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# New Carbohydrate-Based Anti-Cancer And Anti-Bacterial Vaccines

Zhifang Zhou  
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**NEW CARBOHYDRATE-BASED ANTI-CANCER AND ANTI-BACTERIAL VACCINES**

by

**ZHIFANG ZHOU**

**DISSERTATION**

Submitted to the Graduate School

of Wayne State University,

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in partial fulfillment of the requirements

for the degree of

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MAJOR: CHEMISTRY (Analytical)

Approved by:

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Advisor

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Date

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## DEDICATION

This dissertation is dedicated to my dear parents, Licong Zhou, Jianmei Qiu, my brother, Zhiang Zhou, and my lovely daughter, Cara (Meijia) Zhou.

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## TABLE OF CONTENTS

Dedication .....	ii
Acknowledgements .....	iii
List of Tables .....	vii
List of Figures .....	viii
List of Abbreviations .....	xii

### CHAPTER 1 Introduction

1.1 Currently Licensed Vaccines.....	1
1.2 Carbohydrate-Based Vaccines.....	4
1.3 Carbohydrate-Based Cancer Vaccines.....	6
1.3.1 Tumor-associated carbohydrate antigens.....	6
1.3.2 TACA-based cancer vaccines.....	8
1.3.3 A novel cancer immunotherapeutic strategy.....	13
1.4 Carbohydrate-Based Antibacterial and Antifungal Vaccines.....	15
1.5 Conclusion.....	18
1.6 Reference.....	19

### CHAPTER 2 Quantifying the Efficiency of Sialic Acids Metabolic Engineering

2.1 Introduction of Sialic Acid Metabolic Engineering.....	28
2.2 Results and Discussion.....	29
2.3 Conclusion.....	38
2.4 Experimental Section.....	39
2.5 Appendices.....	42
2.6 Reference.....	44

## CHAPTER 3 Development of Anti-Cancer Vaccines Using Monophosphoryl Lipid A as a Carrier Molecule and Built-in Adjuvant

3.1 Introduction of Monophosphoryl Lipid A (MPLA).....	47
3.1.1 Lipopolysaccharide.....	47
3.1.2 Lipid A structure-activity relationships.....	48
3.1.3 Monophosphoryl lipid A.....	52
3.1.4 Introduction to adjuvants.....	53
3.1.5 MPLA-based full-synthetic vaccine.....	54
3.2 Evaluation MPLA-sTnNPhAc Conjugates as Cancer Vaccines and Optimization of the MPLA structure as a Vaccine Carrier.....	58
3.2.1 Introduction.....	58
3.2.2 Results and Discussion.....	59
3.2.3 Conclusion.....	67
3.2.4 Experimental Section.....	68
3.3 MPLA Carrier Applied in Globo H-Based Vaccine.....	72
3.3.1 Introduction.....	72
3.3.2 Results and Discussion.....	74
3.3.3 Conclusion.....	81
3.3.4 Experimental Section.....	82
3.4 Reference.....	85

## CHAPTER 4 Development of KLH- and MPLA-based Vaccines against *Haemophilus Influenzae* Type B (Hib) and *Neisseria Meningitidis* Serotype C

4.1 Vaccines against <i>Haemophilus Influenzae</i> Type B (Hib).....	93
4.1.1 Introduction.....	93
4.1.2 Our Vaccine Designs.....	96

4.1.3 Results and Discussion.....	97
4.1.3.1 KLH Conjugates <b>4.1-4.3</b> as Vaccines.....	97
4.1.3.2 MPLA Conjugates <b>4.7-4.9</b> as Vaccines.....	105
4.1.3.3 Evaluation of the Hib-binding Property of Antisera.....	108
4.1.4 Conclusion.....	111
4.1.4 Experiment Section.....	112
4.2 Vaccines against <i>Neisseria Meningitides</i> Serogroup C.....	119
4.2.1 Introduction.....	119
4.2.2 Our Vaccine Designs.....	121
4.2.3 Results and Discussion.....	122
4.2.3.1 KLH Conjugates <b>4.10-4.13</b> as Vaccines.....	122
4.2.3.2 MPLA Conjugates <b>4.18-4.22</b> as Vaccines.....	129
4.2.4 Conclusion.....	133
4.2.5 Experiment Section.....	133
4.3 Reference.....	137
<b>Abstract.....</b>	<b>141</b>
<b>Autobiographical Statement.....</b>	<b>145</b>



## LIST OF TABLES

Table 1.1:	Examples of licensed vaccines.....	1
Table 1.2:	Expression profiles of TACAs on malignant tissues.....	8
Table 1.3:	Examples of carbohydrate-based anticancer vaccines.....	9
Table 1.4:	Examples of licensed carbohydrate-based vaccines.....	16
Table 2.1:	Recovery analysis of Neu5PhAc from the SKMEL-28 cell culture.....	33
Table 2.2:	Analysis of Neu5Ac and Neu5PhAc mixtures prepared in different ratios..	33
Table 3.1:	Adjuvant types and their mechanisms of action.....	53
Table 3.2:	Licensed adjuvants.....	54
Table 3.3:	Dose of KLH conjugate <b>3.6</b> and MPLA conjugate <b>3.7</b> .....	83
Table 4.1:	The loadings of saccharide moiety in protein conjugates.....	98
Table 4.2:	Influence of cytokines on antibody isotype switching.....	102
Table 4.3:	Doses of KLH conjugates used in animal studies.....	112
Table 4.4:	The loading of saccharide moiety in protein conjugates <b>4.10-4.17</b> .....	123
Table 4.5:	Dose of each KLH conjugate ( <b>4.10-4.13</b> ) used for immunizing mice.....	134
Table 4.6:	Dose of each MPLA conjugate ( <b>4.18-4.22</b> ) used for immunizing mice.....	134

## LIST OF FIGURES

Figure 1.1:	Carbohydrate antigens have been employed for various vaccine designs...5
Figure 1.2:	Examples of TACAs.....7
Figure 1.3:	A novel immunotherapy strategy based on glycoengineering.....15
Figure 1.4:	Anti-bacterial vaccines based on capsular polysaccharides.....18
Figure 2.1:	Structure of Neu5Ac.....28
Figure 2.2:	Metabolic engineering of sialic acids on the cancer surface.....29
Figure 2.3:	Reactions between sialic acids and DMB and structures of the reaction products.....30
Figure 2.4:	A linear correlation between the peak areas of Neu5Ac or Neu5PhAc derivative and the concentrations of Neu5Ac or Neu5PhAc was demonstrated. The coefficient of determination ( $R^2$ ) is displayed for each graph.....31
Figure 2.5:	HPLC diagram of a sample derived from $10^6$ SKMEL-28 cells treated with 30 mM of ManNPhAc for 3 days. The retention times for the DMB derivatives of Neu5Ac and Neu5PhAc <b>2.3</b> and <b>2.4</b> were about 9.90 and 15.50 min, respectively.....34
Figure 2.6:	The amounts of Neu5Ac (A) and Neu5PhAc (B) expressed by SKMEL-28 cells ( $10^6$ ) that were incubated with 0, 5, 10, 20 and 30 mM of ManNPhAc for 3 days, determined by HPLC.....36
Figure 2.7:	The percentages of Neu5PhAc expressed on SKMEL-28 cells incubated with 0, 5, 10, 20 and 30 mM of ManNPhAc for 3 days (A), and the percentages of Neu5PhAc on SKMEL-28 cells incubated with 5 and 20 mM of ManNPhAc for 2, 3, 4 and 9 days (B), respectively.....37
Figure 2.8:	MALDI MS of Neu5PhAc-DMB: (A) Sample was from reaction solution of standard Neu5PhAc after DMB derivatization; (B) HPLC fraction sample of 15.4 min in the cell samples which was treated with 20 mM precursor ManNPhAc for 2 days.....43
Figure 3.1:	The structure of outer layer of outer membrane of Gram-negative bacteria.....47
Figure 3.2:	An example of lipid A structure.....49
Figure 3.3:	Schematic representation of lipid A structure-activity relationships.....50

Figure 3.4:	A new construct of full-synthetic vaccine based on MPLA.....	55
Figure 3.5:	The structure of MPLA-GM3NPhAc and immune results.....	56
Figure 3.6:	The structure of sTn antigen.....	58
Figure 3.7:	The structure of designed MPLA derivatives and their sTn conjugates.....	59
Figure 3.8:	ELISA results of day 38 antisera of mice immunized with <b>3.1</b> alone (A), <b>3.1</b> plus Titermax Gold (B), <b>3.2</b> alone (C), <b>3.2</b> plus Titermax Gold (D), <b>3.3</b> alone (E), <b>3.3</b> plus Titermax Gold (F), <b>3.4</b> alone (G) and <b>3.4</b> plus Titermax Gold (H), respectively.....	62
Figure 3.9:	Competitive ELISA results of the pooled mouse antisera obtained with conjugates <b>3.2</b> , <b>3.3</b> , <b>3.4</b> and <b>3.2</b> plus adjuvant.....	64
Figure 3.10:	Structure of Globo H.....	73
Figure 3.11:	The structure of Globo H-KLH ( <b>3.6</b> ) and Globo H-MPLA ( <b>3.7</b> ).....	74
Figure 3.12:	ELISA results of day 38 antisera of mice immunized with <b>3.6</b> plus CFA adjuvant (A) and <b>3.7</b> in liposome form (B), respectively.....	76
Figure 3.13:	The kinetic ELISA results of antisera induced by conjugates <b>3.6</b> and <b>3.7</b> . ELISA results of Globo H-specific antibody in the antisera pooled from mice immunized with (A) conjugate <b>3.6</b> plus CFA adjuvant and (B) <b>3.7</b> in liposome form, respectively, on day 21 (blue line), day 27 (red line) and day 38 (green line).....	77
Figure 3.14:	FACS results of MCF-7 cells.....	79
Figure 3.15:	FACS results of SKMEL-28 cells.....	80
Figure 3.16:	Overlay plots of FACS results of MCF-7 (A) and SKMEL-28 (B) cell lines stained with normal sera, pooled antisera from mice immunized with <b>3.6</b> plus CFA adjuvant and <b>3.7</b> liposome, respectively.....	80
Figure 4.1:	The structure of polyribosylribitol phosphate (PRP) on Hib surface.....	94
Figure 4.2:	The structure of designed olio-RRPs and their KLH conjugates ( <b>4.1-4.3</b> ), HSA conjugates ( <b>4.4-4.6</b> ), and MPLA conjugates ( <b>4.7-4.9</b> ).....	97
Figure 4.3:	MALDI TOF MS results of HSA conjugates <b>4.4</b> , <b>4.5</b> and <b>4.6</b> .....	99
Figure 4.4:	ELISA results of total antibodies (kappa) of the pooled antisera collected on day 0, day 38 and day 48 from mice immunized with: (A) <b>4.1</b> plus Titermax Gold adjuvant; (B) <b>4.2</b> plus Titermax Gold adjuvant; (C) <b>4.3</b>	

	plus Titermax Gold adjuvant. (D) Calculated antigen-specific total antibody titers of the conjugates.....	100
Figure 4.5:	ELISA results of antigen-specific antibody isotypes in day 48 antisera of mice immunized with: (A) <b>4.1</b> plus Titermax Gold adjuvant, (B) <b>4.2</b> plus Titermax Gold adjuvant, (C) <b>4.3</b> plus Titermax Gold adjuvant, respectively.....	102
Figure 4.6:	ELISA results of the cross reactions between antigens displayed in <b>4.4-4.6</b> and pooled antisera of mice immunized with <b>4.1, 4.2</b> and <b>4.3</b> ....	104
Figure 4.7:	ELISA results of the anti-kappa (total) antibody titers (OD 405 = 0.1) in the pooled antisera of mice immunized with conjugate <b>4.7</b> obtained on day 1, 2, 4, 5, 7 and 9 after the 5th boost immunization.....	106
Figure 4.8:	ELISA results of different isotypes of antigen-specific antibodies in day 2 antisera of mice after final boost immunization with <b>4.7, 4.8</b> and <b>4.9</b> .....	107
Figure 4.9:	ELISA results of the cross reactions between <b>4.4-4.6</b> and the antisera derived from mice immunized with <b>4.7-4.9</b> .....	108
Figure 4.10:	ELISA results of antibody-Hib binding experiments. (A) and (B): results of the 1:100 diluted pooled antisera of conjugates <b>4.1-4.3</b> and <b>4.7-4.9</b> ; and (C) and (D): results of the 1:300 diluted pooled antisera of <b>4.1-4.3</b> and <b>4.7-4.9</b> (d0: the pooled sera before immunization used as negative controls).....	109
Figure 4.11:	Hib competitive ELISA results using: (A) 1:2700 diluted antisera of mice immunized with KLH conjugate <b>4.1</b> ; (B) 1:900 diluted antisera of mice immunized with MPLA conjugate <b>4.7</b> ; (C) 1:300 diluted antisera of mice immunized with <b>4.2</b> ; (D) 1:900 diluted antisera of mice immunized with <b>4.3</b> .....	111
Figure 4.12:	The structures of designed [ $\alpha$ 2-9] linked oligo sialic acids KLH conjugates ( <b>4.10-4.13</b> ), HSA conjugates ( <b>4.14-4.17</b> ), and MPLA conjugates ( <b>4.18-4.22</b> ) .....	122
Figure 4.13:	ELISA results of antigen-specific antibody isotypes in day 38 antisera of mice immunized with (A) <b>4.10</b> plus Titermax Gold adjuvant, (B) <b>4.11</b> plus Titermax Gold adjuvant, (C) <b>4.12</b> plus Titermax Gold adjuvant, (D) <b>4.13</b> plus Titermax Gold adjuvant, respectively.....	124
Figure 4.14:	The antigen-specific antibody titers of kappa (A and B) and IgG2b (C and D) isotypes for all the four glycoconjugates <b>4.10-4.13</b> .....	126
Figure 4.15:	The kinetic plot of glycoconjugates ( <b>4.10-4.13</b> ). The pooled antisera of day 28 was obtained from mice immunized three times with	

glycoconjugates, respectively. The pooled antisera of day 38 was obtained ten days later after the fourth boost from the mice immunized with glycoconjugates, respectively.....127

Figure 4.16: The ELISA results of cross reactions between the pooled antisera obtained from mice immunized with conjugates (4.10-4.13) dimer-, trimer-, tetramer- and pentamer-sialic acid-KLH and the capture reagents (4.14-4.17) dimer-, trimer-, tetramer- and pentamer- sialic acid-HSA.....128

Figure 4.17: The ELISA results of antigen-specific antibody titers in day 35 antisera of mice immunized with (A) 4.18 alone, (B) 4.18 plus Freund's Completed Adjuvant (CFA), (C) 4.19 alone, (D) 4.20 alone, (E) 4.21 alone and (F) 4.22 alone, respectively.....130

Figure 4.18: The ELISA results of cross reactivity of the antisera induced by conjugates (4.18-4.22) dimer-, trimer-, tetramer- and pentamer-sialic acid-MPLA and the different capture reagents (4.14-4.17) from dimer- to pentamer-sialic acid-HSA.....132

## LIST OF ABBREVIATIONS

Ac	acetyl
Ab	antibody
ADCC	antibody-dependent cell-mediated cytotoxicity
AIDS	acquired immunodeficiency syndrome
Alum	aluminum hydroxide
AP	alkaline phosphate
APC	antigen presenting cell
AS03	adjuvant system 03
AS04	adjuvant system 04
ATCC	American Type Culture Collection
BCG	Bacille Calmette Guerin
BSA	bovine serum albumin
c.a.	calculated
CDC	complement-dependent cytotoxicity
CFA	Freund's complete adjuvant
CRM	diphtheria toxin mutant
CP	carrier protein

CPS	capsular polysaccharide
d	day
DMB	1,2-diamino-4,5-methylenedioxybenzene
DMEM	Dulbecco's modified Eagle's medium
DSPC	1,2- distearoyl- <i>sn</i> -glycero-3-phosphocholine
DT	diphtheria toxoid
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagle's Minimum Essential Medium
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
Fuc	fucosyl
Gb3	globosyltrioside
GD	ganglioside antigen
Globo H	globohexaosylceramide
GM	gangliosidoses
GM3PhAc	gangliosidoses 3 with <i>N</i> -phenyl acetyl sialic acid
h	hour

Hib	<i>Haemophilus influenzae</i> type b
HIV	human immunodeficiency virus
HPV	human papillomavirus
HSA	human serum albumin
IFA	Freund's incomplete adjuvant
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
KLH	keyhole limpet hemocyanin
LC	liquid chromatography
Le	Lewis
mAb	monoclonal antibody
ManNAc	<i>N</i> -acetyl-D-mannosamine
ManNPhAc	<i>N</i> -phenyl acetyl-D-mannosamine
MHC	major histocompatibility complex



MPL	monophosphoryl lipid A
MPLA	monophosphoryl lipid A
MS	mass spectrometry
MUC	mucin
ND	not detected at given threshold
Neu5Ac	<i>N</i> -acetyl neuraminic acid
Neu5PhAc	<i>N</i> -phenyl acetyl neuraminic acid
NPhAc	<i>N</i> -phenyl acetyl
OD	optical density
OMP	outer membrane protein
OVA	ovalbumin
Pam <sub>3</sub> Cys	<i>S</i> -[ <i>(R)</i> -2,3-dipalmitoyloxy-propyl]- <i>N</i> -palmitoyl- <i>(R)</i> -cysteine
PBS	phosphate-buffered saline
PBST	PBS with 0.05% Tween-20
PNPP	<i>p</i> -nitrophenylphosphate
PRRs	polyribosylribitol
PS	polysaccharide
PSA	poly sialic acid

RAFT	regioselectively addressable functionalized template
r.t.	room temperature
Sacc	saccharide
s.c.	subcutaneous
sTF	sialic Thomsen-Friedenreich
sTn	sialyl 2-6- $\alpha$ -N-acetylgalactosamine
sTnNPhAc	<i>N</i> -phenyl acetyl sTn (sTnPhAc)
TACA	tumor-associated carbohydrate antigen
TGF	transforming growth factor
TF	Thomsen-Friedenreich
TFA	trifluoroacetic acid
Th	T helper
TLR4	Toll-like receptor 4
Tn	2-6- $\alpha$ -N-acetyl-galactosaminyl
TT	tetanus toxoid

## CHAPTER 1 INTRODUCTION

### 1.1 Currently Licensed Vaccines

Since Edward Jenner's discovery that cowpox could prevent human from the threat of smallpox infection, vaccines have become the most important and successful strategy to protect against infectious diseases.<sup>1-3</sup> Currently, there are several types of licensed vaccines available, including attenuated or killed pathogens, toxoids, proteins or polysaccharides from those pathogens, and so on (**Table 1.1**).<sup>4</sup>

**Table 1.1** Examples of licensed vaccines<sup>1,4</sup>

Vaccine type	Vaccine examples
Live mycob	Tuberculosis
Live attenuated	Influenza (intranasal), Measles, Mumps, Polio (Sabin), Rotavirus, Rubella, Varicella, Yellow Fever
Killed	Hepatitis A, Pertussis (whole cell), Polio (Salk), Rabies,
Killed, subunit	Influenza
Toxoid	Diphtheria toxoid, Tetanus toxoid,
Protein	Hepatitis B, Pertussis (acellular),
PS	Hib PS, Meningococcal PS, Pneumococcal PS, Typhoid PS
PS-protein	Hib glycoconjugates, Meningococcal conjugates, Pneumococcal conjugates
Virus-like-particle	Papillomavirus

Hib: *Haemophilus influenzae* type B; PS: polysaccharide

Vaccines derived from bacterial and viral particles or subunits, such as influenza vaccine<sup>5,6</sup>, Bacillus Calmette-Guerin (BCG)<sup>7</sup> and measles, and from attenuated or killed

pathogens are relatively easily accessed and developed.<sup>8</sup> Hence, once an epidemic surges and the pathogen of the disease is found, it is theoretically quick to design a vaccine based on the pathogen itself. Currently, most of the successful vaccines in clinical applications are developed empirically. For example, the seasonal influenza vaccines are always developed based on the specific influenza virus components which vary year to year.<sup>5,6</sup>

Despite the great success of whole cell and subunit/component-based vaccines, there are still concerns or issues about them. First, the number of vaccines is still limited, and there are still many diseases that have not been covered.<sup>1,9</sup> Second, many of the vaccines have limited immunological efficacy or are only effective in certain populations not including young children who need more protection against pathogens.<sup>10-12</sup> For example, to control the spread of typhoid fever by routine vaccination, three licensed vaccines, parenteral inactivated whole-cell vaccine, oral attenuated *S. typhi* Ty21a vaccine, and parenteral Vi polysaccharide vaccine, confer only about 70% protection in older children and adults and do not protect young children.<sup>11,13</sup> safety issues and the difficulties in quality control are other important issues associated with these vaccines. Potential incomplete attenuation and contamination caused by pathogenic residuals are big impediments to vaccine safety.<sup>14,15</sup>

In recent decades, vaccines derived from some specific antigenic components of pathogens, including both proteins or polysaccharide (PS), have been developed, such as vaccines against Hib<sup>12,16-19</sup>, *N. meningitidis*<sup>10,20</sup> and *S. pneumoniae*<sup>21-24</sup>. Regarding these vaccines, the antigenic components are structurally defined and can be easily accessible from cultured bacteria in purified form. Therefore, these vaccines play an

important role in the study of vaccine immunology and the interactions between antigens and the immune systems. Understanding the mechanism of vaccines and immunological interactions may help us design and optimize vaccines that can elicit more effective and long-term immunity.<sup>25</sup>

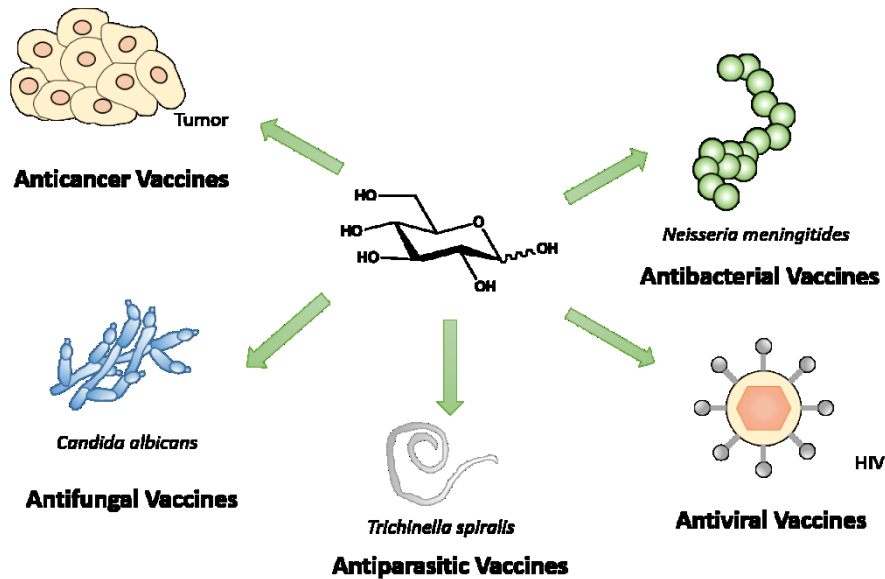
Proteins and carbohydrates of pathogens are two major components that can be attractive targets for vaccine development. Pathogen proteins can often elicit strong and T cell-dependent immune responses, so they have been useful and popular targets for vaccine design. On the other hand, carbohydrates alone typically only introduce short-term and T cell-independent immunity,<sup>26-29</sup> especially in infants and children. Therefore, pathogen polysaccharides have been mainly used as vaccines for adults. To overcome this problem, carbohydrates have been coupled with carrier proteins to form conjugate vaccines.<sup>22</sup> In this way, the immune response induced against carbohydrate antigens can be switched to T cell-dependent immunity which is more potent and functional. In addition, the immune responses induced by conjugate vaccine have long-term effects and better immunological activities.<sup>23,24</sup> In the meantime, carbohydrates are exposed on the cell surface, making them easily recognized by and interacting with the immune system.<sup>30</sup> Consequently, the unique carbohydrates as antigens of both pathogen and cancer cells have become particularly important targets for various immune studies and for vaccine design.<sup>1,30</sup> As the main topic of the present dissertation, carbohydrate-based vaccines will be the focus of the introduction.

## 1.2 Carbohydrate-Based Vaccines

The unique glycans expressed by pathogens, including bacteria, parasites, fungi and viruses, as well as the abnormal glycans on tumor cells, are very attractive markers for the design and development of novel carbohydrate-based vaccines.<sup>1,31-33</sup> First, the glycans expressed by pathogens or the abnormal glycans expressed by tumors often have distinctive chemical structures and are exposed on the cell surface, thus they are easy targets as antigens for the immune system.<sup>1,10,34-37</sup> Second, carbohydrates are typically highly conserved and are often the most abundant antigens on the cell surface. Consequently, development of carbohydrate-based vaccines against various diseases (**Figure 1.1**) has been a hot area in recent decades, and many new and highly effective vaccines have been developed.<sup>1,31,38</sup> For example, as mentioned above, current licensed vaccines against typhoid fever confer only about 70% protection in older children and adults and have no protection to young children.<sup>11,13</sup> To deal with the issue, Lin *et al* developed a new conjugate vaccine based on the capsular polysaccharide of *Salmonella typhi*, which has been demonstrated to be safe and strongly immunogenic and have shown more than 90% efficacy in children two to five years old.<sup>11</sup>

Despite that some specific carbohydrates on the cell surface can be potentially ideal antigens for the development of vaccines, they still have problems and limitations. Most importantly, carbohydrates alone usually have low immunogenicity and are T cell-independent antigens,<sup>30,31</sup> thus even if they are immunogenic, they typically elicit T cell-independent immune responses and fail to activate T cell immunity.<sup>26-29</sup> As a result, the inducing immune responses are often weaker and shorter antibody responses lacking necessary immune memory.<sup>30,31</sup> Moreover, carbohydrates alone usually cannot induce

robust immune responses in children under 5 years old. Therefore, although a number of antibacterial vaccines made of pure polysaccharides have been developed, they are only used in adults in clinic.



**Figure 1.1** Carbohydrate antigens have been employed for various vaccine designs

A widely adopted strategy that has been employed to overcome the problem of low immunogenicity of carbohydrate antigens is to link them to a large, immunologically active protein carrier to form glycoprotein conjugates. This would not only improve the immunogenicity of carbohydrates but also switch them to T cell-dependent antigens and formulate functional vaccines. In this way, many effective carbohydrate-based vaccines have been developed, which will be discussed in detail in the following sections.

However, polysaccharide-protein conjugates as vaccines have complex structure and are difficult to analyze and control in terms of quality and purity.<sup>39-42</sup> To overcome this problem, in recent years, new vaccine designs and strategies have been explored, such as conjugate vaccines derived from chemical synthetic carbohydrate antigens that are

homogeneous and fully synthetic conjugate vaccines with well-defined structure. These vaccines may provide consistent quality as well as improved safety.<sup>1,38</sup> To meet this demand, new carrier molecules, in addition to proteins, have been exploited for the development of synthetic glycoconjugate vaccines.<sup>42-45</sup>

## 1.3 Carbohydrate-Based Cancer Vaccines

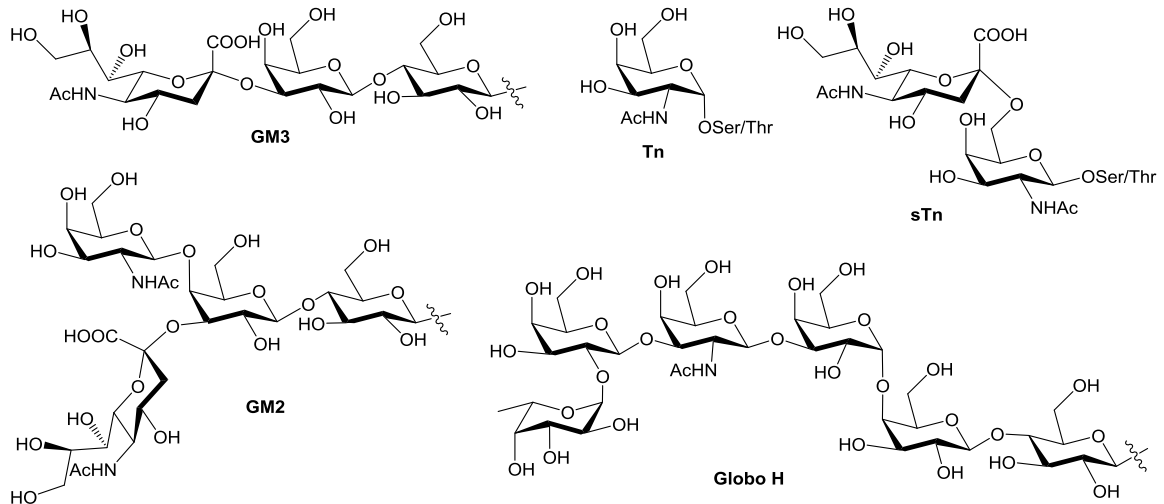
### 1.3.1 Tumor-associated carbohydrate antigens

The association of abnormal glycosylations with tumor progression was first discovered by Meezan *et al.* in 1969 with the demonstration that many glycans on cancer cells differ from that on normal cells.<sup>36,46</sup> The abnormal glycosylations include loss or overexpression of certain glycans, presence of truncated glycans, and insertion of new glycans.<sup>47,48</sup> A variety of abnormal glycans on tumor cells have been identified by immunohistochemical staining with lectin or monoclonal antibody (mAb) or by MS analysis.<sup>49,50</sup> Abnormal glycans expressed on cancer cells are termed tumor-associated carbohydrate antigens (TACAs),<sup>72,92-95</sup> some of which are depicted in **Figure 1.2**.

TACAs are widely detected in the majority of common human cancers, including lung, breast, colorectal, ovarian, prostate, and pancreatic cancers. TACAs have been correlated to specific types of cancers, and each type of malignant tissue is often characterized by a distinct set of TACA expression as shown in **Table 1.2**.<sup>49,50</sup> TACAs are thus employed as important biomarkers for clinical diagnosis of cancer. On the other hand, TACAs are also attractive targets for anti-cancer vaccine and immunotherapy



development because they are usually abundantly expressed by many tumors but rarely or not expressed on normal cell surface.<sup>30,31</sup>



**Figure 1.2** Examples of TACAs

**Table 1.2** Expression profiles of TACAs on malignant tissues<sup>49,50</sup>

Tumor	Tumor-associated carbohydrate antigens													
	sLe <sup>x</sup>	Le <sup>x</sup>	sLe <sup>a</sup>	Le <sup>a</sup>	sTn	Tn	TF	Le <sup>y</sup>	Globo H	PSA	CD <sub>2</sub>	CD <sub>3</sub>	FucosylGM1	GM2
B cell lymphoma											✓			✓
Breast			✓		✓	✓	✓	✓	✓					✓
Colon			✓		✓		✓	✓						✓
Lung	✓				✓			✓	✓					✓
Melanoma											✓	✓		✓
Neuroblastoma										✓	✓	✓		✓
Ovary					✓		✓	✓	✓					✓
Prostate					✓	✓	✓	✓						✓
Sarcoma											✓	✓		✓
Small cell lung			✓						✓	✓			✓	✓
Stomach		✓	✓	✓	✓	✓	✓	✓	✓					✓

### 1.3.2 TACA-based cancer vaccines

TACA-based cancer vaccines have been widely explored despite of the potential problems associated with TACAs as described previously. In general, the production of immunity or antibodies induced by antigens depends on the cooperative function of two types of lymphocytes, B cells and helper T cells. However, the carbohydrate antigens are T cell-independent antigens and can hardly stimulate helper T lymphocytes. As a result, they only elicit B cell immunity which lacks long-term immunological memory and induce IgM antibodies that have low relatively affinity to antigens. On the other hand, the involvement of helper T cells in the immune reactions (T cell immunity) and the induction

of IgG antibodies, which correlate to long-term immunological memory, higher antibody affinity and improved antibody-dependent cell-mediated cytotoxicity,<sup>31,44,48,52</sup> are important for effective cancer therapy.

To formulate effective therapeutic cancer vaccines, the common strategy is to covalently link TACAs to a carrier protein to form conjugate vaccines that can induce T cell immunity. Keyhole limpet hemocyanin (KLH) is one of the most widely used carrier proteins for the design and development of glycoconjugate cancer vaccines because of its outstanding immunostimulatory ability. By coupling TACAs to proteins, the immune responses to TACAs can switch from T cell-independent to T cell-dependent and lead to the production of IgG antibodies with higher affinity.<sup>22,27</sup> The approach has been widely and successfully utilized to develop a number of TACAs-based vaccines. Some of these vaccines, such as Globo H-KLH and sTn-KLH conjugates as well as others, have been tested in clinical trials.<sup>53,54</sup> Some of the vaccines studied and the carrier proteins used are listed in **Table 1.3**.

**Table 1.3** Examples of carbohydrate-based anticancer vaccines

Target antigen	Spacer/other epitope	Carrier	Cancer type
<b>Monomeric vaccine</b>			
Globo H	CH <sub>2</sub> CH <sub>2</sub>	KLH, BSA	Breast <sup>55</sup>
	MMCCH	KLH	Prostate, breast <sup>56,57</sup>
Fuc-GM1	Ceramide-reductive amination	KLH	Small-cell lung <sup>58</sup>
GD2	Ceramide-lactone	KLH	melanoma <sup>59</sup>

GD3	Ceramide-reductive amination	KLH	Melanoma <sup>60,61</sup>
GM2	Ceramide-reductive amination	KLH	Melanoma <sup>62,63</sup>
GM3	Proteoliposome	OMPC	Melanoma <sup>64</sup>
Le <sup>y</sup>	CH <sub>2</sub> CH <sub>2</sub>	KLH	Ovarian <sup>39</sup>
sTn	Crotyl linker	KLH	Breast, ovarian and colorectal <sup>65-68</sup>
PSA, NP-PSA	Reductive amination	KLH	Small-cell lung <sup>69</sup>
Tn	MBS	KLH, PAM	Prostate <sup>70</sup>
TF	MBS	KLH	Prostate <sup>71</sup>
sTn	sTn(c) crotyl linker-MMCCCH	KLH	Breast <sup>72</sup>
Gb3-MUC5Ac	Gb2-norleucine-MUC5Ac-MBS	KLH	Ovarian <sup>73</sup>
GM3NPhAc	Immunotherapy based on glycoengineering	KLH	Melanoma <sup>74,75</sup>
sTnNPhAc	Immunotherapy based on glycoengineering	KLH	Epithelial cells related cancer <sup>76,77</sup>
<b>Polyvalent vaccine (pooled monomeric vaccines)</b>			
GD3, Le <sup>y</sup> , MUC1 and MUC2	(GD2)-reductive amination (Le <sup>y</sup> )-CH <sub>2</sub> CH <sub>2</sub> (MUC1, MUC2)-MBS	KLH	Melanoma Ovarian Breast <sup>78</sup>
GM2, Globo H, Le <sup>y</sup> , Tn and TF MUC1 (32mer)	(GM2)-reductive amination (Le <sup>y</sup> , Globo H)-MMCCCH (MUC1, Tn, TF)-MBS	KLH	Prostate <sup>79</sup>
GM2, Globo H, Le <sup>y</sup> , Tn, TF, sTn and MUC1	(GM2)-reductive amination (Le <sup>y</sup> , Globo H)-MMCCCH	KLH	Epithelial ovarian,

	(MUC1, Tn, TF, sTn)-MBS		Fallopian tube or peritoneal <sup>80</sup>
<b>Unimolecular polyvalent vaccine (consists of multi-antigens on unimolecule)</b>			
Globo H, Tn, sTn, TF, Ley and GM2	Diaminopropyl-MBS	KLH	Breast and prostate <sup>81-83</sup>
Globo H, GM2, Tn, sTn and TF	Diaminopropyl-MUC1-alanine-MBS	KLH	Breast <sup>84</sup>
<b>Multicomponent vaccine</b>			
Fucosyl GM1	FucGM1-norleucine-MHC II binding peptide-MBS	KLH	Small-cell lung <sup>85</sup>
Tn (Two components)	Pam3Cys-aminobutyl-di-Tn <sup>86</sup>		
Ley (two components)	Pam3Cys-peptide-(Ley) <sub>3</sub>		Ovarian <sup>87</sup>
Tn, TF or STF	Pam3CysSK4-ethylene glycol-MUC1		Breast <sup>88</sup>
Tn and TF (three-component branched)	Pan-DR epitope-Lys-MUC1-Tn-Ala-MUC1-TF		Breast <sup>89</sup>
Tn (three component)	TLR-2 ligand –Th epitope-MUC1-Tn		Breast <sup>42,43</sup>
Tn (four component)	TLR-2 ligand –CD8+ epitope (OVA257-264)-CD4+ epitope (Pan-DR)- Tn	RAFT	Breast <sup>90,91</sup>
sTnNPhAc	sTnNPhAc-linker-MPLA; immunotherapy based on glycoengineering		Epithelial cells related cancer <sup>92,93</sup>
GM3NPhAc	GM3NPhAc-linker-MPLA; immunotherapy based on glycoengineering		Melanoma <sup>45</sup>

(c): cluster; Ala: alanine; BSA: Bovine serum albumin; CRM: Diphtheria toxin mutant; Fuc: Fucosyl; Gb3: Globosyltrioside; GD: Ganglioside antigen; Globo H: Globohexaosylceramide; GM: Gangliosidoses; KLH: keyhole limpet hemocyanin; Le<sup>y</sup>: Lewis<sup>y</sup> antigen; MBS: M-maleimidobenzoyl-*N*-hydroxysuccinamide ester; MMCCH: 4-(4-*N*-maleimidomethyl) cyclohexane-1-carbonylhydrazide; MUC: Mucin; MUC5Ac: Mucin 5, subtypes A and C; NP: *N*-propionylated; NPhAc: *N*-phenyl acetyl; OMPC: Neisseria meningitidis serogroup B outer membrane protein complex; OVA: Ovalbumin; PAM: Palmitic acid; Pam3Cys: Tripalmitoyl-*S*-glyceryl-cysteinylserine; Pan-DR: CD4<sup>+</sup> T-helper epitope peptide; PSA: polysialic acid; RAFT: Regioselectively addressable functionalized template; SK4: Serine-lysine-lysine-lysine-lysine; STF: sialic Thomsen-Friedenreich; sTn: Sialyl 2-6- $\alpha$ -*N*-acetylgalactosamine; TF: Thomsen-Friedenreich; Th: T helper; Tn: 2-6- $\alpha$ -*N*-acetylgalactosamine; TT: Tetanus toxoid.

As tumor cells have multiple TACAs expressed on their surface, and in different stages of development, they have different kinds of TACAs. Therefore, polyvalent cancer vaccines containing multiple antigens is desirable for targeting transformed tumor cells. These polyvalent vaccines can be achieved by using a mixture of several monomeric vaccines individually containing one antigen<sup>78-80</sup> or a unimolecular polyvalent vaccine consisting multi-antigens<sup>81-83</sup> (**Table 1.3**). The pooled polyvalent vaccine of GD3-KLH, Le<sup>y</sup>-KLH, MUC1-KLH and MUC2-KLH with QS021 adjuvant was found to elicit strong antigen-specific IgG and IgM antibodies.<sup>78</sup> Another vaccine pooled from GM2-KLH, Globo H-KLH, Le<sup>y</sup>-KLH, TF-KLH, Tn-KLH, sTn-KLH and MUC1-KLH had similar results.<sup>80</sup> However, this mixture vaccine required more amount of carrier proteins and pre-validated vaccine component. To promote the polyvalent vaccine, the unimolecular vaccines consisting multi-antigens were developed. The KLH conjugates with Globo H, Tn, sTn, TF, Le<sup>y</sup> and GM2 was demonstrated to generate antigen-specific IgM and IgG antibodies which was similar as the other unimolecular polyvalent vaccines.<sup>81-83</sup>

Although these semi-synthetic vaccines have potential to be further used in clinic, they have some limitations. The reproducibility of protein conjugates is not consistent from batch-to-batch which induces the difficulty in safety control and quality control.<sup>9,42,43,94</sup> To further overcome these problems, some new construct of vaccines with fully-synthetic chemical structures are in development. The examples of full-synthetic carbohydrate vaccine having robust immunogenicity without the use of a protein carrier or adjuvant were composed of TACAs and an immunologically active lipopeptide, tripalmitoyl-s-glycerol-cysteinylserine (Pam3Cys).<sup>42,43,94</sup> Our group developed novel full-synthetic anti-cancer vaccine using monophosphoryl lipid A (MPLA) as a immunostimulatory carrier and build-in adjuvant and the animal studies demonstrated these vaccines induced excellent immune response, especially T cell immune response.<sup>45,92</sup>

Another strategy for new construct of vaccines was developed by Huang's group that they used tobacco mosaic virus and bacteriophage Qbeta virus-like particles as carrier to stimulate immune response.<sup>95,96</sup> The key advantage of virus-like particles carrier is the ability to present antigens in an organized and high density manner. The antigen-specific antibodies generated by these vaccines included IgG isotypes which reacted strongly with the native Tn antigens on human leukemia cells.<sup>95,96</sup>

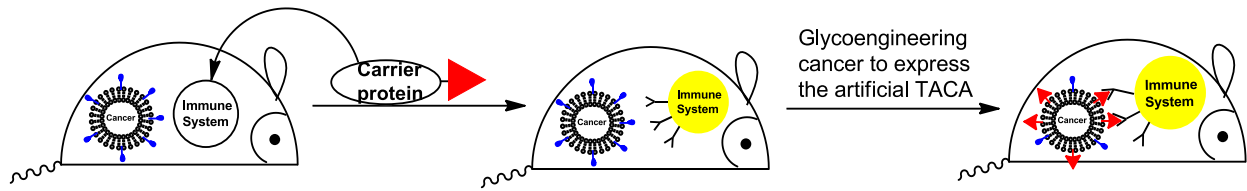
### **1.3.3 A novel cancer immunotherapeutic strategy**

Although promising, most vaccines made of natural TACAs failed in clinical trials, because of the lack of sufficient capability to generate effective immune responses.<sup>31</sup> This problem is mainly caused by the immunotolerance of TACAs resulting from the similar

structure to self-antigens that induces the absence of immunological supervision.<sup>31,36,37</sup> Therefore, an approach to break immunotolerance to TACAs is employing unnatural TACA analogs instead of natural TACAs to induce strong immune response, especially T cell-mediated immunity which is required for successful cancer therapy. This hypothesis was proved by some earlier studies that unnatural TACA analogs really provoked strong immune response including both IgM and IgG antibodies.<sup>97,98</sup> However, the antibodies elicited by the unnatural TACA analogs may have limited cross-reactivity to the natural TACAs on cancer cells which compromised the efficacy of immunotherapy. To overcome this problem, a novel immunotherapy strategy based on artificial TACA vaccine and cell glycoengineering was developed in our group.<sup>31,74,75,93</sup> This novel strategy comprised of two steps. First, a vaccine with artificial glycan resident, such as artificial sialic acid residues, will be used to stimulate the immune response including a strong T cell immunity. Immediately after immune response being elicited, a precursor (mannosamine corresponding to sialic acids) with the same artificial modification will be employed to glycoengineer the target cells and enforce the cells specifically expressing the corresponding glycan antigens on cell surface.<sup>74</sup> Subsequently, the pre-stimulated antibodies can recognized the target cells and kill them with the help of other stimulated components in immune system. **(Figure 1.3)** To carry out this novel immunotherapy strategy, our group have demonstrated that an array of cancer cells were efficiently engineered to express unnatural GM3 and sTn analogs, that is *N*-phenylacetyl GM3 (GM3NPhAc) and sTn (sTnNPhAc) by the treatment of precursor, *N*-phenylacetyl-D-mannosamine (ManNPhAc). The previous studies also revealed that vaccines based on GM3NPhAc and sTnNPhAc can provoked strong T cell-mediated immune responses



which exhibited strong and specific complements-dependent cytotoxicity (CDC) to the pre-glycoengineered melanoma cell lines.<sup>31,45,74-77,93</sup> This strategy was verified as a novel and selective cancer immunotherapy although the selectivity of this strategy for tumor *in vivo* need to be further investigated.<sup>31</sup>



**Figure 1.3** A novel immunotherapy strategy based on glycoengineering

## 1.4 Carbohydrate-Based Antibacterial Vaccines

The polysaccharides (PS), either a capsule or lipopolysaccharide, uniquely expressed on the surface of bacterial pathogens are attractive targets for anti-bacterial vaccine designs and immunotherapies.<sup>1</sup> Recently, since the antibiotic-resistant problems in traditional clinical treatment of bacterial pathogens, the development of capsular polysaccharides-based vaccines have engendered increasing excitement.<sup>20,99-101</sup> For many bacterial infections, some vaccines have been made from the fragment of bacterial capsular polysaccharide, such as *Haemophilus influenza* type b (Hib), *Neisseria meningitides* A, C, Y and W-135, and *Salmonella typhi*, and have been approved for clinical using.<sup>1</sup> (**Table 1.4**)

**Table 1.4** Examples of licensed carbohydrate-based vaccines<sup>1</sup>

Target pathogen	Vaccine	Manufacture (Trade name)
Haemophilus influenzae type b (Hib)	Glycoconjugate, polysaccharide with tetanus toxoid (TT)	Sanofi Pasteur (ActHIB, 1993); GlaxoSmithKline Biologicals (Hiberix, 1998, EU)
	Diphtheria toxoid (DT), TT and acellular pertussis adsorbed, inactivated poliovirus and Hib-TT conjugate vaccine	Sanofi Pasteur (Pentacel)
	Hib conjugate (meningococcal protein conjugate)	Merck & Co (PedvaxHIB, 1990)
	Hib conjugate (meningococcal protein conjugate) and hepatitis B (recombinant) vaccine	Merck & Co (Comvax)
	Synthetic oligosaccharides with TT conjugate	Licensed in Cuba (Quimihib 2004) <sup>33</sup>
Neisseria meningitides A, C, Y and W-135 <sup>102</sup>	Glycoconjugate, meningococcal polysaccharide with DT	Sanofi Pasteur (Menactra, 2005)
	Meningococcal polysaccharide without carrier	GlaxoSmithKline (ACWY Vax, 1981)
	Meningitidis A, C, Y and W-135 polysaccharide with CRM197	Novartis (Menveo, 2010)
Salmonella typhi	Vi capsular polysaccharide	Sanofi Pasteur (Typhim Vi, )
Streptococcus pneumoniae	Capsular polysaccharide with CRM <sub>197</sub>	Wyeth (Prevnar 13, 2000)
	Capsular polysaccharide	Merck & Co (Pneumovax 23, 1983)

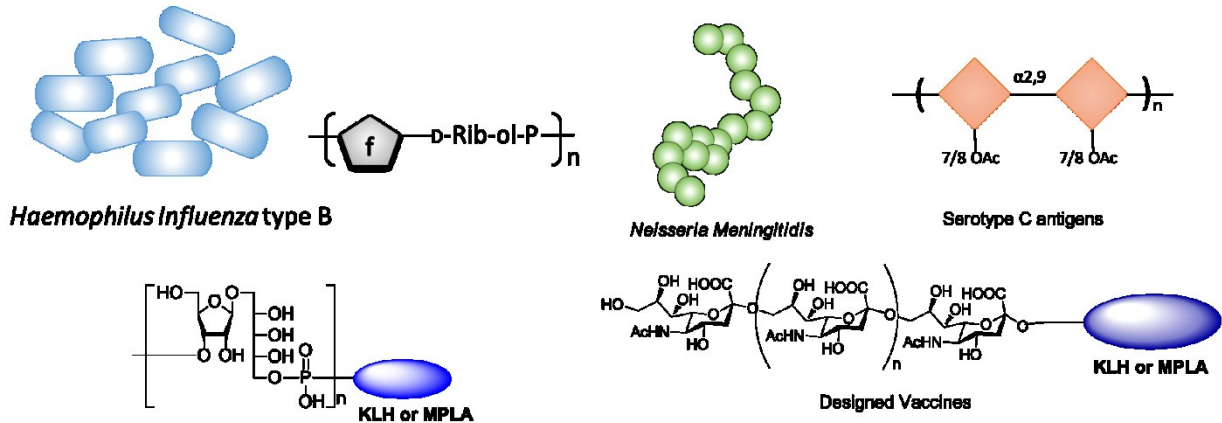
(From the US Food and Drug Administration website and [www.cdc.gov/vaccine](http://www.cdc.gov/vaccine) )

However, the heterogeneity and complexity of capsular polysaccharides cause the difficulties and complicacy in the development of efficient carbohydrate-specific vaccines.<sup>39-42</sup> Moreover, using naturally derived polysaccharides to produce protein conjugate vaccines presents the challenges in quality control and safety standards as described above. Therefore, to overcome these problems, synthetic glycans with well-characterized carrier proteins or full-synthetic vaccines with a well-defined synthetic carrier can be the future directions.<sup>33,103</sup>

The first approved full-synthetic glycan-based vaccine, Quimi-Hib, was developed in Cuba to prevent from Hib infection.<sup>33</sup> The synthetic ribosylribitol-phosphate (RRP) repeat units have been proven efficient in provoking immunity and can be accessed by large-scale good manufacturing practice production. This promising production strategy of vaccine incorporating a synthetic bacterial carbohydrate antigen reduced the safety problems which existed in the purification of saccharide fragments from bacteria culture and provided effective vaccine as already-licensed vaccine for human use. This strategy was therefore feasible and can be further applied in the development of vaccines against other pathogens.<sup>33</sup> However, the reassembling Hib polysaccharide fragments were the mixture of several RRP repeat units but not single structure.

To further study the structure-immunogenicity relationships of saccharide moieties in a conjugate vaccine and optimize a well-defined glycoconjugate vaccine, in our group, two series of oligosaccharide fragments, mimicking the antigens from Hib or *N. meningitidis*, respectively, were synthesized and were conjugated with KLH or MPLA carriers individually. The chapter 4 of this dissertation will focus on the immunological

studies of these semi-synthetic and full-synthetic anti-bacterial vaccines in animal, and the investigation of structure-immunogenicity relationships. (Figure 1.4)



**Figure 1.4** Anti-bacterial vaccines based on capsular polysaccharides

## 1.5 Conclusion

Carbohydrate antigens are attractive targets for immunotherapy and vaccine development against bacteria, fungi, virus and cancers. However, there are a lot of problems existing in the development of efficient vaccines. To overcome these problems, many approaches have been explored. In our lab, a novel immunotherapy strategy based on glycoengineering was developed to overcome the immunotolerance of TACAs. Another novel construct of vaccine based on monophosphoryl lipid A (MPLA) was explored. The achievements based on these two pioneering ideas will be exhibited in this dissertation including the investigation of glycoengineering efficiency, a series of anti-cancer vaccines based on TACAs with MPLA as carrier, and two series of anti-bacterial vaccines.

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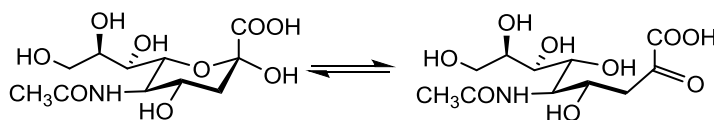
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## CHAPTER 2 Quantifying the Efficiency of Sialic Acids Metabolic Engineering

### 2.1 Introduction of Sialic Acid Metabolic Engineering

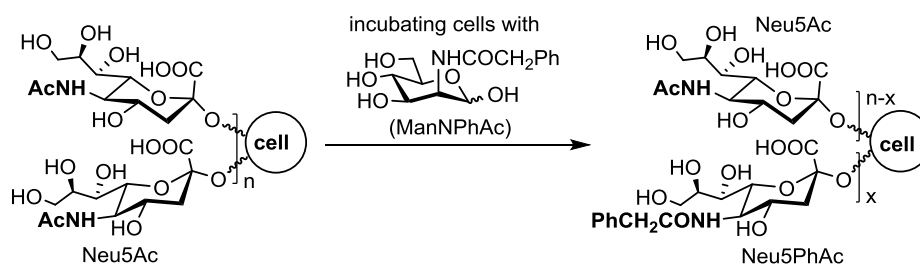
Sialic acids are a family of nine-carbon keto carbohydrates, and the most common member is *N*-acetyl neuraminic acid (Neu5Ac, **Figure 2.1**). Neu5Ac is usually present at the non-reducing end of glycans and plays important roles in various biological processes, such as specific recognition, binding and regulatory events.<sup>1,2</sup> It is also well known that Neu5Ac is typically overexpressed on the surface of cancer cells<sup>3</sup> and many of the uniquely or excessively expressed glycans on cancer cells, known as tumor-associated carbohydrate antigens (TACAs),<sup>4</sup> contain the Neu5Ac residue. Some sialo-TACAs, such as  $\alpha$ -2,8-polysialic acid, sTn antigen, sialy Lewis antigens and the GM and GD gangliosides, are abundantly expressed by a number of tumors. Therefore, sialo-TACAs are useful targets for the design and development of new strategies for cancer diagnosis and immunotherapy.<sup>5-7</sup> However, most native TACAs are poorly immunogenic and tolerated by the patients' immune system; therefore, they may not be able to induce robust antitumor immune responses, especially T cell-dependent immune responses, in cancer patients.



**Figure 2.1.** Structure of Neu5Ac

To deal with the immunotolerance problem of TACAs, we have explored a novel strategy for cancer immunotherapy based on metabolic engineering of cancer cell surface sialo-TACAs.<sup>8,9</sup> A key step of this strategy is to give cancer cells an unnatural analog of

*N*-acetyl-D-mannosamine (ManNAc), the native biosynthetic precursor of Neu5Ac, to bioengineer cancer cells to express an unnatural sialo-TACA analog<sup>10,11</sup> (**Figure 2.2**) that is more distinguishable and immunogenic than the natural TACA to enable effective cancer immunotherapy.<sup>8</sup> Using antigen-specific antibodies combined with bioassays such as ELISA and flow cytometry,<sup>12,13</sup> we have demonstrated that *N*-phenylacetyl-D-mannosamine (ManNPhAc) could metabolically engineer cancer cells to express sialo-TACA analogs, such as analogs of sTn and GM3, carrying unnatural *N*-phenylacetylsialic acid (Neu5PhAc) residues. However, these were only qualitative and semiquantitative analyses. To ultimately determine the efficiency of ManNPhAc to metabolically engineer cancer cells, a reliable method to quantify the ratio of Neu5Ac and Neu5PhAc expressed by cancer cells is required, which is the aim of this research.

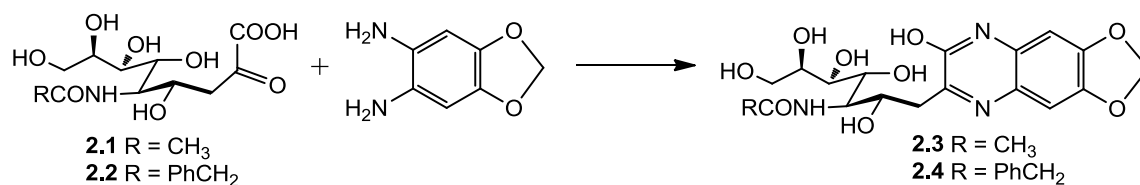


**Figure 2.2** Metabolic engineering of sialic acids on the cancer surface

## 2.2 Results and Discussion

In the literature several methods have been described for the analysis of sialic acids, such as colorimetric and fluorometric assays after derivatization with thiobarbituric acid<sup>14,15</sup> and liquid chromatography (LC) with UV,<sup>16</sup> fluorescence<sup>17-27</sup> and mass spectrometry (MS)<sup>28-32</sup> detection. Except for LC-MS, sialic acids must be derivatized to be photometrically detectable. In addition to thiobarbituric acid,<sup>14,15</sup> a number of reagents

including 3,4-diaminotolene,<sup>32</sup> 1,2-diamino-4,5-dimethoxybenzene,<sup>17</sup> *O*-phenylenediamine,<sup>20</sup> and 1,2-diamino-4,5-methylenedioxybenzene (DMB),<sup>21,22,24-29,33-35</sup> have been used for sialic acid derivatization, taking advantage of the special  $\alpha$ -keto acid functionality. Among these methods, DMB derivatization for HPLC-fluorescence and LC-MS analyses was the most popular due to the high sensitivity and selectivity of the reaction involved. In this research, we aimed at a potentially broadly applicable method that uses simple instrument. Therefore, we became interested in a method that was based on HPLC separation and UV detection. To make sialic acids UV-detectable, we planned to employ DMB for sialic acids derivatization.<sup>29,35,36</sup> As shown in **Figure 2.3**, the resulting DMB derivatives had strong maximum absorbance at 370 nm, which was utilized for quantitative analysis. Consequently, our design for the sialic acid analysis was to release the total sialic acids from cell surfaces under mild acidic conditions, derivatize them with DMB and finally subject the mixture to HPLC analysis with UV detection at 370 nm.

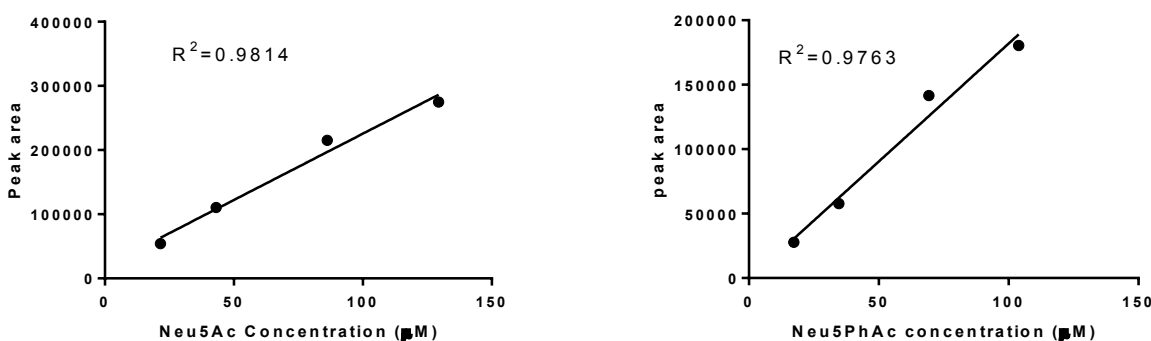


**Figure 2.3** Reactions between sialic acids and DMB and structures of the reaction products

To verify that DMB derivatization of sialic acids can be used for quantitative analysis and that the HPLC signals of corresponding sialic acid derivatives have a linear relationship with the concentrations of Neu5NAc and Neu5PhAc and to establish proper calibration curves, different concentrations of standard Neu5NAc and Neu5PhAc samples



were treated with DMB and then applied to HPLC-UV analysis. It was revealed that the reaction was clean and complete and the HPLC retention times (C18 column, 0.5  $\mu\text{m}$ , 250  $\times$  4.6 mm; eluted with water and acetonitrile containing 0.1% trifluoroacetic acid (TFA): acetonitrile concentration changed from 5% to 25% in 10 min, kept at 25% for 10 min, then from 25% to 70% in 15 min; flow rate: 1mL/min) for the Neu5Ac and Neu5PhAc derivatives **2.3** and **2.4**, which were confirmed with MS, were 9.87 min and 15.45 min, respectively. As shown in **Figure 2.4**, under the tested conditions (i.e., Neu5Ac and Neu5PhAc concentrations in the range of 15-130  $\mu\text{M}$ ), the observed HPLC peak areas of **2.3** and **2.4** were in good linear relationship (coefficient: 0.9814 and 0.9763, respectively) with the Neu5Ac and Neu5PhAc concentrations in the samples. These results have demonstrated that the method could be used for the quantification of Neu5Ac and Neu5PhAc. Equations, HPLC peak area = 2067 [Neu5Ac] + 18,835 and HPLC peak area = 1832 [Neu5PhAc] – 1,141, were used to calculate the concentrations or quantities of Neu5Ac and Neu5PhAc in samples, respectively.



**Figure 2.4** A linear correlation between the peak areas of Neu5Ac or Neu5PhAc derivative and the concentrations of Neu5Ac or Neu5PhAc was demonstrated. The coefficient of determination ( $R^2$ ) is displayed for each graph.

For the analysis of biological samples derived from cells, there are several issues that may be of concern. First, the detachment of sialic acids from the cell surface may not be complete, which can affect the observed amount of sialic acids. However, this is a systematic error that may affect the sample recovery but not the ratio of Neu5Ac and Neu5PhAc, the latter of which is actually our focus. Second, the collected sialic acids need to be derivatized before analysis, while under the condition that the samples contain numerous impurities, whether the derivatization reaction will be affected is another question, despite that the above experiments have proved the reaction efficiency in the absence of impurities. Third, samples derived from metabolically engineered cells will contain variable ratios of Neu5Ac and Neu5PhAc, which can be immensely different. Whether this can be accurately reflected is another question.

To address these issues, we then probed the reliability and accuracy of the proposed method under more complex situations, such as for the analysis of biologic samples or samples with big differences in the Neu5Ac and Neu5PhAc concentrations. To probe the reliability of the method, we added different amounts of Neu5PhAc in the culture of SKMEL-28, a melanoma cell line, and then examined its recovery upon a series of treatments involved in the real sample analysis, including incubation with acetic acid employed to detach sialic acids from cells, lyophilization, reaction with DMB, and eventually HPLC analysis. As shown in **Table 2.1**, the recovery rates of the externally added Neu5PhAc in various samples were excellent (97-99%, average  $98\pm 1\%$ ). In this study, the recovery of Neu5Ac from cells was not examined, as the added Neu5Ac would interfere with naturally existing Neu5Ac. However, we anticipated that its recovery rate should not be lower than that of Neu5PhAc. To probe the accuracy of the method, we

prepared a series of Neu5Ac and Neu5PhAc mixtures in ratios ranging from 100:0 to 100:8 and applied them to analysis by the proposed method. As shown in **Table 2.2**, the experimental results were in good agreement with the expected ratios, and in average the accuracy was above 93%. These results have clearly demonstrated the reliability and accuracy of the proposed method for the analysis of Neu5Ac and Neu5PhAc expressed by metabolically engineered cells.

**Table 2.1** Recovery analysis of Neu5PhAc from the SKMEL-28 cell culture

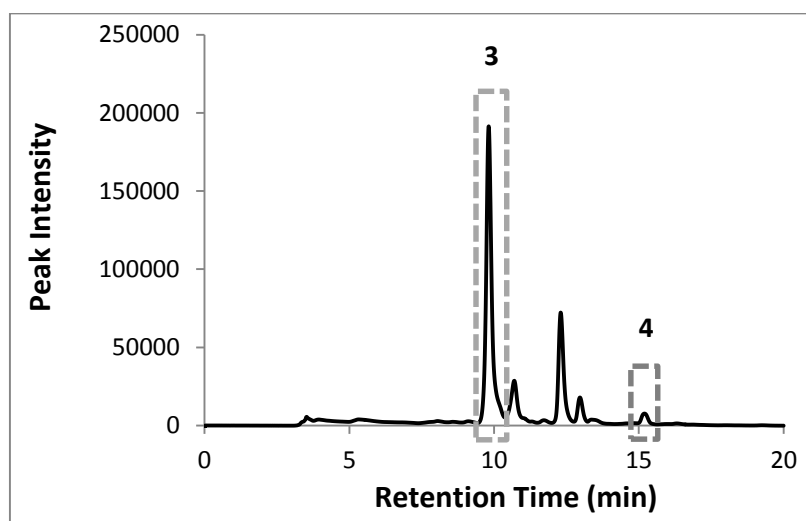
Added Neu5PhAc (nmol)	65	130	130	130
Detected Neu5PhAc (nmol)	63	128	127	129
Recovery rate (%)	97	98	98	99

**Table 2.2** Analysis of Neu5Ac and Neu5PhAc mixtures prepared in different ratios

Sample number	1	2	3	4	5
Standard samples Neu5Ac:Neu5PhAc	100:0	100:2	100:4	100:6	100:8
Experimental results Neu5Ac:Neu5PhAc	100:0	100:1.9( $\pm 0.1$ )	100:3.7( $\pm 0.1$ )	100:5.9( $\pm 0.3$ )	100:7.1( $\pm 0.4$ )

After the proposed method was verified with standard samples under different conditions, it was applied to the analysis of metabolically engineered cancer cells. In these studies, SKMEL-28 cells were incubated with various concentrations of ManNPhAc (0, 5, 10, 20, and 30 mM) for 2-4 days. Then, *ca.*  $1.0 \times 10^6$  cells were harvested from each culture, washed, and treated with 2 M acetic acid at 80 °C for 2 h to release sialic acids from cells. This was followed by lyophilization and reaction with excessive DMB at 50 °C for 3 h.<sup>29</sup> After the reaction mixtures were filtered off through a 0.22  $\mu$ m film, they

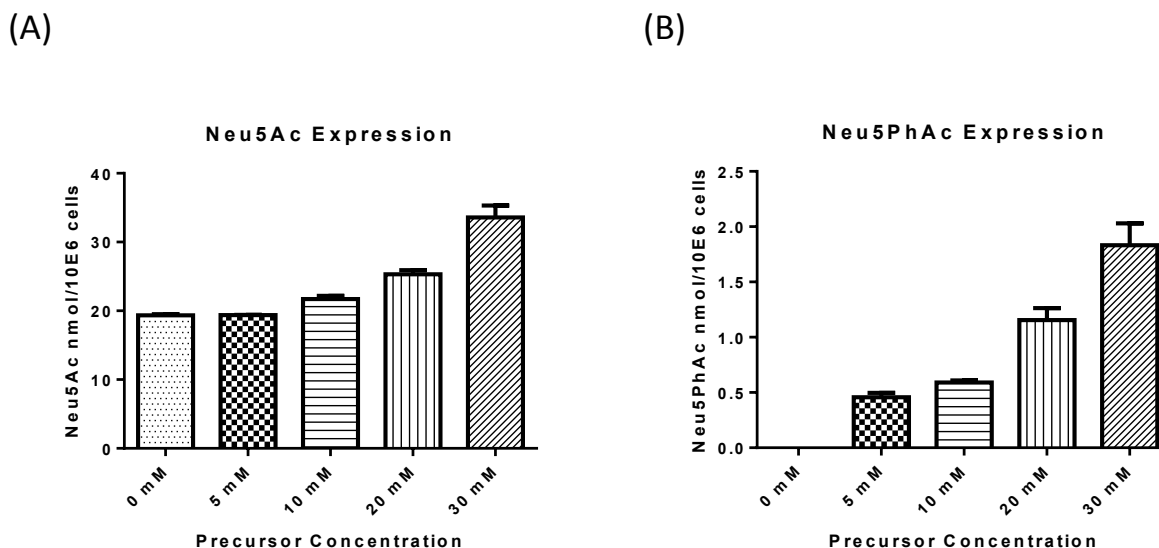
were subjected to analysis by RP-HPLC using gradient eluents of water and acetonitrile containing 0.1% TFA. **Figure 2.5** shows the typical HPLC diagram of a sample. As labeled in the figure, the DMB derivatives of Neu5Ac and Neu5PhAc **2.3** and **2.4** had the same retention times as that of the standard samples. To further prove their identity, **2.3** and **2.4** were collected and subjected to MALDI-MS analysis, which gave the correct masses [m/z: calculated, 425.2 and 501.2; observed, 426.3 and 502.5 (M+H<sup>+</sup>), respectively]. Evidently, the samples also contained a small number and amount of other impurities, which appeared at different positions and did not affect the analysis.



**Figure 2.5** HPLC diagram of a sample derived from  $10^6$  SKMEL-28 cells treated with 30 mM of ManNPhAc for 3 days. The retention times for the DMB derivatives of Neu5Ac and Neu5PhAc **2.3** and **2.4** were about 9.90 and 15.50 min, respectively.

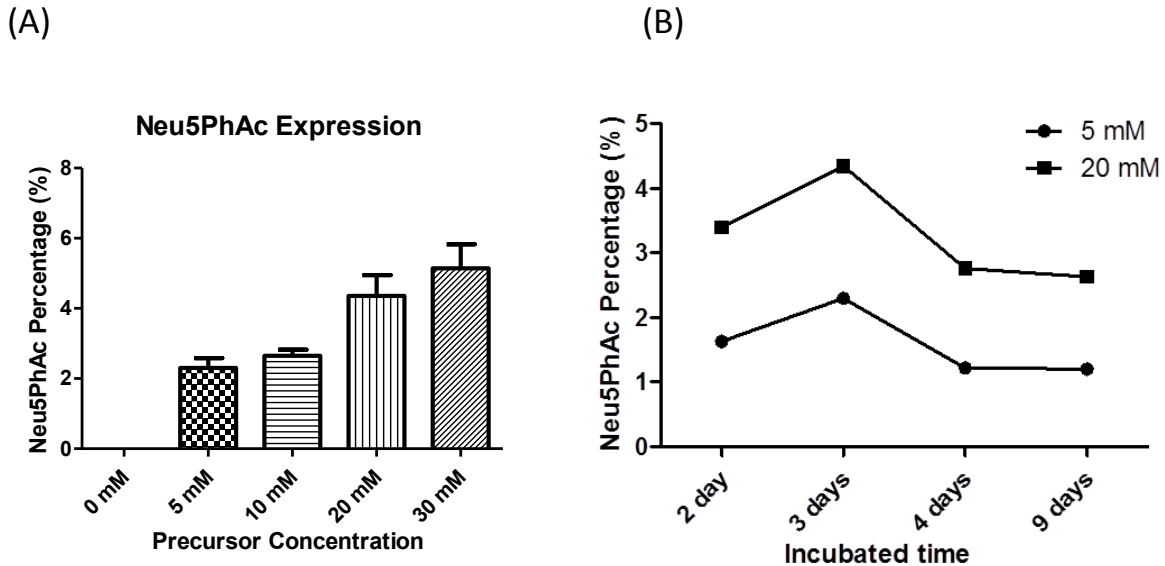
The amounts of Neu5Ac and Neu5PhAc in samples obtained from  $10^6$  SKMEL-28 cells were calculated based on the HPLC peaks of **2.3** and **2.4** according to the standard curves shown in **Figure 2.4**, and the results are presented in **Figure 2.6**. As anticipated, significant Neu5PhAc expression was observed for cells incubated with 5 mM of

ManNPhAc (**Figure 2.6B**), and the amount of expressed Neu5PhAc increased proportionally with the increase of ManNPhAc concentration in the cell culture. However, Neu5PhAc expression was still much lower than that of Neu5Ac even at a quite high concentration (30 mM) of ManNPhAc. Furthermore, it was found that natural Neu5Ac expression was also elevated in the presence of high concentrations of ManNPhAc. Typically, one should expect that natural product biosynthesis would be depressed on the addition of an unnatural biosynthetic precursor due to either competition or inhibition. In contrast, it was very interesting to notice that the presence of ManNPhAc seemed to promote not only Neu5PhAc expression but also Neu5Ac biosynthesis, which is unique and can be of biological significance, so it is worthy further investigation. Conclusively, the results suggested that ManNPhAc could effectively glycoengineer SKMEL-28 cells to express unnatural Neu5PhAc in a concentration-dependent manner in the range of 5-30 mM, whereas Neu5Ac expression was only slightly affected under the same condition. It was also evident that only a small fraction of Neu5Ac on the cell surface was substituted with Neu5PhAc upon metabolic engineering using ManNPhAc, a process that had to compete with the endogenous biosynthetic pathway.



**Figure 2.6** The amounts of Neu5Ac (A) and Neu5PhAc (B) expressed by SKMEL-28 cells ( $10^6$ ) that were incubated with 0, 5, 10, 20 and 30 mM of ManNPhAc for 3 days, determined by HPLC. Error bar represents the standard deviation of three parallel experiments.

To further define the efficiency of cell metabolic engineering using ManNPhAc, the percentage of Neu5PhAc expressed on the cell surface in terms of total sialic acids was calculated according to the following equation,  $\text{Neu5PhAc\%} = \frac{\text{Neu5PhAc}}{\text{Neu5Ac} + \text{Neu5PhAc}} \times 100\%$ , and the results are shown in **Figure 2.7(A)**. The percentage was about 2.2% and 5.8% for cells incubated with 5 mM and 30 mM of ManNPhAc for 3 days, respectively.



**Figure 2.7** The percentages of Neu5PhAc expressed on SKMEL-28 cells incubated with 0, 5, 10, 20 and 30 mM of ManNPhAc for 3 days (A), and the percentages of Neu5PhAc on SKMEL-28 cells incubated with 5 and 20 mM of ManNPhAc for 2, 3, 4 and 9 days (B), respectively. The error bar represents the standard deviation of three parallel experiments.

Finally, we examined the influence of incubation time on the cell engineering efficiency. In these studies, we incubated cancer cells with 5 mM and 20 mM of ManNPhAc for 2, 3, 4 and 9 days, and then examined their Neu5PhAc expression. The results (**Figure 2.7B**) indicated that the percentage of Neu5PhAc increased during the first 3 days to reach peak values at day 3 and then decreased slightly to retain a consistent level of Neu5PhAc after day 4. The reason for decreased Neu5PhAc expression after day 3 was not clear, but it might be due to the change of metabolic activities of the cell, as we found that after 3 days of incubation cell proliferation and growth became slower. Nevertheless, it was ultimately disclosed that cancer cells could be effectively engineered

to express a significant level of unnatural Neu5PhAc to make the cells distinct and that this could persist for a long period.

### 2.3 Conclusion

We have developed a convenient and reliable method for quantitative analysis of sialic acids expressed by cells. This method was based on the specific reaction between DMB and the  $\alpha$ -keto acid functionality of sialic acids to generate products that had strong UV absorption, enabling easy HPLC analysis with UV detection. After the consistency and accuracy of this method were verified with standard samples under various conditions, it was used to analyze metabolically engineered cancer cells, which expressed both natural Neu5Ac and unnatural Neu5PhAc.

The protocols for this analysis were rather straightforward. After incubation with ManNPhAc, cells were subjected to acetic acid-promoted hydrolysis to release sialic acids on the cell surface. Then, the sample was lyophilized and treated with DMB. The reaction mixture was directly applied to HPLC analysis to determine the amounts and ratio of Neu5Ac and Neu5PhAc. The method is not only applicable to the analysis of Neu5Ac and Neu5PhAc but also generally useful for the analysis of other sialic acids.

While this is a simple, reliable and quantitative method, it does have limitations. For example, it is not easily applicable to the analysis of cells incubated with  $\mu\text{M}$  concentration of ManNPhAc, probably because the Neu5PhAc expression level was too low. However, Neu5PhAc expression on these cells was detected by flow cytometry with the help of antibody-labeling.<sup>13</sup> Evidently, flow cytometry is more sensitive, albeit not precisely quantitative, than HPLC analysis. Another problem for the flow cytometry method is that it relies on antigen-specific antibodies that are not always readily available.



Therefore, a combination of these two analytical methods can portrait the whole picture concerning the metabolic engineering of cell surface sialic acids with unnatural ManNAc derivatives as biosynthetic precursors.

This research has revealed for the first time that more than 2% of Neu5Ac was substituted for Neu5PhAc upon metabolic engineering with ManPhNAc. We have previously shown by means of flow cytometry<sup>13</sup> and antibody-mediated cytotoxicity<sup>37</sup> that cancer cells could be engineered to express Neu5PhAc at low  $\mu\text{M}$  concentrations (20-50  $\mu\text{M}$ ) of ManNPhAc. Under these conditions, the expression level of Neu5PhAc should be much lower than 2%, but the engineered cells could be recognized by Neu5PhAc-specific antibodies and undergo functional immune reactions. This information is useful not only for our immunotherapy but also for other studies that are based on metabolic engineering of cell surface sialic acids or other sugars. A particularly interesting but unexpected discovery was that natural sialic acid expression also increased in the presence of ManNPhAc, which could be only disclosed from quantitative analysis. This may be of biological significance, as sialic acid expression is related to various biological activities, and it is worthy further investigation.

## 2.4 Experimental Section

### Materials, reagents and animals

SKMEL-28 cell line, Dulbecco's Modified Eagle's Medium (DMEM) for cell culture, and fetal bovine serum (FBS) were purchased from American Type Culture Collection (ATCC). Penicillin-streptomycin and trypsin-EDTA were purchased from Invitrogen.

ManNPhAc was synthesized by a procedure previously reported by our laboratory.<sup>13</sup> DMB, sodium hydrosulfite,  $\beta$ -mercaptoethnal and other chemicals were purchased from Sigma.

### **Metabolic engineering of tumor cell**

SKMEL-28 cells were cultured in DMEM containing 10% of FBS, 1% of penicillin-streptomycin and various concentrations (0 to 30 mM) of ManNPhAc at 37 °C. After a certain number of days of incubation, cells were harvested upon treatment with trypsin-EDTA solution, and finally washed twice with PBS to be readied for further analysis.

### **Hydrolysis of sialic acids**

A cell sample (containing about  $1.0 \times 10^6$  cells) was dissolved in a 2 M solution of acetic acid (2 mL). The mixture was stirred at 80 °C for 2 h, and then lyophilized to get a powder product that contained sialic acids released from cells.

### **DMB derivatization of sialic acids and HPLC analysis**

The powder product obtained above was mixed with DMB (0.5 mg, excessive) in 1.4 M acetic acid containing 18 mM of sodium hydrosulfite and 0.75 M of  $\beta$ -mercaptoethnal.<sup>29</sup> After the reaction mixture was stirred at 50 °C for 3 h, it was filtered off through a 0.22  $\mu$ m film, and the filtrate was analyzed by RP-HPLC (C18 column, 250  $\times$  4.6 mm, 0.5  $\mu$ m) using gradient eluents of water (A) and acetonitrile (B) containing 0.1% TFA at flow rate of 1 mL/min. Elution started with 5% B, which was increased gradually to 25% B in 10 min. Elution of the column with 25% B was kept for 10 min, followed by gradual increase of B composition to 70% within 15 min, and the elution was kept under this condition to the end of an experiment. Finally, the column was washed and balanced

with 5% B for 40 min before the next experiment. The eluents were monitored with a UV detector at 370 nm, at which wavelength DMB derivatives of sialic acids had the maximum absorption. All of the HPLC peaks were separately collected, and the fractions containing the anticipated DMB derivatives of sialic acids were further analyzed and verified by MALDI-MS.

### **Establishment of standard calibration curves and equations used to quantify Neu5Ac and Neu5PhAc**

A Neu5Ac or Neu5PhAc standard sample (1, 2, 4 or 6  $\mu\text{g}$ ) was mixed with a DMB reaction solution (150  $\mu\text{L}$  containing 0.5 mg DMB, excessive) in 1.4 M acetic acid, 18 mM of sodium hydrosulfite and 0.75 M of  $\beta$ -mercaptoethnal. The reaction mixture was stirred at 50 °C for 3 h, and then filtered off through a 0.22  $\mu\text{m}$  film. The filtrate (10  $\mu\text{L}$ ) was analyzed by RP-HPLC (C18 column, 250  $\times$  4.6 mm, 0.5  $\mu\text{m}$ ) using gradient eluents of water and acetonitrile containing 0.1% TFA at flow rate of 1 mL/min as described above. The eluents were monitored with a UV detector at 370 nm. The peak areas were plotted against the sample concentration to obtain the calibration curves and equations.

### **Determining the recovery rate of Neu5PhAc from cell culture**

SKMEL-28 cell was cultured, harvested, and washed twice with PBS. To the cell sample (about  $1.0 \times 10^6$  cells, in 1 mL PBS solution) was added 65 or 130 nmol of a Neu5PhAc standard sample. The cell sample was added to an acetic acid solution (final volume 2 mL and final concentration 2 M). The mixture was stirred at 80 °C for 2 h. The reaction mixture was lyophilized to obtain a powder product that was reacted with a DMB solution (150  $\mu\text{L}$ , containing 0.5 mg DMB) in 1.4 M acetic acid containing 18 mM of sodium

hydrosulfite and 0.75 M of  $\beta$ -mercaptoethnal. The reaction mixture was finally subjected to HPLC analysis as described above.

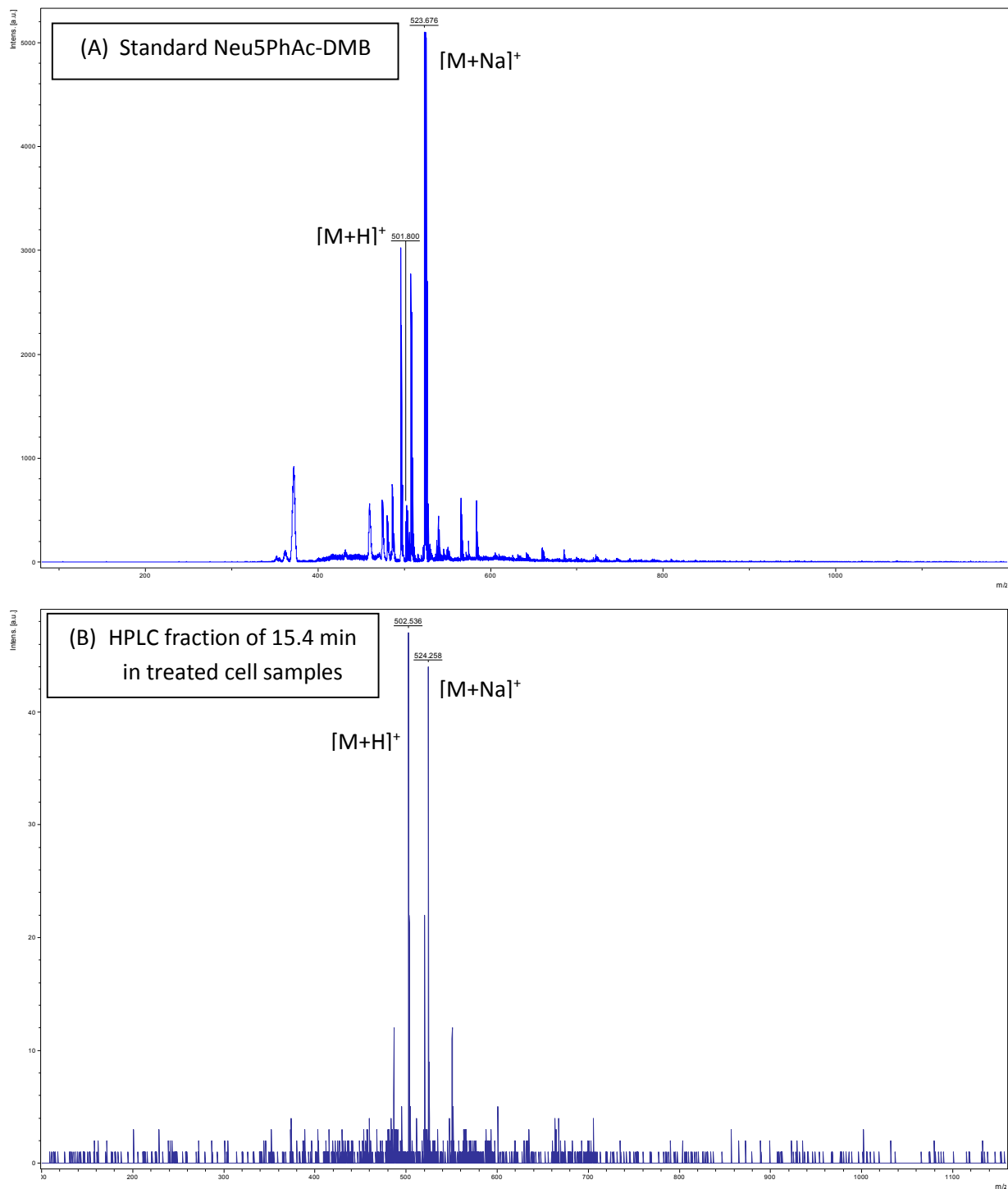
### **Determining the Neu5Ac and Neu5PhAc ratios in standard samples**

A series of standard samples containing different ratios of Neu5Ac and Neu5PhAc (100:0, 100:2, 100:4, 100:6, 100:8 mol/mol) were prepared. About 20 nmol of each sample was added to a DMB solution (150  $\mu$ L, containing 0.5 mg DMB) in 1.4 M acetic acid containing 18 mM of sodium hydrosulfite and 0.75 M of  $\beta$ -mercaptoethnal. After the reaction mixture was stirred at 50 °C for 3 h, it was filtered off through a 0.22  $\mu$ m film, and 10  $\mu$ L of the filtrate was finally subjected to RP-HPLC analysis as described above.

## **2.5 Appendices**

### **Mass Spectrum Confirmation**

The fraction of 15.45 min of HPLC was collected to be further confirmed by MALDI-TOF MS (**Figure 2.8**). In the standard Neu5PhAc-DMB mass spectrum, the MALDI peaks, 501.800 ( $M+H^+$ ) and 523.676 ( $M+Na^+$ ) confirmed that the standard Neu5PhAc was successfully labeled with DMB. In the HPLC fraction of 15.4 min collected from cell samples treated with precursor, the peaks, 502.536 ( $M+H^+$ ) and 524.258 ( $M+Na^+$ ), confirmed that the cells expressed the modified sialic acid as expected. The mass error was due to the system shift of  $m/z$  value in MALDI machine.



**Figure 2.8** MALDI MS of Neu5PhAc-DMB: (A) Sample was from reaction solution of standard Neu5PhAc after DMB derivatization; (B) HPLC fraction sample of 15.4 min in the cell samples which was treated with 20 mM precursor ManNPhAc for 2 days.

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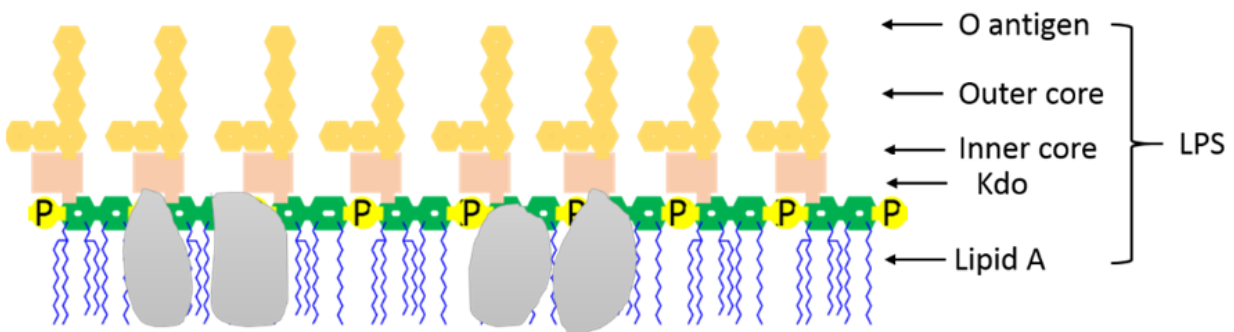


## CHAPTER 3 Development of Anti-Cancer Vaccines Using Monophosphoryl Lipid A as a Carrier Molecule and Built-in Adjuvant

### 3.1 Introduction of Monophosphoryl lipid A (MPLA)

#### 3.1.1 Lipopolysaccharide

The endotoxins were first found in heat-killed bacteria *Vibrio cholera* by Richard Pfeiffer and Robert Koch in the nineteenth century and were associated primarily with Gram-negative bacteria.<sup>1</sup> They are distinguished from exotoxin because the former are not secreted to the culture media by bacteria but only released upon lysis of bacteria cells. The principal endotoxin, lipopolysaccharide (LPS) which is a family of complex glycolipid, is the major component of the outer monolayer of Gram-negative bacteria cell outer membrane.<sup>2</sup> LPS was discovered as a heat-stable and amphiphilic components containing a hydrophobic lipid A as a membrane anchor domain, an oligosaccharide 'core', and a distal polysaccharide (**Figure 3.1**).<sup>3</sup>



**Figure 3.1** The structure of outer layer of outer membrane of Gram-negative bacteria.

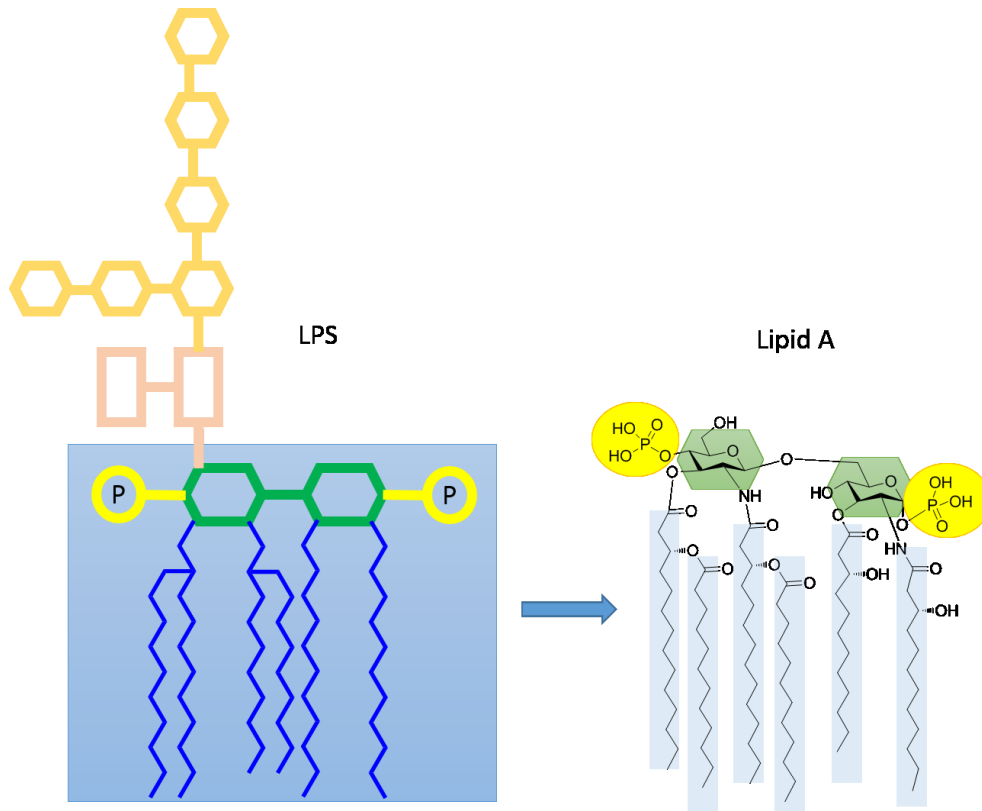
The LPS layer of the outer membrane plays important roles in bacteria activities, such as reacting to the changes of environment and inhibiting toxic compounds (e.g., antibiotics) to protect the bacteria cell, working for nutrient transport, and mediating interaction with the host.<sup>4</sup> The outer membrane with LPS can associate with divalent cations to provide an effective permeability barrier for the bacteria cell against external stress factors.<sup>5-7</sup> Moreover, LPS has been demonstrated to be an extremely powerful immunostimulators and elicit toxic effects by inhibiting cellular functions or killing host cells. According to present knowledge about LPS, it interacts with various types of host cells including mononuclear cells, endothelial cells, smooth muscle cells, polymorphonuclear granulocytes, thrombocytes and so on through its lipid A component. Consequently, LPS has engendered considerable interests for antibiotics development because of its important role in antibiotics resistant and for immunotherapy development because of its strong immunostimulatory ability.

### 3.1.2 Lipid A structure-activity relationships

In general, the lipid A domain of LPS determines its principal immunostimulatory ability because lipid A itself is a potent toxin that can be specifically and sensitively recognized by immune system.<sup>8</sup> Lipid A binds specifically to the toll or interleukin-1 receptor domain-containing adaptor-inducing interferon- $\beta$  (TRIF) of toll-like receptors (TLRs) in association with MyD88 to induce a downstream signaling cascade, stimulate the release of cytokines and chemokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6 and interferon- $\beta$  (IFN- $\beta$ ), and upgrade immune cell

expression.<sup>9</sup> Although the immunostimulatory activity of lipid A can be extremely useful, its pro-inflammatory and septic properties are a serious problem.<sup>10</sup>

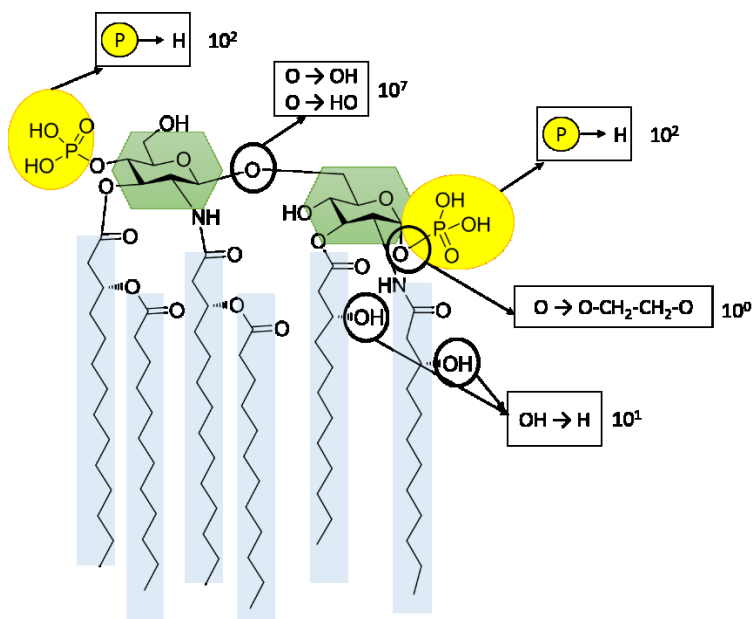
Regardless of the source, all lipid As contain the highly conserved construct as illustrated in **Figure 3.2**. They have several lipids, varying considerably in chain length, saturation, number and distribution, linked to the *N*-2-, *N*-2'-, *O*-3- and *O*-3'- positions and two phosphate groups linked to the *O*-1 and *O*-4'- positions of the  $\beta$ -1,6-linked disaccharide of D-glucosamine (GlcNH<sub>2</sub>).



**Figure 3.2** An example of lipid A structure.

Biological assays demonstrated that the number, structure and position of lipids and the degree of phosphorylation are important factors determining the bioactivity of lipid A.<sup>2,3</sup> To learn the structure-activity relationships of lipid A, studies were performed using

a murine macrophage cell line and human peripheral monocytes. The mediator-inducing capacity of various lipid A derivatives carrying synthetic partial structures were quantitatively analyzed. The results are schematically summarized in **Figure 3.3**.<sup>2-4</sup>



**Figure 3.3** Schematic representation of lipid A structure-activity relationships.

As showed in **Figure 3.3**, once one of the phosphoryl groups in lipid A was removed, the bioactivity was reduced significantly (by a factor of  $10^2$  as compared to parent lipid A). The resulting monophosphoryl lipid A (MPLA) derivatives still had strong immunostimulatory ability but much reduced toxicity, thus they can be very useful. The monosaccharide derivatives had the least activity (reduction of activity by a factor of more than  $10^7$ ). In addition, substituting the  $\alpha$ -anomeric phosphate group with the  $\beta$ -anomer significantly reduced the bioactivity, and modifications in the hydrophobic region of lipid chains had dramatic effects as well.<sup>2-4</sup>

The number of lipid chains also affected the bioactivity significantly.<sup>2-4,11</sup> A typical lipid A that exhibits the full spectrum of immunological and endotoxic activities consists of

six lipids that can be distributed symmetrically (3+3), such as in the lipid A of *Neisseria meningitides*, or asymmetrically (4+2), such as in the lipid A of *Escherichia coli*, on the two GlcNH<sub>2</sub> units. Abstraction of one acyl group (resulting in pentaacyl lipid A) or addition of a fatty acid (resulting in heptaacyl lipid A) reduced the bioactivity by a factor of 10<sup>2</sup>. However, abstraction of two acyl groups (resulting in tetraacyl lipid A) caused complete loss of the mediator-inducing capacity (a factor of > 10<sup>7</sup>). Therefore, tetraacyl lipid A was an antagonist for the signaling activation rather than an agonist. The hydroxide group in the lipid chains also affect the immunostimulatory activity of lipid A by about a factor of 10<sup>1.2,4</sup>

To further investigate the action mechanisms and the structure-activity relationships of lipid A, its interaction with the toll-like receptor 4 (TLR4)-MD2 signaling pathway that is activated by LPS or lipid A binding was analyzed.<sup>12</sup> Most lipid A molecules can be detected at picomolar levels by TLR4, a receptor of the innate immune system, present on macrophages and endothelial cells. TLR4 is a class 1 transmembrane receptor with an extracellular domain, a single membrane spanning helix and a globular cytoplasmic domain, the TIR (Toll interleukin-1 receptor) domain. Another important lipid-binding protein is MD2 which can form a TLR4-MD2 heterodimer.<sup>13</sup> Binding of LPS to TLR4-MD2 is mediated by the intercalation of the acyl chains of lipid A into the hydrophobic core of the MD2 β-sandwich pocket that is similar to that formed by the immunoglobulin domains of antibody molecules.<sup>11-19</sup>

Generally, hexa-acyl lipid A is a TLR4 agonist, whereas tetra-acyl lipid A that has different binding conformation with MD2 is an antagonist.<sup>18,19</sup> For the tetra-acyl lipid A, two of the acyl chains, but four for hexa-acyl lipid A, can fully extended into the binding

pocket of MD2, while two are bent in the center. The tetra-acyl lipid A-MD2 ligand does not induce a conformational change in the receptor, which makes it an antagonist. The rest of lipid A, including the diglucosamine backbones, is fully exposed.<sup>17</sup> The MD2-LPS complex binds TLR4 to form a higher order structure, creating an activation cluster that stimulates the following MyD88-dependent signal pathway. This signal pathway finally leads to production of inflammatory cytokines such as IL-12, IL-1, IL-6 and TNF $\alpha$ .<sup>20</sup>

In conclusion, LPS, especially its lipid A domain, has potent immunostimulatory ability that can be very useful for various immunological studies and immunotherapeutic applications. However, it is worth mentioning that the structure-activity relationship studies of lipid A were performed *in vitro* instead of *in vivo*.

### 3.1.3 Monophosphoryl lipid A

From the structure-activity relationship investigations described above, it is clear that the two phosphate groups in lipid A are essential for its endotoxic activity. However, modification of its O-1-position, for example, removal of the phosphate group at this position to generate monophosphoryl lipid A (MPLA), was shown to greatly reduce its endotoxicity (at most only 0.08% of toxicity remains)<sup>21</sup> with limited impact (by a factor of  $10^2$ )<sup>4</sup> on its immunostimulatory activity. As a result, MPLA has emerged as a potential adjuvant used in human vaccine formulation.<sup>22</sup> The clinical grade of MPLA, called MPL adjuvant<sup>TM</sup> manufactured by GlaxoSmithKline showed approximately 0.1% the toxicity of LPS in pre-clinical rabbit pyrogenicity assays.<sup>23</sup> MPL adjuvant is usually added to an adjuvant system, such as alum and water-oil form adjuvant, instead of replacing the other adjuvant. For example, MPL adjuvant has been widely used in adjuvant systems, such as AS01, AS02, and AS04. AS04 is a licensed adjuvant system containing MPLA and

alum, which elicits a major immune reaction including antibodies and Th1 immune responses (**Table 3.1**).

**Table 3.1** Adjuvant types and their mechanisms of action

Adjuvant type	Examples	Mechanisms of action
<b>Type A</b>	MPLA	Agonists for TLRs, indirectly, activating APCs and triggering the secretion of cytokines <sup>22</sup>
<b>Type B</b>	Alum, MF59, liposomal adjuvants	Non-specific adjuvant, enhancing antigen presentation by building a depot at the injection site, leading to a high local antigen concentration and improving uptake by APCs <sup>24,25</sup>
<b>Type C</b>	Freud's adjuvant, nanoparticles, toxin-derived adjuvant, flagellin	Enhancing signal, co-stimulatory molecular, a novel super agonist, directly stimulating T cells, inducing cytokine storm <sup>26</sup>

### 3.1.4 Introduction to adjuvants

Adjuvants are materials that can improve immune responses. This adjuvant idea was first recognized by William Coley who employed bacterial products to treat cancer patients.<sup>3</sup> Typically, there are three types of adjuvants (**Table 3.1**). MPLA (formulated in AS04) is a type A adjuvant that stimulates TLR4 signal pathway and triggers a polarized Th1 response and has been approved for clinical application in Europe.<sup>27,28</sup>

Currently, there are four licensed adjuvants in human clinical application: Alum, MF59, AS03, and AS04.<sup>29</sup> Despite that these adjuvants have been widely utilized in clinic (**Table 3.2**), there is still an urgent need of new adjuvants for usage with relatively immunologically challenging vaccine, such as cancer vaccines. More importantly, new

adjuvants are needed for the design and development of novel constructs of vaccines. There are also commercial adjuvants, such as Freund's complete/incomplete adjuvant (CFA/IFA), Titermax Gold adjuvant, QS21 and so on, which are widely used in animals and in research laboratories but not in human.<sup>27</sup>

**Table 3.2** Licensed adjuvants<sup>28,30</sup>

Adjuvant	Immune active component	Pattern recognition receptors (PRRs)	Major immune response	Vaccine
Alum	Aluminum salts	NLRPs inflammasome	Ab. Th2(mice) Ab. Th1+Th2 (humans)	Various
MF59	Squalene in emulsion of oilwater	Pro-inflammatory responses	Ab. Th1+Th2	Influenza/ pandemic flu
AS03	Squalene in emulsion of oil-water + a tocopherol	Pro-inflammatory responses	Ab. Th1+Th2	Pandemic influenza
AS04	MPL +Alum	TLR4+NLRP3 inflammasome	Ab. Th1	HBV (Fendrix), HPV (Cervarix)
Liposomes	Oil-water emulsion			HAV, Flu

Ab: antibodies; Alum: aluminum hydroxide; AS03: Adjuvant System 03; AS04: Adjuvant System 04; HBV: hepatitis B virus; HPV: human papillomavirus; MPL: monophosphoryl lipid A; NLRP3: nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing 3; Th: helper cells; TLR4: Toll-like receptor 4.

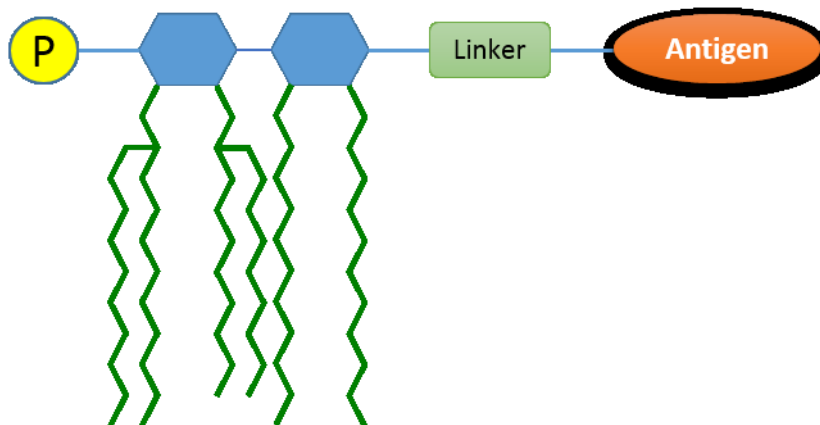
### 3.1.5 MPLA-based full-synthetic vaccine

In addition to the advantages of semi-synthetic glycoconjugate vaccine described in chapter 1, full-synthetic vaccines with a well-defined structure have more improvements.



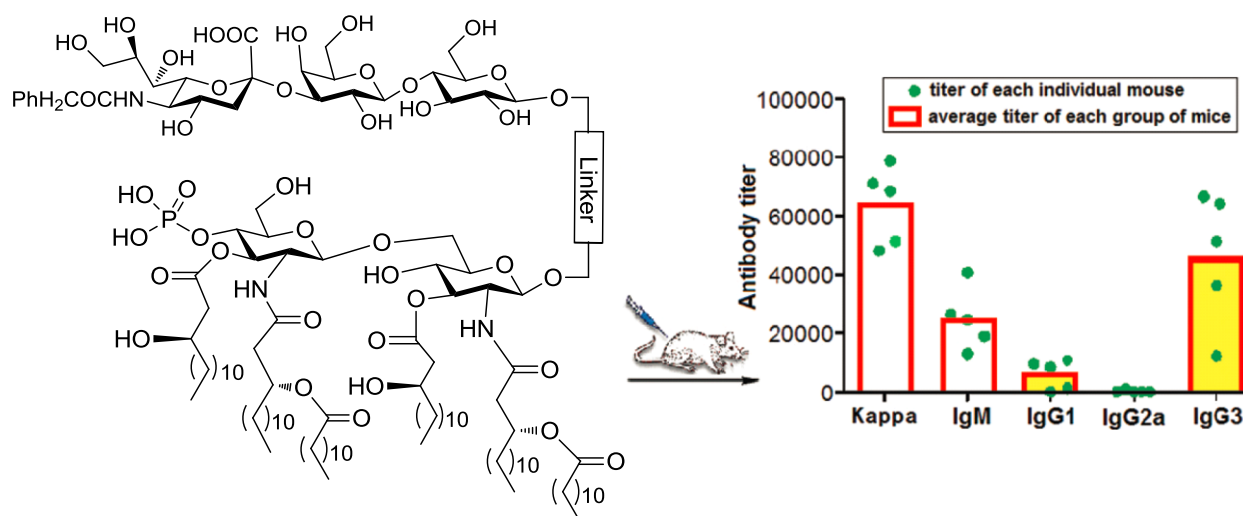
Firstly, glycan-protein conjugates usually have non-consistent loading of saccharides which induces the difficulties in quality control. But the full-synthetic vaccines will have consistent composition. In addition, the protein conjugates always need adjuvant for stimulating the immune response, while the full-synthetic vaccines could have a built-in adjuvant which will be more convenient to preparation and be further improved in vaccine design. With the help from the technology of organic synthesis, the full-synthetic vaccines have much stronger potential to provide immunological prevention against diseases.

Therefore, based on the direction of full-synthetic vaccine, one of our research projects has focused on the development of fully synthetic TACA-based cancer vaccines using MPLA as a carrier. We have been interested in MPLA as a carrier because of its attractive immunostimulatory properties. We anticipated that MPLA would be able to not only improve the immunogenicity of tumor-associated carbohydrate antigens used for vaccine development but also act as a built-in adjuvant to formulate self-adjuvanting vaccines. The new vaccine design was to have the carbohydrate antigen linked to the non-reducing end of MPLA through a spacer as shown in **Figure 3.4**.



**Figure 3.4** A new construct of full-synthetic vaccine based on MPLA

In our previous investigation, a MPLA analog of the lipid A structure derived from *Neisseria meningitides*, was coupled to a GM3 antigen, which is overexpressed on melanoma cancer cell surface, and its synthetic analog *N*-phenylacetyl GM3 (GM3NPhAc).<sup>31,32</sup> The resulting conjugates, such as the MPLA-GM3NPhAc conjugate illustrated in Figure 3.5, were evaluated as vaccines that can be used for cancer immunotherapy based on cell surface sialic acid metabolic engineering.<sup>31,33-37</sup> It was revealed that these conjugates alone could induce robust immune responses in animals in the absence of an external adjuvant (**Figure 3.5**), suggesting that, as an immunostimulant, MPLA was functional not only as a vaccine carrier but also as a built-in adjuvant.<sup>31</sup>



**Figure 3.5** The structure of MPLA-GM3NPhAc and immune results.

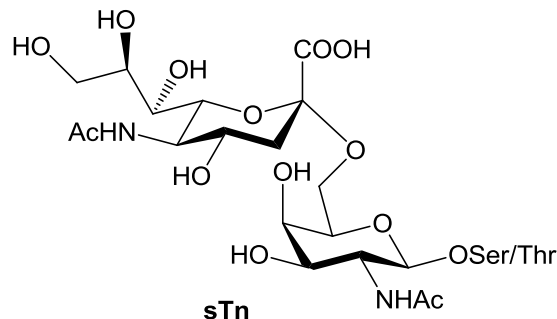
Encouraged by the above interesting discoveries, the present work aimed at: (1) further optimizing the lipid A structure as a vaccine carrier and built-in adjuvant, thus a series of MPLA derivatives were designed and synthesized for systematic structure-activity relationship analysis in the context of conjugate vaccines, and (2) developing new

fully synthetic carbohydrate-based anticancer vaccines with the optimized lipid A as the carrier molecule.

## 3.2 Evaluation of MPLA-sTnNPhAc Conjugates as Cancer Vaccines and Optimization of the MPLA Structure as a Vaccine Carrier

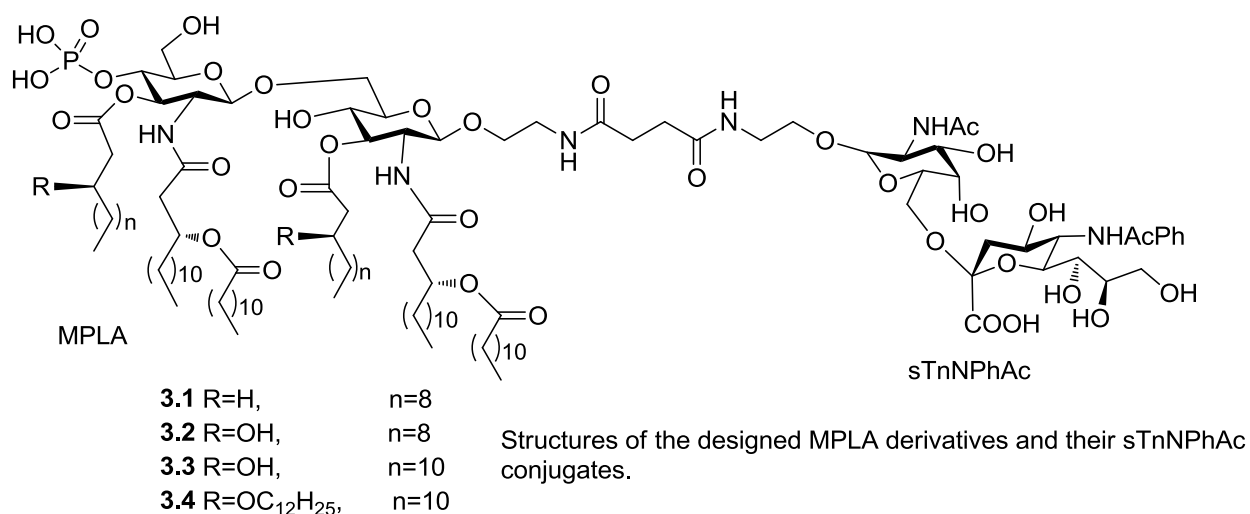
### 3.2.1 Introduction

Sialyl-Tn (sTn, **Figure 3.6**) is a mucin-associated carbohydrate antigen that is overexpressed by a variety of tumors, such as breast, prostate, colorectal, ovarian, gastric and pancreatic carcinoma rather than normal cells,<sup>38-54</sup> and it is relatively cancer-specific as it is rarely expressed on normal cells. In addition, the expression of sTn antigen is an independent indicator for cancer diagnosis.<sup>39,41,43</sup> Moreover, sTn antigen is a predictor of metastatic potential which associates with more aggressive cancer status in breast cancer.<sup>38,39</sup> Therefore sTn is an attractive target for anti-cancer vaccine and immunotherapy development. In 2003, a semi-synthetic cancer vaccine, Theratope®, was developed by Biomira Inc., which had the sTn antigen linked to keyhole limpet hemocyanin (sTn-KLH conjugate). This vaccine was successful in phase I and II clinical trials with excellent results in stimulating antibody- and cell-mediated immune responses against the tumor-associated antigen, but eventually failed in phase III clinical trial, because it eventually failed to induce robust immune responses in cancer patient.<sup>55-61</sup>



**Figure 3.6** The structure of sTn antigen

Inspired by the results of MPLA-GM3 conjugates<sup>31</sup>, here we have designed and synthesized four monophosphoryl analogs (including one from previous exploration) of the lipid A of *N. meningitidis*, containing lipids of different chain lengths and linkages as well as different patterns of lipidation (**Figure 3.7**). To investigate the immunological properties of these MPLA derivatives as vaccine carriers and adjuvants, we conjugated them with *N*-phenyl acetyl sTn (sTnNPhAc), a modified TACA that can be further used in our immunotherapy strategy based on glycoengineering, and examined in mice the specific immune responses induced by the resulting MPLA-sTnNPhAc conjugates **3.1-3.4** (**Figure 3.7**). Through these studies, we anticipated to identify novel vaccine carriers and adjuvants with improved immunological properties useful for the development of fully synthetic glycoconjugate cancer vaccines.



**Figure 3.7** The structures of designed MPLA derivatives and their sTn conjugates.

### 3.2.2 Results and Discussion

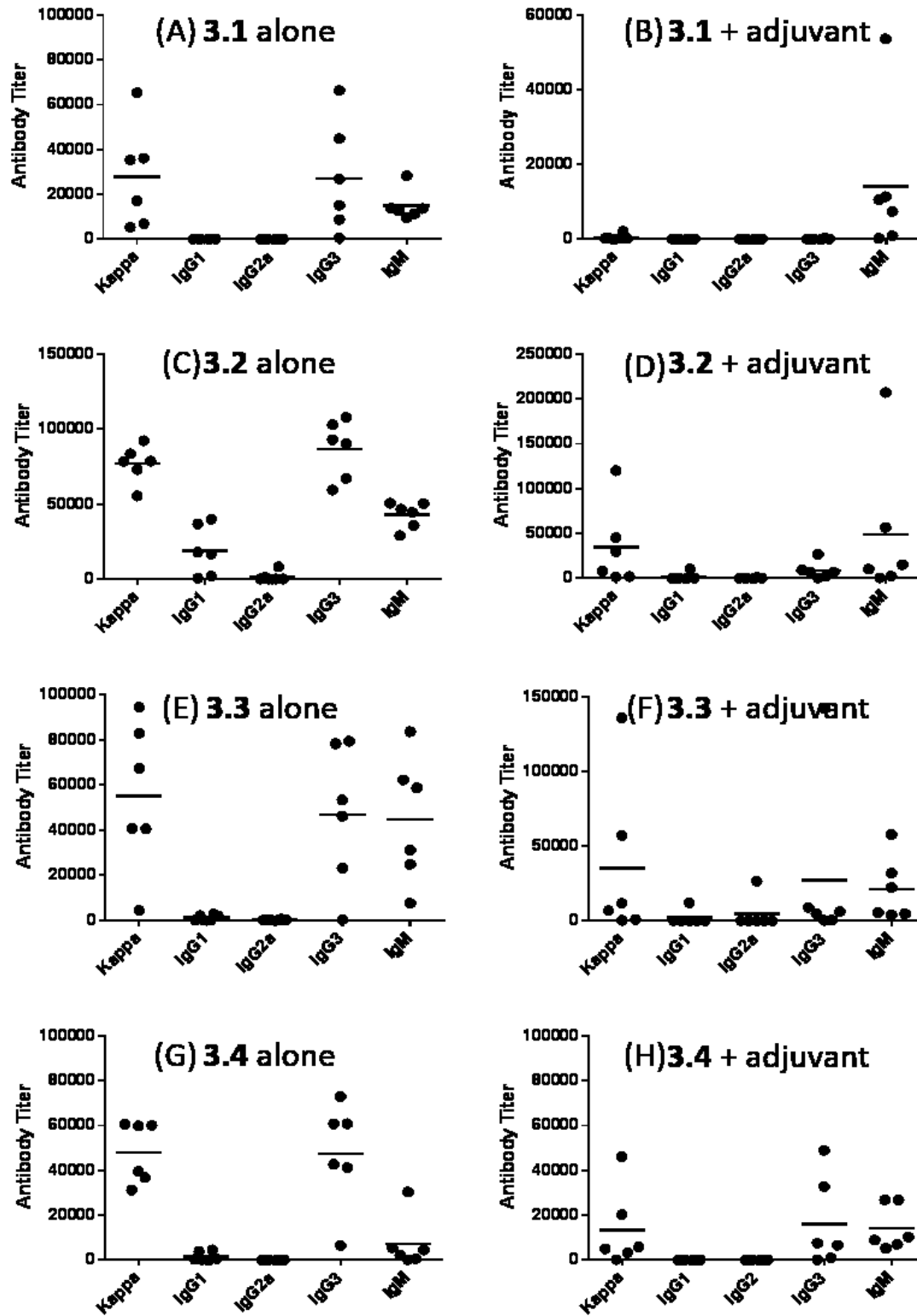
Immunological investigation of the MPLA-sTnNPhAc conjugates **3.1-3.4** was carried out with female C57BL/6J mouse, a well-characterized inbred strain for vaccine

evaluation and comparable to the human in immune reactions.<sup>62</sup> To improve the solubility of these conjugates in buffer, they were incorporated in liposomes formed by using 1, 2-distearoyl-*sn*-glycero-3-phosphocholine and cholesterol. Delivering glycoconjugate vaccines in liposomal forms can further improve their immunogenicity.<sup>63,64</sup> For immunization, the liposomal preparations of **3.1-3.4** were each injected subcutaneously (s.c.) to a group of six mice. In the meantime, groups of mice were simultaneously inoculated with emulsions of **3.1-3.4** liposomes and Titermax Gold adjuvant to evaluate the potential influence of an external adjuvant on the activities of these glycoconjugate vaccines. The adopted vaccination schedule was to inject into each mouse 0.1 mL of a vaccine preparation containing *ca.* 3  $\mu$ g of sTnNPhAc on day 1, 14, 21 and 28, respectively. Each mouse was subjected to bleeding on day 0 before the first injection (used as blank controls) and on day 27 and 38 after immunization. The blood samples were treated according to standard protocols to prepare antisera for the analysis of sTnNPhAc-specific antibodies by enzyme-linked immunosorbent assay (ELISA) with the human serum albumin (HSA) conjugate of sTnNPhAc (sTnNPhAc-HSA)<sup>35</sup> as a capture antigen. In addition to total antibodies, antibody isotypes such as IgG1, IgG2a, IgG3 and IgM were also individually assessed. Antibody titers were calculated from linear regression analysis of the curves of the optical density (OD) value against serum dilution number, and were defined as the dilution number yielding an OD value of 0.2.<sup>31</sup>

The ELISA results of antisera obtained with **3.1** without the use of an external adjuvant (**Figure 3.8A**) revealed that the conjugate itself provoked a strong sTnNPhAc-specific immune response in mice. More importantly, in addition to IgM antibody, a high titer of IgG3 and some IgG1 antibodies were also observed, suggesting a T cell-

dependent immune response.<sup>65,66</sup> Interestingly, **3.1** plus Titermax Gold failed to stimulate a significant antibody response (**Figure 3.8B**), although IgM antibodies were observed. These results suggest that the external adjuvant had an inhibitory effect on the T cell-mediated immune response against **3.1** but a relatively small influence on the B cell response. The results agreed well with our previous report about other MPLA-TACA conjugates.<sup>31</sup>

The ELISA results of **3.2** (**Figure 3.8C** and **3.8D**) were similar to that of **3.1** in terms of the type of immune responses provoked and the influence of Titermax Gold. However, the IgM and IgG antibody titers for **3.2** were much higher than that for **3.1**. Moreover, **3.2** stimulated a strong immune response in all of the mice. Another interesting finding was that **3.2** also induced a significant level of IgG1 antibody which is usually observed with neoglycoprotein vaccines. The ELISA results of **3.3** (**Figure 3.8E** and **3.8F**) and **3.4** (**Figure 3.8G** and **3.8H**) were similar to that of **3.1** and **3.2**, but their antibody titers were lower than that of **3.2** and higher than that of **3.1**. In all of these cases, Titermax Gold showed inhibition of immune responses against the conjugate vaccines.

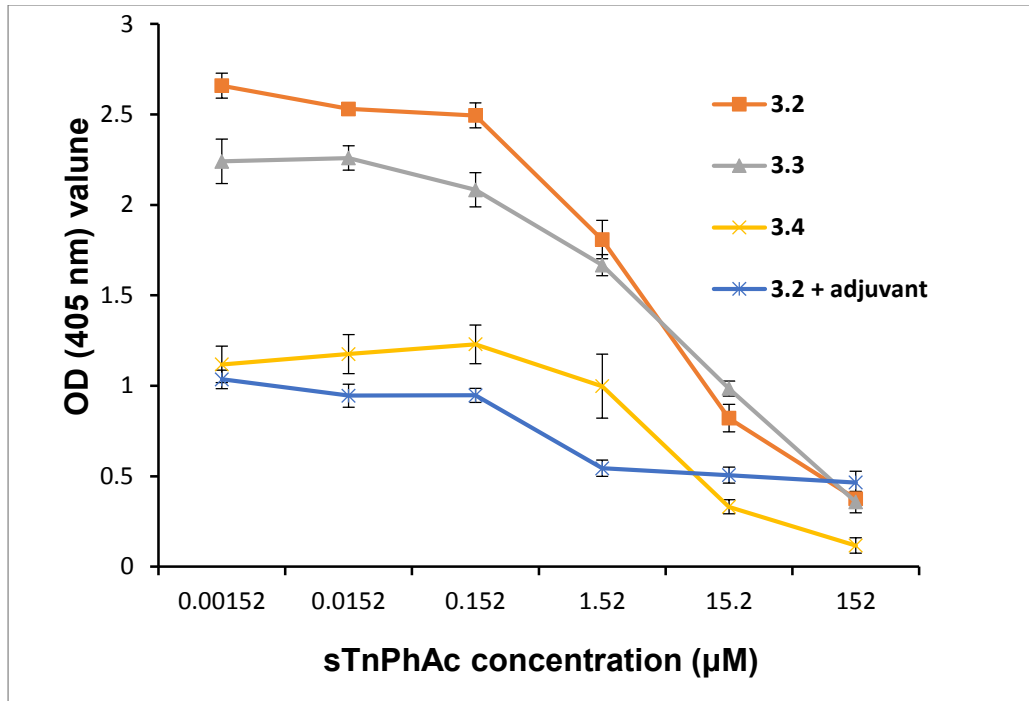


**Figure 3.8** ELISA results of day 38 antisera of mice immunized with 3.1 alone (A), 3.1 plus Titermax Gold (B), 3.2 alone (C), 3.2 plus Titermax Gold (D), 3.3 alone (E), 3.3 plus



Titermax Gold (F), **3.4** alone (G) and **3.4** plus Titermax Gold (H), respectively. The titers of various sTnNPhAc-specific antibodies are displayed. Each dot represents the antibody titer of an individual mouse, and the black bar shows the average antibody titer of a group of six mice.

To further verify that the detected antibodies were indeed sTnNPhAc-specific, a competitive ELISA experiment for the pooled mouse antisera obtained with **3.2**, **3.3**, **3.4** and **3.2** plus adjuvant was performed. In this study, various concentrations (0, 0.00152, 0.0152, 0.152, 1.52, 15.2 and 152  $\mu\text{M}$ ) of free sTnNPhAc were added to compete with sTnNPhAc-HSA attached to the ELISA plate for antibody binding. Again, alkaline phosphatase-linked goat anti-mouse kappa antibody was employed as the secondary antibody. The OD values at 405 nm reflected the levels of total antibodies bound to the sTnNPhAc-HSA-coated plates. These studies (**Figure 3.9**) have revealed that sTnNPhAc had concentration-dependent inhibition of antibody binding to the plates and that, at concentrations higher than 152  $\mu\text{M}$ , sTnNPhAc could essentially completely inhibit the binding, proving that the elicited antibodies were indeed specific to the sTnNPhAc antigen.



**Figure 3.9** Competitive ELISA results of the pooled mouse antisera obtained with conjugates **3.2**, **3.3**, **3.4** and **3.2** plus adjuvant. The OD values at 405 nm reflected the levels of total antibodies bound to the sTnNPhac-HSA-coated ELISA plates in the presence of specified concentrations of sTnNPhAc.

The above ELISA results disclosed that the MPLA conjugates **3.1-3.4** alone, that is, in the absence of any external adjuvant, provoked a robust antigen-specific immune response in mice. More importantly, they stimulated high titers of IgG3 and IgG1 antibodies, indicating a T cell-mediated immune response desirable for cancer immunotherapy.<sup>65,66</sup> Therefore, all of the synthetic MPLA derivatives could act both as a vaccine carrier and as an adjuvant to effectively enhance the immunogenicity of sTnNPhAc and to efficiently promote a T cell-dependent immune response toward TACAs.<sup>20,31,67</sup> These properties of MPLA should be particularly useful for the design and development of new fully synthetic carbohydrate-based conjugate vaccines.

Titermax Gold is a commercial adjuvant that is commonly used with vaccines such as carbohydrate-protein conjugates<sup>37</sup> to improve their immunogenicity. However, we showed in this work that it had an inhibitory impact on the immunological activities of **3.1-3.4**. As Titermax Gold mainly affect the T cell-mediated immune response. We proposed that Titermax Gold might interact with MPLA in the conjugate vaccine to prevent MPLA from binding to cell surface TLRs and affect its engagement in T cell-mediated immunological pathways. Another hypothesis is that Titermax Gold might interact with MPLA to affect vaccine delivery to the lymph system or antigen presenting cells (APCs) required for effective T cell-mediated immunity.<sup>68,69</sup> This discovery and further detailed studies may help understand vaccine adjuvants, such as their binding sites, functional mechanisms and so on.

Despite the fact that **3.1-3.4** stimulated similar patterns of immune responses in mice, they did show differences in immunological activity. For example, conjugate **3.2** elicited much higher and more consistent titers of both total antibodies and IgG2 antibody than **3.1**, **3.3** and **3.4** (**Figure 3.9**). Moreover, all of the mice showed strong immune responses to **3.2** but not to other conjugates. Clearly, **3.2** was the best vaccine among the MPLA conjugates investigated.

Structurally, **3.1-3.4** were different only in their MPLA moiety, and their antisera were obtained by means of the same immunization protocol and schedule. Consequently, any difference in their immunological activity should reflect the impact of the MPLA structure. Conjugate **3.2** contained the monophosphoryl form of natural lipid A of *N. meningitides* (H44/76 strain).<sup>70</sup> In **3.1**, the two free hydroxyl groups on the lipid chains of

the MPLA moiety were removed; **3.3** and **3.4** were different in that their MPLA contained different lengths and different numbers of lipid chains than that of **3.2**.

As all these conjugates had essentially the same immunological profile, it seems that the free hydroxyl groups on the lipid chains and the length and number of lipid chains of MPLA had a quantitative, rather than qualitative, impact on its biological activities. As discussed in **3.1.2** with **Figure 3.3**, it is clear that the hydroxyl groups on the lipid chains play an important role in the lipid A interaction with its receptors so that the endotoxin of lipid a has a reduce factor about 10 comparing with natural form. Although the level of antibody titers is not exactly the same as the endotoxin activities of MPLA, the decreasing trend of immunostimulatory activity of MPLA in conjugate **3.1** compared with that in **3.2** agrees with the trend of lipid A's endotoxin activities. Additionally, elongating the length of lipids at the 3-O- and 3'-O- positions (conjugate **3.3**) seem to have a relatively small impact, although such changes did result in reduced immunological activity and some inconsistency of immune responses in individual mice. Usually, as described in **3.1.2**, lessened or additional one lipid chains linked to these lipids have a reduced impact of endotoxin activities about  $10^{2.2}$ . What's more, lipid A totally loses its endotoxin activity to be a LPS antagonist if two lipid chains are removed.<sup>4</sup> In our case, the additional two lipid chains linked to these lipids as an octa-lipid chains MPLA is not like tetra-lipid chains one that loses immunological activity, but helps to elicit a considerable high level of antibody titers (conjugate **3.4**) which only a little lower than that of **3.2**.

### 3.2.3 Conclusion

In summary, four MPLA derivatives were coupled with sTnNPhAc to form fully synthetic glycoconjugate cancer vaccines. Studies on the resulting MPLA-sTnNPhAc conjugates revealed that they elicited strong and T cell-dependent immune responses without the use of any external adjuvant. Our previous work revealed that antisera derived from mice immunized with MPLA conjugates could effectively bind to and kill cancer cells metabolically engineered to express the corresponding antigen.<sup>31</sup> MPLA has thus been demonstrated to be a useful platform for the development of new vaccine carriers and adjuvants and for the development of novel types of fully synthetic carbohydrate-based cancer vaccines with self-adjuvanting properties. Our results have also revealed that MPLA derivatives containing six lipid chains exhibited more potent immunostimulatory activities than those with eight lipid chains (conjugate **3.4**) and that the lipid structure and length had a significant impact on the immunology of MPLA. The monophosphoryl form of natural *N. meningitides* lipid A was found to have the most promising immunological properties and its sTnNPhAc conjugate elicited the most potent and the most consistent T cell-dependent anti-sTnNPhAc immunity. As a result, the MPLA moiety in **3.2** was identified as the first generation of optimized vaccine carriers and adjuvants that is under further optimization and additional investigation.

On the other hand, Titermax Gold was found to inhibit the immunological activity of MPLA-sTnPhAc conjugates, whereas it has the opposite influence on the activity of protein-sTnNPhAc conjugates.<sup>33,35</sup> It is proposed that Titermax Gold may interact with MPLA to affect its binding to cell surface receptors and/or its delivery to the lymph system or antigen presenting cells. It is anticipated that these issues may be clarified by studies

utilizing labeled MPLA derivatives and conjugates, the results of which should be useful for understanding the immunostimulatory and adjuvant activities and the functional mechanisms of MPLA and Titermax Gold, which is a very important topic in cancer vaccine immunology.

### 3.2.4 Experimental Section

#### Materials, Reagents, and Animals

Glycoconjugates **3.1**, **3.2**, **3.3** and **3.4** conjugates were synthesized by Mohabul Mondal. STnNPhAc-HSA conjugate were synthesized according to the procedures previously reported by our laboratory. Titermax Gold adjuvant, 1, 2-distearoyl-*sn*-glycero-3-phosphocholine, and cholesterol were purchased from Sigma-Aldrich. The SKMEL-28 and B16F0 cancer cell line, Dulbecco's Modified Eagle's Medium (DMEM) for cell culture, and fetal bovine serum (FBS) were purchased from American Type Culture Collection. Penicillin-streptomycin and trypsin-EDTA were purchased from Invitrogen.

Alkaline phosphatase linked goat anti-mouse kappa, IgM, IgG1, IgG2a, and IgG3 antibodies and FITC-labeled goat anti-mouse kappa antibody were purchased from Southern Biotechnology. Female C57BL/6 mice of 6-8 weeks age used for immunological studies were purchased from the Jackson Laboratory.

#### General Procedure for the Preparation of Liposomes of Glycoconjugates **3.1-3.4**

The mixture of a specific MPLA conjugate **3.1-3.4** (60 µg of sTnNPhAc moiety, 0.102 µmol), 1, 2-distearoyl-*sn*-glycero-3-phosphocholine (M.W. 790.15, 0.52 µg, 0.664

$\mu\text{mol}$ ), and cholesterol (M.W. 386.66, 0.197  $\mu\text{g}$ , 0.51  $\mu\text{mol}$ ) (a 10:65:50 molar ratio) was dissolved in  $\text{CH}_2\text{Cl}_2$  and MeOH (1:1, v/v, 2 mL) in a 10 mL vial. Then, the solvents were removed in vacuum to form a thin lipid film on the vial wall, which was hydrated by adding 2.0 mL of HEPES buffer (20 mM, pH 7.5) containing NaCl (150 mM) and shaking the mixture in 40 °C water bath, and then shaking it by vortex mixer for several times to form a milky suspension. The milky suspension was finally sonicated for 1 min to obtain the desired liposomes.

### **General Procedure for the Preparation of Emulsion of liposomes and Adjuvant**

The prepared liposomes with double concentration were mixed with Titermax Gold adjuvant (1:1, v/v) and to form an emulsion according to the manufacturer's protocol.

### **Immunization of Mouse**

Each group of six female C57BL/6 mice were immunized on day 1 by subcutaneous (sc) injection of 0.1 mL of the liposomal solution of a specific glycoconjugate **3.1-3.4** containing 3  $\mu\text{g}$  of the carbohydrate antigen (5.1 nmol) or by injection of an emulsion of the liposomal solution of a specific glycoconjugate vaccine and Titermax Gold adjuvant prepared according to the manufacturer's protocol (each 1 mL of the liposomal solution mixed with 1 mL of Titermax Gold adjuvant). Following the initial immunization, mice were boosted 3 times on day 14, day 21 and day 28 by sc injection of the same conjugate and by the same immunization protocol. Blood samples of each mouse were collected through the leg veins prior to the initial immunization on day 0 and after immunization on day 27 and day 38 and were clotted to obtain antisera that were stored at -80 °C before use.

## Protocols for ELISA

ELISA plates were treated with 100  $\mu$ L of a solution of sTnNPhAc-HAS conjugate (2  $\mu$ g/ml) dissolved in coating buffer (0.1 M bicarbonate, pH 9.6) at 4  $^{\circ}$ C overnight, and then at 37  $^{\circ}$ C for 1 h, which was followed by treatment with blocking buffer (10% BSA in PBS solution with  $\text{NaN}_3$ ) and washing 3 times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST). Thereafter, a pooled or an individual mouse antiserum with serial half-log dilutions from 1:300 to 1:656100 in PBS was added to the coated ELISA plates (100  $\mu$ L/well), which was followed by incubation at 37  $^{\circ}$ C for 2 h. The plates were then washed with PBS and incubated at rt for another 1 h with a 1:1000 diluted solution of alkaline phosphatase linked goat anti-mouse kappa, IgM or IgG2a antibody or with a 1:2000 diluted solution of alkaline phosphatase linked goat anti-mouse IgG1 and IgG3 antibody (100  $\mu$ L/well), respectively. Finally, these plates were washed with PBS and developed with 100  $\mu$ L of p-nitrophenylphosphate (PNPP) solution (1.67 mg/mL in buffer) for 30 min at rt, followed by colorimetric readout using a BioRad 550 plate reader at 405 nm wavelength. The optical density (OD) values were plotted against antiserum dilution values, and a best-fit line was obtained. The equation of the line was employed to calculate the dilution value at which an OD of 0.2 was achieved, and the antibody titer was calculated at the inverse of the dilution value.

## Protocols for Competitive ELISA

The protocols for competitive experiment had the same process as ELISA of antibody investigation described above. The only difference was that free sTnNPhAc saccharide was added to compete with antibody binding of plate-bound sTnNPhAc-HSA. Hence, the ELISA plate was coated with sTnNPhAc-HSA in 4  $^{\circ}$ C overnight and then



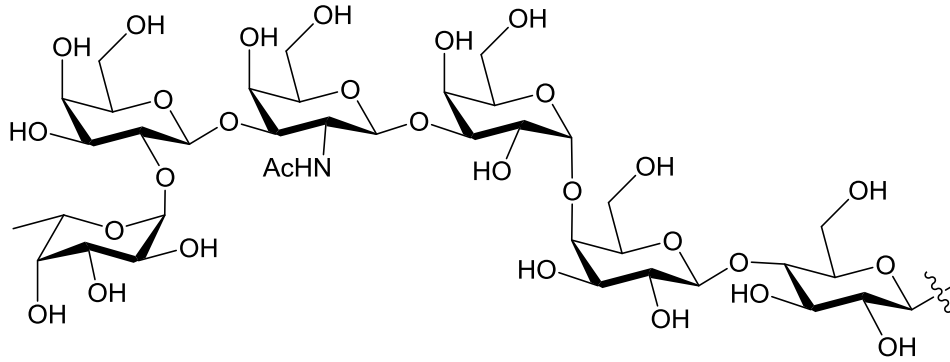
incubated in 37 °C for 1 h. After washed with PBS buffer, the plate was treated with blocking buffer in rt (room temperature) for 1 h. After that, the plate was washed with PBS and then, added 50 µL PBS solution containing 0, 0.003, 0.03, 0.30, 3.0, 30.4 and 304 µM of free sTnNPhAc and 50 µL of pooled antisera (1:150 dilutions) in PBS to each well (the final concentrations of free sTnNPhAc were 0, 0.0015, 0.015, 0.15, 1.5, 15.2 and 152 µM in 1:300 diluted pooled antisera solution) at the same time, and incubated in 37 °C for 2 h. Then, the plate was washed and incubated with alkaline phosphatase linked goat anti-mouse kappa antibody (1:1000 dilution), washed, reacted with PNPP solution and finally collected the reading at 405 nm as described above.<sup>31</sup>

### 3.3 MPLA Carrier Applied in Globo H-Based Vaccine

#### 3.3.1 Introduction

Globo H (GH) is a hexasaccharide specifically overexpressed on a number of cancer cells, such as breast, lung, ovary, stomach and small-cell lung cancer cells but weakly expressed on normal cells.<sup>71-76</sup> It was first discovered from human breast cancer cell line MCF-7 in 1983 by Hakomori.<sup>77-79</sup> The structure of Globo H is showed in **Figure 3.10** which was first assigned by Hakomori<sup>80</sup> and colleagues and was fully synthesized by several groups.<sup>79,81-84</sup> The immunocharacterization of Globo H attracted interests in this antigen which was studied via the monoclonal antibodies (mAb) MBr1 and VK9.<sup>78,85</sup>

As a TACA described before, Globo H is an attractive target for the development of an immunotherapy and anti-cancer vaccines against a variety of cancer such as breast carcinoma, small-cell lung carcinoma, and several malignant cancers. Base on the structure of Globo H, a therapeutic cancer vaccine, full-synthetic Globo H conjugated to keyhole limpet hemocyanin (KLH) with adjuvant QS-21, against breast and prostate cancer, showed hopeful results in clinical trials.<sup>79,83,86</sup> This vaccine was now in Phase III clinical trial with Globo H synthesized by the one-pot method.<sup>84</sup>

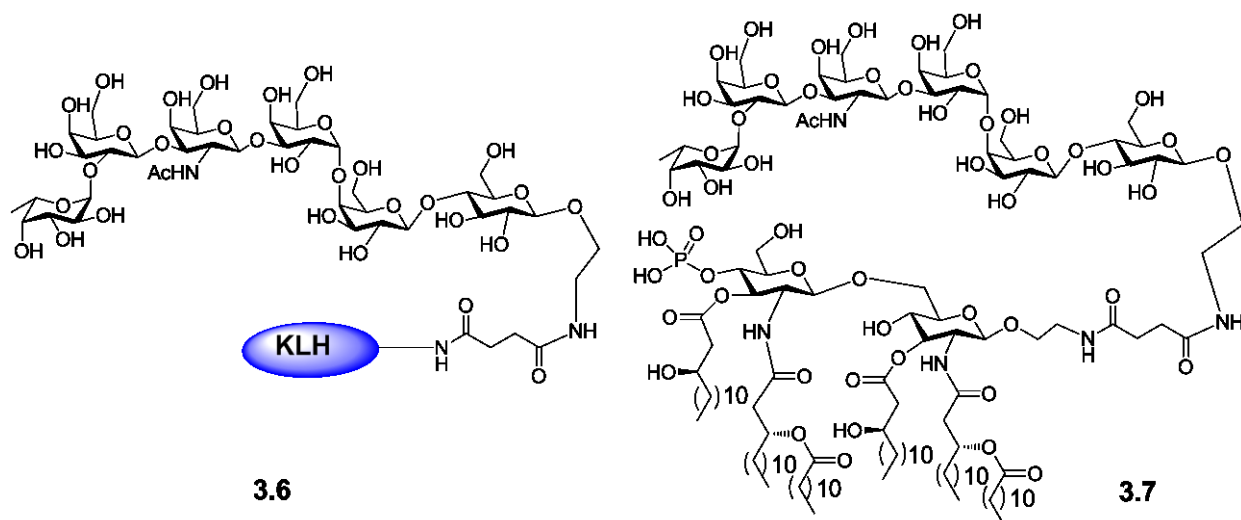


**Figure 3.10** Structure of Globo H

To further improve the efficiency of Globo H conjugated vaccine, in Chi-Huey Wong's group, Globo H was conjugated to different carrier proteins including KLH, diphtheria toxoid cross-reactive material (CRM) 197 (DT), tetanus toxoid (TT), and BSA with the combination of various adjuvants such as QS-21 and  $\alpha$ -galactosylceramide C34.<sup>81</sup> The results showed that, compared with the phase III clinical trial vaccine, Globo H-KLH, the Globo H-DT with C34 adjuvant vaccine elicited higher antibody titer of IgG isotype which has high selectivity for not only Globo H but also the Globo H related epitopes, including stage-specific embryonic antigen 3 (SSEA3) and SSEA4. In addition, they developed a novel vaccine candidate, SSEA4-DT with C34 adjuvant, which can stimulate strong immune response with high IgG antibodies which are highly specific for SSEA4 antigen.<sup>81</sup>

In our group, Globo H was successfully synthesized which can be further linked with carriers as anti-cancer vaccine. Based on the previous investigation, MPLA showed excellent characteristics as a build-in carrier and adjuvant. Therefore, the MPLA was applied in Globo H-based anti-cancer vaccine design. The structure of Globo H-KLH (3.6)

and Globo H-MPLA (**3.7**) were showed in **Figure 3.11**. This Globo H-MPLA conjugate contains the MPLA moiety which is the structure used in conjugate **3.2** which has the best result in our above evaluation.<sup>87</sup>



**Figure 3.11** The structure of Globo H-KLH (**3.6**) and Globo H-MPLA (**3.7**)

### 3.3.2 Results and Discussion

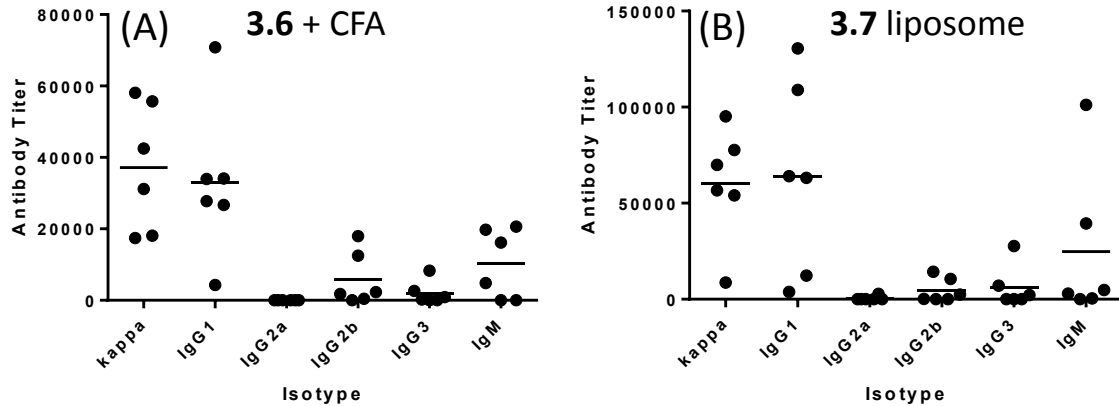
Immunological investigation of Globo H-KLH (**3.6**) and Globo H-MPLA (**3.7**) conjugates was carried out with female C57BL/6J mouse. The KLH conjugate was dissolved in PBS solution and then mixed with Freund's complete adjuvant (CFA) as an emulsion to immunize mice. To improve the solubility of the MPLA conjugate and also improve the immunogenicity of vaccine, the MPLA conjugate was incorporated in liposomes formed by using 1,2-distearoyl-*sn*-glycero-3-phosphocholine and cholesterol the same as described in previous MPLA-sTnNPhAc conjugate project.<sup>63,64</sup> For immunization, the adjuvant preparation of **3.6** and the liposomal preparation of **3.7** was injected subcutaneously (s.c.) to a group of six mice. The inoculation schedule was to inject into each mouse 0.1 mL of a vaccine preparation containing ca. 3  $\mu$ g of Globo H

moiety on day 1, 14, 21 and 28, respectively. The blood of each mouse was collected on day 0 before the first inoculation (used as blank controls) and on day 27 and 38 after immunization. The blood samples were used to prepare antisera according to the protocol. Then the antisera were further analyzed by ELISA the same as previous described in MPLA-sTnNPhAc project. Globo H-HSA was used as capture reagent, and goat anti-mouse antibodies with AP enzyme were used as second antibodies. The total antibody titer and the antibody titers of each isotype, IgG1, IgG2a, IgG2b, IgG3 and IgM were calculated from the curves of the optical density (OD) value against serum dilution number, and the titers were defined as the dilution number yielding an OD value of 0.1.<sup>31,87</sup>

The ELISA results of antisera induced by **3.6** with CFA as an external adjuvant (**Figure 3.12**) indicated that Globo H-KLH (**3.6**) conjugate provoked a strong Globo H-specific antibody response (kappa: about 38,000) in mice. Moreover, the high antibody titer (average about 35,000) of IgG1 and some positive antibody titer of IgG2b and IgG3 suggested that T cell-dependent immunity was involved. Generally, six out of six mice had strong Globo H specific antibody titers which means every mouse had been immunized successfully. In addition, five out of six mice had high IgG1 antibody titer.

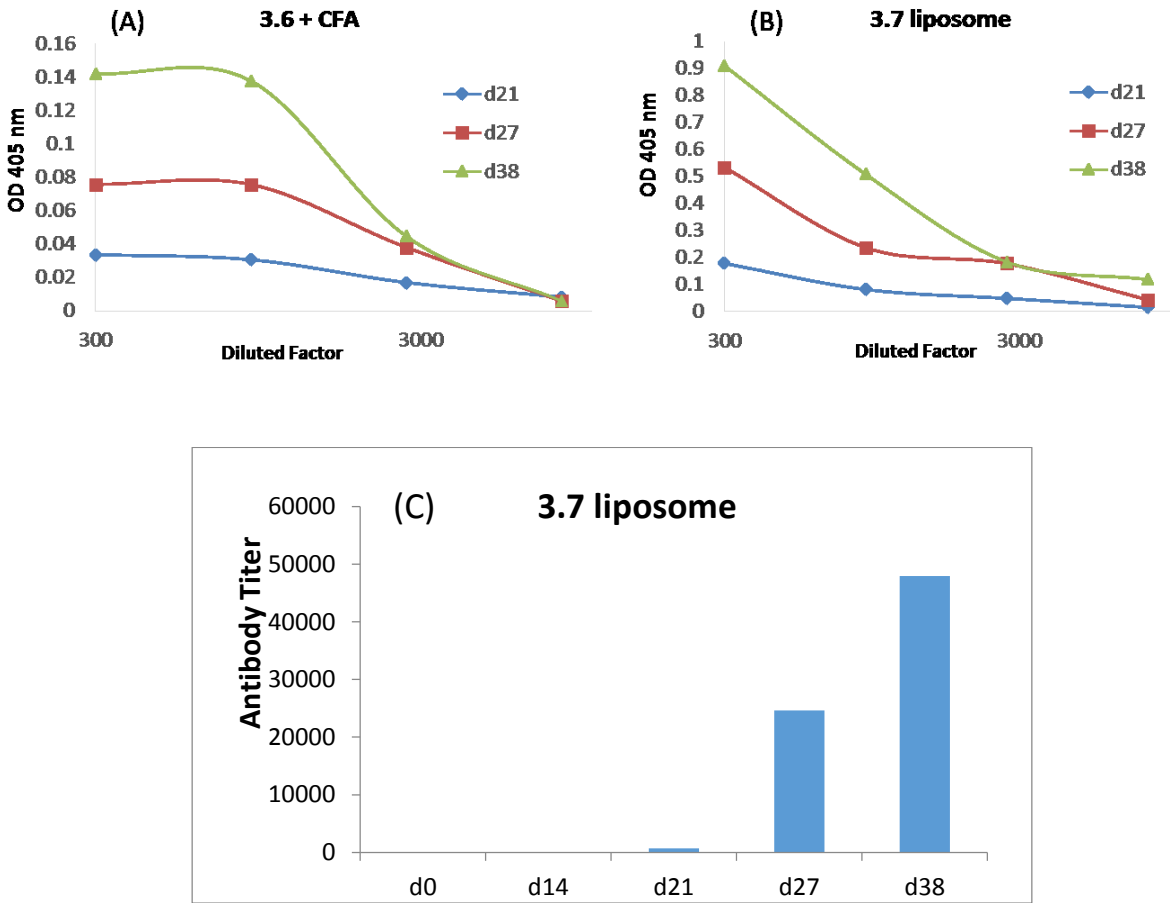
The ELISA results of antisera elicited by Globo H-MPLA (**3.7**) had similar antibody response (the average total antibody titer was about 60,000) including high antibody titer of IgG1 isotype ( about 64,000) which indicated that Globo H-MPLA (**3.7**) had excellent immunogenicity to stimulate immune response especially T cell immunity. Excitingly, the total antibody and IgG1 isotype of Globo H-MPLA (**3.7**) had even higher antibody titer than that elicited by Globo H-KLH (**3.6**) conjugate. Additionally, five out of six mice (one had 8741 much lower than others) in this group had been successfully immunized by

conjugate **3.7** indicated by the high kappa antibody titer. And, five out of six mice (one had low antibody titer 3853) in this group had high antibody titer of IgG1 isotype.



**Figure 3.12** ELISA results of day 38 antisera of mice immunized with **3.6** plus CFA adjuvant (A) and **3.7** in liposome form (B), respectively. The titers of Globo H specific antibodies are displayed. Each dot represents the antibody titer of an individual mouse, and the black bar shows the average antibody titer of a group of six mice.

These results suggested that the MPLA conjugate had an excellent potential for design as a build-in adjuvant and carrier. This phenomenon was discovered here that a small molecule vaccine can work as well as large molecule vaccine with protein carrier. What's more, the protein carrier usually with T cell epitopes can help provoked T cell immunity like the results here provoked by Globo H-KLH (**3.6**) (**Figure 3.12**). However, in our new discovery, MPLA, usually considered as a TLR-based adjuvant,<sup>18,19</sup> can serve as a stimulator to elicit T cell-mediate immunity as well.



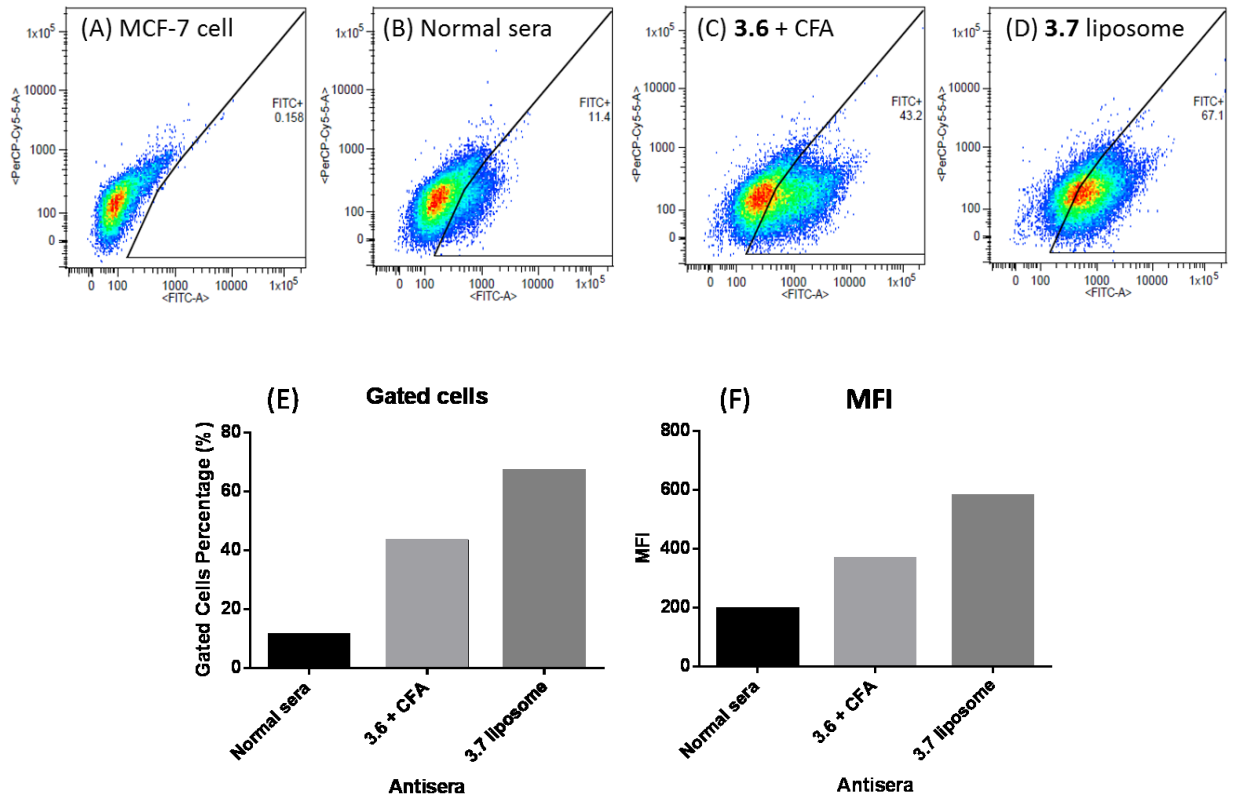
**Figure 3.13** The kinetic ELISA results of antisera induced by conjugates **3.6** and **3.7**. ELISA results of Globo H-specific antibody in the antisera pooled from mice immunized with (A) conjugate **3.6** plus CFA adjuvant and (B) **3.7** in liposome form, respectively, on day 21 (blue line), day 27 (red line) and day 38 (green line). X-axis is the diluted factor from 300 to 8100 in logarithmic scale, and y-axis is the OD value at 405 nm reading each well after 30 min PNPP reaction. (C) Calculated antibody titer of pooled antisera obtained from mice immunized with **3.7** in liposome form on day 0, day 14, day 21, day 27 and day 38.

The time courses of the induction of total antibody pooled from six mice immunized by **3.6** or **3.7** were shown in **Figure 3.13**. The antibody titers generally increased with the more immunization boost. Day 27 antisera were obtained after three boosts and day 38 were obtained after four boosts. Three boosts of conjugates both **3.6** and **3.7** can already

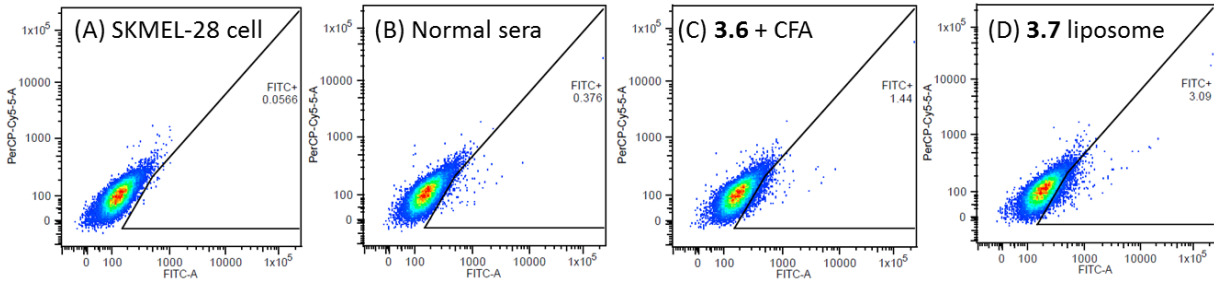
elicit significant immune response. The fourth boost could improve the immune response significantly, nearly twice higher than day 27 result. Therefore, both the vaccines required four times immunization injection.

Cell surface reactivity of these antisera obtained from mice immunized with **3.6** plus CFA adjuvant or **3.7** in liposome form was evaluated by fluorescence-activated cell sorting (FACS) technology on Globo H positive MCF-7 cell line (**Figure 3.14**) and Globo H negative SKMEL-28 cell line as a negative control (**Figure 3.15**). In these studies, both kinds of cells were incubated with normal mouse sera or pooled antisera obtained from mice immunized with **3.6** or **3.7**, respectively. Thereafter, the cells were cultured with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse kappa antibody, followed by FACS analysis.

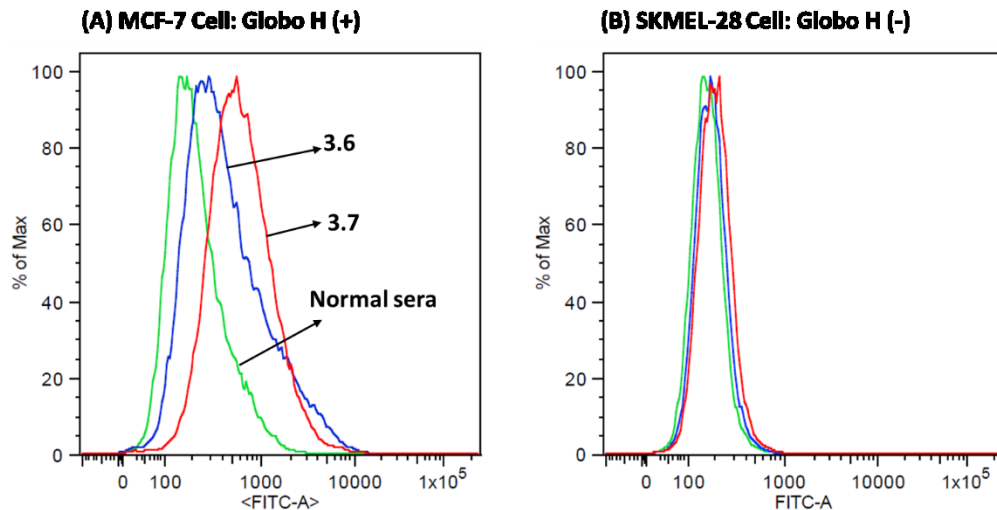




**Figure 3.14** FACS results of MCF-7 cells. (A) MCF-7 cell without any treatment; (B) MCF-7 cells stained with normal sera and FITC labeled 2<sup>nd</sup> antibody; (C) MCF-7 cells stained with antisera obtained from mice immunized with **3.6** plus CFA adjuvant; (D) MCF-7 cells stained with antisera obtained from mice immunized with **3.7** in liposome form; (E) Gated cells percentage of positive cells and (F) Mean fluorescent intensity of cells stained with normal sera and the other two antisera.



**Figure 3.15** FACS results of SKMEL-28 cells. (A) SKMEL-28 cell without any treatment; (B) SKMEL-28 cells stained with normal sera and FITC labeled 2<sup>nd</sup> antibody; (C) SKMEL-28 cells stained with antisera obtained from mice immunized with **3.6** plus CFA adjuvant; (D) SKMEL-28 cells stained with antisera obtained from mice immunized with **3.7** in liposome form.



**Figure 3.16** Overlay plots of FACS results of MCF-7 (A) and SKMEL-28 (B) cell lines stained with normal sera (green), pooled antisera from mice immunized with **3.6** plus CFA adjuvant (blue) and **3.7** liposome (red), respectively.

From the FACS results (**Figure 3.16**) of MCF-7 cell line and SKMEL-28 cell line which were stained with normal sera and antisera obtained from mice immunized with conjugate **3.6** and **3.7**, the conclusion that both conjugate **3.6** and **3.7** can elicit strong immune response and Globo H-specific antibodies was further confirmed. The MCF-7 cells with Globo H expressed on cell surface can be significantly stained with the antisera

containing Globo H-specific antibodies, while the SKMEL-28 cell had negative results. What's more, the gated cells percentage of positive cells of antisera induced by **3.7** was significantly higher than that of antisera induced by **3.6** plus CFA adjuvant. This phenomenon suggested that **3.7** conjugates may have better immunological characteristics than that of conjugate **3.6** which was consistent with the results of ELISA. In addition, the MFI of cells stained with antisera induced by **3.7** are significantly higher than that of **3.6** which further confirmed the results of ELISA and gated cells percentage. Therefore, both ELISA and FACS results confirmed that both conjugates can elicit strong immune response and MPLA conjugate **3.7** may have even better results than KLH conjugate **3.6**.

### 3.3.3 Conclusion

In summary, both Globo H-KLH and –MPLA conjugates can provoke strong Globo H-specific immune response, especially the T cell immunity. Moreover, Globo H-MPLA was proved that have better capability to generate antibodies than that of KLH conjugate. It's the first time we discovered that small molecular glycoconjugate with MPLA carrier had better immunological capability than that of glycoprotein. Although the mechanism of how MPLA conjugate elicited stronger immune response was not clear, the MPLA-based new construct of vaccine really had charming immunological properties, such as external adjuvant-free and the ability of stimulate T cell-mediated immunity. Some of the Globo H-KLH conjugate was already in phase III clinical trial for the therapy of breast and prostate cancers. Therefore, our new construct vaccine, Globo H-MPLA which showed better ELISA and FACS results than KLH conjugate, should have magnificent hope to be further

applied in clinical trial. For future directions, it is feasible and interesting to prepare Globo H-specific monoclonal antibodies (mAbs) using in the further immunotherapeutic studies. The improvement of the novel construct using MPLA as carrier is also another interesting direction. The construct may be promoted by coupling with other components, such as T cell epitopes, multivalent antigens and so on. What's more, the successful strategy of vaccine designs with a TLR ligands also can be moved forward to other TLR ligands, lectin ligands and so on.

### 3.3.4 Experimental Section

#### Materials, Reagents, and Animals

Glycoconjugates **3.6**, **3.7** and Globo H-HSA were synthesized by **Guochao Liao**. Freund's complete adjuvant (CFA), 1, 2-distearoyl-*sn*-glycero-3-phosphocholine, and cholesterol were purchased from Sigma-Aldrich. The MCF-7 and SKMEL-28 cancer cell line, Dulbecco's Modified Eagle's Medium (DMEM) for cell culture, and fetal bovine serum (FBS) were purchased from American Type Culture Collection (ATCC). Penicillin-streptomycin and trypsin-EDTA were purchased from Invitrogen.

Alkaline phosphatase (AP) linked goat anti-mouse kappa, IgM, IgG1, IgG2a, IgG2b and IgG3 antibodies and FITC-labeled goat anti-mouse kappa antibody were purchased from Southern Biotechnology. Female C57BL/6 mice of 6-8 weeks age used for immunological studies were purchased from the Jackson Laboratory.

#### Protocol of vaccine preparation

The loading of Globo H-KLH is about 8%. According to KLH m.w. 350~400 kDa, each KLH molecule has 29~33 Globo H residents.

**Table 3.3** Dose of KLH conjugate **3.6** and MPLA conjugate **3.7**

Conjugate	Globo H amount/ ug	Globo H amount/nmol	1 Dose amount/ $\mu$ g	30 Dose/ $\mu$ g
<b>3.6</b>	3	2.83	37.5	1125
<b>3.7</b>	6	5.7	16	500

Preparation of liposomes of glycoconjugates **3.7**. The protocol was similar to that reported in the literature.<sup>31,87</sup> Briefly, the mixture of a specific conjugate **3.7** (0.5 mg, 0.176  $\mu$ mol), 1, 2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) (0.872 mg, 1.1  $\mu$ mol), and cholesterol (0.33 mg, 0.85  $\mu$ mol) (in a 10:65:50 molar ratio) was dissolved in CH<sub>2</sub>Cl<sub>2</sub>, MeOH and H<sub>2</sub>O (about 3:3:1, v/v, 2 mL) in a vial. The solvents were then removed in vacuum to form a thin lipid film on the vial wall, which was hydrated by adding 3.0 mL of HEPES buffer (20 mM, pH 7.5) containing NaCl (150 mM) and shaking the mixture by vortex.

Preparation of emulsion of KLH conjugate **3.6**. The protocol was similar to that reported in the literature. Generally, conjugate 2 (1.125 mg) was dissolved in 1.5 mL 1X PBS solution and mixed with 1.5 mL Freund's Complete Adjuvant (CFA) following the manufacturer's protocol to form an emulsion.

### Immunization of mouse

Each group of six female C57BL/6J mice were inoculated on day 1 by subcutaneous (s.c.) injection of 0.1 mL of the liposomal solution or CFA emulsion of a

specific glycoconjugate. Following the initial immunization, mice were boosted 3 times on day 14, day 21, and day 28 by s.c. injection of the same conjugate and by means of the same immunization protocol. Mouse blood samples were collected prior to the initial immunization on day 0 and after immunization on day 21, day 27 and day 38, and were clotted to obtain antisera that were stored at -80 °C before use. The animal protocol (#A 02-10-14) for this investigation was approved by the Institutional animal Care and Use Committee (IACUC) of Wayne State University, and all animal experiments were performed in compliance with the relevant laws and institutional guidelines.

### **ELISA protocol**

ELISA plates were treated with 100 µL of a solution of Globo H-HSA conjugate (2 µg/mL) dissolved in coating buffer (0.1 M bicarbonate, pH 9.6) at 37 °C for 1 h, which was followed by treatment with a blocking buffer and washing 3 times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST). Then, the pooled or an individual mouse antiserum with serial half-log dilutions from 1:300 to 1:656100 in PBS was added to the coated plates (100 µL/well) and incubated at 37 °C for 2 h. The plates were washed with PBST and incubated at rt for another hour with a 1:1000 diluted solution of alkaline phosphate (AP) linked goat anti-mouse kappa, IgG1, IgG2a, IgG2b, IgG3 and IgM antibody (100 µL/well), respectively. Finally, the plates were washed with PBST and developed with 100 µL of a *p*-nitrophenylphosphate (PNPP) solution (1.67 mg/mL in buffer) for 30 min, 1 h or 2 h (Due to the low reading, the longer reaction time made the reading higher; all groups will be compared in the same reaction condition) at rt for colorimetric readout using a microplate reader at 405 nm wavelength. For titer analysis, OD values were plotted against antiserum dilution values, and a best-fit logarithm line was obtained.

The equation of this line was used to calculate the dilution value at which an OD of 0.1 (this value will be further modified according the data to get better plots) was achieved, and the antibody titer was calculated at the inverse of the dilution value.

### **Protocols for FACS Assay**

MCF-7 cell line (which can expressed Globo H antigen) was chose to be performed this experiment, and SKMEL-28 cell line (which was confirmed that Globo H antigen negative) was chose to be as negative group. MCF-7 cell line was incubated in ATCC-formulated Eagle's Minimum Essential Medium (EMEM) containing 10% FBS and 1% antibiotics. SKMEL-28 cell line was incubated in ATCC-formulated Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 1% antibiotics.

Then these cells were harvested by being treated with trypsin-EDTA solution. Cells (about  $1.0 \times 10^6$ ) were washed twice with FACS buffer (PBS containing 5% FBS) and incubated with 50  $\mu$ L of normal mouse sera (1:10 dilution) at 4 °C for 15 min. Then the cell were washed with FACS buffer and incubated with 50  $\mu$ L certain pooled antisera of day 38 (1:10 dilution) at 4 °C for 30 min. After that, cells were washed again with FACS buffer and incubated with FITC-linked goat anti-mouse kappa antibody (2  $\mu$ L in 50  $\mu$ L FACS buffer) at 4 °C for 30 min. Finally, cells were washed and suspended in 0.8 mL of FACS buffer, and then sent to FACS analysis on a Becton Dickinson LSR II Analyzer.

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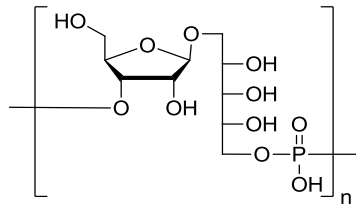
## CHAPTER 4 Development of Novel Carbohydrate-Based Vaccines against *Haemophilus Influenzae* Type B and *Neisseria Meningitidis* Serotype C

### 4.1 Vaccines against *Haemophilus Influenzae* Type B

#### 4.1.1 Introduction

*Haemophilus influenzae* type b (Hib) is an important human pathogen that can cause various diseases, such as bacterial meningitis and pneumonia, and it usually strikes children under the age of 5 years old. Before Hib vaccine available, bacterial meningitis caused by Hib was a life-threatening disease among young children. ([www.cdc.gov/vaccines](http://www.cdc.gov/vaccines)) Hib-caused disease have been under control in developed countries by the introduction of successful conjugate vaccines beginning from 1990s.<sup>1</sup> However, according to the estimation of the World Health Organization (WHO), Hib still remains a life-threatening disease in developing country that causes approximately three million serious illnesses and an estimation of 386,000 deaths per year among children under the age of 5 years old.<sup>2,3</sup> (World Health Organization website (2005): <http://www.who.int/mediacentre/factsheets/fs294/en/>)

It has been well known for many years that the capsular polysaccharide (CPS) of Hib, a polymer of repeating ribosyl ribitol phosphate (RRP) units<sup>4</sup> (**Figure 4.1**), is an attractive target for developing vaccines against Hib. Currently, the commercial available Hib vaccines were made from the CPS extracted from bacteria by conjugation with carrier proteins such as tetanus toxoid (TT) (ActHib, 1993, Sanofi Pasteur; Hiberix, 1998, EU) and diphtheria toxoid (DT) (Pentacel, Sanofi Pasteur), which are protective against the diseases caused by Hib.



**Figure 4.1** The structure of polyribosylribitol phosphate (RRP) on Hib surface.

However, the Hib RRP polysaccharides used for vaccine production, which were isolated from the supernatants of bacterial cell cultures, are often heterogeneous and contaminated with other antigenic components that may cause safety problems and quality control problems. To deal with the issue, conjugate vaccines based on well-defined synthetic carbohydrate antigens have attracted great interest. After decades of development and optimization, the first semi-synthetic glycoconjugate vaccine against Hib, Quimi-Hib™ (Heber Biotech), which was derived from the conjugation of synthetic polyribosylribitol phosphate and tetanus toxoid (TT), was approved and commercialized in Cuba and became a part of Cuba's national vaccination program in 2004.<sup>5-7</sup> This vaccine was also confirmed to stimulate higher long-term protective antibody titers than that of licensed products composed with polysaccharide extracted from Hib bacteria.<sup>5,6</sup> This research has provided a proof of principle for the development of effective bacterial vaccines based on oligosaccharides in addition to CPSs. The great success of Quimi-Hib™ has encouraged the exploration of a series of vaccines against *Neisseria meningitidis* serogroup C<sup>8</sup> and W135<sup>9</sup> and *Streptococcus pneumoniae*<sup>10</sup>.

Actually, as early as in 1990s, Peeters' group already synthesized a series of Hib CPS derivatives, including trimeric and tetrameric RRP, and conjugated them to carrier



proteins such as DT and TT via a thioether linkage.<sup>11</sup> Immunological studies indicated that all of these conjugates elicited RRP-specific antibody responses with an increasing IgG/IgM antibody ratio in both mice and monkeys. Moreover, the tetramer conjugates elicited higher RRP-specific antibody titers than the trimer conjugates, suggesting that the tetrameric RRP was a better immunogen. In addition, the results of adult monkeys immunized with tetrameric RRP-TT conjugate were as good as that of oligosaccharide-CRM197 conjugate, which elicited protective levels of serum antibodies in human infants after two or three boosts.

Additional investigation about synthetic RRP conjugate vaccine against Hib was carried out by Chong's group.<sup>12</sup> They coupled synthetic oligosaccharides carrying RRP repeating units to synthetic peptides, which contained potent T-helper cell determinants and B-cell epitopes of Hib outer membrane proteins (OMPs), P1, P2 and P6. Animal studies of these conjugates revealed that some of them could elicit high titers of both RRP-specific and OMP-specific IgG antibodies. Moreover, they investigated the factors that might affect the conjugate immunogenicity. They found that the magnitude of RRP-specific antibody responses significantly depended on the relative spatial orientations of RRP and T-cell determinants and that the antibody response increased when a multiple antigenic peptide carrier was used. It was found that three RRP repeating units were optimal to elicit RRP-specific antibodies. Lipidation of the peptide-RRP conjugates was found to have a minimal effect on the immune response.

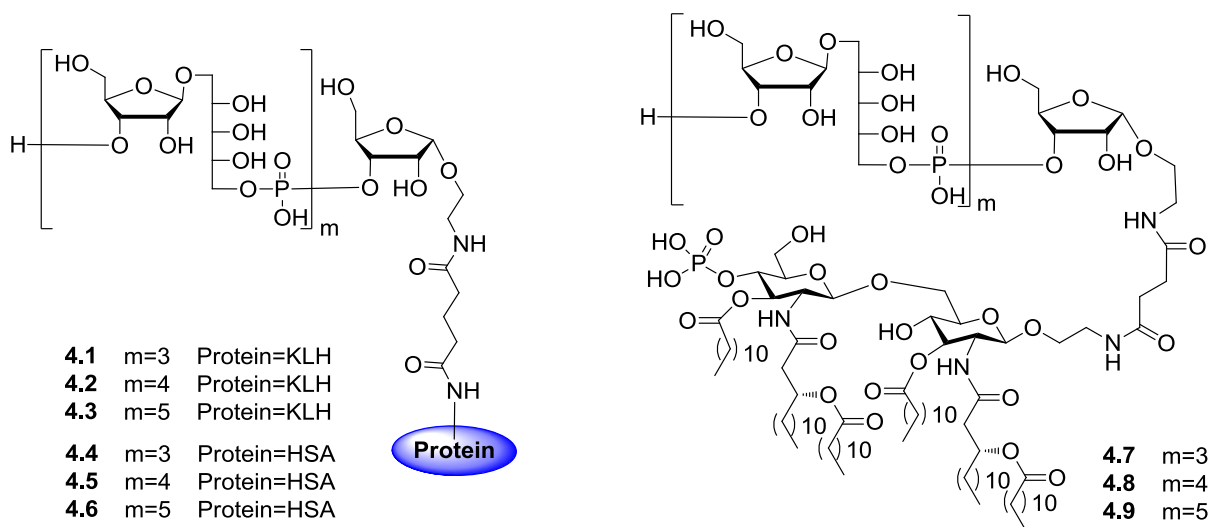
In conclusion, the above studies have clearly demonstrated that a carbohydrate hapten conjugated with a peptide can provoke strong immune response, and especially help elicit T cell-dependent immune response. Furthermore, the antisera raised by these

conjugates were confirmed to be protective against Hib infection in an infant rat model.

#### 4.1.2 Our Vaccine Designs

The main aims of this project were to systematically explore the structure-activity relationships of oligo-RRP as antigens and develop effective RRP-based Hib vaccines. According to the literature, Hib-derived capsular polysaccharides used in some licensed vaccines can contain oligosaccharides as short as five RRP repeating units.<sup>13</sup> Moreover, trimeric and tetrameric RRP were demonstrated to have excellent immunostimulatory ability.<sup>11,12</sup> For example, in Quimi-Hib™, the saccharide moiety has an average of eight RRP repeating units.<sup>5</sup>

Based on the above discoveries, our group designed tri-, tetra- and pentameric RRP, which were successfully synthesized by Dr. Guochao Liao, and linked them to KLH and MPLA to form conjugates **4.1-4.3** and **4.7-4.9** (**Figure 4.2**). In the meantime, these oligosaccharides were also coupled with HSA to get conjugates **4.4-4.6** that were utilized as the capture reagents in ELISA experiment. The new RRP-KLH conjugates **4.1-4.3** and RRP-MPLA conjugates **4.7-4.9** were evaluated as vaccines in mice.



**Figure 4.2** The structure of designed oligo-RRPs and their KLH conjugates (**4.1-4.3**), HSA conjugates (**4.4-4.6**), and MPLA conjugates (**4.7-4.9**)

### 4.1.3 Results and Discussion

#### 4.1.3.1 KLH Conjugates 4.1-4.3 as Vaccines

After the KLH conjugates **4.1**, **4.2** and **4.3** and HSA conjugates **4.4**, **4.5**, and **4.6** were synthesized, their weight% loadings of the saccharide moiety were examined, and the results were showed in **Table 4.1**. The saccharide loadings of HSA conjugates were estimated by means of MS. In this case, the average molecular weights of HSA and HSA conjugates were obtained by MALDI TOF MS (**Figure 4.3**). Then, the saccharide loadings of were calculated according to the following equation:

$$\% \text{ saccharide loading} = [\text{conjugate MW} - \text{HSA MW}] / \text{conjugate MW}$$

where the HSA MW was 67 kDa. The calculated saccharide loadings of **4.4**, **4.5** and **4.6** were about 13.7%, 8.4% and 7.4%, respectively. Their saccharide loadings were also examined by a chemical method,<sup>14,15</sup> which gave slightly different results, namely that the loadings of **4.4**, **4.5** and **4.6** were about 8%, 8.6% and 9.6%, respectively. The differences may be caused by the systematic errors of the chemical method, in which the presence

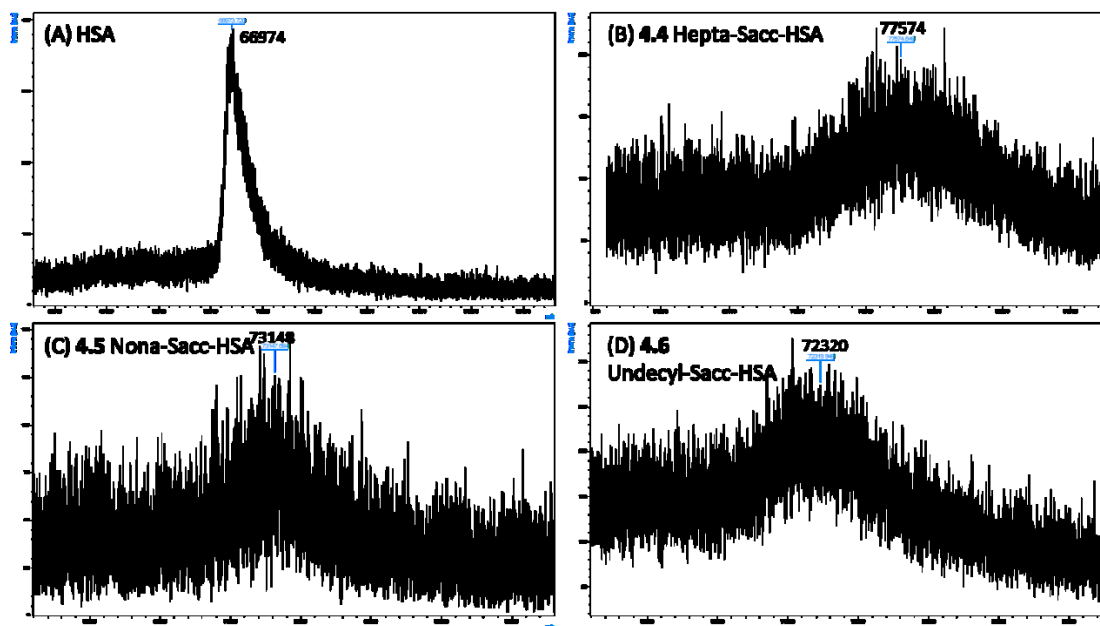
of protein and the existence of saccharides in the form of conjugate might have some impacts on the carbohydrate analysis. Nevertheless, the results suggested that resultant glycoconjugates had the proper saccharide loadings as vaccines, as it has been observed that glycoconjugates with sugar loading in the range of 5-20% produce the optimal immunological results. For the KLH conjugates, as KLH is huge and exists in difficult forms, which makes its measurement by MS very difficult, their loadings were only examined by the chemical method.<sup>14,15</sup> Their loadings were also in the acceptable range of 8~9%.

**Table 4.1** The loadings of saccharide moiety in protein conjugates \*

Conjugates	Loading (wt %)	Average saccharide residues per protein
<b>4.1</b> (Hepta-Sacc-KLH)	8.4	27
<b>4.2</b> (Nona-Sacc-KLH)	8.4	21.5
<b>4.3</b> (Undecyl-Sacc-KLH)	9.0	19.2
<b>4.4</b> (Hepta-Sacc-HSA)	7.88 (13.7)**	8
<b>4.5</b> (Nona-Sacc-HSA)	8.60 (8.4)**	3.7
<b>4.6</b> (Undecyl-Sacc-HSA)	9.57 (7.4)**	2.7

\* Data provided by Dr. Guochao Liao.

\*\*The values in ( ) were calculated according to the MALDI TOF MS data (Figure 4.4).



**Figure 4.3** MALDI TOF MS results of HSA conjugates **4.4**, **4.5** and **4.6**.

Immunological evaluations of RRP-KLH conjugates **4.1-4.3** were carried out with female C57BL/6J mouse. For initial immunization, each KLH conjugate containing ca. 3  $\mu\text{g}$  of the carbohydrate antigen mixed with Titermax Gold adjuvant as an emulsion (0.1 mL) was injected subcutaneously (s.c.) to each mouse in a group of six. Four more boost immunizations were carried out in these mice on day 14, day 21, day 28 and day 38, respectively, using the same vaccines.

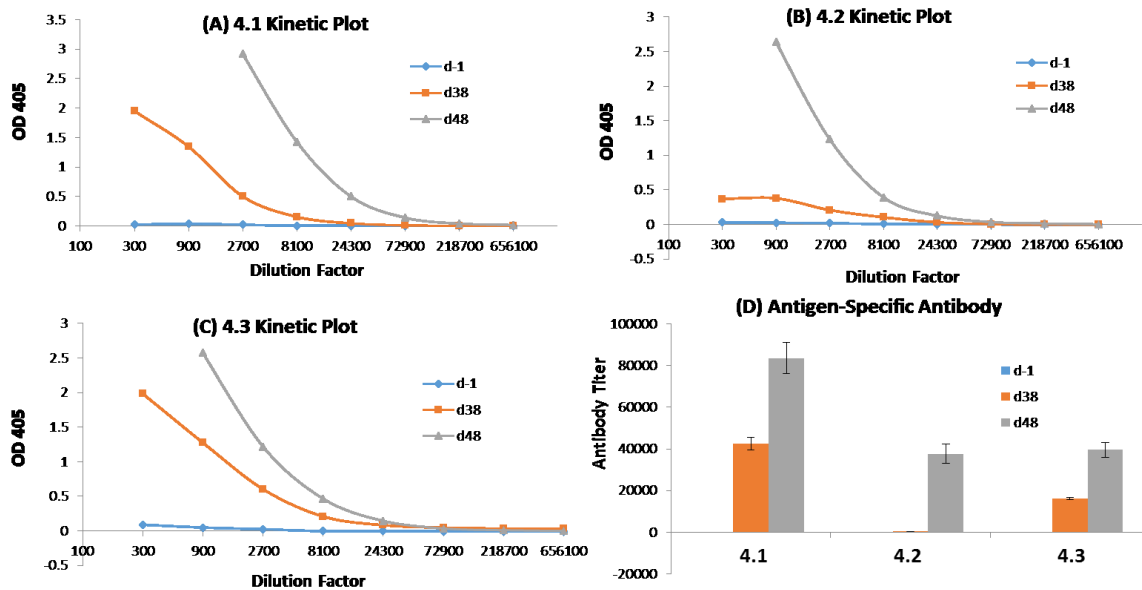
The immunological responses to conjugates **4.1-4.3** were evaluated by analyzing the antigen-specific antibody titers in the mouse sera by ELISA. For this purpose, each mouse was subjected to bleeding on day 0 before the initial injection (used as the blank control) and on day 27, day 38 and day 48 after immunization. The blood samples were treated to prepare sera according to standard protocols and the antisera were stored at  $-80\text{ }^{\circ}\text{C}$  before further analysis by ELISA with the corresponding HSA conjugates **4.4-4.6** as capture antigens. Antibody titers were calculated from the logarithm fit of the curves of

the OD value against serum dilution number, and were defined as the dilution number yielding an OD value of 0.5.

### Kinetic results of the immune responses

To investigate the changes in immune responses upon each boost immunization, we analyzed the total antibody (kappa) titers in the sera obtained at different date points.

**Figure 4.4** shows the antigen-specific total antibody titers of pooled sera. Clearly, all of the three conjugates elicited strong immune response after 4 boost immunizations, and the antibody titers of the day 48 antisera were significantly higher than that of day 38 antisera. Most significantly, some of the conjugates, e.g. **4.2**, did not stimulate strong responses until the 4<sup>th</sup> boost immunization.



**Figure 4.4** ELISA results of total antibodies (kappa) of the pooled antisera collected on day 0, day 38 and day 48 from mice immunized with: (A) **4.1** plus Titermax Gold adjuvant; (B) **4.2** plus Titermax Gold adjuvant; (C) **4.3** plus Titermax Gold adjuvant. (D) Calculated antigen-specific total antibody titers of the conjugates.

It is worth pointing out that conjugate **4.2** was somehow not well soluble in 1X PBS.

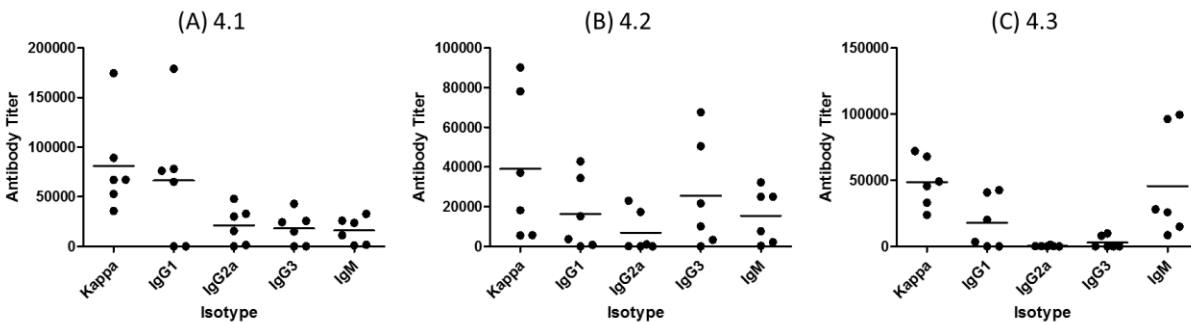
The low antibody titer of **4.2** after first 3 boost immunizations might be due to this problem. Thereafter, the method to prepare vaccines for immunization was improved. The conjugates were first dissolved in 10X PBS (high ionic concentration can help the conjugates to dissolve) and then diluted to 2X PBS buffer, which were finally mixed with adjuvant. Furthermore, for the initial and first 3 boost immunization, the route of vaccine administration was subcutaneous, but the 5<sup>th</sup> boost immunization was intramuscular. All these changes might have helped conjugate **4.2**.

In conclusion, conjugates **4.1-4.3** could elicit robust immune responses, and the induced immune responses were dependent on the frequencies of boost immunization. With the increase in the number of boost immunization, the immune responses became stronger. The results also suggested that immune responses might be dependent on the antigen structure, as the 3 conjugates were different. In addition, it seems that the preparation and administration methods of the vaccines also had some impact on the immune response, which is an interesting question worth further investigation.

### **Isotypes of antibodies**

In addition to the total antibody titer which shows the overall immune response to a specific vaccine, we have also examined the titers of various antibody isotypes of the antiserum obtained with each vaccine to show the quality of the immune response. The ELISA results of day 48 antisera are depicted in **Figure 4.5**. Each dot represents the antibody titer of an individual mouse, and the black bar represents the average antibody titer of a group of six mice. It is evident that, in addition of IgM antibodies, all of the three conjugates **4.1-4.3** could elicit high levels of IgG1 antibodies, indicating the involvement of T cell-mediated immunity. In the case of **4.1** and **4.2**, significant levels of IgG2a and

IgG3 antibodies were also elicited. More detailed analyses revealed that the two mice with lower IgG1 antibody titers had higher IgG2a antibody titers and that the mice with higher IgG1 antibody titers always had lower IgG2a antibody titers. This phenomenon was consistent with the influence of cytokines on antibody isotype switching (**Table 4.2**). Nevertheless, each mouse showed high titer of at least one kind of IgG antibody. On the other hand, the elicitation of high titers of IgG1 antibody indicated the production of IL-4 cytokine.



**Figure 4.5** ELISA results of antigen-specific antibody isotypes in day 48 antisera of mice immunized with: (A) **4.1** plus Titermax Gold adjuvant, (B) **4.2** plus Titermax Gold adjuvant, (C) **4.3** plus Titermax Gold adjuvant, respectively. Each dot represents the antibody titer of an individual mouse, and the black bar represents the average antibody titer of a group of six mice.

**Table 4.2** Influence of cytokines on antibody isotype switching<sup>16</sup>

Cytokine	IgG1	IgG2a	IgG2b	IgG3	IgM
IL-4	Induces	Inhibits		Inhibits	Inhibits
IFN- $\gamma$	Inhibits	Induces		Induces	Inhibits
TGF- $\beta$			Induces	Inhibits	Inhibits

Previously, we found that KLH-carbohydrate conjugates usually elicited IgG1 antibodies whereas lipid A-carbohydrate conjugates typically elicited IgG1 and IgG3

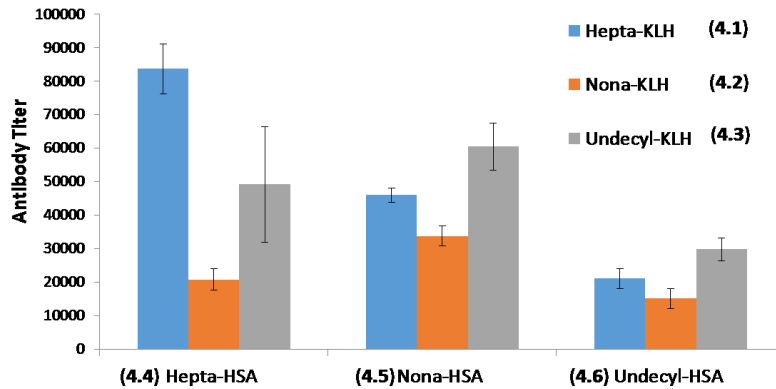


antibodies.<sup>14,17-19</sup> However, the KLH conjugates here elicited various isotypes of IgG antibodies. We proposed that different administration routes of vaccines might be one of the factors that influence the immune responses. Depending on whether the vaccine is administered by s.c. or i.m. injection, the antigen will meet different sets of antigen presenting cells (APCs) in the dermal area and in the muscle. As a result, the APCs will present the antigenic determinant in MHC-II molecule complex to the T helper cells in the nearest local draining lymph nodes which are different as well.<sup>20</sup>

Although **4.1-4.3** elicited similar immune responses, the IgG1, IgG2a and IgG3 antibody titers induced by the higher oligomer **4.3** (pentamer of the RRP repeating unit) were significantly lower than that induced by the other two conjugates. Literature results also indicated that the trimer and tetramer of the RRP repeating unit showed good immunogenicity.<sup>11,12</sup> These results combined suggest that the immunogenicity of a polysaccharide is not necessarily increased by simply increasing its length. This information should be especially useful and helpful for the design and development of fully synthetic conjugate vaccines based on oligosaccharide antigens.

### **Cross reactions**

To further investigate the properties of antisera induced by different conjugates, cross reactions between different antisera and antigens were carried out. The results are shown in **Figure 4.6**.



**Figure 4.6** ELISA results of the cross reactions between antigens displayed in 4.4-4.6 and pooled antisera of mice immunized with 4.1, 4.2 and 4.3.

In this study, the antisera were pooled for each group of 6 mice immunized with conjugates 4.1, 4.2 and 4.3, respectively. Conjugates 4.4, 4.5 and 4.6 were used to coat plates for ELISA analysis. It is clear that the antisera induced by the three conjugates had significant cross reactions, meaning the antibodies induced by each vaccine can at different levels recognize and bind the other two oligosaccharide antigens as well. Thus, they share some common structural motifs. On the other hand, the reactivities did show some differences. In general, antisera showed higher titers to the carbohydrate antigens same to the ones in the vaccines used to induce antisera. In addition to the structural factor, the loading percent of saccharides in the HSA conjugates and the concentrations of antibodies in the antisera may also affect the results shown in **Figure 4.6**. This makes it complex to derive any firm conclusion.

Nevertheless, this high level of cross reactions indicated that the structural motif of the polysaccharide repeat unit was probably the antigen determinant, and at least a part of the antibodies in the antisera were against this common motif. Alternatively, the results may be interpreted that the oligosaccharide antigens might have been degraded

*in vivo*, so that antibodies against different lengths of saccharides have been elicited. In the latter case, the antisera induced by **4.3** should show higher cross reactions to **4.4** and **4.5** than that of **4.1** to **4.5** and **4.6**, which is not conclusive. However, degradability of vaccines is indeed an important factor influencing vaccine immunity. Usually proteins vaccines are digested into pieces by APCs to become suitable for loading onto the MHC molecules for presentation to the T cells. However, how carbohydrate antigens are processed in the immune system is largely unclear presently.

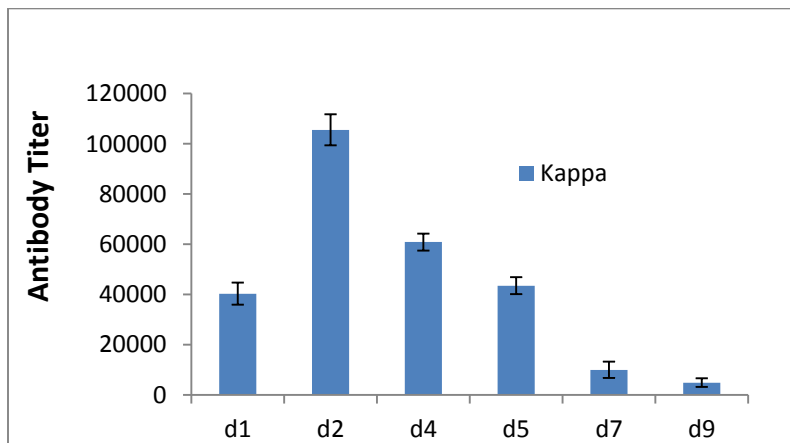
#### **4.1.3.2 MPLA Conjugates 4.7-4.9 as Vaccines**

Structurally well-defined and characterized conjugates **4.7-4.9** were synthesized by Dr. Guochao Liao in the Guo laboratory. Immunological studies of these conjugates were performed using female C57BL/6 mice, following the protocols described for conjugates **4.1-4.3**, except that different vaccine preparations and adjuvant schedules were involved. Because MPLA conjugates had low solubility in aqueous buffer, they were administered in the form of liposomes, prepared with 1,2-distearoyl-*sn*-glycero-3-phosphocholine and cholesterol. According to literature,<sup>17,21</sup> this not only can improve the solubility of vaccines but also can increase their immunogenicity, as the liposomes may help vaccine delivery *in vivo*. Again, antibody titers were calculated from the logarithm fit of the curves of OD value against serum dilution number, and were defined as the dilution number yielding an OD value of 0.1.

#### **Kinetic results of the immune responses**

Experiment to examine the antibody response kinetics against MPLA conjugates was performed with **4.7**. Thus, 1 day to 9 days after the 5th boost immunization, mouse

antisera were obtained and antigen-specific total antibodies were evaluated by ELISA to give normalized antibody titers. The results are shown in **Figure 4.7**. Clearly, 1 day after the boost immunization, high titers of antibodies started to appear, and it reached the maximum on day 2. Thereafter, the antibody titers started to decline, and at about day 9, the antibody titer became relatively small, even though significant immune response remained. These results demonstrated that the immune responses induced by previous immunizations were memorized and immediate and strong immune responses could be generated in the mice upon contact of the same antigen.

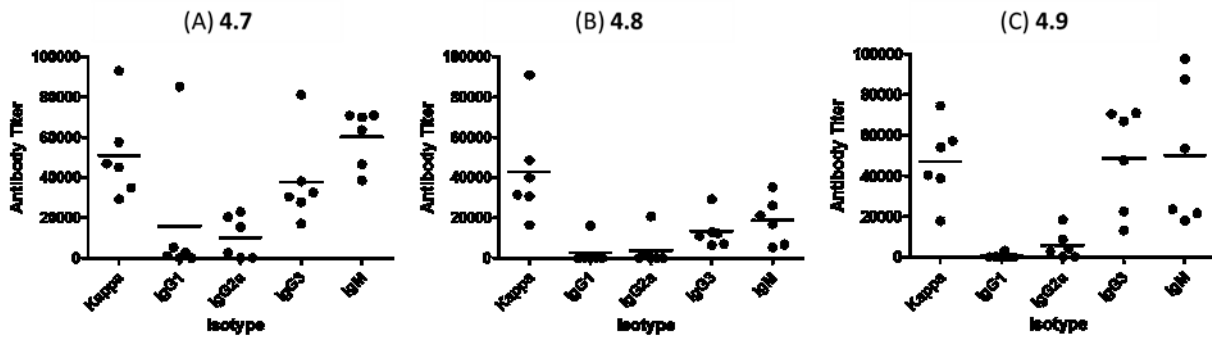


**Figure 4.7.** ELISA results of the anti-kappa (total) antibody titers (OD 405 = 0.1) in the pooled antisera of mice immunized with conjugate **4.7** obtained on day 1, 2, 4, 5, 7 and 9 after the 5<sup>th</sup> boost immunization.

### Isotypes of antibodies

The titers of antibody isotypes in the antisera obtained from mice two days after the 5<sup>th</sup> immunization with **4.7-4.9** were analyzed according to protocols described previously. As shown in **Figure 4.8**, all conjugates induced high titers of IgG3 antibodies, which is consistent with the pattern of immune responses to glycolipid conjugates.<sup>17,18</sup> In the meantime, significant levels of IgG1 and IgG2a antibodies were also observed in the

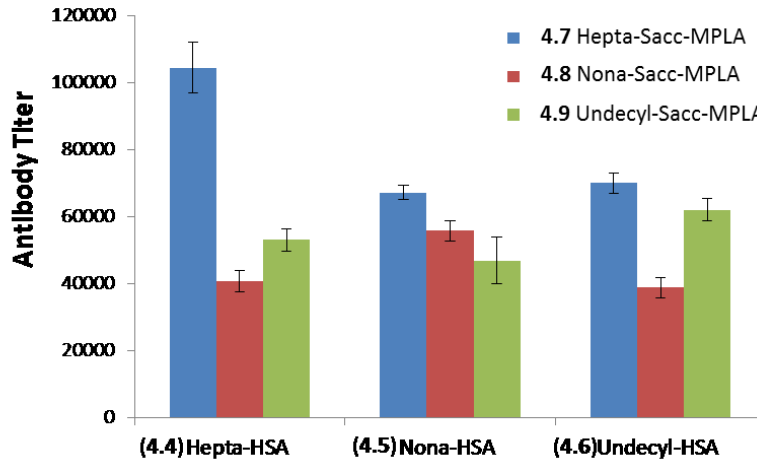
antisera. These results indicated T cell-mediated immune responses, which is important for the vaccines. There were some differences in terms of antibody titers among these conjugates, but the differences were much less obvious than that observed with KLH conjugates 4.1-4.3. Overall, conjugate 4.7 showed the highest IgG antibody, once again suggesting that longer saccharides are not necessarily better antigens/immunogens or induce stronger immune responses.



**Figure 4.8.** ELISA results of different isotypes of antigen-specific antibodies in day 2 antisera of mice after final boost immunization with 4.7, 4.8 and 4.9. Each dot represents the antibody titer of an individual mouse, and the black bar represents the average antibody titer of a group of six mice.

### Cross reactions

**Figure 4.9** shows the cross reactions of HSA conjugates 4.4-4.6 with all three groups of pooled antisera derived from mice immunized with MPLA conjugates 4.7-4.9. The results were very similar to that of the KLH conjugates (**Figure 4.6**). Once again, all antisera had significant cross reactions with all three different oligosaccharide antigens, suggesting that the three vaccines 4.7-4.9 might share some common antigenic motifs in terms of the RRP structure.

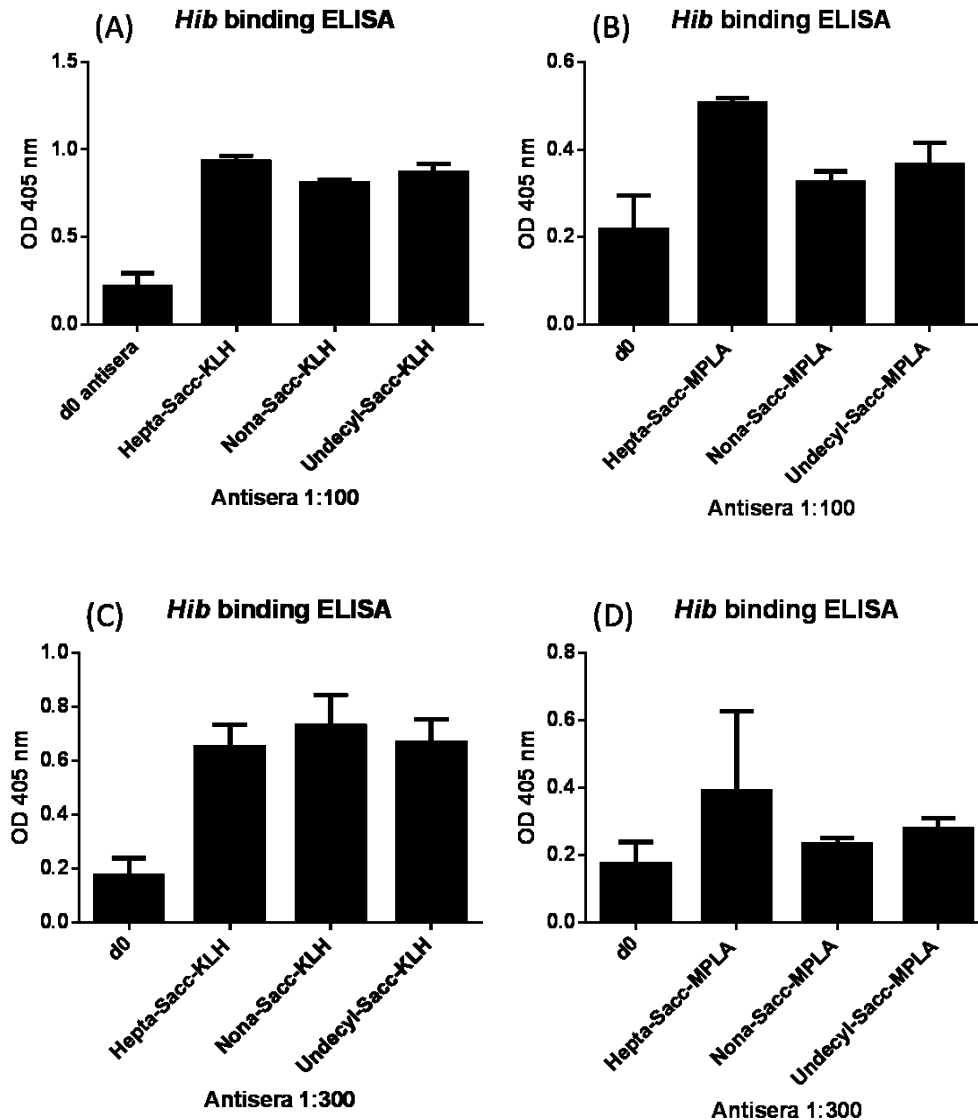


**Figure 4.9** ELISA results of the cross reactions between **4.4-4.6** and the antisera derived from mice immunized with **4.7-4.9**.

#### 4.1.3.3 Evaluation of the Hib-binding Property of Antisera

Although we have demonstrated that the synthetic vaccines could induce strong antigen-specific immunities, the coating antigens utilized in the ELISA experiments were respective oligosaccharides. The key question is whether the antibodies can recognize the carbohydrate antigens on the bacterial cell surface and interact with bacterial cells. To answer this question, the cell binding properties of these antisera were evaluated by two different experiments. One is the direct binding experiment, which was very similar to the regular ELISA except that Hib cells were used as the capture reagents. The other experiment was competitive ELSA. For the former experiment, Hib (ATCC 10211) cells instead of HSA conjugates were utilized to coat 96-well plates and then pooled mouse antisera obtained from mice immunized with conjugate **4.1-4.3** and **4.7-4.9** were added to the plates. After washing, AP-linked goat anti-mouse kappa antibody was added for

detecting antibodies bound to Hib cell-coated plates, reflected by the OD values at 405 nm. The ELISA results are showed on **Figure 4.10**.



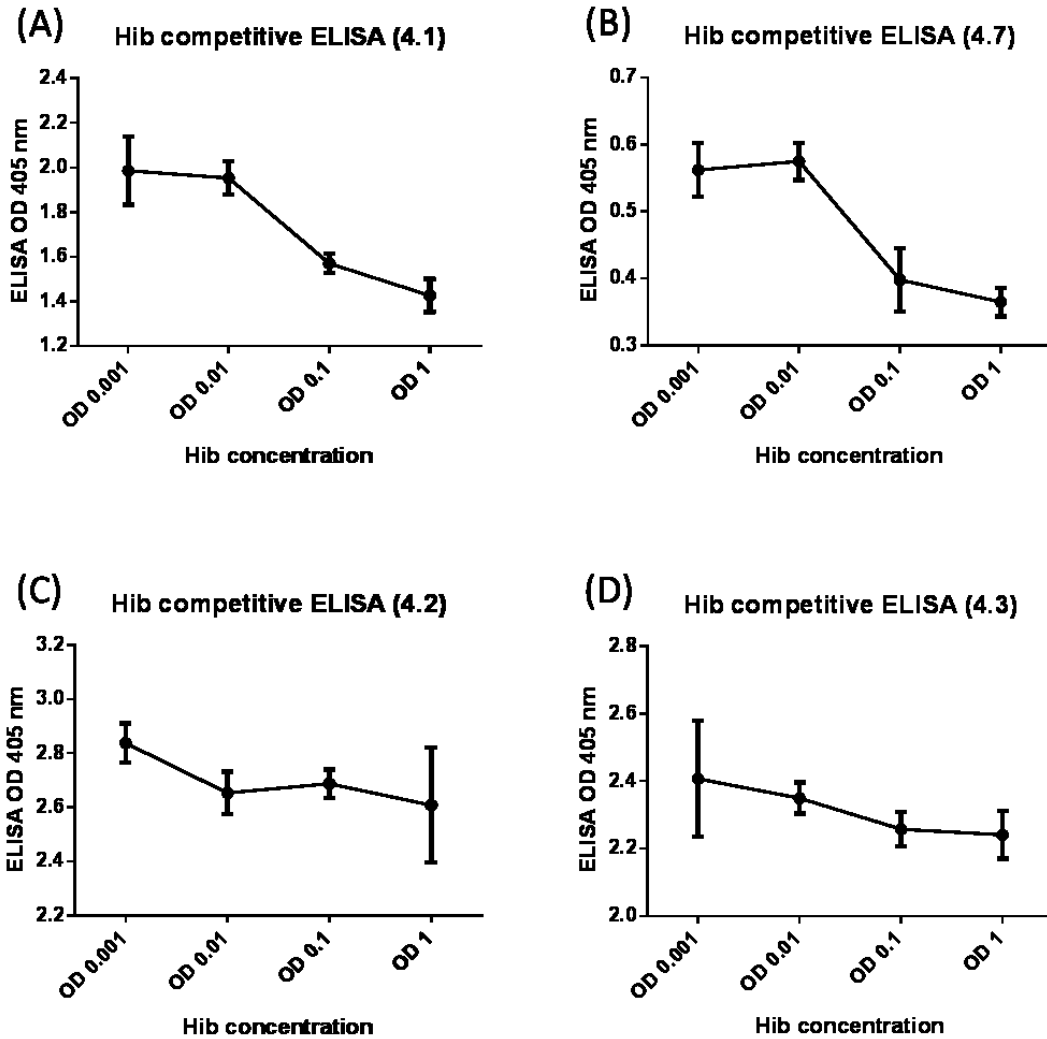
**Figure 4.10** ELISA results of antibody-Hib binding experiments. **(A)** and **(B)**: results of the 1:100 diluted pooled antisera of conjugates **4.1-4.3** and **4.7-4.9**; and **(C)** and **(D)**: results of the 1:300 diluted pooled antisera of **4.1-4.3** and **4.7-4.9** (d0: the pooled sera before immunization used as negative controls). The error bars represent standard deviations of three parallel experiments.

The pooled antisera before immunization (d0) were used as blank controls which had an OD value below 0.2. For the pooled antisera derived from all KLH conjugates, the

OD values were similar, ca. 0.85-0.95 (1:100 diluted sera) and 0.6-0.8 (1:300 diluted sera). The result confirmed that the antisera against KLH conjugates could successfully recognize and bind to Hib cells. For the antisera of MPLA conjugates, at 1:100 dilution, significantly higher OD values (0.3-0.5) than that of the d0 antisera were observed, but the difference was not significant with 1:300 diluted sera. These results correspond well with the observed antibody titers induced by various conjugate vaccines (**Figures 4.5 and 4.8**). In conclusion, these results have demonstrated that antisera elicited by KLH and MPLA conjugates could bind to Hib cells.

Competitive ELISA experiment was performed to confirm above antibody binding to Hib cells and verify its specificity. The experimental protocols were similar to that for regular ELISA except that, in the step of adding diluted antisera solution to coated plates, Hib cells were mixed to bind with the antibodies in the antisera solution to compete with the HSA-antigen conjugates. The results (**Figure 4.11**) showed that with the increase of Hib cells added to the plate well to compete with HSA conjugates coated in the plates, the plate OD values (representing antibodies bound to plates) decreased significantly in a concentration dependent manner. The antisera of two KLH conjugates **4.1-4.3** and **4.7** had similar results, though it seems that the competition for **4.1** is more effective than the others. Therefore, the competitive ELISA results have verified the specific binding of antisera obtained from mice immunized with our synthetic vaccines to Hib cells.





**Figure 4.11.** Hib competitive ELISA results using: (A) 1:2700 diluted antisera of mice immunized with KLH conjugate **4.1**; (B) 1:900 diluted antisera of mice immunized with MPLA conjugate **4.7**; (C) 1:300 diluted antisera of mice immunized with **4.2**; (D) 1:900 diluted antisera of mice immunized with **4.3**. The amounts of Hib cells in the experiments are represented by the OD value (X-axis) of cells at 600 nm, and higher OD means higher cell concentrations. Error bars are the standard deviation of three parallel experiments.

#### 4.1.4 Conclusions

In summary, KLH conjugates **4.1-4.3**, and MPLA conjugates **4.7-4.9**, can elicit strong immune response, especially the T cell-mediated immune response. The capability of conjugates to generate antibodies followed the order: **4.1**>**4.3**≈**4.2**, and **4.7**≥**4.9**>**4.8**,

respectively. Therefore, the Hepta-saccharide had the best results both in KLH conjugate and MPLA conjugate. This capability order also suggested that longer saccharides are not necessarily better antigens/immunogens or induce stronger immune responses. The cross reaction between the three structures was significant which revealed that vaccines might share some common antigenic motifs in terms of the RRP structure. The future works about this project can focused on the investigation about minimum antigen determinant which can provide a principle to direct the design of more effective vaccines. To achieve this purpose, the technology of monoclonal antibodies (mAbs) can be employed to produce RRP-specific mAb for the study of binding properties with different oligomer antigens. This kind of mAb binding studies was well-established in the exploration of fungal vaccines by Bundle's group.<sup>22</sup>

#### 4.1.4 Experiment Section

##### KLH conjugates

**Table 4.3** Doses of KLH conjugates used in animal studies

Conjugates	Loading (%)	Each mouse ( $\mu\text{g}$ )	6 mice ( $\mu\text{g}$ )	6mice 4 times ( $\mu\text{g}$ )
Hepta-KLH	8.4	35.7	214.2	856.8
Nona-KLH	8.4	35.7	214.2	856.8
Undecyl-KLH	9.0	33.3	199.8	799.2

All glycoconjugates were synthesized by Dr. Guochao Liao. Titermax Gold adjuvant, 1, 2-distearoyl-*sn*-glycero-3-phosphocholine, and cholesterol were purchased from Sigma-Aldrich. Alkaline phosphatase linked goat anti-mouse kappa, IgM, IgG1, IgG2a, and IgG3 antibodies and FITC-labeled goat anti-mouse kappa antibody were

purchased from Southern Biotechnology. Female C57BL/6J mice of 6-8 weeks age used for immunological studies were purchased from the Jackson Laboratory. The doses of KLH conjugates were showed in **Table 4.3**.

### **General Procedure for the Preparation of Emulsion of Conjugates**

Each conjugate (30 doses) was dissolved in small 0.3 ml 10× PBS buffer and then was diluted to 1.5 ml 2× PBS solution. The solution was mix with 1.5 ml Titermax Gold adjuvant (1:1, v/v) and to form an emulsion according to the manufacturer's protocol. (Usually, 1× PBS buffer works well to dissolve the KLH conjugates. However, these conjugated are difficultly dissolved in 1× PBS buffer. The high ionic concentration of 10× PBS buffer may help the conjugates to dissolve in water-based buffer.)

### **Immunization of Mouse**

Each group of six female C57BL/6 mice were immunized on day 1 by subcutaneous (sc) injection of 0.1 mL of the emulsion of the conjugate vaccine and Titermax Gold adjuvant prepared according to the manufacturer's protocol. Following the initial immunization, mice were boosted 4 times on day 14, day 21, day 28 and day 38 by sc injection of the same conjugate and by the same immunization protocol. Blood samples of each mouse were collected through the leg veins prior to the initial immunization on day 0 and after immunization on day 27, day 38 and d 48 were clotted to obtain antisera that were stored at -80 °C before use.

### **Protocols for ELISA**

ELISA plates were treated with 100 ul of a solution of individual conjugates (**4.4 - 4.6**) (2 µg/ml) dissolved in coating buffer (0.1 M bicarbonate, pH 9.6) at 4 °C overnight, and then at 37 °C for 1 h, which was followed by treatment with blocking buffer (10% BSA

in PBS solution with  $\text{NaN}_3$ ) and washing 3 times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST). Thereafter, a pooled or an individual mouse antiserum with serial half-log dilutions from 1:300 to 1:656100 in PBS was added to the coated ELISA plates (100  $\mu\text{L}$ /well), which was followed by incubation at 37 °C for 2 h. The plates were then washed with PBS and incubated at rt for another 1 h with a 1:1000 diluted solution of alkaline phosphatase linked goat anti-mouse kappa, IgM or IgG2a antibody or with a 1:2000 diluted solution of alkaline phosphatase linked goat anti-mouse IgG1 and IgG3 antibody (100  $\mu\text{L}$ /well), respectively. Finally, these plates were washed with PBS and developed with 100  $\mu\text{L}$  of p-nitrophenylphosphate (PNPP) solution (1.67 mg/mL in buffer) for 30 min at rt, followed by colorimetric readout using a BioRad 550 plate reader at 405 nm wavelength. The optical density (OD) values were plotted against antiserum dilution values, and a best-fit line was obtained. The equation of the line was employed to calculate the dilution value at which an OD of 0.5 was achieved, and the antibody titer was calculated at the inverse of the dilution value.

### **MPLA conjugates**

<b>4.7</b> (Hepta-Sacc-MPLA):	MW=2959.22	14.8 $\mu\text{g}$ /mouse,
<b>4.8</b> (Nona-Sacc-MPLA):	MW=3305.45	16.53 $\mu\text{g}$ /mouse,
<b>4.9</b> (Undecyl-Sacc-MPLA)	MW=3651.68	18.26 $\mu\text{g}$ /mouse,

Dose:  $5.0 \times 10^{-9}$  mol/mouse, about 9.4  $\mu\text{g}$  saccharide per mouse.

### **General Procedure for the Preparation of Liposomes of Glycoconjugates 4.7-4.9.**

The mixture of each specific MPLA conjugate (**4.7-4.9**) (75.2  $\mu\text{g}$  of saccharide moiety, 0.04  $\mu\text{mol}$ ), 1, 2-distearoyl-sn-glycero-3-phosphocholine (M.W. 790.15, 200  $\mu\text{g}$ ,

0.26  $\mu\text{mol}$ ), and cholesterol (M.W. 386.66, 77  $\mu\text{g}$ , 0.20  $\mu\text{mol}$ ) (a 10:65:50 molar ratio) was dissolved in  $\text{CH}_2\text{Cl}_2$  and MeOH (1:1, v/v, 2 mL) in a 10 mL vial. Then, the solvents were removed in vacuum to form a thin lipid film on the vial wall, which was hydrated by adding 0.80 mL of HEPES buffer (20 mM, pH 7.5) containing NaCl (150 mM) and was finally sonicated for 1 h to obtain the desired liposomes. (This kind of conjugates were difficultly dissolved in water-based buffer. So, a long time sonication was need for making milk-like liposome form.)

### **Immunization of Mouse**

Each group of six female C57BL/6 mice were immunized on day 1 by subcutaneous (sc) injection of 0.1 mL of the liposomal solution of a specific glycoconjugate 1-3 containing 9.4  $\mu\text{g}$  of the carbohydrate antigen (5.0 nmol) or by injection of an emulsion of the liposomal solution of a specific glycoconjugate vaccine. Following the initial immunization, mice were boosted 3 times on day 14, day 21 and day 28 by sc injection of the same conjugate and by the same immunization protocol. Blood samples of each mouse were collected through the leg veins prior to the initial immunization on day 0 and after immunization on day 27 and day 38 and were clotted to obtain antisera that were stored at  $-80\text{ }^\circ\text{C}$  before use.

The ELISA results of day 27 and day 38 antisera showed that the antibody titer was very low which may be caused by that antibody may disappear on day 7 and day 10 after the boost. The maximum antibody titer may appear on the earlier days after the boost. Therefore, the blood was collected again on day 1, 2, 4, 5, 7, and day 9 after another boost and the antisera were analyzed.

## Protocols for ELISA

ELISA plates were treated with 100  $\mu$ l of a solution of HSA conjugates **4.4-4.6** (2  $\mu$ g/ml) dissolved in coating buffer (0.1 M bicarbonate, pH 9.6) at 4 °C overnight, and then at 37 °C for 1 h, which was followed by treatment with blocking buffer (10% BSA in PBS solution with  $\text{NaN}_3$ ) and washing 3 times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST). Thereafter, a pooled or an individual mouse antiserum with serial half-log dilutions from 1:300 to 1:656100 in PBS was added to the coated ELISA plates (100  $\mu$ L/well), which was followed by incubation at 37 °C for 2 h. The plates were then washed with PBS and incubated at rt for another 1 h with a 1:1000 diluted solution of alkaline phosphatase linked goat anti-mouse kappa, IgM or IgG2a antibody or with a 1:2000 diluted solution of alkaline phosphatase linked goat anti-mouse IgG1 and IgG3 antibody (100  $\mu$ L/well), respectively. Finally, these plates were washed with PBS and developed with 100  $\mu$ L of p-nitrophenylphosphate (PNPP) solution (1.67 mg/mL in buffer) for 30 min at rt, followed by colorimetric readout using a BioRad 550 plate reader at 405 nm wavelength. The optical density (OD) values were plotted against antiserum dilution values, and a best-fit line was obtained. The equation of the line was employed to calculate the dilution value at which an OD of 0.1 was achieved, and the antibody titer was calculated at the inverse of the dilution value.

## Protocols for Hib binding ELISA experiments

*Haemophilus influenzae* type b (Hib, ATCC 10211) was chose to be performed this experiment. Hib was incubated in ATCC #814 agar medium in 37 °C with 5%  $\text{CO}_2$  atmosphere for 24 h.

Bacterial cells were collected and resuspended in 10% buffered formalin phosphate (4% w/w formaldehyde, 0.4% w/w sodium phosphate monobasic monohydrate, 0.65% w/w sodium phosphate dibasic anhydrous, 1.5% w/w methanol) for 15 min. Then the fixed bacteria solution was centrifuged in 1000 rpm for 5 min. The chocolate agar medium precipitated in the bottom was removed, and the supernate containing bacterial cells was used to coat the 96-well plates. Each well of 96-well plate was coated with 100  $\mu$ L bacteria solution which had an OD value of 1 at 600 nm. The 96-well plate was dried off under biological cabinet in room temperature and formed a thin bacterial film on the bottom of the well.

The bacteria-coated plate was then treated with BSA blocking buffer (200  $\mu$ L/well) in r.t. for 1 h and then washed three times with PBS containing 0.05% Tween-20 (PBST). Then, the pooled antisera from mice immunized with **4.1-4.3** or **4.7-4.9** with dilutions 1:100 or 1:300 in PBS were added to the coated plate (100  $\mu$ L/well), respectively, and incubated at 37 °C for 2 h. The plates were washed with PBST and incubated at r.t. for another one hour with a 1:1000 diluted solution of alkaline phosphate (AP) linked goat anti-mouse kappa antibody (100  $\mu$ L/well). Finally, the plates were washed with PBST and developed with 200  $\mu$ L of a p-nitrophenylphosphate (PNPP) solution (1.67 mg/mL in PNPP buffer). After reacting for 30 min at r.t., 100  $\mu$ L solution obtained from each well was used for colorimetric readout using a microplate reader at 405 nm wavelength. OD values represented the binding ability of antisera to bacteria.

### **Protocol for Hib Competitive ELISA**

The protocol for this experiment was the same as for regular ELISA described in above except that Hib solution (in PBS) was utilized to compete with plate-bound antigen-HSA (4.4-4.5) for antibody binding. Thus, after an ELISA plate was treated with different antigen-HSA and the blocking solution sequentially and then washed with PBS, 100  $\mu$ L of the pooled antisera obtained from mice immunized with conjugates (1:300, 1:900 or 1:2700 dilution) in PBS containing different concentration Hib (OD 600 nm value at 0.001, 0.01, 0.1, and 1) was added to each well of the ELISA plate, respectively. Then, the plate was incubated in 37 °C for 2 h and washed. Finally, the plate was treated with alkaline phosphatase linked goat anti-mouse kappa antibody (1:1000 dilution), washed, developed with PNPP, and finally subjected to colorimetric readout at 405 nm wavelength, as described above.



## 4.2 Vaccines against *Neisseria Meningitides* Serogroup C

### 4.2.1 Introduction

*Neisseria meningitides* remains a leading cause of bacterial meningitis and septicaemia in the United States and other countries.<sup>23-26</sup> According their capsular polysaccharides, they are classified into 13 serogroups.<sup>27,28</sup> In worldwide, most cases of meningococcal diseases were caused by serogroup A, B, C, Y and W-135.<sup>27</sup> Right now, some multivalent polysaccharide vaccines, such as MPSV4 (Menomune; Sanofi-Pasteur, 1981), ACWY Vax (GlaxoSmithKline) and MCV4 (Menactra; Sanofi Pasteur, 2005), are available to provide protection against serogroup A, C, Y and W-135.<sup>28</sup>

In 1981, the first meningococcal vaccine, MPSV4 (Menomune; Sanofi Pasteur), based on polysaccharides from serogroup A, C, Y and W-135, was licensed in US.<sup>26</sup> However, generally, the polysaccharide vaccines have some marked limitations, such as lacking of induction of immunological memory, relatively short duration of protection and low immunogenicity in infants.<sup>26,29</sup>

To overcome the limitation of low immunological efficiency of vaccines, conjugate vaccines in which polysaccharides extracted from *Neisseria meningitidis* are covalently linked to a carrier protein have been developed. Therefore, in 2005, the first such product, the quadrivalent meningococcal vaccine MCV4 (Menactra; Sanofi Pasteur) containing polysaccharides from *N. meningitidis* serogroup A, C, Y and W-135 conjugated individually to diphtheria toxoid (DT), has been approved in United States.<sup>26,29</sup>

The second such product, MenACWY-CRM (Menveo; Novartis Vaccines and Diagnostics), another quadrivalent conjugate vaccine, containing *N. meningitidis* serogroup A, C, Y and W-135 oligosaccharides conjugated individually to

Corynebacterium diphtheria cross-reactive material (CRM<sub>197</sub>) was approved by the US FDA in 2010, to prevent invasive meningococcal disease in people aged 11-55 years.<sup>29,30</sup>

For serogroup B, the polysaccharide of capsular is  $\alpha$ [2,8]-linked polysialic acid that is identical structure to polysialic acid in fetal neural tissue and therefore may be immunotolerated in human immune system.<sup>27</sup> One of the general strategy to overcome this immunotolerance problem is to modify the carbohydrate antigen to be a foreign structure to the host as a vaccine using for immunization. Then the antibody elicited by the modified carbohydrate-based vaccine may further cross-react with the natural glycan on the pathogen surface. Bruge's group developed a group B meningococcal vaccine, consisting of N-propionylated (NPr) B capsular polysaccharide conjugated to tetanus toxoid (TT) carrier protein resulting in moderately higher NPr B polysaccharide-specific antibody titers with a certain cross reaction with B polysaccharide antigen.<sup>31</sup> However, the induced antibodies lack functional activity *in vitro*. Thus, further investigation about this strategy are still needed.

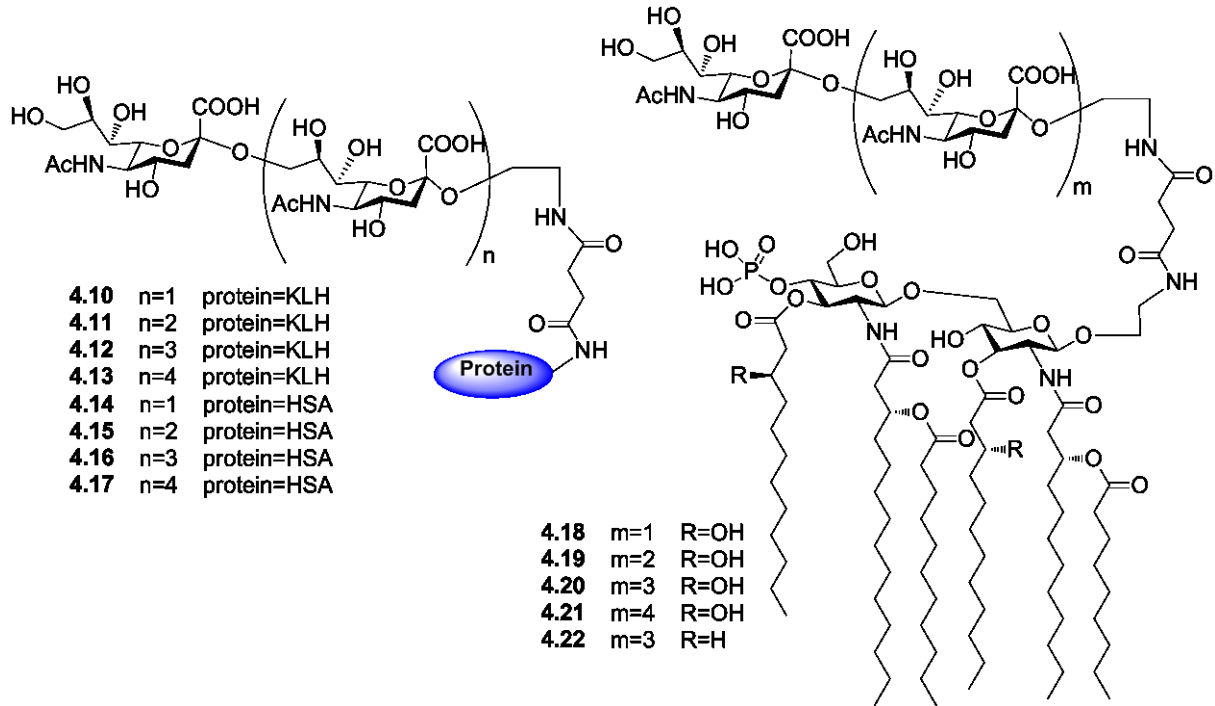
The other method to develop the vaccine against serogroup B *Neisseria meningitidis* is based on the surface-exposed proteins expressed by the genome sequence. Rappuoli's group and their collaborators developed a total of 350 candidate antigens expressed in *Escherichia coli*, purified and used to immunize mice.<sup>32</sup> The surface exposed proteins conserved in sequence across a range of strains can induce a bactericidal antibody response which may be not only against group B *N. meningitidis* but also against other serogroups and species of pathogenic *Neisseria*.<sup>32</sup>

In conclusion, the above studies have clearly demonstrated that a carbohydrate hapten conjugates with a peptide are effective vaccine design against *meningitidis*. To

overcome the immunotolerance of serogroup B *N. meningitidis* antigen,  $\alpha$ [2-8]-linked polysialic acids, the vaccines based on modified carbohydrate antigens, or the proteins expressed on pathogen surface were explored.

#### 4.2.2 Our Vaccine Designs

The previous development of full-synthetic carbohydrate-based vaccines with protein carrier or MPLA carrier to against cancer and Hib, encouraged us to develop the vaccines against *meningitidis* based on the capsular polysaccharide antigens of serogroup C,  $\alpha$ [2-9]-linked polysialic acids. Therefore, our group designed dimer, trimer, tetramer and pentamer sialic acids, which were successfully synthesized by Guochao Liao, and linked them to KLH and MPLA to form conjugates **4.10-4.13** and **4.18-4.22**. (**Figure 4.12**). In the meantime, these oligosaccharides were also coupled with HSA to get conjugates **4.14-4.17** that were utilized as the capture reagents in ELISA experiment. These new glycoconjugates, **4.10-4.13** and **4.18-4.22** were evaluated as vaccines in mice.



**Figure 4.12** The structures of designed  $[\alpha 2-9]$  linked oligo sialic acids KLH conjugates (4.10-4.13), HSA conjugates (4.14-4.17), and MPLA conjugates (4.18-4.22)

## 4.2.3 Results and Discussion

### 4.2.3.1 KLH Conjugates 4.10-4.13 as Vaccines

After the KLH conjugates 4.10-4.13 and HSA conjugates 4.14-4.17 were synthesized, their weight% loading of the saccharide moiety were examined by the same method described in Hib vaccines, and the results were showed in **Table 4.4**.

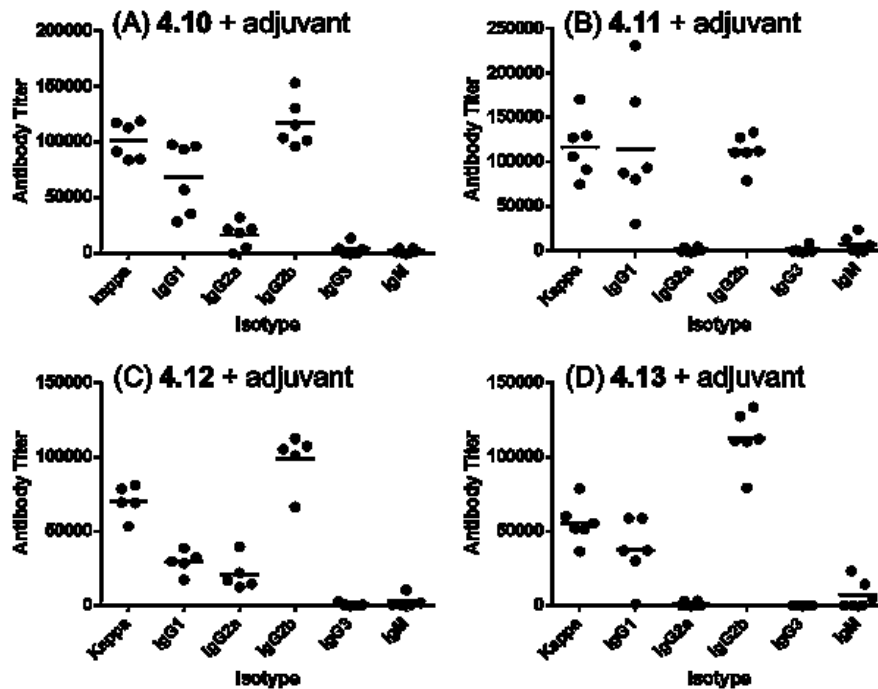
**Table 4.4** The loading of saccharide moiety in protein conjugates **4.10-4.17**

Conjugates	Loading (wt %)
<b>4.10</b> (Dimer-KLH)	7.52
<b>4.11</b> (Trimer-KLH)	11.49
<b>4.12</b> (Tetramer-KLH)	7.89
<b>4.13</b> (Pentamer-KLH)	6.83
<b>4.14</b> (Dimer-HSA)	8.58
<b>4.15</b> (Trimer-HSA)	11.48
<b>4.16</b> (Tetramer-HSA)	10.92
<b>4.17</b> (Pentamer-HSA)	7.79

Immunological studies of oligo-sialic acid-KLH conjugates (**4.10-4.13**) were carried out with female C57BL/6J mouse. For initial immunizations, each glycolconjugate containing  $4.7 \times 10^{-9}$  mol saccharide residues mixed with Titermax Gold adjuvant as an emulsion (0.1 mL) was injected intramuscular (i.m.) in mice (3  $\mu$ g Dimer-sialic acid moiety per mouser, see experiment section). More boost immunization were carried out in these mice by subcutaneous (s.c.) injection on day 14, day 21 and day 28, using the same vaccines. Each mouse was subjected to bleeding on day 0 before the first injection (used as blank controls) and on day 27 and day 38 after immunization. The blood samples were treated according to standard protocols to prepare antisera for the analysis of antigen-specific antibodies by ELISA with the HSA conjugates **4.14-4.17** as capture reagents. In addition to total antibodies, antibody isotypes such as IgG1, IgG2a, IgG2b, IgG3 and IgM were also individually assessed. Antibody titers were calculated from linear regression

analysis of the curves of the optical density (OD) value against the logarithmic scale of serum dilution number, and were defined as the dilution number yielding an OD value of 0.2.

**Figure 4.13** showed the antibody titers of antigen-specific antibodies in d38 antisera obtained from mice immunized with **4.10-4.13**. Each dot represents the antibody titer of an individual mouse, and the black bar represents the average antibody titer of a group of six mice. The results revealed that all the conjugate **4.10-4.13** can elicit strong immune responses, especially the high level of IgG1, IgG2a and IgG2b antibodies indicating the involvement of T cell-mediated immunity. In additionally, all the mice had similar antibody titer and antibody isotype. The ratio of successful immunization could be considered as 100% that means all individual can be successfully provoked to produce immune response by the vaccine.



**Figure 4.13** ELISA results of antigen-specific antibody isotypes in day 38 antisera of mice immunized with (A) **4.10** plus Titermax Gold adjuvant, (B) **4.11** plus Titermax Gold

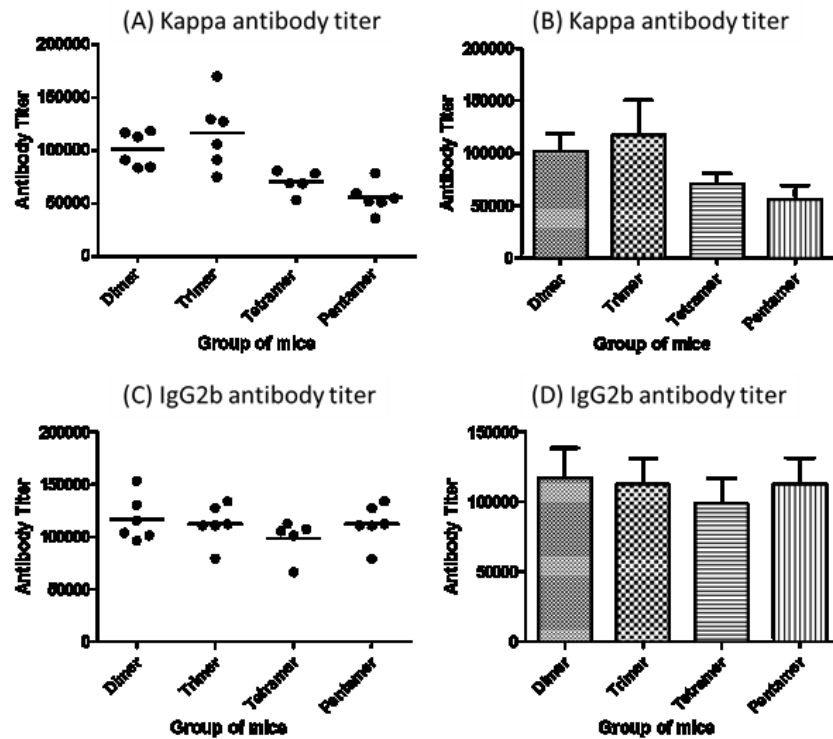
adjuvant, (C) **4.12** plus Titermax Gold adjuvant, (D) **4.13** plus Titermax Gold adjuvant, respectively. Each dot represents the antibody titer of an individual mouse, and the black bar shows the average antibody titer of a group of five or six mice.

In all the isotypes, IgG2b was extremely higher than the other isotype for all the six mice. According **Table 4.2**, it indicated that TGF- $\beta$  cytokine was involved in this immune response. The IgG1 was also showed good antibody titers which may indicated that IL-4 cytokines was involved. The corresponding T cell immune pathway can be figure out according this relationship between cytokines and antibody isotype switching (**Table 4.2**).<sup>16</sup> Falk Nimmerjahn and Jeffrey V. Ravetch<sup>33</sup> found out that different isotype of IgG can bind to different Fcy receptors which included activation and inhibitory receptors. Based on the activating-to-inhibitory (A/I) ratio of the receptors IgG binding to, the hierarchy of activity for the IgG subclasses was thus IgG2a $\geq$ IgG2b>IgG1>>IgG3.<sup>33</sup> Therefore, in this result, the high antibody titer of IgG2b elicited by the vaccines could be considered that antibody IgG2b has very strong immune activities.

Furthermore, among IgG subclasses, IgG 2a and 2b are generally considered to be the most potent for activating effector responses and dominate antiviral immunity, which also supported the application potential of this anti-bacteria vaccine.<sup>34-38</sup>

In another aspect, carrier protein KLH should make an important role in IgG isotype switch because that protein antigens usually elicit a thymus-dependent response generally represented by the IgG1, 2a and 2b, whereas carbohydrate antigens can elicit thymus-independent responses that induce IgG3 antibody secretion.<sup>39</sup> Hence, in the carbohydrate antigen vaccine, carrier protein was very important to improve the immunogenicity. Moreover, the adjuvant, Titermax Golden adjuvant, worked well with poly-sialic acids KLH conjugates.

Kappa antibody isotype standing for the total antibodies, and IgG2b isotype representing T cell-mediated immunity, were selected to compare the immunogenicity of conjugates (**Figure 4.14**). There was no statistical difference between group Dimer **4.10** and Trimer **4.11**. Interestingly, the kappa antibody titers induced by the higher oligomer (**4.12** and **4.13**) were significantly lower than that induced by **4.11**, but the IgG2b antibody titers were almost the same for all the conjugates. The saccharide loading of HSA conjugates was closed to each other which may hardly affect the antibody titers. Hence, the trend of kappa antibody titer may due to the immunogenicity of oligo-sialic acids antigen only. Therefore, the capability of conjugates to generate the kappa antibodies followed the order  $4.11 \approx 4.10 > 4.12 \approx 4.13$ , while the capability to generate IgG2b was similar.

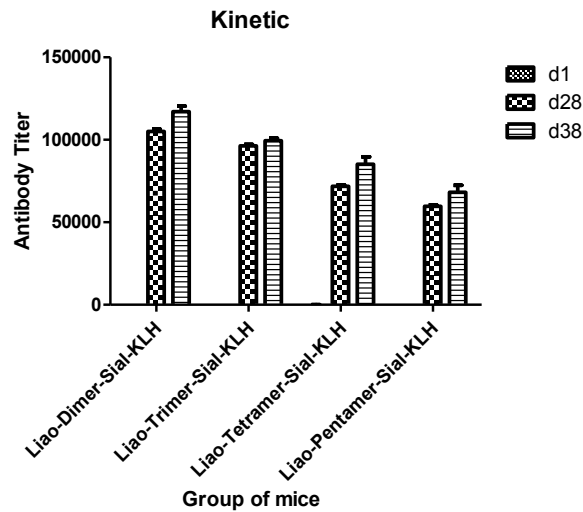


**Figure 4.14** The antigen-specific antibody titers of kappa (A and B) and IgG2b (C and D)



isotypes for all the four glycoconjugates **4.10-4.13**. In (A) and (C), each dot represents the antibody titer of an individual mouse, and the black bar shows the average antibody titer of a group of five or six mice. In (B) and (D), the error bar represented the standard deviation of six or five individual mice.

### Kinetic plots



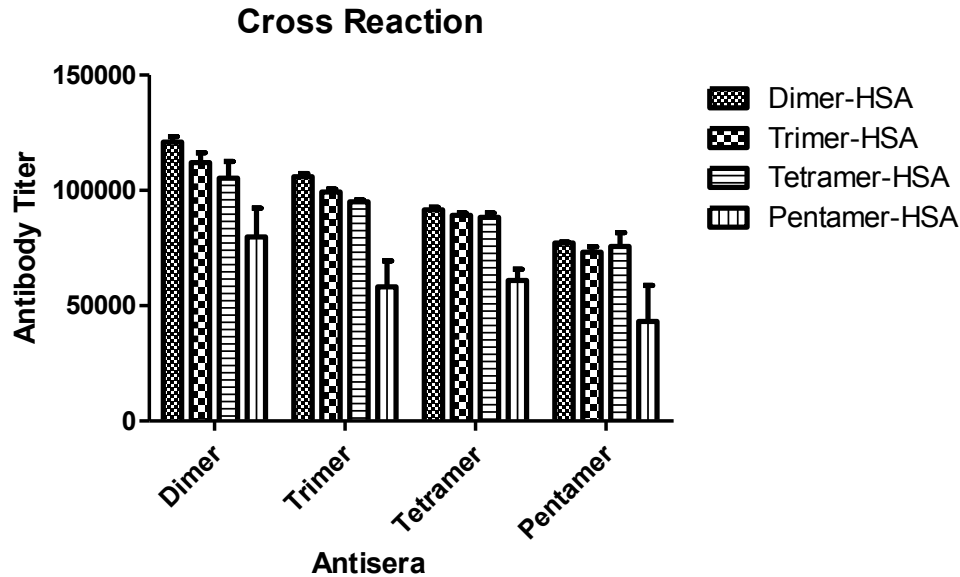
**Figure 4.15** The kinetic plot of glycoconjugates (**4.10-4.13**). The pooled antisera of day 28 was obtained from mice immunized three times with glycoconjugates, respectively. The pooled antisera of day 38 was obtained ten days later after the fourth boost from the mice immunized with glycoconjugates, respectively.

For all the four groups, antibody titer of kappa on day 38 was slightly higher than day 28. After third or fourth administration, the antibody titer had already reached a high level which indicated that three or four times injection was sufficient for the immunization.

### Cross reaction

To further investigate the properties of antisera induced by different conjugates, cross reactions between different antisera and antigens were carried out. The protocol of

cross reaction was similar as that in anti-Hib vaccines. The results are shown in **Figure 4.16**.



**Figure 4.16** The ELISA results of cross reactions between the pooled antisera obtained from mice immunized with conjugates (4.10-4.13) dimer-, trimer-, tetramer- and pentamer-sialic acid-KLH and the capture reagents (4.14-4.17) dimer-, trimer-, tetramer- and pentamer-sialic acid-HSA.

It is clear that the antisera induced by the four conjugates had significant cross reactions, meaning the antibodies induced by each vaccine can recognize and bind the other oligosaccharide antigens as well. Thus, they share some common structural motifs that should be dimer-sialic acid. This high level of cross reactions indicated that the structural motif of dimer-sialic acid was probably the minimum antigen determinant, and at least a part of the antibodies in the antisera were against this common motif.

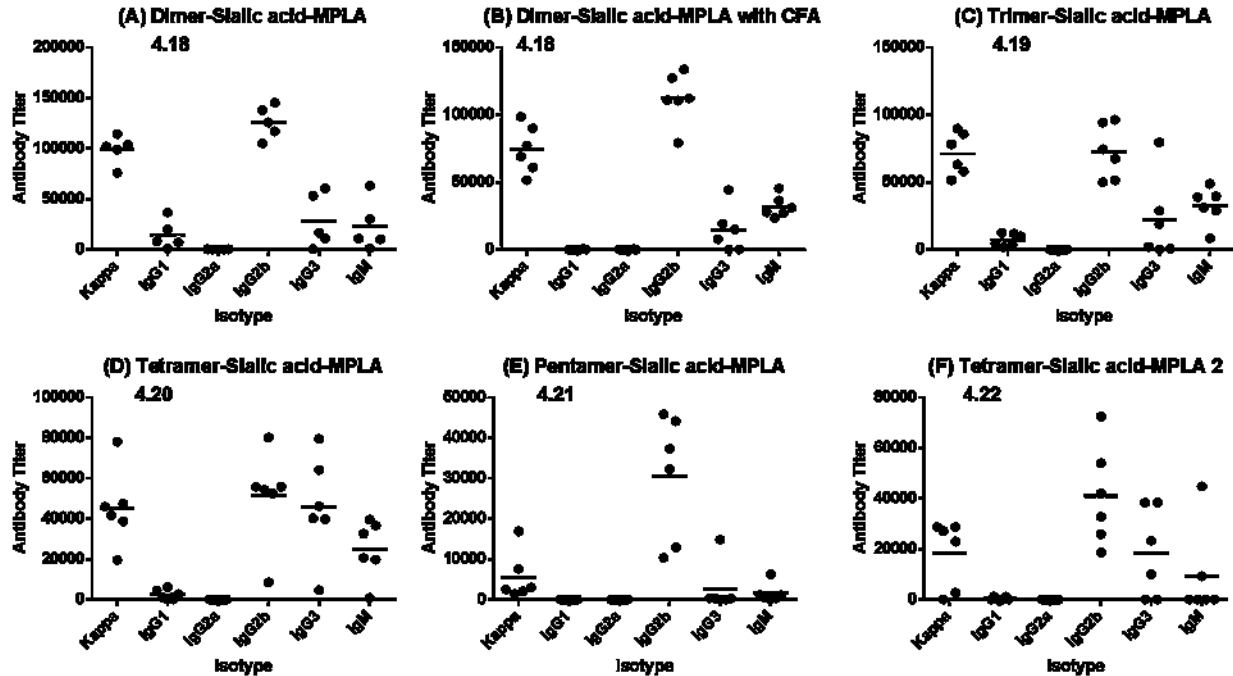
#### 4.2.3.2 MPLA Conjugates 4.18-4.22 as Vaccines

The MPLA conjugates **4.18-4.22**, as structural well-defined and characterized constructs, were synthesized by Dr. Guochao Liao in our laboratory. Immunological studies of these conjugates were performed using female C57BL/6J mice, following the similar protocols described for anti-Hib MPLA conjugates **4.7-4.9**.

All the conjugates **4.18-4.22** were incorporated in liposomes formed by using 1,2-distearoyl-*sn*-glycero-3-phosphocholine and cholesterol to improve the solubility in buffer and further improve the immunogenicity. For immunization, the liposomal preparations of conjugate **4.18-4.22** were each injected subcutaneously (s.c.) to a group of five or six mice. Each female C57BL/6J mouse was injected with 0.1 mL of a vaccine preparation containing ca. 14.0 nmol of saccharide moiety on day 1, 14, 21 and 28, respectively. Each mouse was subjected to bleeding on day 0 before the first injection (used as blank controls) and on day 27 and 35 after immunization. The blood samples were treated according to standard protocols to prepare antisera for the analysis of antigen-specific antibodies by ELISA with the HSA conjugates (**4.14-4.17**) as capture antigens. In addition to total antibodies, antibody isotypes including IgG1, IgG2a, IgG2b, IgG3 and IgM were also individually analyzed. Antibody titers were calculated from linear regression analysis of the curves of the optical density (OD) value against logarithmic scale of serum dilution number, and were defined as the dilution number yielding an OD value of 0.2.

#### Isotypes of antibodies

**Figure 4.17** shows the isotypes of antibodies in the antisera obtained from mice immunized with conjugate **4.18-4.22**.



**Figure 4.17** The ELISA results of antigen-specific antibody titers in day 35 antisera of mice immunized with (A) **4.18** alone, (B) **4.18** plus Freund's Completed Adjuvant (CFA), (C) **4.19** alone, (D) **4.20** alone, (E) **4.21** alone and (F) **4.22** alone, respectively. Each dot represents the antibody titer of an individual mouse, and the black bar shows the average antibody titer of a group of five or six mice.

The ELISA results of antibody titers in antisera obtained with **4.18-4.22** without the use of an external adjuvant (**Figure 4.17** except (B)) suggested that all the conjugates provoked a strong antigen-specific immune response in mice. Moreover, the high antibody titer of IgG2b isotype and some IgG1 and IgG3 antibody isotypes revealed that a T cell-dependent immune response was involved.<sup>17,18</sup> According to previous discussion, IgG2b has an extremely high immunological activity which further confirmed that this conjugate had strong immunogenicity and could be further developed to be a vaccine.<sup>33,37,38</sup> In addition, no significant difference between the antisera obtained from mice immunized with conjugate **4.18** alone and plus CFA adjuvant either in kappa or in IgG isotypes. This result confirmed the great adjuvant function of MPLA as a build-in

carrier and the possibility of adjuvant-free vaccine preparations.<sup>17,18</sup>

Tetramer-sialic acid saccharide was conjugated with two kinds of MPLA including the best one we optimized in chapter 3 and another one without two hydroxyl groups in the acetyl-chains (**Figure 4.12**).<sup>18</sup> The ELISA results of antisera obtained from mice immunized with **4.20** and **4.22** indicated that both the conjugates can provoke strong immune response in mice. However, the antibody titers of antisera obtained with **4.20** were significantly higher than that of **4.22** which revealed that the MPLA structure without two hydroxyl groups had much lower immunostimulatory ability.<sup>17,40</sup> In another aspect, this result confirmed that MPLA as a build-in adjuvant and as a carrier played an extremely important role in the immunostimulatory pathway that even a small change in the MPLA structure can induced a significant decrease in the final antibody production. To enhance the immunogenicity of MPLA conjugates, the optimized structure was the best choice for vaccine design.

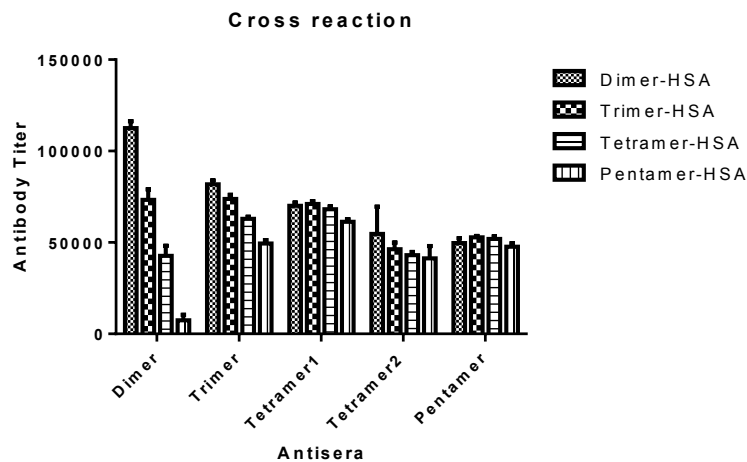
The ELISA results of antisera obtained from mice immunized with conjugate **4.21** were interesting that the total antibody titer was significantly decreased but the antibody titer of IgG2b isotype was almost the same as the other conjugates. The phenomenon was similar as that in KLH conjugates. This means that the pentamer-sialic acid-MPLA conjugate may have a comparable potential to be designed as a vaccine.

In conclusion, all these conjugates without external adjuvant can elicit strong immune response, especially the T cell-dependent immunity. The MPLA as a build-in adjuvant and a carrier plays an extremely important role in the immunostimulatory pathway although the details of antibody production and antibody isotype switching were not clear. Moreover, with the length of saccharide moiety increasing, the total antibody

titer was decreased slightly but the antibody titer of IgG2b isotype was consistent.

### Cross Reaction

To investigate the recognition pattern of provoked antibodies, the cross reactions between different length saccharides and the antisera obtained from mice immunized with different saccharides conjugates were carried out (**Figure 4.18**). Antisera of **4.18** had the highest antibody titer with Dimer-sialic acid-HSA, but had a significant lower cross reactivity with longer sialic acid-HSA. This phenomenon revealed that, although dimer-sialic acid did induce anti-dimer antibodies, these antibodies may be not protective due to the low reactivity with other oligomer antigens which should be more popular on bacteria surface. However, the cross reactivity of other conjugates (**4.19-4.22**) were strong and similar as that in KLH conjugates (**4.10-4.13**).



**Figure 4.18** The ELISA results of cross reactivity of the antisera induced by conjugates (**4.18-4.22**) dimer-, trimer-, tetramer- and pentamer-sialic acid-MPLA and the different capture reagents (**4.14-4.17**) from dimer- to pentamer-sialic acid-HSA.

The antibodies elicited by vaccines should be more effective if they can cross react with wild-spectrum oligosaccharide antigens. Thus the proper number of oligomer in this kind vaccine construct should be higher than 2. Combining with the conclusion in above

isotype discussion, trimer-sialic acid conjugates was the best design with best capability to generate effective antibodies. The cross reactivity was an important topic and worth to be further investigated.

#### 4.2.4 Conclusion

In summary, all the oligo-sialic acid-KLH and -MPLA conjugates can stimulate extremely strong immune response, especially high level of IgG2b antibody titer which indicated T cell-dependent immunity successfully involving. The IgG2b considered as one of the most powerful antibody isotypes was particularly concentrated. In each group, all five or six mice can be successfully immunized. Among these conjugates, trimer-sialic acid was the best candidate for both KLH and MPLA constructs. Dimer-sialic acid may be too short for a protective vaccine because the low cross reactivity of antibodies. The results of MPLA conjugates further confirmed the potential immunostimulatory ability of MPLA which can be developed to be an efficient carrier and build-in adjuvant. In future works, the deeply investigation of structure-activity relationships of oligo-sialic acid antigens should be an interesting and meaningful direction. For this purpose, the monoclonal antibodies (mAb) can be prepared and the interactions of mAb and oligomer antigens can be further explored by NMR binding studies.<sup>22</sup>

#### 4.2.5 Experiment Section

##### Materials, reagents and animals

All glycoconjugates were synthesized by Dr. Guochao Liao. Titermax Gold adjuvant, 1, 2-distearoyl-*sn*-glycero-3-phosphocholine, and cholesterol were purchased

from Sigma-Aldrich. Alkaline phosphatase linked goat anti-mouse kappa, IgM, IgG1, IgG2a, and IgG3 antibodies and FITC-labeled goat anti-mouse kappa antibody were purchased from Southern Biotechnology. Female C57BL/6J mice of 6-8 weeks age used for immunological studies were purchased from the Jackson Laboratory.

***N.M* group C vaccine poly-sialic acid-KLH conjugate:**

Saccharide about  $4.66 \times 10^{-9}$  mol per mouse (3  $\mu$ g Dimer-sialic acid moiety per mouse per time to scale up). The loading percentages are shown in **Table 4.4** above.

**Table 4.5** Dose of each KLH conjugate (4.10-4.13) used for immunizing mice

Dose	Saccharide/ $\mu$ g	Conjugates/ $\mu$ g	24 doses/ $\mu$ g	30 doses/mg
Dimer (4.10)	3.00	39.89	957.36	1.1967
Trimer (4.11)	4.35	37.89	909.36	1.1367
Tetramer (4.12)	5.71	72.376	1737	2.171
Pentamer (4.13)	7.07	103.47	2483	3.104

***N.M* group C vaccine poly-sialic acids-MPLA conjugates:**

$13.98 \times 10^{-9}$  mol (9  $\mu$ g dimer sialic acid moiety, 33.59  $\mu$ g conjugate) per mouse

**Table 4.6** Dose of each MPLA conjugate (4.18-4.22) used for immunizing mice

Dose	nmol	M.W.	1 dose/ $\mu$ g	30 doses/ $\mu$ g
Dimer (4.18)	13.98	2402.9432	33.59	1.007
Trimer (4.19)	13.98	2694.1978	37.66	1.1298
Tetramer 1 (4.20)	13.98	2985.4523	41.736	1.2521
Tetramer 2(4.22)	13.98	2953.4535	45.81	1.239
Pentamer (4.21)	13.98	3276.7069	41.29	1.3743



### **General Procedure for the Preparation of Emulsion of KLH Conjugates**

Each conjugate (30 doses, 1.19 mg of **4.10**, 1.14 mg of **4.11**, and 2.17 mg of **4.12**, and 3.10 mg of **4.13**, respectively) was dissolved in 1.5 ml 1× phosphate-buffered saline (PBS) buffer. The solution was mix with 1.5 ml Titermax Gold adjuvant (1:1, v/v) and to form an emulsion according to the manufacturer's protocol.

### **General Procedure for the Preparation of Liposomes of MPLA Glycoconjugates**

The mixture of a specific MPLA conjugate (**4.18-4.22**) (13.98 nmol for each dose), 1, 2-distearoyl-*sn*-glycero-3-phosphocholine (M.W. 790.15), and cholesterol (M.W. 386.66) (a 10:65:50 molar ratio) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and MeOH (1:1, v/v, 2 mL) in a 10 mL vial. Then, the solvents were removed in vacuum to form a thin lipid film on the vial wall, which was hydrated by adding 2.0 mL of HEPES buffer (20 mM, pH 7.5) containing NaCl (150 mM) and shaking the mixture in 40 °C water bath, and then shaking it by vortex mixer for several times to form a milky suspension. The milky suspension was finally sonicated for 1 min to obtain the desired liposomes.

The liposome of MPLA conjugates with double concentration of the front one, was mixed with the same volume of Completed Freud's adjuvant (CFA) to form a uniform formula according to the manufacturer's protocol.

### **Immunization of Mouse**

**For KLH conjugates:** Each female C57BL/6J mice in a group of six was immunized on day 1 by intramuscular (i.m.) injection of 0.1 mL of the emulsion of the conjugate vaccine and adjuvant prepared according to the manufacturer's protocol. Following the initial immunization, mice were boosted 3 times on day 14, day 21, and day 28 by subcutaneous (sc.) injection of the same conjugate prepared by the same protocol. Blood samples of

each mouse were collected through the leg veins prior to the initial immunization on day 0 and after immunization on day 28 and d 38 were clotted to obtain antisera that were stored at  $-80^{\circ}\text{C}$  before use.

**For lipid A conjugates:** Each female C57BL/6J mice in a group of six was immunized on day 1 by subcutaneous (sc.) injection of 0.1 mL of the liposome of the conjugate vaccine with/without adjuvant prepared according to the manufacturer's protocol. Following the initial immunization, mice were boosted 3 times on day 14, day 21, and day 28 by subcutaneous (sc.) injection of the same conjugate prepared by the same protocol. Blood samples of each mouse were collected through the leg veins prior to the initial immunization on day 0 and after immunization on day 18, day 28 and d 35 were clotted to obtain antisera that were stored at  $-80^{\circ}\text{C}$  before use.

### **Protocols for ELISA**

ELISA plates were treated with 100  $\mu\text{l}$  of a solution of HAS conjugate (2  $\mu\text{g}/\text{ml}$ ) dissolved in coating buffer (0.1 M bicarbonate, pH 9.6) at  $4^{\circ}\text{C}$  overnight, and then at  $37^{\circ}\text{C}$  for 1 h, which was followed by treatment with blocking buffer (10% BSA in PBS solution with  $\text{NaN}_3$ ) and washing 3 times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST). Thereafter, a pooled or an individual mouse antiserum with serial half-log dilutions from 1:300 to 1:656100 in PBS was added to the coated ELISA plates (100  $\mu\text{L}/\text{well}$ ), which was followed by incubation at  $37^{\circ}\text{C}$  for 2 h. The plates were then washed with PBS and incubated at rt for another 1 h with a 1:1000 diluted solution of alkaline phosphatase linked goat anti-mouse kappa, IgM, IgG2a and IgG2b antibody or with a 1:2000 diluted solution of alkaline phosphatase linked goat anti-mouse IgG1 and IgG3 antibody (100  $\mu\text{L}/\text{well}$ ), respectively. Finally, these plates were washed with PBS and

developed with 100 uL of p-nitrophenylphosphate (PNPP) solution (1.67 mg/mL in buffer) for 30 min at rt, followed by colorimetric readout using a BioRad 550 plate reader at 405 nm wavelength. The optical density (OD) values were plotted against antiserum dilution values, and a best-fit line was obtained. The equation of the line was employed to calculate the dilution value at which an OD of 0.2 was achieved, and the antibody titer was calculated at the inverse of the dilution value.

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**ABSTRACT****NEW CARBOHYDRATE-BASED ANTI-CANCER AND ANTI-BACTERIAL VACCINES**

by

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The unique carbohydrates expressed on the surface of cancer, bacterial, viral and fungal cells are excellent target antigens for the design of therapeutic or preventive vaccines. However, as antigens carbohydrates have problems. First, carbohydrates usually have low immunogenicity. Second, even if immunogenic, carbohydrates typically elicit T cell-independent immune responses. To overcome these problems and design useful vaccines based on carbohydrate antigens, they are usually coupled with carrier proteins to form conjugates to enhance the immunogenicity of the antigens. However, there are still some issues existing in glycoprotein vaccines, such as poor reproducibility of the conjugates, difficulties in quality control and so on. To deal with these issues, our group explored a strategy to utilize synthetic carbohydrate antigens with well-defined structures for the construction of glycoprotein vaccines. In the meantime, our group has also developed new vaccine carriers, such as monophosphoryl lipid A (MPLA), to construct full-synthetic carbohydrate-based vaccines that have well-defined structures and improved immunological properties. The main aims of this dissertation are to study

and evaluate these semi- and full-synthetic glycoconjugates and develop carbohydrate-based vaccines against cancer and bacteria.

The first part of this dissertation (Chapters 2 and 3) is focused on antitumor vaccines targeting at tumor-associated carbohydrate antigens (TACAs). For TACAs, in addition to the problems associated with carbohydrate antigens mentioned above, there is another problem, namely immunotolerance, due to their structural similarity to normal carbohydrates on normal cells. To overcome the immunotolerance problem, our group developed a novel immunotherapeutic strategy based on glycoengineering of sialo-TACAs on cancer cells. An important requirement for this strategy to work is to engineer cancer cells to express unnatural sialo-TACAs. In Chapter 2, a convenient method was developed for the quantification of various sialic acids expressed by cells and used to analyze the efficiency of *N*-phenylacetyl-D-mannosamine (ManNPhAc) to metabolically glycoengineer SKMEL-28 cancer cell. In specific, after cancer cells were cultured with ManNPhAc, the cells were treated with 2M acetic acid to release sialic acids and then with 1,2-diamino-4,5-methylenedioxybenzene (DMB). Sialic acids could react with DMB to form the corresponding derivatives that had strong UV absorptions. The reaction mixture was then applied to HPLC-UV analysis to determine the amounts and the ratios of natural sialic acid and its unnatural analog. It was confirmed that after incubation with ManNPhAc, the SKMEL-28 cell was effectively glycoengineered to express a significant amount of unnatural sialic acid.

Another requirement for the new cancer immunotherapeutic strategy is to have effective vaccines made of TACAs that contain the correspondingly modified sialic acid. In Chapter 3, a new construct of carbohydrate-based cancer vaccines with MPLA as the



carrier molecule and a build-in adjuvant, which are full-synthetic and potentially possess strong and self-adjuvanting immunological activities, were investigated. For this purpose, four MPLA analogs were prepared and immunologically evaluated to identify the ideal vaccine carrier. It was confirmed MPLA conjugates of chemically modified sTn antigen induced robust immune responses, thus they can be used as effective vaccines for the new cancer immunotherapeutic strategy. Furthermore, the optimized MPLA was used to develop Globo H-based anti-breast cancer vaccine. The immunological results of Globo H-keyhole limpet hemocyanin (KLH) and Globo H-MPLA conjugates indicated that the Globo H-MPLA conjugate had better immunogenicity than that of Globo H-KLH, including the capability of stimulating a T cell-mediated immunity. Therefore, Globo H-MPLA had the potential for being further developed into clinically useful vaccines.

The second part of this dissertation (Chapter 4) is focused on the development of antibacterial vaccines based on their capsular polysaccharide antigens. Instead of using polysaccharides isolated from bacterial cells, oligomers of the polysaccharide antigen repeating units were synthesized and then conjugated with a carrier protein, such KLH, or a MPLA derivative to form semi- or full-synthetic vaccines. The resultant conjugates were evaluated in mice and their structure-activity relationships were analyzed to identify the proper repeating unit oligomers for vaccine development. In this dissertation, two types of bacteria *Haemophilus Influenzae* type b (Hib) and group C *Neisseria meningitidis* were studied. The target antigen for Hib was the repeating ribosylribitol phosphate (RRP) polysaccharide, and for group C *N. meningitides*, the target antigen was  $\alpha$ [2,9]-linked polysialic acid. Immunological studies of these conjugates suggested that they all can stimulate strong T-cell mediated immune responses. Most importantly,

it was concluded that short oligomers of bacterial polysaccharide antigens can be highly immunogenic and induce immune responses that can recognize and bind the target pathogens.

In conclusion, in this dissertation, a new method was developed for quantitative analysis of cell surface sialic acids and analysis of the efficiency of sialic acid metabolic engineering. This method can be broadly useful for various cells and sialic acid analogs. In this dissertation, two different vaccine strategies, which could lead to semi- and fully synthetic vaccines, against cancer and bacteria have been investigated in great details. These vaccines showed promising properties and are worth further investigation. More importantly, the results of this dissertation have provided proof of principle for the new strategies, which may be widely applicable to other cancer and bacteria.

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- 3) Wang, Q.; Zhou, Z.; Tang, S.; Guo, Z.\*. Carbohydrate-Monophosphoryl Lipid A Conjugates are Fully Synthetic Self-Adjuvanting Cancer Vaccines Eliciting Robust Immune Responses in the Mouse. *ACS Chemical Biology*, **2012**, 7(1), 235-240. [13 citations] Cover Article and Introduced Author

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Zhou, Z.; Wang, Q.; Tang, S.; Guo, Z.\*. Carbohydrate-Monophosphoryl Lipid A Conjugate Cancer Vaccines.

- a. Graduate Exhibition of Wayne State University, Detroit, MI, March 18, **2014**. (poster presentation)
- b. The 9th Annual Midwest Carbohydrate Research Symposium, Toledo, OH, October 11, **2013**. (poster presentation)
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