.8 but using various concentrations of sodium chloride, calcium chloride, sodium sulfate, sodium acetate (pH 7.0) and sodium phosphate (pH 7.0). The stock solutions of all solvents were always prepared at a 110% strength, and the DNA and TriPL solutions were always prepared in deionized water at a 10 fold strength over the concentration
required. Mixing of 1 part of the DNA or TriPL in water with 9 parts of stock solvent resulted in the desired concentration. For control experiments, TriPL was substituted with PL or Agal-TriPL.

Assays for Monitoring DNA-Carrier Complexes

TriPL-pCMVL "solubility" was determined by analyzing an aliquot (1 μg DNA) of the complex before and after centrifugation at 13000 g for 4 min at room temperature. The aliquots were diluted to 1 ml in appropriate solvent (saline, HBS, mannitol, or various salt solutions) and the DNA in solution measured by absorbance at 260 nm. The ratio of the values obtained after and before centrifugation (and multiplied by 100) was used as a measure of "percent solubility".

Complexation was monitored by a fluorescence assay based on exclusion of intercalating dye from DNA by TriPL or PL. An aliquot of TriPL-pCMVL (1 μg DNA, in 25 μl - 500 μl) was diluted to 0.5 ml in solvent and then diluted with 3 ml of solvent containing 0.117 μM thiazole orange\(^{129}\) (from a 0.1 mg/ml stock in 1% methanol, \(\epsilon_{476} = 30,000 \text{ M}^{-1} \text{ cm}^{-1}\)). Fluorescence of the intercalated dye was measured using excitation at 500 nm and emission at 530 nm, with the slits set at 15 and 20 nm respectively to maximize sensitivity. In the case of fluorescence measurements made in the presence of ethidium bromide (1.2 μM), emission and excitation monochromators were set to 595 and 530 nm, with slits of 10 and 2.5 nm respectively.
Complexes were also measured by light scattering in order to monitor DNA condensation. Traces of dust were removed from sample tubes by means of 0.1 μm filtered pressurized air and solvents were filtered through 0.2 μm surfactant free cellulose acetate syringe filters. TriPL-pCMVL (1 μg DNA) was diluted to 3.5 ml in solvent and scattered light intensity at 90° was measured by keeping both monochromaters at 350 nm (2.5 nm slits). Light scattering synchronous scans were obtained by the simultaneous scanning of emission and excitation monochromaters paired at the same wavelength.

Additionally, band retardation assay was used to monitor complexation. TriPL-pCMVL (200 ng DNA) was mixed with gel loading buffer and electrophoresed on a 1% agarose gel at 70 V and DNA was visualized post-run by ethidium bromide staining and UV detection.

Transfection and Gene Expression

HepG2 cells were plated on 6×35 mm wells (0.5-1×10⁶ cells per well) and grown to 40-70% confluency in minimum essential media (MEM) 90%, with 10% Fetal Bovine Serum (FBS). The MEM used was supplemented with Earle's balanced salts, non-essential amino acids, sodium pyruvate, and a penicillin-streptomycin antibiotic mix. Transfections were performed in MEM (2 ml per 35 mm well) with 2% FBS, with or without 100 μM chloroquine. TriPL-pCMVL complexes (0.1-20 μg DNA, in 0.5 ml) were added dropwise to triplicate wells. After 5 h incubation at
37°C, the media was replaced with MEM supplemented with 10% FBS.

Luciferase expression was determined at 24 h (19 h post transfection), with some modifications of a published method\(^{130}\). Cells were washed twice with ice-cold phosphate buffered saline (calcium, magnesium free) and then treated with 0.5 ml of ice-cold lysis buffer (25 mM tris chloride pH 7.8, 1 mM EDTA, 8 mM magnesium chloride, 1% triton X-100, with 1 mM DTT added fresh) for 10 min. The cell lysate mixture was scraped, transferred to 1.5 ml microcentrifuge tubes, and centrifuged for 7 min at 13000 g at 4°C to pellet debris.

Lysis buffer (350 μl), sodium-ATP (4 μl of a 180 mM solution, pH 7, 4°C) and cell lysate (100 μl, 4°C) were combined in a test tube, briefly mixed and immediately placed in the luminometer. Luciferase light units were recorded with 10 sec integration after automatic injection of 100 μl of 0.5 mM D-luciferin (prepared fresh in lysis buffer without DTT). Relative light units (RLU) of luciferase activity were converted to femtomoles of luciferase (after subtraction of background counts, typically 150-200 RLU) based on a standard curve which was obtained with known triplicate concentrations of firefly luciferase in lysis buffer in the presence of untransfected HepG2 cell lysate (Figure 20). The standard curve was linear between 200 and 10^6 RLU and each femtomole was equivalent to 740,000 RLU.

Protein concentration in the cell lysate was measured by Bradford assay\(^{131}\) using bovine serum albumin as a standard (the sample size of the cell lysate was 50 μl or less, and no interference from triton X-100 was observed). The femtomoles of
Figure 20. Luciferase Standard Curve and Assay Validation. A. Known amounts of luciferase were spiked into HepG2 cell lysate at 4°C and luciferase activities measured in a luminometer. The response observed was linear over five orders of magnitude. B. Luciferase activity was stable for the duration of the assay in the presence of cell lysate at 4°C in the lysis buffer described.
luciferase in each sample were normalized to mg protein, and the mean and standard deviation obtained from each triplicate.

RESULTS AND DISCUSSION

Complexation between DNA and carrier is a critical parameter for receptor mediated gene delivery. Unfortunately, complexation also frequently results in the precipitation of DNA, limiting the concentration of DNA that can be used\textsuperscript{29,113}. In the case of TriPL-pCMVL complexation, precipitation occurred even at low DNA concentrations (5 \( \mu \)g/ml) in solvents like saline. Therefore it was necessary to find conditions under which TriPL-pCMVL complexes were reasonably soluble. This necessitated the use of a rapid assay to monitor the solubility of complexes, and a centrifugation assay was used for this purpose. TriPL-pCMVL complexes were defined as "soluble" if centrifugation under the conditions selected failed to remove DNA from the supernatant, and the term "solubility" has been used only in this limited context. The assay established that the solvent used to prepare complexes dramatically influenced their solubility. Complexation of pCMVL with TriPL in saline or HBS resulted in precipitates at DNA concentration of 20 \( \mu \)g/ml (Figure 21A), but very little precipitation was observed when complexes were prepared in a solution of mannitol, a non-toxic carbohydrate which can be used \textit{in vivo} (Figure 21B). This effect was unrelated to the higher viscosity of the mannitol formulation, since the addition of small amounts of sodium chloride caused precipitation of the
Figure 21. Influence of Solvent on the Solubility of TriPL-pCMVL Complexes. A. Insolubility of complexes was observed in saline as evidenced by absorbance of DNA complexes before (dashed line) and after (solid line) centrifugation. B. Complexes prepared in a solution of mannitol remained soluble at various ratios of carrier to DNA (at a DNA concentration of 20 μg/ml), as evidenced by absorbance scans of the supernatant after centrifugation.
complexes while there was no change in viscosity. Further experimentation led to the conclusion that the solubility of carrier-DNA complexes was dependent upon the presence of low or non-ionic conditions, and was not dependent on mannitol. However, the presence of mannitol was necessary to avoid hypotonicity. The formation of TriPL-pCMVL precipitates correlated with greatly diminished transfection efficiencies. For example complexes prepared in saline or HBS were 5-12% soluble and resulted in gene expression levels two orders of magnitude lower than complexes prepared in mannitol which were typically 90% soluble (Figure 22).

DNA complexes were prepared in a solution of mannitol for all the transfection studies reported in this chapter. However, the effect of ionic strength on TriPL-pCMVL complexes was investigated further by monitoring their solubility in the presence of five different salts at varying concentrations. The percent solubility measurements were plotted against molarity of salt, and nonlinear curve fitting performed using PCNONLIN™ (SCI software, Lexington, KY). From the fitted curves, the values of salt concentration required to cause 50% precipitation were estimated and denoted as SC₅₀ values. Figure 23 shows the plots obtained for sodium chloride, sodium acetate and sodium sulfate, while the estimated SC₅₀ values (rounded off) for all the salts are as follows: sodium chloride ~ 4.5 mM; sodium acetate ~ 6.0 mM; calcium chloride ~ 2.5 mM; sodium sulfate ~ 0.3 mM; sodium phosphate ~ 0.4 mM. The first three salts have univalent anions, while the last two have divalent anions (phosphate is trivalent when fully ionized, but divalent at neutral pH).
Figure 22. Effect of Solvent on Solubility and Gene Transfection. TriPL-pCMVL complexes (pCMVL 20 μg/ml and TriPL 16 nmol/ml) were prepared in saline, HBS, or mannitol solution and were analyzed for solubility, and transfection competency in HepG2 cells in the presence of chloroquine. The shaded bars represent percent of DNA remaining in solution after centrifugation, while the solid bars represent luciferase reporter activity obtained at 24 h.
Figure 23. Effect of Salt Concentration on the Solubility of TriPL-pCMVL complexes. Sodium sulfate, providing divalent anions, caused precipitation at a ten-fold lower concentration compared to sodium chloride or sodium acetate that provided monovalent anions. The dashed lines indicate the estimation of the $SC_{50}$ values, the salt concentrations that resulted in 50% solubility.
Therefore, the divalent anions have ten-fold higher precipitating power than the univalent anions. On the other hand, calcium chloride, which provides a divalent cation, does not cause a lowering of SC_{50} compared to sodium chloride; the 2-fold reduction in this case can be explained on the basis that each calcium chloride molecule provides two chloride anions.

Although the set of salts studied is small, the above data indicates that anionic strength is an important determinant of insolubility of TriPL-DNA complexes. A possible explanation of this phenomenon arises from the consideration that TriPL-DNA complexes at the ratio studied have a calculated amine/phosphate ratio of ~ 5, and carry a net positive charge. Therefore the presence of anions, sandwiched between cationic complexes, may help in the formation of aggregates in a concentration dependent manner. Further, divalent anions cause precipitation at lower concentration than univalent anions, since they are able to form salt bridges with opposing strands of complexes more easily. This model leads to some important considerations for the improved formulation of cationic carrier-DNA complexes: if salt anions are to be included in the formulation (as in buffered formulations), divalent or multivalent anions should be excluded, and the total anionic strength should be kept low. Further, anions on bulky molecules that are sterically hindered in forming salt bridges may provide better solubility of complexes.

The process of complexation between DNA and carrier is usually monitored by an electrophoretic band retardation assay on an agarose gel^{13,17,29}, and this method
was used for the initial evaluation of DNA-TriPL complexation. A fixed amount of pCMVL was titrated with increasing amounts of TriPL resulting in complexes which showed complete retardation on a 1% agarose gel when the carrier to DNA ratio was 0.2 nmol/µg or more (Figure 24). It should be noted that a carrier to DNA ratio of 0.2 nmol/µg approximates the calculated ratio for neutralization of assumed unit charges on DNA by unit charges on TriPL.

In order to further characterize DNA complexes, two spectroscopic assays were developed, involving fluorescence and light scattering. Fluorescent dye displacement assays utilize dyes that show increased quantum yield of fluorescence when intercalated within nucleic acids and are usually utilized to monitor binding of competing intercalating molecules. Experimentation showed that this type of assay may also be used to monitor ionic binding of polycations to DNA, even though the modes of binding of dye and polycation are different. Preliminary experiments were carried out in the presence of the intercalating dye ethidium bromide. The addition of PL or TriPL was found to prevent the intercalation of the dye, and resulted in fluorescence quench (Figure 25). In order to increase the sensitivity of the assay, thiazole orange\textsuperscript{129} was used for further experiments, and a fluorescence quench titration upon adding increasing amounts of carrier to DNA is shown in Figure 26. This assay indicated 45% fluorescence quench at a carrier-DNA ratio of 0.2, and a 95% quench at a ratio of 0.8.
Figure 24. Band Retardation Assay for Monitoring Carrier-DNA Complex Formation. TriPL formed complexes with pCMVL that did not show any electrophoretic migration on an agarose gel when the ratio of carrier to DNA was 0.2 nmol/µg or more, whereas plasmid DNA alone migrated normally (lane 1, Carrier-DNA ratio 0.0).
Figure 25. Fluorescence Quench of Intercalated Ethidium Bromide upon DNA Complexation. Fluorescence scans of ethidium bromide (1.2 μM) in presence of 3 μg of plasmid showed increasing quench of fluorescence (scans labelled from a to h) as TriPL was added in 0.4 nmol increments.
Figure 26. Fluorescence Assay for Monitoring DNA Complexation. A. TriPL-pCMVL complexation in mannitol solution was evaluated at different ratios of carrier to DNA by fluorescence quench of intercalated thiazole orange. The fluorescence was quenched by 95% at a carrier-DNA ratio of 0.8 nmol/µg or more. B. The structure of thiazole orange.
Thiazole orange was selected as intercalating probe due to its almost non-fluorescent nature when unbound, and moderate affinity for DNA which allowed facile displacement of the dye. However, other dyes fulfilling these criteria may also be used. One of the advantages of the fluorescence assay is that complexation can be monitored in the same solvent that the complexes are prepared in, and the assay may be performed even in biological media, whereas this is not be possible with the electrophoresis assay. Further, the fluorescence assay allows a quantitative estimate of the extent of complexation and allows the sensitive comparison of the DNA binding of different types of carriers. However, it may not be valid to use this assay to analyze absolute binding constants, since the modes of binding of the dye and carrier are different. Also, this assay would not be useful to monitor very weak binding carriers in which case the presence of the dye would interfere with the complexation process.

An important consequence of complexation between polycations and DNA is condensation of DNA into compact structures\textsuperscript{39,40}. Band retardation assay cannot be used to evaluate DNA condensation. However, previously published studies indicate that total intensity laser light scattering may be used to evaluate extent of condensation while quasi-elastic laser light scattering may be used for size analysis of condensates\textsuperscript{132,133}. Based on this information, light scattering measurements at 90° were performed in a fluorimeter to monitor DNA-carrier complexation. Synchronous scans showed that the highest intensity of scattered light was obtained at 350 nm, and
this was probably due to the energy output profile of the source lamp (Figure 27A). Complexes prepared with increasing ratios of TriPL to pCMVL resulted in increased light scattering at 350 nm until an asymptote was reached (Figure 27B). At the DNA concentrations used in the study (20 μg/ml), light scattering may result from DNA condensation as well as some aggregation. Nevertheless, the results with light scattering measurements complement those obtained with the dye displacement assay, and indicate that DNA condensation occurs as complexation of the DNA with TriPL proceeds, as evidenced by the sudden elevation of scattered light intensity.

Light scattering was nearly at solvent background at a carrier-DNA ratio of 0.2, and at 95% of the asymptote at a ratio of 0.8 nmol of carrier per μg DNA. Similarly, fluorescence quench of intercalated thiazole orange showed a 95% quench at a ratio of 0.8. Both light scattering and fluorescence intensity could be measured for the same sample by adjusting slit widths and monochromators since the presence of thiazole orange did not produce any significant difference in the scattered light intensity. Further, the entire procedure of adjusting instrument parameters for each sample was automated by means of programming the computer interfaced fluorimeter (Appendix).

HepG2 cells were transfected with complexes containing different ratios of TriPL to pCMVL in either the presence or absence of chloroquine. The level of reporter gene expression was amplified by more than 2 orders of magnitude when the carrier-DNA ratio was increased from 0.2 to 0.8 nmol/μg (Figure 28). Further
Figure 27. Light Scattering Assay for Monitoring DNA Condensation. A. Plasmid DNA showed light scattering with a maximum at 350 nm when complexed with TriPL. B. Upon titrating DNA with increasing amounts of TriPL, light scattering at 350 nm reached 95% of its maximal value at a carrier-DNA ratio of 0.8 nmol/µg.
Figure 28. Influence of Carrier-DNA Complexation Ratio upon Gene Transfection. Complexes were prepared in mannitol solution at increasing ratios of TriPL to pCMVL (10 μg DNA) and their transfection competency was analyzed in HepG2 cells in the presence of chloroquine. The bars show luciferase expression at 24 h. The spectroscopic assays (Figures 26 and 27) were predictive of the optimal stoichiometries for transfection.
increase in the carrier-DNA ratio to 1.2 caused only a modest 2-fold increase in reporter gene expression. Gene expression was also observed when TriPL was substituted with PL; however this was two orders of magnitude lower than that observed with TriPL. In either case, an enhancement of gene expression (1 to 2 orders of magnitude) was obtained when the transfection was carried out in the presence of chloroquine, consistent with a receptor mediated endocytosis process.

Based on the above transfection results, an optimized ratio of 0.8 nmol of TriPL carrier per μg of DNA (approximate molar ratio of 3000; positive:negative charge ratio of 5) was selected for further experiments. It is significant that fluorescence quench and light scattering assays (Figure 26, 27) also indicated 95% complexation or condensation at a carrier-DNA ratio of 0.8 whereas the electrophoresis band retardation assay indicated complete complexation at a TriPL-pCMVL ratio (nmol/μg) of 0.2 or more (Figure 24). Thus spectroscopic assays were more predictive of optimal transfection activity compared to the band retardation assay.

The specificity of gene delivery was investigated according to the ligand recognition characteristics of the ASGP-R\textsuperscript{63,66}. To confirm that the enhancement of gene expression was due to specific receptor recognition of terminal galactose residues, TriPL was substituted with Agal-TriPL in optimized DNA-carrier complexes. The resulting gene expression was approximately equivalent to that seen with PL (Figure 29). In addition, TriPL-pCMVL complexes provided the same
Figure 29. Influence of Carrier Type on Receptor-Mediated Gene Delivery to Hepatoma Cells. TriPL-pCMVL complexes were prepared with 10 μg pCMVL and 8 nmol of PL, Agal-TriPL, or TriPL in mannitol solution, and transfected into HepG2 cells in the absence (shaded bars) and presence (solid bars) of 80 μM chloroquine. Complexes prepared with TriPL showed luciferase expression two orders of magnitude above those made with PL (polylysine dp19). However this enhancement was dependent only on the presence of terminal galactose residues on TriPL which were recognized by the asialoglycoprotein receptor on hepatocytes since Agal-TriPL-pCMVL complexes showed expression similar to PL-pCMVL complexes.
background gene expression as PL-pCMVL when transfected into HeLa cells, a human cell line lacking the ASGP-R.

Millimolar concentrations of galactose can inhibit binding of galactose terminated triantennary to the ASGP-R\textsuperscript{63}; however, expression levels were not inhibited by transfection in the presence of 100 mM free galactose. This may be due to the very high affinity of complexes for the ASGP-R when multiple oligosaccharide residues are clustered on condensed DNA.

The influence of fetal bovine serum (FBS) concentration on transfection in the presence of chloroquine was investigated. Transfections in the presence of serum free media or in 10% FBS resulted in similar luciferase levels, and incubation in the presence of 2% FBS resulted in a modest two fold increase of transfection (Figure 30). This latter concentration was chosen for all the other experiments to optimize activity. The results demonstrate that no dramatic inhibition of gene transfection is obtained with these complexes in the presence of FBS, which can be a significant problem for gene transfection with cationic liposomes\textsuperscript{134}.

An experiment was carried out to obtain information on the time course of expression. The results demonstrated that expression was obtained as early as 12 hours, peaked at 3 days, and was at a low level by the seventh day. This is consistent with earlier reports on receptor mediated gene delivery to HepG2 and other cells showing transient gene expression\textsuperscript{29}. Although peak level of expression was at 3 days, luciferase activity was routinely assayed at 24 h for rapidity. Dose response
Figure 30. Effect of Serum Concentration upon Transfection with Carrier-DNA Complexes. TriPL-pCMVL complexes were prepared at a carrier-DNA ratio of either 0.4 (shaded bars), or 0.8 (solid bars), and transfected into HepG2 cells in the presence of either 0, 2, or 10% fetal calf serum. For both types of complexes, transfection was higher in the presence of 2% serum. Transfection in media with 10% serum did not cause any inhibition of gene delivery as compared to serum free media.
Figure 31. Effect of TriPL-pCMVL Dose on Reporter Gene Expression. TriPL-pCMVL (0.1-20 μg DNA) complexes were transfected into HepG2 cells at a carrier-DNA ratio 0.8 nmol/μg in the presence of chloroquine and the transfection competency analyzed at 24 h. Increasing levels of expression were obtained with increasing dose of DNA.
experiments established a non-linear increase in reporter gene expression with increasing dose of TriPL-pCMVL up to the maximum of 20 μg DNA dose tested (Figure 31). In the presence of chloroquine, luciferase expression was observed even with 0.1 μg pCMVL. Increasing the dose from 1 to 10 μg resulted in a 1000-fold increase in luciferase expression, while further increasing the dose to 20 μg led to a 2.5-fold increase in luciferase levels. The highest level of expression seen with 20 μg DNA in the presence of chloroquine corresponds to an average of $7 \times 10^6$ light units from the entire 35 mm well containing approximately 1 million cells ($37 \times 10^6$ light units per mg protein). Although strict comparisons cannot be made, levels of luciferase expression obtained in this study are either similar to or better than those observed in previous studies where high molecular weight carriers were used for chloroquine enhanced, ASGP-R mediated gene delivery to HepG2 cells$^{15,17}$. The non-linearity seen in dose-response has also been observed previously$^{17}$. These results emphasize the importance of achieving high solubility of plasmid complexes in order to maximize expression levels.

CONCLUSION

A low molecular weight glycopeptide carrier was studied for the purpose of receptor mediated gene delivery. The glycopeptide carrier complexed with plasmid DNA as evidenced by immobility of complexed DNA upon agarose gel electrophoresis, and displacement of intercalated dye. Further, light scattering
measurements indicated condensation of fully complexed DNA. DNA-carrier complexes were endocytosed into HepG2 cells via the asialoglycoprotein receptor due to recognition of terminal galactose residues on the oligosaccharide. The resulting luciferase reporter gene expression was dramatically influenced by the solubility of complexes, the extent of complexation, and by the presence of the lysosomotropic agent chloroquine.

The results obtained from this study indicate that it is possible to achieve receptor mediated gene delivery with low molecular weight glycopeptide carriers. Oligosaccharide ligands or synthetic analogs designed to bind with high affinity to target receptors may be linked to polycationic oligopeptides of known sequence to obtain completely defined carrier structures. The use of highly characterized carriers will lead to greater reproducibility in gene delivery and expression. Furthermore, it will allow rational manipulation of carrier design for stability and solubility, modulation of DNA and receptor binding characteristics, and incorporation of additional effector molecules like fusogenic and nuclear targeting peptides.
CHAPTER V

Second Generation, Structurally Defined Peptide and Glycopeptide Carriers

INTRODUCTION

In the previous chapter it was demonstrated that low molecular weight glycopeptide carriers can provide receptor mediated gene delivery. This result is very significant, since the use of low molecular weight glycopeptides allows a high level of carrier purification and characterization not possible with high molecular weight glycoprotein or neoglycoprotein conjugates. Further, the use of small glycopeptides should allow the rational, stepwise modification of carrier structure in order to study and optimize DNA-carrier complexes for gene delivery.

However, in order to clearly elucidate structure-activity relationships, the rational design of DNA carriers requires the use of fully defined chemical structures. Despite its high level of characterization, the glycopeptide carrier TriPL does not fulfil the criterion of structural homogeneity. This is due to the fact that the polylysine used has inherent polydispersity, and further, the oligosaccharide is randomly coupled to one of the amines on the polylysine chain. Since the oligosaccharide itself is structurally defined, these problems can be remedied by the use of fully defined
peptides rather than polylysine, and by the use of selective coupling chemistry so that the oligosaccharide is linked to the peptide at a specific site.

The use of such structurally defined glycopeptides would allow for the facile substitution of oligosaccharide structures in order to modulate cell-surface receptor binding activity, and also to potentially target different receptors and cell types. Further, the peptide portion of the conjugate could be rationally constructed in order to modulate and optimize DNA binding activity resulting in defined DNA-carrier complexes having the desired attributes of structure and function.

With these long term objectives in mind, a series of second generation carriers was prepared, with the initial goal of determining the number of lysine residues required for optimal DNA binding and transfection. The series is based on defined peptides of the general formula Cysteine-Tryptophan-Lysine_x (CWK_x), where "x" represents a specific, but variable number of lysine residues. The conjugation of the galactose terminated triantennary oligosaccharide to these peptides using site specific linkage at the side chain of the cysteine residue provides an analogous series of defined glycopeptides.

MATERIALS AND METHODS

N-terminal Fmoc protected amino acids, and all other reagents for peptide synthesis were obtained from Advanced Chemtech, Lexington, KY. Sephadex G25, DL-dithiothreitol, iodoacetic acid, iodoacetamide, N-hydroxysuccinimide, and
dicyclohexyl carbodiimide were purchased from Sigma Chemical, St. Louis, MO. Ethanedithiol (EDT) was purchased from Aldrich Chemical, Milwaukee, WI; and trifluoroacetic acid (TFA) from Fisher Scientific, Pittsburgh, PA. Peptide purification was performed on a Vydac semi-preparative 10 µm silica column (Vydac, Hesperia, CA) operated with computer interfaced HPLC equipment and fraction collector from ISCO (Lincoln, NE). Silica C18 (5 µm) HPLC columns (Microsorb MV) were purchased from Rainin.

**Synthesis of CWKₙ peptides:**

CWKₓ peptides were prepared by solid phase peptide synthesis (SPPS)⁴⁰ on Fmoc-L-Lysine-Wang resin (p-benzzyloxybenzyl alcohol resin¹⁴¹,¹⁴², 1% divinyl benzene crosslinked, 100-200 mesh) at a 100 µmol scale (0.68 mmol peptide/g resin). SPPS was accomplished using a computer interfaced Model 90 synthesizer from Advanced Chemtech, Lexington, KY. Lysine and tryptophan side chains were boc protected and the sulfhydryl side chain of cysteine was protected with the trityl group. N-terminal Fmoc protected amino acids (6 molar excess over resin attached amino acid) were activated in situ in the reaction vessel by the addition of equimolar diisopropylcarbodiimide (DIC) and N-hydroxybenzotriazole (HoBt). Coupling was carried out for 1 h with rapid mixing, and each coupling cycle was followed with a "capping" cycle for 30 min with 10% acetic anhydride in the presence of 1% diisopropylethylamine to acetylate any unreacted amine terminii. Fmoc deblocking
was performed with 25% piperidine for 12 min. All reagents were dissolved in dimethyl formamide (DMF).

At completion of syntheses, the resin conjugated peptides were washed with dichloromethane, dried and weighed. Final cleavage was performed in a solution of TFA:EDT:water (95:2.5:2.5 v/v) for 30 min at room temperature, which simultaneously deprotected the amino acid side chains. The peptide solution was extracted with diethyl ether to remove cleavage products; and the aqueous phase was concentrated by roto-evaporation, followed by freeze drying. Lyophilized crude peptides were dissolved in degassed and nitrogen purged 0.1% TFA and purified by HPLC using a Vydac semi-preparative C-18 reverse phase silica column in 3 - 4 μmol injections, while monitoring absorbance at 280 nm, 1.0 AUFS. The elution conditions for all the peptides are summarized in Table 4. Purified peptides were concentrated by roto-evaporation, lyophilized, and stored dry at -20°C.

Preparation and Purification of Cys-Alkylated AlkCWK\(_x\) Peptides

Each cysteine containing lyophilized peptide (1 μmol) was dissolved in 1 ml of 50 mM tris hydrochloride buffer (pH 7.5) that had been degassed and nitrogen purged. The peptide was treated with 250 μl of 100 mM dithiothreitol (DTT) prepared in the same buffer and incubated at room temperature for 30 min. After reduction of disulfide bonds, 25 mg of solid iodoacetamide (~ 5 fold excess over DTT) was dissolved in the reaction mixture and incubated for 1 h at room temperature. Disulfide
bond reduction and subsequent alkylation was monitored by RP-HPLC by injecting 1 nmol of the reaction mixture on a C18 silica analytical reverse phase column equilibrated with 5% acetonitrile in 0.1% TFA. The acetonitrile concentration was linearly increased to 30% at 25 min while detecting absorbance at 280 nm, and the progress of the reactions monitored by noting successive decrease in retention time of the peptide peak upon completion of each step. The alkylated peptides were acidified with TFA and purified by HPLC using a semi-preparative C-18 reverse phase silica column, using elution conditions summarized in Table 4.

Preparation and Purification of Iodoacetylated Triantennary Oligosaccharide

The N-hydroxysuccinimide ester of iodoacetic acid (NHS-IA) was prepared by the method of Rector et al. with modification of reaction solvent. Briefly, 2.9 g of iodoacetic acid and 1.84 g of N-hydroxysuccinimide (equimolar) were dissolved in 250 ml ethyl acetate and the reaction initiated by dissolving 3.3 g of dicyclohexyl carbodiimide (DCC). The reaction was stirred in a covered beaker at room temperature for 1 h, and the precipitated dicyclohexyl urea removed by filtration. The filtrate was dried by roto-evaporation, dissolved in warmed chloroform (55°C), and crystallized on ice for 3 h. The harvested crystals were recrystallized from chloroform overnight on ice, dried under vacuum, and used without further purification.
Table 4. Reverse Phase HPLC Elution Conditions for Peptide and Glycopeptide Carriers.

<table>
<thead>
<tr>
<th>Peptide or Glycopeptide</th>
<th>Reverse Phase HPLC Elution conditions (Vydac &quot;218TP&quot; C18 Column, 2.2×25cm)</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CWK3</td>
<td>Linear gradient of 5% to 10% acetonitrile developed over 30 min.</td>
<td>23</td>
</tr>
<tr>
<td>CWK8</td>
<td>Linear gradient of 6% to 12% acetonitrile developed over 30 min.</td>
<td>21</td>
</tr>
<tr>
<td>CWK13</td>
<td>Linear gradient of 8% to 20% acetonitrile developed over 30 min.</td>
<td>17</td>
</tr>
<tr>
<td>CWK18</td>
<td>Linear gradient of 5% to 12% acetonitrile over 15 min, elution continued at 12%.</td>
<td>27</td>
</tr>
<tr>
<td>AlkCWK3</td>
<td>Isocratic elution with 5% acetonitrile.</td>
<td>31</td>
</tr>
<tr>
<td>AlkCWK8</td>
<td>Isocratic elution with 6% acetonitrile for 25 min, step up to 7% acetonitrile at 26 min.</td>
<td>37</td>
</tr>
<tr>
<td>AlkCWK13</td>
<td>Isocratic elution with 8% acetonitrile.</td>
<td>40</td>
</tr>
<tr>
<td>AlkCWK18</td>
<td>Isocratic elution with 9% acetonitrile for 30 min, step up to 10% acetonitrile at 31 min.</td>
<td>43</td>
</tr>
<tr>
<td>TriCWK3</td>
<td>Isocratic elution with 7% acetonitrile</td>
<td>33</td>
</tr>
<tr>
<td>TriCWK8</td>
<td>Isocratic elution with 7% acetonitrile for 30 min, step up to 8% acetonitrile at 31 min.</td>
<td>48</td>
</tr>
<tr>
<td>TriCWK13</td>
<td>Isocratic elution with 9% acetonitrile.</td>
<td>35</td>
</tr>
<tr>
<td>TriCWK18</td>
<td>Isocratic elution with 9% acetonitrile for 30 min, step up to 10% acetonitrile at 31 min.</td>
<td>53</td>
</tr>
</tbody>
</table>
Iodoacetylated triantennary oligosaccharide (I-Tri) was prepared as follows. Amine terminus deprotected triantennary oligosaccharide (5 μmol) was prepared as described in Chapter I, and was dissolved in 5 ml sodium bicarbonate buffer (100 mM, pH 8.0, unadjusted). A 40 fold molar excess of NHS-IA (55mg in 0.5 ml DMF) was added in small amounts while mixing to avoid high local concentrations. The reaction was incubated for 3 h at room temperature, and another 55 mg of NHS-IA added in 0.5 ml DMF, followed by overnight incubation at room temperature. The reaction was acidified with 0.5 ml 10% acetic acid and purified by gel filtration chromatography on a G-25-50 Sephadex column (2.5 cm × 45 cm) eluted with 0.1% acetic acid while detecting absorbance at 280 nm. The first eluted peak between 95 and 135 ml was collected, concentrated by rotary evaporation, and freeze dried.

Preparation and Purification of TriCWKα Glycopeptides

An agarose column (1 ml bed volume) with immobilized reductant (Reduce-Imm Column™, Pierce Chemical Co.) was prepared for disulfide bond reduction by washing with a 10 mM solution of DTT according to the manufacturer's directions except that tris hydrochloride buffer (50 mM, pH 7.5, degassed and nitrogen purged) was used. CWKα peptides (1.1 μmol) were individually prepared in 900 μl Tris hydrochloride buffer; loaded onto the reducing column, and the column frit washed with 100 μl of buffer. After incubation for 2 h, the reduced peptide was eluted with 3 ml of buffer directly into 1 μmol of I-Tri prepared in 1 ml of the same buffer, and
the reaction incubated at room temperature overnight. The reaction progress could be followed by injecting 4 µl of the acidified reaction mixture on a C18 silica analytical RP-HPLC column equilibrated with 5% acetonitrile in 0.1% TFA. The acetonitrile concentration was linearly increased to 30% at 25 min while detecting absorbance at 280 nm, and the progress of the reaction monitored by noting the elution of the glycopeptide at a slightly decreased retention time compared to the peptide, and by the concomitant disappearance of the peptide and l-Tri. Upon completion of the reaction, the reaction mixture was acidified with TFA and the glycopeptides purified by HPLC using a semi-preparative C-18 reverse phase silica column, using elution conditions summarized in Table 4.

Characterization of Purified Peptides and Glycopeptides

Absorbance profiles of purified AlkCWKₜ peptides and TriCWKₜ glycopeptides were obtained a Beckman DU640 scanning spectrophotometer. The presence of tryptophan was determined in both peptides and glycopeptides by fluorescence scanning on a Perkin Elmer LS 50B spectrometer using excitation at 280 nm and emission at 350 nm (5 nm slit widths). The glycopeptides were additionally analyzed by monosaccharide analysis. All of the purified peptides and glycopeptides were rechromatographed on an analytical C18 HPLC column to test for structural homogeneity and uniqueness under identical elution conditions (a linear gradient of 8 to 16% acetonitrile in 0.1% TFA developed over 40 min). The purified peptides and
glycopeptides were further characterized by time of flight mass spectrometry using matrix assisted laser desorption ionization (MALDI-MS) with insulin as an internal standard. The matrix used was 2,5 dihydroxybenzolic acid (from Biomolecular Separations, Reno, NV) in a 100mM methanolic solution. A Laser Science VSL-337 ND nitrogen laser (337 nm, with a 3 ns pulse width) was used for desorption, and the analysis was performed using a Vestee-2000 (LaserTec Research, Houston, TX) time of flight mass spectrometer, equipped with 1.2 m flight tube and operated at 23 kV accelerating voltage.

RESULTS AND DISCUSSION

A series of four homologous peptides was prepared with the general formula CWK_x, each with a cysteine and a tryptophan, and possessing either 3, 8, 13, or 18 lysine residues. These peptides were conjugated with either triantennary oligosaccharide, to produce TriCWK_x glycopeptides, or were alkylated with iodoacetamide, to produce corresponding control AlkCWK_x peptides. In both the alkylated peptides and the glycopeptides, the reactive side chain of cysteine is blocked with a thioether linkage, and the chemical schemes for preparing the two types of carriers are shown in Figures 32 and 33 respectively.

Solid phase peptide synthesis was initially attempted with preactivation of the amino acids with DIC and HoBt, but better results were obtained with in situ activation in the reaction vessel. Also, synthesis in DMF provided higher yields than
**Figure 32.** Chemical Scheme for the Preparation of AlkCWK<sub>x</sub> Peptide Carriers. Peptides of the general formula cysteine-tryptophan-lysine<sub>x</sub> (where <i>x</i> represents 3, 8, 13, or 18 lysines) were prepared by solid phase peptide synthesis. The CWK<sub>x</sub> peptides were purified and reacted with iodoacetamide to alkylate the sulfhydryl group of cysteine with a stable thioether linkage resulting in AlkCWK<sub>x</sub> peptides.
Figure 33. Chemical Scheme for the Preparation of TriCWK_\_ Glycopeptide Carriers. Tyrosinamide triantennary oligosaccharide with a deprotected amine terminus was reacted with the NHS ester of iodoacetic acid to obtain Iodoacetylated Tri. The latter was conjugated to each of the four CWK_x peptides (Figure 32) with a stable thioether linkage, to obtain TriCWK_x glycopeptides.
in N-methyl pyrrolidone (NMP), a solvent that is reported to sometimes increase coupling efficiencies due to better resin solvation\textsuperscript{144,145}. Each synthesis was controlled by a computer program operated with ACT\textsuperscript{90}™ software (Advanced Chemtech, Lexington, KY) in which each individual step of the synthesis was specified.

The time course of the cleavage reaction to release peptide chains from the resin was monitored by RP-HPLC, resulting in the selection of a 30 min time period. Crude CWK\textsubscript{x} peptides were obtained with greater than 50% yield based on tryptophan absorbance. Peptides with multiple lysine residues are difficult to purify, but the hydrophobicity imparted by the tryptophan residue allowed purification using reverse phase HPLC. However, after HPLC purification, the yield was 15 - 25% of the starting resin bound amino acid. This was due to both the presence of deletion peptides and contaminants as well as loss of peptides due to adsorption on the reverse phase column. With respect to the latter, a semipreparative C18 column ("218TP") from Vydac (Hesperia, CA) provided ~ 70% recovery of glycopeptides and peptides compared to less than 10% recovery obtained with C8 and C18 ("Spherisorb") reverse phase columns from PhaseSep (Norwalk, CT) or C18 ("Microsorb") columns from Rainin (Emeryville, CA). Polymeric reverse phase columns ("PRP-1") from Hamilton (Reno, NV) afforded good recovery but lacked the resolution to allow reasonable purification.

CWK\textsubscript{x} peptides dimerized in solution due to the formation of disulfide bonds between cysteine residues over a period of few days even when stored at -20°C. The
dimerization could be greatly reduced by lyophilizing the peptides from a degassed and nitrogen purged solution of 0.1% TFA and storing the dried peptides at -20°C. However some dimerization still occurred over a period of weeks to months, necessitating the routine use of a disulfide reduction step to obtain optimal yields in the synthesis of cys-alkylated peptides and glycopeptides. In the case of the former, reduction was conveniently performed in solution using DTT, and alkylation achieved in the presence of DTT by using an excess of iodoacetamide, an inexpensive reagent.

TriCWK₅ glycopeptides were prepared by covalently conjugating triantennary oligosaccharide to CWK₅ peptides by a thioether linkage. This necessitated the preparation of iodoacetylated triantennary oligosaccharide (I-Tri, Figure 33) by reacting amine deprotected tyrosinamide triantennary with the N-hydroxysuccinimide ester of iodoacetic acid (NHS-IA). I-Tri was purified away from excess reagent on a gel filtration column with greater than 90% yield, and quantitated from the absorbance of the tyrosine residue. I-Tri however, did not display any fluorescence, presumably due to quenching by iodide. Proton NMR (500 MHz) of purified I-Tri showed all the characteristic anomic resonances of the triantennary oligosaccharide.

For the preparation of glycopeptides, the reduction of dimerized peptides was achieved on an agarose column with an immobilized reductant, which allowed greater than 95% recovery of the peptide in the monomer form without contamination with the reducing agent. Monomeric peptides with free sulfhydryl groups were eluted directly into a solution of I-Tri which allowed coupling to proceed immediately. This
strategy allowed use of stoichiometric amounts of the oligosaccharide and peptide, and the use of degassed and nitrogen purged buffer allowed the reaction to proceed to greater than 95% completion with preferential formation of glycopeptide compared to the peptide dimer, as evidenced by RP-HPLC. Although the glycopeptide formation was complete in two hours, the reactions were incubated overnight to allow the slight excess of peptide monomer (10% over oligosaccharide) to dimerize, since this facilitated subsequent HPLC purification.

The purification of alkylated peptides and glycopeptides could not be achieved with gravity flow gel filtration using Sephadex G-25 (Pharmacia), or P-6 (BioRad) resins, necessitating repurification by HPLC. A semi-preparative Vydac C18 column afforded yields of 55-65% with respect to the purified starting materials. However, the presence of multiple lysines on the carriers necessitated the purifications to be carried out with small injections (0.25 - 1 µmol) to avoid peak overloading. Purified alkylated peptides and glycopeptides were rechromatographed using analytical RP-HPLC to establish homogeneity and uniqueness of structure as shown in Figures 34 and 35.

Sensitive quantitation of the purified CWKx peptides and glycopeptides was obtained by absorbance measurement at 280nm, utilizing the presence of tryptophan in the peptides and the presence of tryptophan as well as tyrosine in the glycopeptides (ε280, peptide : 5600 M⁻¹cm⁻¹; ε280, glycopeptide : 6930 M⁻¹cm⁻¹). The presence of tryptophan was confirmed in both peptides and glycopeptides by fluorescence scanning to obtain
Figure 34. Analytical RP-HPLC Characterization of AlkCWK<sub>x</sub> Peptides. The four purified AlkCWK<sub>x</sub> peptides were chromatographed on a C18 reverse phase HPLC column under identical conditions (described in text). Analytical RP-HPLC was used to monitor progress of cysteine alkylation reactions and to establish homogeneity upon purification.
Figure 35. Analytical RP-HPLC Characterization of TriCWKₙ Glycopeptides. The four purified TriCWKₙ glycopeptides were chromatographed on a C18 reverse phase HPLC column under identical conditions (described in text). Analytical RP-HPLC was used to monitor progress of glycopeptide formation and to establish homogeneity upon purification.
Figure 36. Absorbance and Fluorescence Spectra of AlkCWK₁₈ and TriCWK₁₈. A. The absorbance spectrum of AlkCWK₁₈ (at a concentration of 20 nmol/ml, dotted line) was characteristic of the absorbance of tryptophan, with a maximum at 280 nm. TriCWK₁₈ (at a concentration of 20 nmol/ml, solid line) possessed higher absorbance due to the presence of both tryptophan and tyrosine, and also displayed a maximum at 280 nm. B. The same samples of AlkCWK₁₈ (dotted line) and TriCWK₁₈ (solid line) showed fluorescence excitation and emission at 280 nm and 350 nm respectively, with enhanced fluorescence in the latter case.
Figure 37. MALDI-TOF mass spectrometry of TriCWK$_{18}$. Mass spectrometry of the glycopeptide TriCWK$_{18}$ was performed with insulin as internal standard, and the singly protonated mass ion of the glycopeptide was within 1 amu of the calculated mass.
Table 5. Calculated Masses for Peptide and Glycopeptide Carriers and Observed Mass Ions by MALDI-TOF Mass Spectrometry.

<table>
<thead>
<tr>
<th>Peptide or Glycopeptide</th>
<th>Calculated Mass Ion (M+H⁺)</th>
<th>Observed Mass Ion (M+H⁺)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlkCWK₃</td>
<td>750.0</td>
<td>750.2</td>
</tr>
<tr>
<td>AlkCWK₄</td>
<td>1390.9</td>
<td>1391.1</td>
</tr>
<tr>
<td>AlkCWK₁₃</td>
<td>2031.8</td>
<td>2031.1</td>
</tr>
<tr>
<td>AlkCWK₁₈</td>
<td>2672.7</td>
<td>2672.5</td>
</tr>
<tr>
<td>TriCWK₃</td>
<td>2901.9</td>
<td>2902.3</td>
</tr>
<tr>
<td>TriCWK₈</td>
<td>3542.8</td>
<td>3543.6</td>
</tr>
<tr>
<td>TriCWK₁₃</td>
<td>4183.7</td>
<td>4184.0</td>
</tr>
<tr>
<td>TriCWK₁₈</td>
<td>4824.6</td>
<td>4824.4</td>
</tr>
</tbody>
</table>

*Average Mass
excitation and emission maxima at 280 and 350 nm respectively. Fluorescence scans of the glycopeptides did not show any tyrosine emission at 305 nm, and variable enhancement of tryptophan fluorescence in the four glycopeptides was observed over the corresponding peptides. The enhancement of tryptophan emission in the glycopeptides probably occurs due to fluorescence energy transfer between the tyrosine and the tryptophan, since the emission of tyrosine at 305 nm falls just within the outer limits of tryptophan's excitation band. Absorbance and fluorescence spectra for AlkCWK₁₈ and TriCWK₁₈ are shown in Figure 36.

Conclusive evidence of the structures of the alkylated peptides and glycopeptides was obtained by time of flight mass spectrometry, which provided singly protonated mass ions within one atomic mass unit of the calculated masses. The MALDI-MS result for TriCWK₁₈ is shown in Figure 37, and numerical values for the observed and calculated mass ions for all of the peptides and glycopeptides are summarized in Table 5.

CONCLUSION

A homologous series of four AlkCWKₓ peptides was prepared, with each peptide possessing one cysteine, one tryptophan, and either 3, 8, 13 or 18 lysine residues. From these peptides, an analogous series of four TriCWKₓ glycopeptides was obtained by conjugating the triantennary galactose terminated oligosaccharide at the cysteine side chain. The peptides and glycopeptides were purified to homogeneity,
and evidence of their structure confirmed by mass spectrometry.

The function of the cysteine residue in the CWK\textsubscript{x} peptides is to provide a handle for site specific linkage of the oligosaccharide, whereas the tryptophan residue provides hydrophobicity for RP-HPLC purification in addition to allowing sensitive detection of peptides and glycopeptides by both absorbance and fluorescence. A further reason for inclusion of the tryptophan in these carriers is to provide a sensitive spectroscopic handle for monitoring of carrier-DNA complexation by tryptophan fluorescence. The number of lysine residues was deliberately varied between 3 and 18 to allow an investigation of this parameter with respect to DNA binding and transfection efficiency, providing a first step towards the optimization of rationally designed and structurally defined glycopeptide (and peptide) carriers for gene delivery.
CHAPTER VI

Spectroscopic Characterization, and Biological Activity of Defined Peptide and Glycopeptide Carriers

INTRODUCTION

One of the major limiting factors for the widespread application of gene therapy is the development of safe, efficient, and well characterized DNA delivery methods for routine transfection \textit{in vivo}^{3,5,6,8}. Accordingly, the approach taken in this thesis project has been focussed on the development of highly characterized carriers whose structure and function can be rationally designed and controlled. It is felt that this approach is necessary to allow a high level of control over gene delivery and expression, as well as crucial to probing the various parameters that need to be optimized in order to make receptor mediated gene transfer safe, efficacious, and reliable enough for routine use in clinical practice.

Therefore, a major effort in this thesis project has been to prepare carrier molecules whose structure is unambiguously known, and whose design is sufficiently modular and flexible to allow the systematic variation of structure. Parallel to these aspects are the requirements of testing the effects of structure on activity in order to
obtain clues about how the rational optimization of carrier design should proceed. Accordingly, another major focus of this thesis project has been to explore the physico-chemical characterization of not only the carrier molecules, but also of the DNA-carrier complexes, in order to understand the factors that can be correlated with biological activity.

In the previous chapter, the preparation and characterization of a series of AlkCWK\(_x\) peptides and TriCWK\(_x\) glycopeptides was described. In this chapter, the spectroscopic characterization and the transfection activity of DNA complexes formed with these carriers will be discussed.

MATERIALS AND METHODS

The experiments detailed in this chapter were performed using the same materials, procedures and instrument models as described in Chapter IV. However, due to relocation of the laboratory (to Ann Arbor, Michigan) with consequent acquisition of new instrumentation, and due to further optimization of protocols, some modifications were made. The modified procedures are detailed below.

Preparation of Carrier-DNA Complexes.

Carrier-DNA complexes were prepared with the final DNA concentration fixed at 20 \(\mu g/ml\) and carrier-DNA ratios (nmol carrier/ \(\mu g\) DNA) varying from 0.1 to 1.5. The term "carrier" in this chapter refers to either the AlkCWK\(_x\) peptides or the
TriCWKₙ glycopeptides, and "DNA" refers to the plasmid pCMVL as described in Chapter IV.

Complexes were prepared by adding carrier (2 - 30 nmol, in 500 µl solvent) to pCMVL (20 µg, in 500 µl solvent) while vortexing, followed by incubation at room temperature for 30 min. These amounts and volumes were linearly scaled for preparing different amounts of complex containing 2.5 - 120 µg DNA. The solvent used was hepes buffered mannitol (HBM, consisting of 0.27 M or 5% w/v mannitol, and sodium hepes, 5 mM, pH 7.5) unless otherwise indicated. The DNA and carriers were individually prepared in deionized water, combined with 9 volumes of solvent at 110% strength, and then finally mixed together to obtain the final concentrations. Upon transfection of DNA, luciferase light units were converted into femtomoles using a new standard curve which was linear between $10^1$ and $10^7$ relative light units (RLU) and each femtomole was equivalent to 273,000 RLU.

For freeze drying studies, complexes were prepared using either deionized water, HBM, 5% mannitol, 10% mannitol, 5% glucose, 10% glucose, 9.8% lactose, or 19.6% lactose (strengths indicated are w/v; and are either isotonic or twice the isotonic concentrations for the sugars used). After a 30 min incubation period, complexes were frozen in dry ice for 10 min, and lyophilized overnight.
Assays for Monitoring Carrier-DNA Complexes

The terms "soluble" and "solubility" are used here with the same limited meaning as described in Chapter IV. Solubility was determined by analyzing a 50 µl aliquot (1 µg DNA) of the complex before and after centrifugation at 13000 g for 4 min at room temp. The aliquots were diluted with 1 ml water and the DNA in solution measured by UV absorbance at 260 nm. The ratio of the values obtained after and before centrifugation (and multiplied by 100) was used as a measure of "percent solubility".

DNA complexation was monitored by a fluorescence assay based on the exclusion of intercalating dye from DNA by peptide or glycopeptide carriers. DNA condensation was monitored simultaneously by measuring scattered light at 90°. For fluorescence and light scattering measurements, solvents were always filtered through 0.2 µm surfactant free cellulose acetate filters, and if necessary, traces of dust were removed from sample tubes by means of 0.1 µm filtered pressurized air. An aliquot of carrier-DNA complex (1 µg DNA, in 50 µl) was diluted to 1.0 ml in solvent containing 0.105 µM thiazole orange (from a 0.1 mg/ml stock in 1% methanol, ε₄₇₆ = 30,000 M⁻¹ cm⁻¹)¹²⁹. Fluorescence of the intercalated dye was measured in a microcuvette (with 1 cm pathlength in the excitation beam) using excitation at 500 nm and emission at 530 nm, with the slits set at 15 and 20 nm and photomultiplier gain set to 700 volts. Light scattering intensity was measured for the same sample by adjusting both monochromaters to 500 nm and decreasing slit widths to 2.5 nm.
Instrument control of slit widths and monochromator settings (for automated recording of fluorescence and scattering data in performing titrations) was accomplished using a custom written computer program run by FLDM™ software (Perkin Elmer, UK) (Appendix). Fluorescence and scattered light intensity resulting from dye dissolved in solvent (blank) was subtracted from all values before data analysis.

The tryptophan fluorescence of peptides and glycopeptides was measured in complexes prepared with 1 nmol carrier and 10 μg DNA (carrier-DNA ratio of 0.1). Paired controls were also prepared in which the DNA was omitted, and the carriers were mixed with the buffer alone. The 500 μl samples were diluted with buffer to 1 ml after the 30 min incubation period and tryptophan emission determined at 350 nm with the excitation set at 280 nm (band widths of 10 nm and 5 nm respectively). For each set of triplicated samples, the total intensity I and the polarized intensities "I_v,v" and "I_v,h" were measured. The subscripted letters refer to the orientation of the polarizers in the excitation and emission beams respectively; thus I_v,h refers to an emission measurement with a vertical polarizer in the excitation beam, and a horizontal polarizer in the emission beam. The measured intensities of the complexes and the paired controls were corrected for blank values resulting from buffer with and without DNA, respectively. Since the excitation and emission monochromators transmit polarized light with an efficiency dependent upon the angle of polarization, a correction factor "G" (calculated as the ratio of I_h,v and I_h,h) is required for
anisotropy calculations\textsuperscript{146}. For the wavelengths and slit widths used, $G$ was determined to be 1.11165.

The percent fluorescence quench ($q$) was calculated from the total emission intensity of the carrier in the absence ($I_d$) and presence ($I_b$) of DNA as follows:

$$q = [1 - (I_b/I_d)] \times 100 \quad (2)$$

Anisotropy ($r$) was calculated from the polarized intensities according to equation 3.

$$r = (I_{V,V} - G \cdot I_{V,II}) / (I_{V,V} + 2G \cdot I_{V,II}) \quad (3)$$

**Electron Microscopy studies**

Samples for transmission electron microscopy were prepared on carbon coated copper grids (3 mm diameter, 400 mesh; from Electron Microscopy Sciences, Fort Washington, PA). Grids were glow discharged, and carrier-DNA complexes (3 µl) were placed on the grids for 5 min. The samples were stained by floating the grids for 2 min on each of three 100 µl drops of uranyl acetate (1%, in 95% ethanol). The grids were then rinsed with 0.4% detergent solution (PhotoFlo, Kodak), and blotted dry. Samples were examined on a Philips CM-10 PC electron microscope operated at 60 kV potential and using a 30 µm aperture.
RESULTS AND DISCUSSION

Formulation of Carrier-DNA Complexes

The formulation of carrier-DNA complexes using the first generation glycopeptide TriPL is described in Chapter IV. Based on the results obtained from those studies, it was clear that the solvent used for preparing carrier-DNA complexes should be of low ionic strength. Another conclusion reached from those studies was that non-ionic excipients such as mannitol did not influence solubility, but were necessary to avoid hypotonicity relative to physiologic fluids.

TriPL-DNA complexes for transfection and spectroscopic studies were prepared in an unbuffered 0.72 M mannitol formulation (Chapter IV). In the present study, this formulation was improved by using an isotonic strength of mannitol (5% w/v, or 0.27 M), and by including a buffer to stabilize the pH at 7.5. Previous results suggested that the buffer should have monovalent anions at a low concentration. Accordingly, sodium hepes (N-[2-hydroxyethyl]-piperazine-N'-2-ethanesulfonic acid, neutralized with sodium hydroxide) was selected due to its pKa (7.5), and compatibility with biological systems\textsuperscript{147,148}. Based on experiments with AlkCWK\textsubscript{18} and TriCWK\textsubscript{18}, a hepes concentration of 5 mM was selected since it provided an anionic strength sufficient to buffer complexes, which was also low enough to allow reasonable solubility.
Solubility of Carrier-DNA Complexes.

The percent solubility of carrier-DNA complexes was analyzed in hepes buffered mannitol, at a DNA concentration of 20 µg/ml, and at carrier-DNA ratios of 0.1 up to 1.5 nmol/µg (Figure 38). The two smallest carriers, AlkCWK₃ and TriCWK₃, did not result in any insolubility of DNA. As will be shown later, this is due to the inability of these carriers to bind DNA appreciably at the ratios tested. However, for all the other carriers, complexes of varying solubility were obtained, depending upon the ratio of carrier to DNA. Glycopeptide carriers resulted in DNA complexes that were more soluble than peptide-DNA complexes, consistent with the solubilizing effect of hydrophilic oligosaccharides in the former.

Characteristic minima were observed in the solubility profiles for all the DNA binding carriers. Complexes formed from either AlkCWK₁₈ or TriCWK₁₈ displayed their minima near the carrier-DNA ratio where charge neutral complexes would be expected (0.16 nmol/µg), based on the calculated stoichiometry of positively charged amines to negatively charged phosphates. This strongly suggests that minimum solubility occurs due to charge neutralization. In comparison, for peptides and glycopeptides with 13 and 8 lysines, charge neutralization (as evidenced by solubility minima) occurred at approximately twice the calculated neutral stoichiometry.

Increasing the ratio of carrier to DNA ratio beyond the solubility minimum led to progressive increases in solubility, presumably due to the binding of additional carrier, which resulted in the formation of increasingly cationic and hydrophilic
Figure 38. Solubility Profiles of Peptide-DNA and Glycopeptide-DNA Complexes. pCMVL plasmid was complexed with each of the AlkCWKₙ peptides (A) or the TriCWKₙ glycopeptides (B) in hepes buffered mannitol at a DNA concentration of 20 μg/ml and at varying carrier to DNA ratios. Solubility of the complexes varied as a function of carrier-DNA ratio, and characteristic solubility minima were obtained for all the carriers except AlkCWK₃ and TriCWK₃.
complexes. This explains why DNA complexation with smaller carriers results in decreased solubility compared to the larger carriers (beyond the minima, Figure 38). Smaller carriers possess fewer amines, and at any given carrier-DNA ratio, complexes with these carriers are less cationic (and more hydrophobic) compared to complexes with longer carriers. Further, smaller carriers bind DNA with lower affinity as demonstrated by spectroscopic measurements (to be discussed later). The presence of unbound carrier may be the reason that for carriers with 8 and 13 lysines, charge neutralization stoichiometry is observed at ratios that are higher than predicted by simple calculation. Likewise, DNA complexes with either AlkCWK\textsubscript{3} or TriCWK\textsubscript{3} were completely soluble since due to weak or no affinity, enough carrier was not bound to DNA to allow charge neutralization at any of the ratios tested.

Spectroscopic Studies of Carrier-DNA Complexes.

Complexes were monitored at different stoichiometries of carrier to DNA using both fluorescence quench of DNA intercalating dye, as well as light scattering. Both the fluorescence and scattering measurements were made in a microcuvette, which allowed reducing the sample volume from 3 ml (Chapter IV) to 1 ml. In the case of light scattering, the wavelength of detection was also changed, from 350 nm to 500 nm. This was found necessary due to the much higher sensitivity of the new fluorimeter used, resulting in off-scale measurements at 350 nm even at the minimum photomultiplier gain.
Thiazole orange fluorescence quench profiles for carrier-DNA titrations are shown in Figure 39. At the stoichiometries tested, no evidence of DNA complexation was seen with either AlkCWK₃ or TriCWK₃. In contrast, quenching of intercalating dye was observed for each of the other three members of the peptide and glycopeptide series. Each pair of matched peptide and glycopeptide resulted in very similar fluorescence quench profiles, suggesting that the covalent attachment of the oligosaccharide to the peptide did not significantly influence the affinity of DNA binding. The fluorescence quench appears typically biphasic, initially declining rapidly as more and more carrier is added, and then showing an asymptotic region. It is significant that for AlkCWK₁₈ and TriCWK₁₈, the asymptote was reached at a carrier-DNA stoichiometry of 0.2 nmol/µg, close to the calculated charge neutralization stoichiometry. This suggests approximately 1:1 binding between the amines of these carriers and the phosphates of the DNA. Analogous to the results from solubility measurements, the peptides and glycopeptides with 8 or 13 lysines resulted in asymptotes at approximately twice the calculated charge neutralization stoichiometries.

The occurrence of an asymptote with 99% fluorescence quench in the titration of DNA with AlkCWK₁₈ or TriCWK₁₈ suggests that upon reaching the asymptote, all of the surface exposed ionic binding sites on the DNA are completely covered with carrier molecules. Any additional binding of the carrier occurs in a different mode, maybe in the mode of "atmospheric binding" by means of partial ionic interactions.
Figure 39. Complexation Titrations of DNA with Peptide and Glycopeptide Carriers, Monitored by Fluorescence Quench of Thiazole Orange. pCMVL plasmid was complexed with each of the AlkCWKx peptides (solid symbols) or the TriCWKx glycopeptides (open symbols) in hepes buffered mannitol at a DNA concentration of 20 µg/ml and at varying carrier to DNA ratios. Complexation was evaluated by fluorescence quench of thiazole orange. Each pair of matched glycopeptide and peptide carriers displayed similar quench profiles, and decreasing complexation was noted as the number of lysines in the carriers decreased.
One might surmise that in fact no additional binding occurs beyond the point of charge neutralization, but this is contrary to the solubility evidence discussed above, which is best explained by the assumption of continued binding of carrier molecules, leading to positively charged complexes that are more hydrophilic and more soluble.

The binding of the carriers to DNA is primarily a function of the number of lysine residues. At physiologic pH, each lysine provides a protonated amine side chain to interact with the negatively charged DNA phosphate groups (one more amine is provided by the N-terminal cysteine in all of the carriers, which however may not be optimally positioned for binding). Thus the reduced number of lysines in the smaller carriers may be compensated for by using higher stoichiometries. This indeed is the case since fluorescence quench asymptotes for the carriers with 13 or 8 lysines are reached at progressively higher carrier-DNA ratios. However, there is also a clear effect of increasing binding cooperativity of lysines as their number is increased in either the peptide or glycopeptide series. Therefore, carriers with 13 or 8 lysines produce asymptotes of 95% and 85% quench respectively, compared to 99% for AlkCWK<sub>18</sub> or TriCWK<sub>18</sub>. This may reflect the length dependent weaker binding affinity of the smaller carriers, and/or a faster on-off rate, allowing the dynamic exchange of a percentage of dye molecules even in the presence of excess carrier.

It is also interesting to note that the maximal fluorescence quench obtained with the first generation glycopeptide TriPL was about 95%, and at a carrier to DNA ratio (nmol/μg) of 0.8 (Chapter IV). TriCWK<sub>18</sub>, which has approximately the same
number of amines as TriPL, achieves a 99% quench of dye fluorescence, at a 2.7 fold lower concentration (0.3 nmol/µg), displaying a much stronger binding interaction. The inefficiency of DNA binding by TriPL may be due to the fact that the oligosaccharide is randomly conjugated to the one of the amine side chains in TriPL, causing local disruption in the binding of neighbouring lysines. However, this hypothesis does not explain the analogous results seen with polylysine of dp 19 (PL_{19}) and AlkCWK_{18} which display maximal dye quench at stoichiometries of 0.8, and 0.3 nmol/µg respectively. Since these carriers are devoid of pendant oligosaccharides, the presence of the either alkylated cysteine or tryptophan is implicated as the cause of increased DNA binding affinity of AlkCWK_{18} or TriCWK_{18} relative to PL_{19} or TriPL. However, tryptophan is most likely to be the cause of increased binding since it has a planar indole ring that can potentially intercalate between the base pairs of DNA. This hypothesis is supported by the finding that the fluorescence of tryptophan changes upon DNA binding, as will be described later. Intercalation of the indole ring of tryptophan into DNA may enhance carrier binding by providing an additional, hydrophobic anchor that may prevent "walking" or "sliding" of the carriers along the phosphate backbone.

The light scattering titrations of DNA complexation with each of the peptides and glycopeptides is shown in Figure 40. All the carriers formed complexes with DNA that caused sudden elevation of light scattering as the carrier to DNA ratio was increased, except for AlkCWK_{3} and TriCWK_{3}. For each of these carriers, the upward
Figure 40. Complexation Titrations of DNA with Peptide and Glycopeptide Carriers, Monitored by Light Scattering. pCMVL plasmid was complexed with each of the AlkCWK₃ peptides (solid symbols) or the TriCWK₃ glycopeptides (open symbols) in hepes buffered mannitol at a DNA concentration of 20 µg/ml and at varying carrier to DNA ratios. Complexation was evaluated by light scattering. Except for AlkCWK₃ and TriCWK₃, light scattering indicated DNA condensation with all the carriers. However there was a dramatic difference in magnitude of light scattering between complexes formed with peptides or glycopeptides.
inflection of the light scattering curve was complementary to the downward inflection of the fluorescence quench curve (Figure 39), followed by an asymptotic plateau. The light scattering inflection points were consistent with the solubility (Figure 38) and dye quench measurements. However, in striking contrast to the fluorescence quench curves, the magnitude of light scattering in the plateau region was dramatically higher (3 - 4 fold) for peptide-DNA complexes compared to glycopeptide-DNA complexes.

As discussed in Chapter IV, light scattering resulting from carrier-DNA complexation is diagnostic of DNA condensation. The light scattering titrations indicate that DNA condensation occurs only upon complexation with either peptides or glycopeptides containing 8 lysines or more, consistent with a lack of binding by the smaller carriers. A detailed comparison of the scattering curves of the DNA binding carriers is difficult, given that light scattering is affected by a variety of factors, and also due to the presence of variable levels of aggregation of complexes, as indicated by the solubility profiles. However, the dramatic difference in the magnitude of light scattering between the peptide and glycopeptide complexes indicates large differences in the shape, size or extent of the DNA condensates formed depending upon the absence or presence of the thioether linked oligosaccharide.
Tryptophan Fluorescence Studies

As mentioned in Chapter V, tryptophan was included in the CWKₙ peptides not only to provide a sensitive chromophore and the ability to carry out purifications by reverse phase HPLC, but also to provide an additional spectroscopic handle for the study of carrier-DNA complexes using tryptophan's fluorescence. This may provide additional information useful to the characterization of carrier-DNA complexes, with the advantage of using an intrinsic fluorophore.

Preliminary experiments with AlkCWK₁₈ showed that the fluorescence of tryptophan was quenched upon binding to DNA, but only to about 40%. This provides an advantage over fluorescence quench studies with intercalated dye from which only limited binding parameters can be extracted. Since tryptophan's fluorescence is incompletely quenched, the remaining signal can be used to probe carrier-DNA complexation.

Further experiments with AlkCWK₁₈ showed that the fluorescence quench of tryptophan was dependent upon the carrier-DNA ratio. It was decided to investigate carrier binding by tryptophan fluorescence at a fixed carrier to DNA ratio of 0.1 nmol/µg. This ratio was deliberately chosen to examine the fluorescence properties of bound carriers without introducing complications resulting from DNA condensation or partial insolubility. Further the excess of DNA favors carrier binding, facilitating an assessment of the relative affinity of the different carriers for DNA.
The tryptophan quench observed upon DNA binding with AlkCWK\textsubscript{18} and TriCWK\textsubscript{18} is shown in Figure 41, and a comparison of quench and anisotropies for all the carriers is detailed in Table 6. A quench of 22\% was observed for the fluorescence of TriCWK\textsubscript{18} in the presence of DNA, and this value was 38\% in the case of AlkCWK\textsubscript{18}. TriCWK\textsubscript{13} and AlkCWK\textsubscript{13} showed a slight increase from these values. However a large increase of quench was noted for the next two smaller members of both types of carriers, increasing to about 40\% percent for the glycopeptides, and to about 50\% for the peptides.

The general trend of increasing quench for the smaller carriers is proposed to be related to tryptophan's intercalation within the base pairs of DNA. Larger carriers bind to DNA with greater affinity, and result in greater intercalation of the indole ring, reducing exposure to quenching interactions. In comparison, smaller carriers are less effective in hydrophobic shielding of the tryptophan but still interact with DNA, resulting in higher quench.

It is probable that multiple modes of carrier binding are present, some involving tryptophan's intercalation, and some not, resulting in varying effects on quench. Further, the tryptophan fluorescence of glycopeptides is modified due to the presence of tyrosine as noted in Chapter V. Therefore the analysis of quench measurements is complicated by many influences. In contrast, the anisotropy values provided a much clearer comparison of the relative binding affinities of the various carriers.
Figure 41. Fluorescence Quench of Tryptophan upon Carrier-DNA Complexation. The fluorescence of tryptophan was evaluated in carrier-DNA complexes at a carrier to DNA ratio of 0.1 nmol/μg. In the presence of DNA, a 22% quench was observed for TriCWK<sub>18</sub> (a - glycopeptide alone; b - with DNA) and a 38% quench was observed for AlkCWK<sub>18</sub> (c - peptide alone; d - with DNA).
Table 6. Tryptophan Fluorescence Measurements for Peptide-DNA and Glycopeptide-DNA Complexes.

<table>
<thead>
<tr>
<th>Carrier</th>
<th>%Tryptophan Quench$^b$</th>
<th>Anisotropy, Unbound$^c$</th>
<th>Anisotropy, with DNA$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlkCWK3</td>
<td>48.7 ± 2.3</td>
<td>0.007 ± 0.002</td>
<td>0.013 ± 0.004</td>
</tr>
<tr>
<td>AlkCWK8</td>
<td>48.7 ± 2.4</td>
<td>0.020 ± 0.003</td>
<td>0.055 ± 0.008</td>
</tr>
<tr>
<td>AlkCWK13</td>
<td>40.9 ± 2.4</td>
<td>0.020 ± 0.003</td>
<td>0.089 ± 0.004</td>
</tr>
<tr>
<td>AlkCWK18</td>
<td>38.1 ± 1.8</td>
<td>0.020 ± 0.002</td>
<td>0.119 ± 0.007</td>
</tr>
<tr>
<td>TriCWK3</td>
<td>39.7 ± 1.6</td>
<td>0.014 ± 0.004</td>
<td>0.014 ± 0.004</td>
</tr>
<tr>
<td>TriCWK8</td>
<td>38.9 ± 2.6</td>
<td>0.028 ± 0.002</td>
<td>0.044 ± 0.007</td>
</tr>
<tr>
<td>TriCWK13</td>
<td>23.8 ± 1.9</td>
<td>0.029 ± 0.005</td>
<td>0.054 ± 0.001</td>
</tr>
<tr>
<td>TriCWK18</td>
<td>22.2 ± 1.0</td>
<td>0.027 ± 0.001</td>
<td>0.067 ± 0.002</td>
</tr>
</tbody>
</table>

$^a$ All measurements were performed in hepes buffered mannitol, and with complexes consisting of 1 nmol carrier and 10 μg DNA (carrier-DNA ratio of 0.1). The error shown for each measurement represents one standard deviation.

$^b$ The quench observed for tryptophan fluorescence when carriers were mixed with DNA compared to the fluorescence intensity when equal amounts of carriers were present in buffer alone (Equation 2, Pg. 123).

$^c$ The anisotropy of tryptophan fluorescence observed for carriers in solution in the absence of DNA (Equation 3, Pg. 123).

$^d$ The anisotropy of tryptophan fluorescence observed for carriers in solution in the presence of DNA (Equation 3, Pg. 123).
The anisotropies for all the unbound carriers in buffer were close to zero, as can be expected from freely tumbling carrier molecules, although slight increases in anisotropy were apparent as the size of the carriers increased. The comparison of anisotropies for the carriers in the presence of DNA shows significant increases in the anisotropy indicating reduced rotation of the carrier molecules. This reduced tumbling is a result of binding to DNA. Since the total anisotropy is a direct summation of the anisotropies of the unbound and bound fractions, a greater anisotropy indicates a larger fraction of the carrier in the bound state. The ordering of anisotropies in the presence of DNA indicates that for both peptides and glycopeptides, binding affinity decreases stepwise as the number of lysines is decreased from 18 to 13 to 8, whereas for AlkCWK$_3$ and TriCWK$_3$, the anisotropy remains close to the unbound state. These observations are consistent with the results observed from dye quench measurements which indicate binding in the order AlkCWK$_{18}$ $\sim$ TriCWK$_{18}$ $>$ AlkCWK$_{13}$ $\sim$ TriCWK$_{13}$ $>$ AlkCWK$_8$ $\sim$ TriCWK$_8$.

The anisotropies of the peptides and glycopeptides should not be cross-compared, since there is one fluorophore in the peptides, compared to two fluorophores (tyrosine and tryptophan) in the glycopeptides. It is likely that there is fluorescence energy transfer between the tyrosine and tryptophan residues in the glycopeptides (Pg. 116, Chapter V); and this is expected to influence the anisotropy recorded$^{146}$. However, anisotropy measurements at varying carrier-DNA stoichiometries (low enough to avoid condensation) should allow the determination
of the DNA binding constant of each individual carrier for the sake of direct
comparison between the peptides and glycopeptides.

**Transfection Activity of Carrier-DNA Complexes.**

The transfection activity of DNA complexes was studied in HepG2 cells to
ascertain delivery and expression of the reporter gene luciferase. Peptide-DNA and
glycopeptide-DNA complexes were prepared with increasing stoichiometries of
carrier to DNA in order to obtain the optimal complexation ratio for each individual
member of the series.

No transfection activity was obtained with the smallest peptide (AlkCWK3),
or its glycosylated counterpart (TriCWK3), consistent with the previous discussion.
Relatively low levels of activity were obtained with the next larger peptide and
glycopeptide (AlkCWK8, and TriCWK8, Figure 42). The highest transfection levels
were obtained with the peptides AlkCWK13, and AlkCWK18. Surprisingly, the
glycopeptides TriCWK13 and TriCWK18 showed activities that were 1 to 2 orders of
magnitude lower than obtained with the peptides (Figures 43 and 44).

For both the peptide and glycopeptide carriers that showed significant
transfection activity, the asymptotic regions of the fluorescence quench and light
scattering profiles described earlier were accurate predictors of the carrier-DNA
stoichiometries at which maximum transfection occurred. Further, the order of
binding affinities predicted by anisotropy and dye quench measurements correlated
with the order of transfection activities within each series of peptide or glycopeptide carriers. A summary of the correlations between spectroscopic measurements and transfection activities is shown in Table 7.

The results obtained with the glycopeptide-DNA complexes were unexpected, since the presence of the oligosaccharide was expected to increase DNA delivery by endocytosis via specific binding to the asialoglycoprotein receptor on the hepatocytes. Even more unexpected was the finding that DNA complexes prepared with the peptides $\text{AlkCWK}_{13}$ and $\text{AlkCWK}_{18}$ provided high transfection activities. Polylysine of average degree of polymerization 19 has one more lysine than $\text{AlkCWK}_{18}$ (and six more lysines than $\text{AlkCWK}_{13}$), but does not provide DNA complexes with high transfection activity, as shown in Chapter IV. Therefore, there is evidently some distinct feature of the peptide-DNA complexes that leads to high transfection activity. Furthermore, the glycopeptide-DNA complexes possess some property that does not allow proper receptor binding or endocytosis. This difference in transfection activity between peptide and glycopeptide complexes was investigated further in an experiment in which DNA was complexed with add-mixtures of $\text{TriCWK}_{18}$ and $\text{AlkCWK}_{18}$ while keeping the total carrier-DNA stoichiometry constant at 0.5 nmol/μg. The results demonstrated conclusively that upon increasing the ratio of the glycopeptide relative to the peptide, transfection activity was inhibited (Figure 45).
Figure 42. Transfection Activity of DNA Complexed with AlkCWK₈ and TriCWK₈ Carriers. The plasmid pCMVL was complexed with either AlkCWK₈ (solid bars) or TriCWK₈ (open bars) carriers at varying carrier to DNA ratios, and transfected into HepG2 cells in the presence of chloroquine. Low reporter gene activity was obtained at all ratios, and with both carriers.
Figure 43. Transfection Activity of DNA Complexed with AlkCWK<sub>13</sub> and TriCWK<sub>13</sub> Carriers. The plasmid pCMVL was complexed with either AlkCWK<sub>13</sub> (solid bars) or TriCWK<sub>13</sub> (open bars) carriers at varying carrier to DNA ratios, and transfected into HepG2 cells in the presence of chloroquine. The peptide carrier provided reporter gene activity one to two orders of magnitude greater than the glycopeptide carrier. Fluorescence quench and light scattering assays were good predictors of the carrier-DNA ratios providing optimal transfection.
Figure 44. Transfection Activity of DNA Complexed with AlkCWK₁₈ and TriCWK₁₈ Carriers. The plasmid pCMVL was complexed with either AlkCWK₁₈ (solid bars) or TriCWK₁₈ (open bars) carriers at varying carrier to DNA ratios, and transfected into HepG2 cells in the presence of chloroquine. The peptide carrier provided reporter gene activity one to two orders of magnitude greater than the glycopeptide carrier. Fluorescence quench and light scattering assays were good predictors of the carrier-DNA ratios providing optimal transfection.
Figure 45. Inhibition of Transfection by TriCWK_{18} relative to AlkCWK_{18}. The plasmid pCMVL was complexed with mixtures of AlkCWK_{18} and TriCWK_{18} in which the carrier to DNA ratio was kept constant at 0.5 nmol/µg, but the proportion of peptide to glycopeptide was varied. Transfection into HepG2 cells was progressively higher as the proportion of AlkCWK_{18} was increased.
Table 7. Correlation of Spectroscopic Measurements with Transfection Efficacy of Peptide and Glycopeptide Carrier-DNA Complexes.

<table>
<thead>
<tr>
<th>Peptide or Glycopeptide</th>
<th>Neutral Ratio$^a$</th>
<th>Titration Minima$^b$</th>
<th>Transfection Maxima$^c$</th>
<th>Relative Binding$^d$</th>
<th>Relative Transfection$^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlkCWK3</td>
<td>0.77</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TriCWK3</td>
<td>0.77</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AlkCWK8</td>
<td>0.34</td>
<td>0.6</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>TriCWK8</td>
<td>0.34</td>
<td>0.6</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>AlkCWK13</td>
<td>0.22</td>
<td>0.4</td>
<td>$\geq$0.4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>TriCWK13</td>
<td>0.22</td>
<td>0.4</td>
<td>$&gt;0.4$</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>AlkCWK18</td>
<td>0.16</td>
<td>0.2</td>
<td>$&gt;0.2$</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TriCWK18</td>
<td>0.16</td>
<td>0.2</td>
<td>$&gt;0.2$</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

$^a$ The calculated stoichiometry of carrier to DNA (nmol/μg) at which the number of amines (positive charges) contributed by the carrier equal the number of phosphate groups (negative charges) on DNA.

$^b$ The carrier-DNA stoichiometry for which a minimum was observed in the solubility profiles of carrier-DNA complexes; or the smallest carrier-DNA ratio for which asymptotic regions were achieved in the dye fluorescence and light scattering measurements.

$^c$ The carrier-DNA stoichiometries for which maximum transfection activity of luciferase was achieved (within the same order of magnitude).

$^d$ The relative order of DNA binding affinity of carriers within either the glycopeptide or the peptide series (italicised numbers) as observed by either tryptophan anisotropy or fluorescence quench of intercalated dye. Smaller numbers reflect higher binding.

$^e$ The relative order of gene transfection efficacy provided by carriers within either the glycopeptide or the peptide series (italicised numbers) as judged by the maximal luciferase activities achieved. Smaller numbers reflect higher efficacy.
The only spectroscopic determination that clearly separated DNA complexes prepared with either the peptides or the glycopeptides was revealed by the differences in light scattering. This evidence suggested that the unique properties of these complexes were related to the macromolecular structure of the DNA condensates. Therefore, it was decided to compare AlkCWK₁₅-DNA and TriCWK₁₈-DNA complexes by electron microscopy.

**Electron Microscopy of Carrier-DNA complexes.**

Results obtained with transmission electron microscopy provided a reasonable explanation of the unexpected transfection results obtained with the CWK₉ peptides and glycopeptides. Representative electron micrographs are presented in Figures 46 and 47. AlkCWK₁₅-DNA complexes appeared monodisperse and oval to spherical in shape, with average diameters in the range of 40 to 70 nm. These diameters are smaller than those reported for polylysine-DNA complexes by Wagner and colleagues⁴⁹, and further these complexes do not show a toroidal or "donut" shape with a hole in the center. This smaller size and more condensed nature is probably responsible for the high light scattering observed with these complexes. The compact and monodisperse nature of the condensates is probably also the reason for the high transfection activities, since smaller complexes can be internalized more efficiently by pinocytosis.
In contrast, the DNA complexes obtained with the glycopeptide \( \text{TriCWK}_{18} \) (Figure 47) show much larger condensates that appeared aggregated. Instead of the dense, spherical complexes obtained with the \( \text{AlkCWK}_{18} \), the glycopeptide complexes sometimes assumed toroidal or "donut" shapes with diameters in the range of 60 - 90 nm. Further, the majority of the DNA appeared to be in elongated stem and rod like condensates that formed interconnected networks. The less condensed nature of these complexes is probably the reason for decreased light scattering relative to the peptide-DNA complexes, and it is reasonable to assume that the elongated and interconnected nature of the complexes prevents them from being efficiently internalized into cells. It is not clear how the presence of the oligosaccharide provokes the formation of these dramatically different DNA condensates. The possibilities could include disruption of tryptophan intercalation due to the presence of a large substituent on the next amino acid; a sterically unfavorable orientation of the pendant oligosaccharide when linked to the cysteine side chain by a thioether linkage; or even hydrogen bonding between the sugar residues and the nucleotides.

Accordingly, strategies for preparing glycopeptides that do not interfere with DNA condensation may include conjugating the oligosaccharide at a different position, relatively separated from the tryptophan. Alternatively, a different type of linkage, or a longer and more flexible spacer between the oligosaccharide and the peptide may be sterically compatible with dense compaction. Peptides that bind DNA with much higher affinities than \( \text{AlkCWK}_{18} \) may function as anchors that are
Figure 46. Electron Microscopy of Peptide-DNA Complexes. AlkCWK<sub>18</sub> was combined with pCMVL at a carrier to DNA ratio of 0.5 and the complexes examined under the electron microscope after staining with uranyl acetate. A. At 21,000 fold magnification (bar represents 500 nm). B. At 105,000 fold magnification (bar represents 50 nm).
Figure 47. Electron Microscopy of Glycopeptide-DNA Complexes. TriCWK_{18} was combined with pCMVL at a carrier to DNA ratio of 0.5 and the complexes examined under the electron microscope after staining with uranyl acetate. A. At 21,000 fold magnification (bar represents 500 nm). B. At 105,000 fold magnification (bar represents 50 nm).
relatively uninfluenced by pendant oligosaccharides. Another possibility may be the use of smaller oligosaccharide ligands that still display high affinity binding to their receptors.

**Freeze Drying of Carrier-DNA Complexes.**

A portion of this study was devoted to obtaining carrier-DNA complexes in a freeze dried form. Freeze dried products offer many general advantages over liquid formulations in stability on storage, flexibility in packaging, more efficient space utilization, and convenience in transport. In the case of carrier-DNA complexes, stability is a prime concern since in solution, the complexes aggregate over time and form insoluble precipitates. Further, the formation of complexes of uniform quality is technique dependent, and differences in mixing of carrier and DNA seem to result in variability. Therefore, the use of stable, freeze dried complexes would lead to more uniformity in preparation and testing, and the same batch of stored complex could be conveniently tested at different times and under different conditions. Further in the case of clinical settings, complexes could be conveniently prepared just before use by simple reconstitution in an aqueous solvent.

The solubility of lyophilized and reconstituted complexes was analyzed to screen for optimal formulations. It is well known that many carbohydrates act as stabilizers during freeze drying and cryopreservation\textsuperscript{149,150}. Accordingly, the effect of freeze drying in solutions of three simple sugars (mannitol, glucose, and lactose; at
isotonic and twice the isotonic strength) was investigated for DNA complexes formed with AlkCWK$_{18}$ and TriCWK$_{18}$. In addition, the complexes were also freeze dried in deionized water and hepes buffered mannitol. The results for AlkCWK$_{18}$-DNA complexes are shown in Figure 48, and similar results were obtained with TriCWK$_{18}$ (not shown). In all cases DNA complexes prepared in sugar formulations were recovered in solution upon reconstitution with water, as measured by absorbance at 260 nm. However, upon centrifugation, only complexes prepared in glucose and lactose solutions were found to be fully soluble, and complexes prepared in mannitol or hepes buffered mannitol were found to be partially soluble. The use of twice the isotonic strength of the sugars did not improve solubility in any of the treatment groups. However, the presence of sugars was necessary because when the complexes were prepared and freeze dried in deionized water, no DNA was recovered in solution upon reconstitution.

The transfection efficacy of freeze dried complexes formed with AlkCWK$_{18}$ in isotonic sugar solutions was tested after overnight lyophilization. The results (Figure 49) demonstrate that in all cases, freeze dried and reconstituted complexes provided equivalent transfection activity when compared to complexes that were freshly prepared. Interestingly, the partial solubility observed with freeze dried mannitol formulations did not impair the transfection activity. However, two fold higher activity was obtained in glucose and lactose formulations compared to mannitol formulations, both with freshly prepared as well as freeze-dried complexes.
Figure 48. Solubility of Peptide-DNA Complexes After Freeze-Drying. AlkCWK$_{18}$ was combined with pCMVL at a carrier to DNA ratio of 0.5 nmol/µg in the solvents indicated and frozen in dry ice. After overnight lyophilization, the complexes were reconstituted in water and DNA concentration estimated by UV absorbance before (solid bars) and after centrifugation (shaded bars). Each bar shown is average of two samples.
Figure 49. Transfection activity of Peptide-DNA Complexes Before and After Freeze-Drying. AlkCWK₁₈ was combined with pCMVL at a carrier to DNA ratio of 0.5 nmol/μg in hepes buffered mannitol, and in unbuffered isotonic solutions of mannitol, glucose and lactose. After overnight lyophilization, the complexes were reconstituted in water and compared for transfection activity (open bars) against similar complexes that were freshly prepared (solid bars).
These initial studies show the feasibility of freeze-drying carrier-DNA complexes, and indicate that sugar additives stabilize the complexes during lyophilization. The data indicates that glucose and lactose formulations may be superior to mannitol formulations, although the latter provided better bulking of lyophilized complexes, as evidenced by their visual appearance. Also, the presence of even low concentration of buffer ions led to decreased solubility upon freeze-drying. In the present study, lyophilized complexes were used the next day after freeze-drying, and formulations with partial loss in solubility did not show any decrease in transfection activity. However, in these cases, solubility might be more severely affected over long term storage, resulting in impaired transfection activity.

CONCLUSION

A series of defined peptides and an analogous series of defined glycopeptides was prepared to allow the stepwise optimization of carrier structure. These peptides and glycopeptides were tested by spectroscopic assays and by an in vitro biological assay to observe transfection of a luciferase reporter gene into HepG2 cells. The studies have led to several interesting findings.

It was found that carriers possessing 3 lysine residues did not show significant binding with DNA, and were also inactive in transfection. Carriers possessing 8 lysines showed DNA binding by spectroscopic assays, but also resulted in complexes with poor solubility, and low transfection activity. Carriers possessing 13 or 18
lysines were the best binders and resulted in significant transfection of the reporter gene. A transfection enhancement of approximately only 3-fold was seen with carriers possessing 18 lysines as compared to those possessing 13 lysines, whereas the latter resulted in transfection activity that was 50-fold greater than carriers with 8 lysines.

The presence of the oligosaccharide on DNA complexes did not increase the transfection activity by ASGP-R targeting, but instead led to an inhibition of activity. This unexpected result was due to the formation of macromolecular aggregates in the case of glycopeptide-DNA complexes, as evidenced by electron microscopy. These aggregates did not precipitate in a centrifugation assay, but were evidently too large to be efficiently internalized into cells, even if receptor targeting occurred.

Equally surprising was the observation that peptide-DNA complexes led to high transfection activity, whereas complexes between comparable length polylysine and DNA show only low activity. The uniqueness of the peptide-DNA complexes seems to be related to the presence of a tryptophan residue, whose presence results in modified binding interactions. The altered binding of these peptides resulted in highly condensed DNA complexes which are apparently internalized with relatively high efficiency into cells. In all likelihood, this activity is not receptor mediated, but results from non-specific interactions between the cationic complexes and negatively charged cell surfaces, leading to efficient pinocytosis. These data indicate that further optimization of peptide carriers to modulate DNA binding interactions may result in complexes with very high transfection activities. Further, this discovery may represent
a novel approach to non-specific gene delivery into a variety of cell types, and
peptide-DNA complexes may allow important advantages over currently used non-
specific DNA vectors. The ability of these complexes to retain activity upon freeze-
drying is likely to be one of these advantages.

The study also points the way towards the continued rational development of
efficient and selective, receptor targeted glycopeptide carriers. One of the critical
aspects elucidated for rational carrier design relates to the understanding gained that
the conjugation strategy or the structure of the receptor ligand needs to be compatible
with the formation of highly condensed, monodisperse DNA complexes. Accomplishment of this goal may not only afford targeted gene delivery, but may
allow the efficacy of transfection to be increased dramatically over the present levels
due to tapping into the route of receptor mediated internalization.

Apart from elucidating important aspects of carrier design, this thesis project
has demonstrated the utility of spectroscopic assays in characterizing carrier-DNA
complexes and in providing clues to their optimal biological activity. These types of
assays may serve as valuable research and formulation tools in the study and
optimization of carrier-DNA complexes.
APPENDIX

Computer Programs

In the following pages, the computer programs "OligoMass", "SacCombo", and "FlScat" are included. OligoMass (Pg. 158-169) was used to calculate the molecular weight of oligosaccharides, peptides, and glycopeptides. SacCombo (Pg. 170-174) was used to predict the monosaccharide composition of an oligosaccharide from its molecular weight (as obtained from mass spectrometry). FlScat (Pg. 175-178) was used within a suite of linked programs for the automated control of a Perkin Elmer fluorimeter (LS50B) for fluorescence and light scattering measurements, and for recording the data obtained within a computer file.
The Computer Program "OligoMass"

REM Program "OLIGOMASS" by Manpreet S. Wadhwa
REM Version 1.1, first prepared in summer of '93 to assist Dwight C. Ware for his project.
REM Version 1.2, updated 12/3/93 to include biotin, naphthyl, dansyl and ethylene diamine
REM Version 1.3, updated 2/28/95 to include iodoacetylated oligosaccharides.
REM Version 1.4, updated 3/1/95 to include peptides and glycopeptides.
REM Version 1.5, updated 3/21/95 to include periodate treatment of naphthyl, dansyl oligos.
REM Version 1.6, updated 4/4/95 to include iodoacetamide alkylation of cysteine residues

start:
OPEN "mw.out" FOR OUTPUT AS #1
CLS
PRINT ; PRINT "MOLECULAR WEIGHT CALCULATOR FOR OLIGOSACCHARIDES AND GLYCOPEPTIDES"
PRINT "******************************************************************************"
PRINT
PRINT "Note: Whenever a yes/no response is required, please use 'y' for YES"
PRINT "and 'n' for NO. Pressing 'enter' will result in the default response"
PRINT "indicated in square brackets."
PRINT
DIM pcp$(25)
DIM npcp(25)
mw = 0; temw = 0; tamw = 0; omw = 0; gmw = 0; pmw = 0; ptmw = 0; gtmw = 0; otmw = 0
near = 0; nhyd = 0; nnit = 0; noxy = 0; nsifi = 0; nidn = 0; ndtr = 0; nres = 0; tres = 0
pepcount = 1 'nlys=0
seq$ = ""

INPUT "Name of the Oligosaccharide or Glycopeptide or Peptide? [J", name$
PRINT "Is "; name$; " an unglycosylated peptide? [No] "; INPUT "", peponly$
IF peponly$ = "y" OR peponly$ = "Y" THEN
species = 1
GOTO peptide
ELSE
GOTO glycopeptide
END IF

peptide:
PRINT ; PRINT "Proceeding with peptide calculations.." ; PRINT
PRINT "Please enter name of amino acid residue (use single letter) 
PRINT "Presently, only K, C, and W accepted ": INPUT "", pep$(pepcount)
IF pep$(pepcount) = "" THEN GOTO pependmsg
GOTO numpep

peptide2:
pepcount = pcpcount + 1
PRINT : PRINT "Please enter name of next amino acid residue (use single letter)"
PRINT "(press enter alone if no more residues)": input "", pcp$(pepcount)
IF pcp$(pepcount) = "" THEN GOTO pepmenu
numpep:
PRINT "Please enter number of": pcp$(pepcount); " residues": input "", npcp(pepcount)

iodoacetamide:
IF pcp$(pepcount) = "e" OR pcp$(pepcount) = "C" THEN
INPUT "Are the cysteines in this peptide alkylated with iodoacetamide? [No]": iodoac$
IF iodoac$ = "y" OR iodoac$ = "Y" THEN
pcp$(pepcount) = pcp$(pepcount) + "(acetamido)"
END IF
END IF
GOTO peptidc2

pepmenu:
FOR assign = 1 TO (pepcount - 1)
p$ = pcp$(assign): nrcs = npcp(assign)
tres = tres + nrcs: seq$ = seq$ + " " + p$ + STRS(nrcs)
IF p$ = "k" OR p$ = "K" THEN
pmw = pmw + 146.2 * nrcs
near = near + 6 * nrcs: nhyd = nhyd + 14 * nrcs: nnit = nnit + 2 * nrcs: noxy = noxy + 2 * nrcs
REM nlys = nlys + nrcs
ELSEIF p$ = "c" OR p$ = "C" THEN
pmw = pmw + 121.2 * nrcs
near = near + 3 * nrcs: nhyd = nhyd + 7 * nrcs: nnit = nnit + 1 * nrcs: noxy = noxy + 2 * nrcs: nslf = nslf + 1 * nrcs
ELSEIF p$ = "c(acetamido)" OR p$ = "C(acetamido)" THEN
pmw = pmw + 121.2 + 184.96 - 1.01 - 126.9 = iodine
near = near + 5 * nrcs: nhyd = nhyd + 10 * nrcs: nnit = nnit + 2 * nrcs: noxy = noxy + 3 * nrcs: nslf = nslf + 1 * nrcs
ELSEIF p$ = "w" OR p$ = "W" THEN
pmw = pmw + 204.2 * nrcs
near = near + 11 * nrcs: nhyd = nhyd + 12 * nrcs: nnit = nnit + 2 * nrcs: noxy = noxy + 2 * nrcs
ELSE
PRINT p$: " is an unrecognized residue and will be ignored in calculation"
END IF
NEXT assign
nplink = tres - 1
pmw = pmw - 18.02 * nplink 'nplink is number of peptide bonds
nhyd = nhyd - 2 * nplink: noxy = noxy - nplink
ptmw = pmw 'peptide molecular weight
IF species = 1 THEN
GOTO exactwt
ELSE
gtmw = pmw - 1.01 + gmw' 
gtycopcptidc molecular weight
nhyd = nhyd - 1'(hydrogen lost from cysteine upon coupling to iodo-oligo)
GOTO exactwt
END IF

pependmsg:
PRINT "No aminoacid residues specified."
GOTO continue

glycopeptide:
PRINT "Is " ; name$; " a glycopeptide? [No] " ; : INPUT "", glypep$
IF glypep$ = "y" OR glypep$ = "Y" THEN
PRINT
PRINT "Proceeding with glycopeptide calculations.."
PRINT
species = 2
ELSE
PRINT "Is " ; name$; " an oligosaccharide? [Yes] " ; : INPUT "", oligoS
IF oligoS = "n" OR oligoS = "N" THEN GOTO continue
PRINT
PRINT "Proceeding with oligosaccharide calculations.."
PRINT
species = 3
END IF

PRINT "Please enter the number of monosaccharide residues"
REM assignments
hex = 180.16 'wt of hexose
nah = 221.21 'wt of N-acetyl-hexosamine
fuc = 164.16 'wt of fucose
sea = 309.28 'wt of sialic acid
hxn = 179.17 'wt of hexosamine

INPUT "Hexose"; nhex
mw = mw + nhex * hex
REM Mol formula is C6H12O6

INPUT "N-acetyl-hexosamine"; nnah
mw = mw + nnah * nah
REM Mol formula C8H15NO6

INPUT "Hexosamine"; nhxn
mw = mw + nhxn * hxn
REM Mol formula C6H13NO5

INPUT "Fucose"; nfuc
\[ m_w = m_w + n_{fuc} \times fuc \]

REM Mol formula C6H12O5

INPUT "Sialic Acid", nsca
\[ m_w = m_w + nsca \times sca \]
REM Mol formula C11H19NO9

nmon = n_{hex} + n_{nah} + n_{fuc} + nsca + n_{hx}n \quad \text{total number of monosaccharides.}

nlink = n_{mon} - 1 \quad \text{total number of linkages}

IF nmon = 0 THEN
nlink = 0
GOTO continue
END IF

\[ m_w = m_w - (nlink \times 18.02) \quad \text{weight of water condensation subtracted} \]

omw = mw

IF species = 2 THEN
\[ gmw = m_w + 203.22 \quad \text{weight of iodoacetylated tyrosine (minus OH, minus Iodine)} \]

n_{car} = 11; noxy = 3 - 1; nnit = 2; nhyd = 12 - 1
GOTO peptide
END IF

PRINT

INPUT "Is this a reducing oligosaccharide? [No]", rcd$
IF rcd$ = "y" OR rcd$ = "Y" THEN
chemstate$ = "Reducing"
GOTO oligowt
END IF

INPUT "Is this an oligosaccharide glycosylamine? [No] ", gly$
IF gly$ = "y" OR gly$ = "Y" THEN
derivative = 16.02 - 17.01
REM 17.01 = wt. of OH, 16.02 = wt. of NH2
noxy = -1 : nhyd = 2 - 1 : nnit = 1
REM 1 oxygen and 1 hydrogen subtracted since OH is replaced.
chemstate$ = "Glycosylamine"
GOTO oligowt
END IF

INPUT "Is this oligosaccharide conjugated with t-BOC tyrosine? [No] ", tboctyr$
IF tboctyr$ = "y" OR tboctyr$ = "Y" THEN
derivative = 279.32 - 17.01
REM 279.32 = wt. of conjugated t-BOC tyrosine : C14 O4 H19 N2
noxy = 4 - 1 : nhyd = 19 - 1 : n_{car} = 14 : nnit = 2
chemstate$ = "t-BOC tyrosine conjugate"
INPUT "Is this oligosaccharide conjugated with tyrosine (deprotected)? [No] ", tyr$
IF tyr$ = "y" OR tyr$ = "Y" THEN
  derivative = 179.2 - 17.01
  REM 179.2 = wt. of conjugated tyrosine: C9 O2 H11 N2
  noxy = 2 - 1 : nhyd = 11 - 1 : ncar = 9 : nnit = 2
  chemstate$ = "Tyrosine conjugate"
GOTO oligowt
END IF

INPUT "Is this oligosaccharide conjugated with biotin? [No] ", bln$.
IF bln$ = "y" OR bln$ = "Y" THEN
  derivative = 518.65 - 17.01
  REM 518.65 = wt. of conjugated biotin (biotin + tyrosine): C25 O5 H37 N5 S1
  noxy = 5 - 1 : nhyd = 37 - 1 : ncar = 25 : nnit = 5 : nslf = 1
  chemstate$ = "Biotin conjugate"
GOTO oligowt
END IF

INPUT "Is this oligosaccharide conjugated with naphthyl? [No] ", nph$
IF nph$ = "y" OR nph$ = "Y" THEN
  derivative = 184.22 - 17.01
  REM 184.22 = wt. of conjugated naphthyl: C12 O1 H10 N1
  noxy = 1 - 1 : nhyd = 10 - 1 : ncar = 12 : nnit = 1
  chemstate$ = "Naphthyl conjugate"
GOTO oligowt
END IF

INPUT "Is this oligosaccharide conjugated with dansyl and naphthyl? [No] ", dnp$
IF dnp$ = "y" OR dnp$ = "Y" THEN
  INPUT "Please enter number of dansyl moieties [0] ", dnn
  derivative = 184.22 - 17.01 'to account for naphthyl at reducing end
  derivative = derivative + dnn * (292.38 - 17.01)' to account for dansyl(s)
  REM 184.22 = wt. of conjugated naphthyl: C12 O1 H10 N1
  REM 292.38 = wt. of conjugated dansyl: C14 O2 H18 N3 O2 S
  noxy = 0 + dnn * 1 'oxygens from naphthyl (-1) and dansyl (-1)
  nhyd = 9 + dnn * 17 'hydrogens from naphthyl (-1) and dansyl (-1)
  ncar = 12 + dnn * 14 : nnit = 1 + dnn * 3 : nslf = 0 + dnn * 1
  dnn$ = STRS(dnn)
  chemstate$ = "Dansyl (" + dnn$ + ") and Naphthyl conjugate"
GOSUB periodate
GOTO oligowt
END IF

INPUT "Is this oligosaccharide conjugated with ethylene diamine (NR end)? [No] ", etd$
IF ctd$ = "y" OR cdt$ = "Y" THEN
INPUT "Please enter number of ethylene diamine moieties [0] ", cdn

specify:
INPUT "Please specify whether reducing end has TYR (1) or t-BOC-TYR (2) ", rend
IF rend = 1 OR rend = 2 THEN
  rend$ = "OK"
ELSE
  PRINT
  PRINT "Please specify either 1 or 2"
  PRINT
  GOTO specify
END IF

IF rend = 1 THEN
  rend$ = "Tyrosine"
  derivative = 179.2 - 17.01
  REM 179.2 = wt. of conjugated tyrosine : C9 O2 H11 N2
  noxy = 2 - 1 : nhyd = 11 - 1 : ncar = 9 : nnit = 2
  GOTO ethylenediamine
END IF

IF rend = 2 THEN
  rend$ = "t-Boc-Tyrosine"
  derivative = 279.32 - 17.01
  REM 279.32 = wt. of conjugated t-BOC tyrosine : C14 O4 H19 N2
  noxy = 4 - 1 : nhyd = 19 - 1 : ncar = 14 : nnit = 2
  GOTO ethylenediamine
END IF

ethylenediamine:
derivative = derivative + cdn * (59.09 - 17.01) ' to account for ethylene diamine(s)
REM 59.09 = wt. of conjugated ethylene diamine : C2 H7 N2
noxy = noxy - cdn * 1 'one oxygen lost per e.d. conjugated
nhyd = nhyd + cdn * 6 'hydrogens from e.d. (-1)
near = near + cdn * 2 : nnit = nnit + cdn * 2
cdn$ = STRS(cdn)
chemstate$ = "Ethylene Diamine (" + cdn$ + ") and " + rend$ + " conjugate"
GOTO oligowt
END IF

INPUT "Is this oligosaccharide succinylated? [No] ", succ$
IF succ$ = "y" OR succ$ = "Y" THEN
  derivative = 279.27 - 17.01
  REM 280.28 = wt. of succinic group linked to tyrosine : C13 O5 H15 N2
  noxy = 5 - 1 : nhyd = 15 - 1 : ncar = 13 : nnit = 2
  chemstate$ = "Succinylated"
  GOTO oligowt
END IF
INPUT "Is this oligosaccharide iodo-acetylated? [No] ", iodo$:  
IF iodo$ = "y" OR iodo$ = "Y" THEN  
derivative = 347.13 - 17.01  
REM 347.13 = wt of iodoacetyl group liked to tyrosine : C11 O3 H12 N2 I1  
noxy = 3 - 1 : nhyd = 12 - 1 : ncar = 11 : nnit = 2 : nidn = 1  
chemstate$ = "Iodoacetylated"  
GOTO oligowt  
ELSE  
PRINT  
PRINT "You answered 'NO' to all the above questions"  
PRINT "Sorry, this program cannot handle your molecule"  
GOTO continue  
END IF  

PERIODATE:  
PRINT "Has "; name$: " been periodate oxidized and reduced? [No] "; : INPUT "", period$:  
IF period$ = "y" OR period$ = "Y" THEN  
INPUT "How many ring openings with no loss of carbon? [0] ", clim0  
INPUT "How many ring openings with loss of 1 carbon? [0] ", clim1  
REM aldehydes formed  
aldehyd = 2 * (clim0 + clim1)  
REM atoms lost in oxidation:  
derivative = derivative - (clim0 * 2.02 + clim1 * 32.04)  
ncar = ncar - clim1 : noxy = noxy - clim1 : nhyd = nhyd - (2 * clim0 + 4 * clim1)  
spc$ = " 
: spc2$ = " 

PRINT "Are the "; aldehyd; " aldehydes reduced with hydrogen? [No] "; : INPUT "", hydald$:  
IF hydald$ = "y" OR hydald$ = "Y" THEN  
REM hydrogens gained back on reduction:  
derivative = derivative + (2 * aldehyd * 1.01)  
nhyd = nhyd + 2 * aldehyd  
chemstate$ = chemstate$ + spc$ + "(periodate oxidized, hydrogen reduced)"  
ELSE  
PRINT "Are the "; aldehyd; " aldehydes reduced with deuterium? [No] "; : INPUT "", dtrald$:  
IF dtrald$ = "y" OR dtrald$ = "Y" THEN  
REM deuteriums gained back on reduction  
derivative = derivative + (aldehyd * 2.02 + aldehyd * 1.01)  
ndtr = ndtr + aldehyd : nhyd = nhyd + aldehyd  
chemstate$ = chemstate$ + spc$ + "(periodate oxidized, deuterium reduced)"  
ELSE  
chemstate$ = chemstate$ + spc$ + "(periodate oxidized, unreduced)"  
END IF  
END IF
chemstate$ = chemstate$ + spc2$ + "|" + STRS(elim0 + elim1) + " ring openings;" + STRS(elim1) + " with 1-carbon elimination |
END IF
RETURN

oligowt:
olm w = omw + derivative

exactwt:
near = near + nhex * 6 + nnah * 8 + nhxn * 6 + nfuc * 6 + nsca * 11 'no. of carbons
nhyd = nhyd + nhex * 12 + nnah * 15 + nhxn * 13 + nfuc * 12 + nsca * 19 - nlink * 2 'no. of hydrogens
noxy = noxy + nhex * 6 + nnah * 6 + nhxn * 5 + nfuc * 5 + nsca * 9 - nlink * 1 'no. of oxygens
nnit = nnit + nhex * 0 + nnah * 1 + nhxn * 1 + nfuc * 0 + nsca * 1 'no. of nitrogens
REM monosaccharides included do not have sulfur or iodine or deuterium, so nothing added to NSLF or NIDN.
tcmw = near * 12# + nhyd * 1.007825037# + noxy * 15.99491464# + nnit * 14.003074008# + nsca * 31.9720718#+ nslf * 31.9720718# + nidn * 126.904477# + ndtr * 2.015650074#

report:
CLS
PRINT: PRINT
PRINT "OLIGOMASS MOLECULAR WEIGHT REPORT"
PRINT "**************************************************************************" : PRINT

IF species = 3 THEN
PRINT "Oligosaccharide: "; nameS
PRINT "Chemical State: "; chemstateS
ELSEIF species = 2 THEN
PRINT "Glycopeptide : "; nameS
PRINT "Linkage: Oligosaccharide-Tyrosine-Thioether-Peptide"
PRINT
PRINT "Number of amino acids: "; tres
PRINT "Peptide sequence (as entered)"; seqS
PRINT
ELSEIF species = 1 THEN
PRINT "Peptide: "; nameS
PRINT "Number of amino acids: "; tres
PRINT "Peptide sequence (as entered)"; seqS
PRINT
END IF

IF species = 3 OR species = 2 THEN
PRINT "Hexose: "; nhex
PRINT "N-acetyl hexosamine: "; nnah
PRINT "Hexosamine: "; nhxn
printfilen:

PRINT #1,
PRINT #1, "OLIGOMASS MOLECULAR WEIGHT REPORT"
PRINT #1, "******************************";
PRINT #1,

IF species = 3 THEN
PRINT #1, "Oligosaccharide: "; name$
PRINT #1, "Chemical State: "; chemstate$
ELSEIF species = 2 THEN
PRINT #1, "Glycopeptide: "; name$
PRINT #1, "Linkage: Oligosaccharide-Tyrosine-Thioether-Peptide"
PRINT #1,
PRINT #1, "Number of amino acids: "; tres
PRINT #1, "Peptide sequence (as entered) "; sqc$
PRINT #1,
ELSEIF species = 1 THEN
PRINT #1, "Peptide: "; name$
PRINT #1, "Number of amino acids: "; tres
PRINT #1, "Peptide sequence (as entered) "; sqc$
PRINT #1,
END IF

IF species = 3 OR species = 2 THEN
PRINT #1, "Hexose: "; nhex
PRINT #1, "N-acetyl hexosamine: "; nnah
PRINT #1, "Hexosamine: "; nhx$
PRINT #1, "Fucose: "; nfuc
PRINT #1, "Sialic Acid: "; nsca
PRINT #1,
END IF

PRINT "Average Molecular Weight: ";
IF species = 1 THEN
PRINT ptmw
ELSEIF species = 2 THEN
PRINT gtmw
ELSEIF species = 3 THEN
PRINT otmw
ELSE
GOTO continue
END IF

PRINT #1, "Average Molecular Weight: ",
IF species = 1 THEN
PRINT #1, ptmw
ELSEIF species = 2 THEN
PRINT #1, gtmw
ELSEIF species = 3 THEN
PRINT #1, otmw
ELSE
GOTO continue
END IF
PRINT #1, "Exact Molecular Weight"; tcmw
PRINT "The Molecular formula is "; "C"; near; " H"; nhyd; " O"; noxy; " N"; nmit; " S"; nslf; " I"; nidn;
" D"; ndtr
PRINT
PRINT #1, "Exact Molecular Weight"; tcmw
PRINT #1, "The Molecular formula is "; "C"; near; " H"; nhyd; " O"; noxy; " N"; nmit; " S"; nslf; " I"; nidn;
" D"; ndtr
PRINT #1,

na = 22.98977
k = 38.96371
h = 1.00783

speciesamw(1) = ptmw
speciesamw(2) = gtmw
speciesamw(3) = otmw

FOR speciesnum = 1 TO 3
	tamw = speciesamw(speciesnum)
	IF speciesnum = species THEN
	PRINT "M+1 (proton) average mass:"; tamw + h
	PRINT "M+23 (sodium) average mass:"; tamw + na
	PRINT "M+24 (sodium+proton) average mass:"; tamw + na + h
	PRINT "M+39 (potassium) average mass:"; tamw + k
	PRINT "M+46 (two sodiums) average mass:"; tamw + 2 * na
	PRINT
	PRINT "M+1 (proton) exact mass:"; tcmw + h
	PRINT "M+23 (sodium) exact mass:"; tcmw + na
	PRINT "M+24 (sodium+proton) exact mass:"; tcmw + na + h
	PRINT "M+39 (potassium) exact mass:"; tcmw + k
PRINT "M+46 (two sodiums) exact mass:"; tcmw + 2 * na
PRINT "------------------------------------------------------------------------"

PRINT #1, "M+1 (proton) average mass:"; tamw + h
PRINT #1, "M+23 (sodium) average mass:"; tamw + na
PRINT #1, "M+24 (sodium+proton) average mass:"; tamw + na + h
PRINT #1, "M+39 (potassium) average mass:"; tamw + k
PRINT #1, "M+46 (two sodiums) average mass:"; tamw + 2 * na
PRINT #1,
PRINT #1, "M+1 (proton) exact mass:"; tcmw + h
PRINT #1, "M+23 (sodium) exact mass:"; tcmw + na
PRINT #1, "M+24 (sodium+proton) exact mass:"; tcmw + na + h
PRINT #1, "M+39 (potassium) average mass:"; tcmw + k
PRINT #1, "M+46 (two sodiums) exact mass:"; tcmw + 2 * na
PRINT #1, "------------------------------------------------------------------------"
END IF
NEXT speciesnum

CLOSE #1

INPUT "Do you want to print the output? [No] ", prt$  
IF prt$ = "y" OR prt$ = "Y" THEN  
OPEN "mw.out" FOR INPUT AS #1  
DO WHILE NOT EOF(1)  
LINE INPUT #1, p$  
LPRINT p$  
LOOP  
LPRINT CHR$(12)  
CLOSE #1  
END IF

PRINT
INPUT "Do you want to save the report? [No] ", sav$  
IF sav$ = "y" OR sav$ = "Y" THEN  
PRINT  
PRINT "Please enter filename. Note that if you do not specify a complete path,"  
PRINT "the file will be saved under c:\qbasic"  
PRINT  
INPUT "Filename": filnam$  
NAME "mw.out" AS filnam$  
ELSE  
KILL "mw.out"  
END IF

continue:
PRINT
INPUT "Do you want to use the program again? [Yes] ", start$
IF start$ = "" OR start$ = "y" OR start$ = "Y" THEN
CLOSE
CLEAR
GOTO start
ELSE
SYSTEM
END IF

progend:
END
The Computer Program "SacCombo"

REM Program "SACCOMBO" by Manpreet S. Wadhwa
REM Version 1.1, updated from the previous version on 10/14/93
REM Version 1.0, written in the summer of '93 to assist Dwight C. Ware for his project.

REM this program uses exact weights for calculations

start:
OPEN "molx.out" FOR OUTPUT AS #1
CLS
PRINT
PRINT "MONOSACCHARIDE ANALYSIS PROGRAM FOR OLIGOSACCHARIDES"
PRINT "***********************************************************************************"

progstart:
REM DIM ahx(IOO), ahxn(100), afu(100), asa(100)
INPUT "name of the oligosaccharide? (optional) ", oligoS$  
INPUT "molecular wt": mw
INPUT "plus/minus range? (default 1) ", diff
IF diff= 0 THEN diff= 1


tboctyr = 0 : tyr = 0 : succ = 0 : cnum = 0

PRINT
PRINT "Note: Whenever a yes/no response is required, please use 'y' for YES"
PRINT "and 'n' for NO. Pressing 'enter' will result in the default response"
PRINT "indicated in square brackets."
PRINT

INPUT "is this a reducing oligosaccharide? [No] ", red$  
IF red$ = "y" OR red$ = "Y" THEN
    chemstate$ = "Reducing"
GOTO startcal
END IF

INPUT "is this a t-boc-tyrosinated oligosaccharide? [No] ", tboc$  
IF tboc$ = "y" OR tboc$ = "Y" THEN
    tboctyr = 279.134 - 17.003
    chemstate$ = "t-BOC tyrosine conjugate"
GOTO startcal
END IF

INPUT "is this a tyrosinated (deprotected) oligosaccharide? [No] ", dep$  
IF dep$ = "y" OR dep$ = "Y" THEN
    tyr = 179.082 - 17.003
171

```
chmstate$ = "Tyrosine conjugate (deprotected)"
GOTO startcal
END IF

INPUT "is this a succinylated oligosaccharide? [No] ", succ$
IF succ$ = "y" OR succ$ = "Y" THEN
succ = 279.098 - 17.003
chmstate$ = "Succinylated"
GOTO startcal
ELSE
PRINT
PRINT "you replied NO to all the previous 4 questions"
PRINT "this program cannot handle your oligosaccharide"
GOTO continue
END IF

startcal:
CLS
PRINT
PRINT "SACCOMBO MONOSACCHARIDE ANALYSIS REPORT"
PRINT "***************************************"
PRINT
PRINT "Oligosaccharide: oligoS"
PRINT "Chemical State: "; chmstate$
PRINT "Molecular weight entered: "; mw
PRINT "Molecular weight range scanned: "; mw - diff; "-"; mw + diff
PRINT
PRINT "Hexose NAc-hexsmn Fucose Sialic Acid Calc MW Match % Adduct"
PRINT "__________________________________________________________________________
PRINT #1, "SACCOMBO MONOSACCHARIDE ANALYSIS REPORT"
PRINT #1, "***************************************"
PRINT #1, "Oligosaccharide: "; oligoS
PRINT #1, "Chemical State: "; chmstate$
PRINT #1, "Molecular weight entered: "; mw
PRINT #1, "Molecular weight range scanned: "; mw - diff; ";"; mw + diff
PRINT #1, "Hexose NAc-hexsmn Fucose Sialic Acid Calc MW Match % Adduct"
PRINT #1, "__________________________________________________________________________

derivatives = tboctyr + tyr + succ

FOR m = 1 TO 6
PRINT
PRINT #1,
```

SELECT CASE m
CASE IS = 1
ion = 1.008
ion$ = "H"
CASE IS = 2
ion = 22.99
ion$ = "Na"
CASE IS = 3
ion = 38.964
ion$ = "K"
CASE IS = 4
ion = 23.998
ion$ = "H+Na"
CASE IS = 5
ion = 45.98
ion$ = "2 Na"
CASE IS = 6
ion = 61.953
ion$ = "Na+K"
END SELECT

mwc = mw - 910.328 - derivatives - ion

LET fu = 164.068 - 18.011
LET hx = 180.063 - 18.011
LET hxn = 221.09 - 18.011
LET sa = 309.106 - 18.011

limit = CINT(mwc / 146.057)

FOR i = 0 TO limit

    nhx = i

    FOR j = 0 TO (limit - i)

        nsa = j

        FOR k = 0 TO (limit - i - j)

            nfu = k

            LET nhxn = (mwc - (hx * nhx + sa * nsa + fu * nfu)) / hxn

            LET rhxn = CINT(nhxn)

            LET cnum = cnum + 1

    NEXT k

NEXT j

NEXT i
LET calcmw = hx * nhx + sa * nsa + hxn * rhxn + fu * nfu + 910.328 + derivatives + ion
LET match = calcmw / mw * 100

IF ABS(calcmw - mw) <= diff AND rhxn >= 0 THEN
PRINT nhx + 3; "; rhxn + 2; "; nfu; "; nsa; "; calcmw; "; match; "; ion$
PRINT #1, nhx + 3; "; rhxn + 2; "; nfu; "; nsa; "; calcmw; "; match; "; ion$
END IF

loopend:
NEXT k
NEXT j
NEXT i
NEXT m
PRINT
PRINT "__________________________________________________________"
PRINT "Total combinations screened: "; cnum
PRINT #1,
PRINT #1, "__________________________________________________________"
CLOSE #1

PRINT
INPUT "Do you want to print the output? [No] ", prt$
IF prt$ = "y" OR prt$ = "Y" THEN
OPEN "molx.out" FOR INPUT AS #1
DO WHILE NOT EOF(1)
   LINE INPUT #1, PS
   LPRINT PS
LOOP
LPRINT CHR$(12)
CLOSE #1
END IF

PRINT
INPUT "Do you want to save the report? [No] ", sav$
IF sav$ = "y" OR sav$ = "Y" THEN
PRINT
PRINT "Please enter filename. Note that if you do not specify a complete path,"
PRINT "the file will be saved under c:\qbasic"
PRINT
INPUT "Filename": filnam$
NAME "molx.out" AS filnam$
ELSE
KILL "molx.out"
END IF
continue:

PRINT
INPUT "Do you want to use the program again? [Yes] ", start$
IF start$ = "" OR start$ = "y" OR start$ = "Y" THEN
CLOSE
CLEAR
GOTO start
ELSE
SYSTEM
END IF

progend:
END
The Computer Program "FLScat"

REM program "FLSCAT" by Manpreet S. Wadhwa, summer of '94
REM written in FLDM - OBEY (part of polaroad37)
REM measure fluorescence intensity and light scattering
REM new feature added 10/14/94,
REM program writes "temp.dat" for every measurement, useful backup!!
REM file modified 3/11/95, read light scattering at 500nm since intensity at 350
REM was too high with the new instrument, also forced PMT volts constant at 700v
REM for light scattering measurements.

filter 7 700
heading$="Smp# Fluorescence Scatter(500nm) Info"
fmt$="### ###.###  ###.###  

10 sampnum=0
cls:print "Current Sample Read Parameters"
print "Method: ";meth$;" Date: ";date$;" Time: ";time$" print "Method Info: ";info$" print 
print "Ex Wl: ";ex$;" Em Wl: ";em$;" Ex Slit: ";exsl$;" Em Slit: ";emsl$;" Integration Time (sec): ";int$;" PMTvolts: 700
print "Light Scat at 500nm, no Polarisers." print "Number of Replicates:";rep;" Accessory: ";acc$;" print print":.print"

20 input "; Press ENTER to Continue, or (R)eturn to Main Menu...",a$ if a$="" then goto 25 if a$="r" or a$="R" then goto 3000

25 input "Any 'equilibration time' needed for your sample? (y/n) ",yesno$ if yesno$="Y" or yesno$="y" then input "Please enter equilibration time in seconds ",equil else equil = 0 end if

30 cls
print ": print "Please enter experimental information (max 80 characters)"
input ",expinfo$" print "Please enter additional info if required (max 80 characters)"
input ",expinfo2$"
d$=p$+fil$+".dat"
open d$,"a+t"
write "Method: "+meth$+" Date: "+date$+" Time: "+time$ to d$
write "Experiment Info: "+expinfo$ to d$
write expinfo2$ to d$
write "Ex Wl: "+ex$+" Em Wl: "+cm$+" Ex Slit: "+exsl$+" Em Slit: "+emsl$ to d$
write "Integration Time (sec): "+int$+" PMT volts: 700 EquilTime(s) "+str$(equil) to d$
write "Light Scattering at 500nm, No Polarisers" to d$
write "Number of Replicates: "+str$(rep)+" Accessory: "+acc$ to d$
write "-----------------------------------------------" to d$
write heading$ to d$
window "size" 0,0,1000,400
openwin "W2:" 0,420,1000,580
redirect output to "W2:"
print heading$
redirect output to "W1:"

40 cls
if sampnum=0 then
print "Please enter information for sample 1"
input "",sampinfo$
else
print chr$(7): print chr$(7): print chr$(7): print chr$(7): print chr$(7)
cls
print "Completed Analysis of Sample":sampnum
print "Please enter information for sample":sampnum+1
input "",sampinfo$
end if
print "Press ENTER to Read Sample, or R to run new series of samples"
input "Or press any other key to Quit...",a$
if a$="" then goto 100 else goto 500

100 cls
set timer 1 for equil seconds
print "Waiting for";equil," seconds"
wait for timer 1
sampnum=sampnum+1
print "Analyzing Sample":sampnum
print "Measuring Fluorescence Intensity... "
readord
valint=cxtrint

152 print "Measuring Light Scattering..."
EXSLscat$ = "2.5" : EMSLscat$ = "2.5"
wav$="500"
lightscat
wav4scat=extrint
REM calculations
valint$=str$(valint)
wav4scat$=str$(wav4scat)
write " +str$(sampnum)+" +valint$+" +wav4scat$+" +sampinfo$ to d$
REM data backup procedure, added 10/14/94. Look for "temp.dat" if you lose data!
close d$
copy d$ "c:\data\manpreet\expt\temp.dat"
open d$, "a+t"

redirect output to "W2:"
print using fmt$, sampnum; valint; wav4scat; ; print " ;print sampinfo$
redirect output to "W1:"
print ""
goto 40

500
if a$="r" or a$="R" then
input "Are you sure you want to start new series";yesno$
if yesno$="y" or yesno$="Y" then
write "-------------------------------------------------------------" to d$
write " " to d$ :close d$
closewin "W2:":window "size" 100 30 800 950
goto 10
close
end if
end if

input "Are you sure you want to quit? (y/n) ",yesno$
if yesno$="y" or yesno$ ="Y" then
goto 2000
close if yesno$="n" or yesno$="N" then
goto 40
close
end if
goto 500
end if

procedure readord
ORD EX$ EMS EXSLS EMSLS INTS "PRORD"
open e$,"r"
read header from e$;read header from e$
read rdata from e$
cxtrint=val(mid$(rdata,35,15))
temp=val(mid$(rdata,116,6))
close e$;delete e$
end procedure
REM light scattering measurement

procedure lightscat
ORD wav$ wav$ EXSLscat$ EMSLscat$ INT$ "PRORD"
open c$,"r"
read header from c$:read header from c$
read rdata from c$
extrint=val(mid$(rdata,35,15))
tcmp=val(mid$(rdata,116,6))
close c$:delete c$
end procedure

2000 write "-------------------------------------------------------------------------------------" to d$
write "" to d$
close d$

2100 input "Save output file? (y for yes) ",sav$
if sav$ = "y" or sav$="Y" then
print
print "Output file will be saved as 2 copies under the EXPT subdirectory"
print "and these will have the extension .DA and .DAT"
print "(*.dat file is for further editing to use with grapher etc.)"
print
2150 input "Please input Filename to save to (max 8 characters) ",savfil$
if savfil$ ="" then goto 2150
savp$="c:\data\manpreet\exp1"
savfilnam$=savp$+savfil$+.da"
savfilnam2S=savpS+savfil$+.dat",
Print "Saving files..."
copy d$ savfilnam$
copy d$ savfilnam2S
goto 2500
else if sav$="n" or sav$="N" then
print "Output file will be deleted"
input "press 'Y' to confirm delete without save" con$
if con$="Y" then goto 2500 else goto 2100
else
goto 2100
end if

2500 Print "Deleting source file..."
delete d$
closewin "W2:.window "size" 100 30 800 950
FILTER "7 -1"
3000 end
BIBLIOGRAPHY


