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Recent Progress in Glycotherapy

Editor:
Qun Zhou

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Recent Progress in Glycotherapy

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FOREWORD

This book includes a timely collection of topics addressing important advances in glycotechnology that are being exploited in the development of glycotherapeutics.

This area is rooted in the rich history of the chemistry of carbohydrates that contributed to defining the structures of oligosaccharides and developed the complex synthesis of these exquisite molecules. In addressing the functions of these structures in animal cells glycobiologists, using the major advances in biochemistry and molecular biology developed in the last decades of the 20th century, unraveled the complex process involved in the biosynthesis, assembly, and processing of glycoproteins, glycolipids and proteoglycans. One of the seminal observations that initiated the general area of glycobiology was Victor Ginsburg's discovery in the early 1960's that neuraminidase digestion of lymphocytes altered their normal trafficking pattern, which ultimately led to the discovery of the animal cell lectins, and we now know that the functions of mammalian cell glycans are somehow associated with either direct or indirect protein-glycan interactions making them ideal candidates for glycotherapeutic studies with many examples already available.

The obvious challenge now resides in the continued search for the roles of glycans in normal physiology and disease; and these advances a coming rapidly in the field we now refer to as Glycomics, which has recently been recognized as a strategic area for NIH Common Fund support. The future is indeed bright, and the opportunities are ripe for the investigators to exploit this rapidly expanding area for glycotherapy.

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PREFACE

There are many therapies being developed through biotechnology for treating diseases. They include protein therapy, gene therapy, cell therapy, and glycotherapy. As one of the important disease treatment approaches, glycotherapy provides solution for some unmet medical needs using glycoengineering. It has been used in clinics for decades.

Heparin, a glycosaminoglycan and blood thinner, acts as an anticoagulant and is one of World Health Organization's lists of essential medicines. The sialic acid analogues, oseltamivir and zanamivir, are widely used anti-influenza drugs. Polysaccharide vaccines including Menactra, Prevnar, and Typhim Vi, have been used to stimulate immunity against infection. In addition to therapy using sugar or sugar analogues, therapeutic proteins, such as darbepoetin alfa and imiglucerase, are also generated using glycoengineering *in vivo* or *in vitro* to enhance therapeutic index.

During the last two decades, significant progress has been made in glycotherapy with glycoengineering and glycan mimics although there are many challenges. The glycans are chemoenzymatically modified or conjugated to small molecular weight drugs, proteins, and nucleic acids for increasing pharmacokinetics and pharmacodynamics. Although there is an excellent review published recently by Hudak and Bertozzi (Chemistry & Biology 2014), glycotherapy has not been extensively appreciated in books and unknown to many readers outside this particular field. The current book attempts to fill the gap and provide more recent information related to the exciting progress in this important area of biotechnology.

The book includes reviews on glycotherapy which focus on modification of protein or small molecular weight drug using recombinant and chemoenzymatic approaches. The progress on bioconjugation of glycan using hydrophilic polymers is also covered.

Chapters 1 and 2 focus on the development of vaccines and antibodies against tumor-associated carbohydrate antigen for cancer treatment, as well as the use of glycan for viral inhibition. Chapters 3 and 4 describe the progress in glycoPEGylation and hyaluronic acid conjugation for increasing therapeutic index in treating diseases. The glycoengineering of therapeutic proteins is reviewed in chapters 5, 6 and 7. They provide overviews of recent advances in modification of glycans in proteins or antibodies using recombinant, chemoenzymatic or bioconjugation methods.

Although only a few topics of glycotherapy are being reviewed here, this book aims to provide readers with overview of the researches which have been actively pursued during recent years. We expect to have more excellent books or reviews on this important therapeutic

area in the future.

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CHAPTER 1

Vaccine and Antibody Therapy Against Thomsen-Friedenreich Tumor-Associated Carbohydrate Antigens

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Abstract: The Thomsen Friedenreich (TF) carbohydrate antigens are a group of short *O*-glycans overexpressed on most carcinomas that have been correlated with cancer progression and poor prognosis. Usually associated with immunosuppressive tumor environment, there is a number of potential immunotherapeutics against TF antigens have been developed, which comprise vaccines and antibodies. As therapeutic vaccination, TF antigens already entered into clinical trials, but with limited success due to low patient's response. Novel vaccine design, with multiantigenicity and pointing towards the cellular immune responses arises as a potent stratagem to overcome the low ability of TF antigens to boost immune responses, typical of carbohydrates. The development of antibodies against TF antigens boosted even before vaccine development. These are mainly used for diagnostics, but so far no such antibody entered into clinical trials in patients. Increasing the specificity and the therapeutic efficiency of existing antibodies and developing novel antibodies are still necessary. The vast array of methodologies and engineering techniques available today will allow rapid development and novel formats for both vaccines and antibodies.

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Vaccines and antibodies targeting the same epitopes can function in synergy both to protect and to clear patient's cancer cells. Whilst on one hand, TF antigens dampen immune responses against tumor cells, it is anticipated that the challenge is overcome by applying our increasing knowledge of the mechanisms behind to improve molecular design. Novel solutions are also envisaged by combining anti-TF therapies with other immunotherapies.

Keywords: Clinical trials, Glycan-based vaccines, IgG, Immune response, Immunological memory, Immunotherapy, Mimetic vaccines, Monoclonal antibodies, Mucins, O-glycans, Self-adjuvanting vaccines, Sialic acid, Sialyl-Tn antigens, T antigen, Therapeutic antibodies, Theratope, Thomsen Friedenreich antigens, Tn antigen, Tumor associated carbohydrates.

INTRODUCTION

Structure and Biosynthesis of Thomsen–Friedenreich Antigens

Thomsen–Friedenreich antigens are *O*-linked carbohydrate antigens found on membrane glycoproteins, especially on serine and threonine rich and tandem repeated domains of mucins [1]. The Thomsen–Friedenreich antigens are originated during the first steps of mucin glycosylation, resulting from a defect in the elongation of *O*-glycan chains in cancer cells.

The first described Thomsen-Friedenreich antigen (called T or TF antigen) was initially found on red blood cells and consists in the disaccharide Galβ1-3GalNAc α-linked to serine or threonine residue (Galβ1-3GalNAcα1-Ser/Thr), forming the core 1 structure of mucin *O*-glycans. T antigen is normally the precursor of core 2 *O*-glycans but can be unmasked when cancer cells have lost the ability to synthesize the core 2 (Fig. **1**). The second described TF antigen called Tn antigen is the cryptic precursor of the core 1 and consists in the single *N*-acetyl-galactosamine (GalNAc) residue α-linked to serine or threonine residue (GalNAcα1-Ser/Thr) that can remain unmasked if the ability to synthesize core 1 is lost (Fig. **1**) [2]. The expression of Tn antigen on blood cells is responsible for the Tn-syndrome, which is a rare autoimmune hematological disorder [3]. The Tn antigen can be also sialylated on C6 position of GalNAc, resulting in the disaccharide Neu5Acα2-6GalNAcα1-Ser/Thr, known as sialyl-Tn (STn) antigen.

STn is almost absent in normal healthy tissues but can be detected at various frequencies in almost all kind of carcinomas [4].

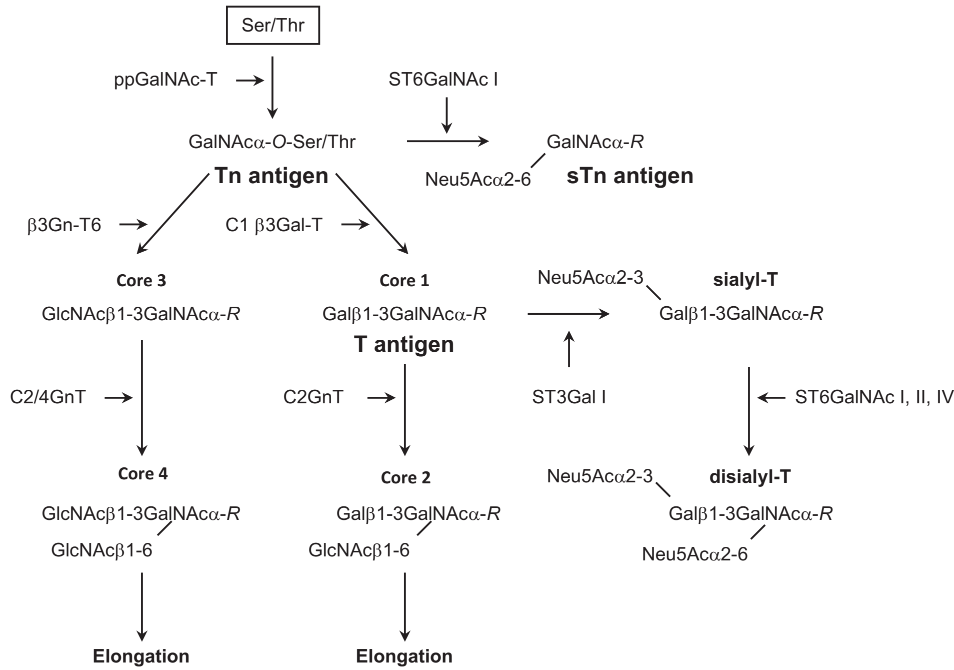

Fig. (1). Biosynthesis of *O*-glycan chains in normal and cancer cells. The figure illustrates mechanisms by which *O*-glycans expressed in normal cells can be turned down to Thomsen–Friedenreich antigens (T, Tn and STn antigens). Gal: Galactose, GalNAc: *N*-acetyl-galactosamine, GlcNAc: *N*-acetyl-glucosamine, Neu5Ac: *N*-acetyl-neuraminic acid, Fuc: Fucose.

The biosynthesis of mucin *O*-glycan chains is a step-by-step process occurring in the Golgi apparatus. *O*-glycans are synthesized by the sequential action of several glycosyltransferases, each transferring a monosaccharide *e.g.*, *N*-acetyl-galactosamine (GalNAc), galactose (Gal), *N*-acetyl-glucosamine (GlcNAc), or *N*-acetyl-neuraminic acid (Neu5Ac) from a donor nucleotide-sugar (*e.g.*, UDP-GalNAc, UDP-Gal, UDP-GlcNAc or CMP-Neu5Ac) to an acceptor that is the glycan being synthesized. Glycosyltransferases are membrane-bound enzymes of which the level of expression, substrate specificity and localization in Golgi compartments are responsible for the pattern of *O*-glycans expressed in a given cell or carried by a given glycoprotein. The initiation step is the transfer of a GalNAc residue on a serine or a threonine of the tandem repeat sequences of

CHAPTER 2

Cell Surface Glycans as Viral Entry Factors and Targets for Broadly Acting Antivirals

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Abstract: Cellular glycans play key roles in the infection process of many human viruses. Viral attachment to heparan sulfate (HS) or sialic acid (SA) moieties in cell surface glycans is a critical and conserved step for the entry of many human viruses, including clinically important human pathogens such as hepatitis B virus, hepatitis C virus, human immunodeficiency virus and influenza virus. As such, glycans are attractive targets for broadly acting antivirals. Molecules that mimic HS or SA interfere with viral attachment by competing for binding of virion glycoproteins to cellular glycans. Modulation of the levels of cellular glycans also affects viral attachment. These approaches show great promise based on their broad-spectrum activities, but the molecules identified so far often possess undesirable pharmacological properties resulting in potential adverse effects. Here, we describe the mechanisms involved in glycan binding, discuss broadly acting glycan-targeted antiviral strategies, and provide perspectives for the rational design of broad-spectrum small molecule entry inhibitors with broad-spectrum activities and appropriate pharmacological properties.

Keywords: Antivirals, Broad-spectrum antiviral therapy, Chemical biology, Glycan mimetics, Glycosaminoglycans, Glycotherapy, Heparan sulfate proteoglycans, Heparin, Hepatitis B virus, Hepatitis C virus, Herpes simplex virus,

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Human immunodeficiency virus, Influenza virus, Polyphenols, Sialic acid, Sialoglycans, Small molecule entry inhibitors, Viral attachment, Viral entry, Virus-host interactions.

INTRODUCTION

Cell surface glycans mediate the initial infection steps of many pathogens, including most human viruses. An overwhelming majority of these viruses initiate infection of their target cells by low-affinity binding to cell surface glycans, such as glycosaminoglycans (GAGs) or sialoglycans (SGs). GAGs and SGs are ubiquitous on cells, and many microbes have evolved to exploit them for initial attachment to their target cells [1]. Among the GAGs, heparin/heparan sulfate (HS) is the most commonly used viral attachment site. The high density of cell-surface HS allows microbes to bind and increase their concentration at the cell surface to facilitate subsequent entry and infection steps. Other viruses recognize and bind to sialic acid (SA), which is abundantly expressed in SGs on the cell surface [2]. These interactions with glycans are critical to capture virions from extracellular spaces and concentrate them in the vicinity of other entry factors and signalling molecules to allow high affinity binding and uptake into the cell. As such, glycans represent attractive targets for broad-spectrum antiviral therapies.

GLYCAN STRUCTURE

GAGs are unbranched polysaccharides comprised of repeating disaccharide units of an amino sugar (*N*-acetylglucosamine or *N*-acetylgalactosamine) and either a uronic sugar (either glucuronic acid or iduronic acid) or galactose [3] and usually linked to proteins *via* *O*-linked or *N*-linked glycosylation. GAGs are classified into four groups that reflect the composition of the disaccharide unit. These include (HS), chondroitin/dermatan sulfate, keratan sulfate or hyaluronic acid [4]. The repeating disaccharide unit of GAGs is variably sulfated [5], leading to high diversity and complexity and allowing for specific binding with distinct GAG-binding proteins. Indeed, interactions between GAGs and a diverse spectrum of GAG-binding proteins regulate many biological processes, including cell growth and proliferation pathways, cell adhesion or migration, and tissue hydration [4]. GAG-binding proteins interact with negatively charged sulfates and carboxylates in GAGs *via* a binding pocket of positively charged basic amino acids. A

consensus sequence of XBBXBX or XBBBXXBX (where B is a basic lysine or arginine residue) is typically found in proteins that bind to HS [6]. Binding affinity depends on the overall shape and conformation of GAGs and is affected by the number and orientation of the sulfate group charges [3].

SGs are a distinct group of cellular glycans, comprised of (SA) attached to the termini of *N*-linked and *O*-linked glycans. The carboxylate group at the 1-carbon position of SA is ionized at physiological pH, allowing for interactions with basic residues of saccharide-binding proteins such as lectins [7]. Binding between SGs and SG-binding proteins is predominantly mediated by extensive hydrogen bonding between the carboxylate, hydroxyls and N-acetyl group of SA and polar amino acid residues.

HEPARAN SULFATE-BINDING VIRUSES

A large and diverse group of human viruses bind to HS proteoglycans, *via* interactions between viral glycoproteins and negatively charged HS moieties in cellular GAGs. These viruses are summarized in Table **1**. Most hepatotropic viruses such as hepatitis C virus (HCV) [8 - 10], hepatitis B virus (HBV) and its satellite, hepatitis D virus [11 - 14], as well as hepatitis E virus [15], require a primary attachment step to HS. Moreover, other viruses that infect a wide range of cell types similarly require HS for binding. These include viruses with RNA genomes, such as human immunodeficiency virus (HIV) [16], dengue virus [17], filoviruses [18], Sindbis virus (SINV) [19], respiratory syncytial virus (RSV) [20 - 22] and other paramyxoviruses [23, 24], Rift Valley fever virus [25], severe acute respiratory syndrome-associated coronavirus [26], as well as DNA viruses, including herpes simplex virus 1 and 2 (HSV-1/-2) [27 - 30], human cytomegalovirus (CMV) [31], varicella zoster virus [32], human herpesvirus 8 [33, 34], vaccinia virus (VACV) [35], adenovirus (AdV) types 2 and 5 [36, 37], some strains of norovirus [38] and human papillomavirus (HPV) [39]. Merkel cell virus initially interacts with HS-containing GAGs, although SA is also required [40]. Other viruses, including the rhabdovirus vesicular stomatitis virus (VSV), are thought to also require HS for binding [41], although the specific details of the interactions remain unclear.

CHAPTER 3

Carbohydrate PEGylation in Chemotherapy

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Abstract: Improvement of drug delivery by covalent modification with polyethylene glycol (PEG) has been widely applied to proteins and low molecular weight pharmaceuticals. Protein therapeutic agents have the drawback of short circulating half-life due to their lability towards proteolytic enzymes and rapid clearance by kidney filtration. Moreover, their recognition by the immune system results in the production of neutralizing antibodies. Several strategies have been used for the PEGylation of proteins at the amino group of lysine but the product is heterogeneous due to random PEGylation. More recently, selective PEGylation of proteins was achieved by introduction of a PEGylated carbohydrate in an *O*-glycosylation site. This technique is called glycoPEGylation and is used for the production of several therapeutics, some of them are currently in advanced clinical trials. Improvement of the delivery system rather than the drug itself has led to the optimization of the therapeutic properties of existing drugs by minimizing their side effects. In this respect, active targeting to receptors present in cancer tissues prevents healthy ones from being damaged. Since carbohydrates have been recognized as playing an important role in the interaction with cell receptors, PEGylated carbohydrates have been used for active targeting of drugs. In this review we discuss different applications of sugar-PEGylation.

Keywords: Active targeting, Bioavailability, Carbohydrates, Chemotherapy, Drug carriers, Drug delivery, Gene delivery, Gene therapy, Glycan delivery, GlycoPEGylation, Ligands, Multiarm PEGs, O-GlcNAc quantification, PEG, PEG-sialic acid, PEGylated nanoparticles, PEGylated polysaccharides,

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PEGylation, siRNA, Sugar PEGylation, Targeted delivery.

INTRODUCTION

Bioavailability of drugs in circulation remains a crucial issue in chemotherapy since most of them are degraded by metabolic processes or are poorly soluble in aqueous systems. The most well-established and widely employed way to increase their circulation half-life without affecting their activity is by conjugation with a water soluble biocompatible polymer. In this regard, poly(ethyleneglycol) (PEG) has been extensively used due to its lack of toxicity and immunogenicity. These characteristics earned PEG its approval by the United States Food and Drug Administration (USFDA) for its use as excipient in pharmaceutical formulas [1].

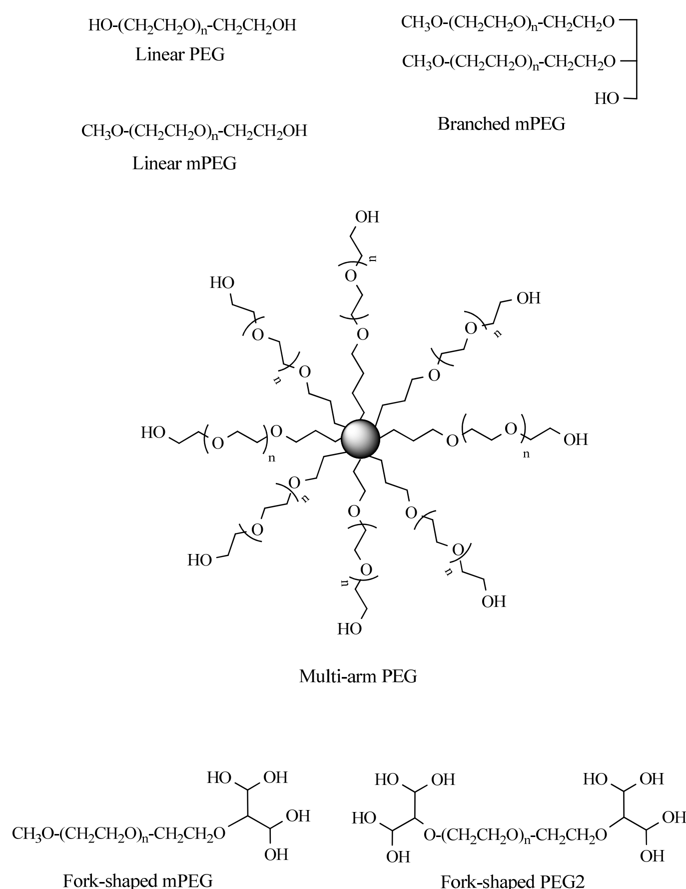

Fig. (1). Types of PEG used for the derivatization of drugs and proteins.

PEG reagents are commercially available in different lengths, shapes and chemistries, allowing them to react with particular functional groups for their covalent attachment. PEGs may be arranged in linear or branched structures, including multiarm PEGs designed to increase the loading of the active molecule (Fig. **1**).

PEG derivatives were first developed for the attachment to the amino groups in proteins [2] and bovine serum albumin was one of the first to be linked to a methoxypolyethylen glycol (mPEG) chain [3]. In this and some other early works the amino groups at the N terminus or in lysine side chains were used for PEG attachment with activated carboxyl or aldehyde functionalized PEGs. Currently, there are many monofunctional and homo and heterobifunctional PEGs available in the market for amide linking including *N*-hydroxysuccinimidyl esters (PEG-NHS), carboxylic acids (PEG-COOH) for *in situ* activation with carbodiimide (EDC or DCC), and *p*-nitrophenylcarbonates (PEG-NPC) (Fig. **2**).

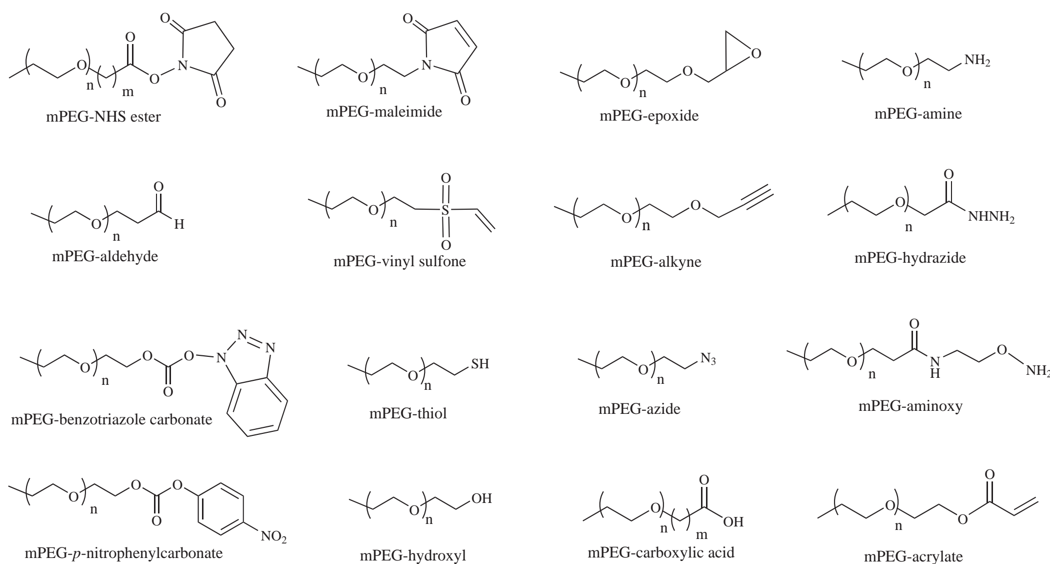

Fig. (2). PEG derivatives for conjugation with drugs.

However, the abundance of lysine and its usual surface location lead to complex mixtures differing in the number and site of PEGylation [4]. To avoid these shortcomings, PEGylation has evolved towards site-specific amino acid

CHAPTER 4

Recent Developments in Hyaluronic Acid-Based Nanomedicine

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Abstract: Originally considered simply another naturally occurring component of a number of tissues, breakthrough findings later disclosed hyaluronan's (HA) extraordinary biological properties and paved the way for a new era of HA-associated medical applications. HA is, in fact, recognized by many cellular receptors, it can mediate cell migration, proliferation, cell-cell aggregation, it has been shown to promote angiogenesis, *and the list goes on.* This plethora of activities initially moved attention away from a polymer that was considered a simple carrier of biomolecules and towards its potential use in both treating many diseases and conditions and being involved in drug delivery. Given these premises, medical applications exploiting HA's different roles have been developed. The focus of this chapter is directed towards the chemical conjugation of HA with small drugs, peptides and proteins. Reviewing the vast body of literature dedicated to this field, an extraordinary range of applications will be outlined. Although HA cannot be considered a polymer that is appropriate for all uses, conjugates specifically designed to exploit some of its biological properties and for numerous specific applications will no doubt enjoy an advantage over other polymeric conjugates.

Keywords: Anti-TNF-α, Anticancer drug, Anticancer therapy, CD44, Doxorubicin, Drug conjugates, Drug delivery, Human growth hormone, Hyaluronan, Hyaluronic acid, Insulin, Interferon alpha, Osteoarthritis, Paclitaxel,

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Polymer conjugation, Polysaccharide, Protein conjugation, Protein delivery, RHAMM, RNase A, SN38.

INTRODUCTION

The term "polymer therapeutics" was coined by Ruth Duncan in the 1990s to describe families of polymer constructs to which a drug is covalently bound [1]. This class of substances, which falls under the definition of "new chemical entities", can be distinguished from those drug delivery systems that "per se" only entrap a free drug. Polymer-drug conjugates, polymer-protein/peptide conjugates, polymer-oligonucleotide conjugates, polymeric drugs and block-copolymer-drug micelles all represent sub-classes of polymer therapeutics. Already in 1975, Helmut Ringsdorf introduced the concept of polymer-drug conjugates and the idea of exploiting polymers to increase the solubilization of hydrophobic drugs, to protect drugs from degradation, to prolong their pharmacokinetic profile, and to achieve controlled delivery to cells, tissues and/or organs [2]. Among the several polymers that have been extensively studied, polyethylene glycol (PEG) has proved to be one of the most successful in view of its wide applicability. It has, in fact, already yielded numerous products widely and commonly used in clinical practice such as conjugates with proteins, peptides and oligonucleotides or PEG-modified liposomes [3]. PEG has made a significant contribution to polymer therapeutics, and several new polymers and their derivatives are presently being investigated in the context of preclinical and clinical trials as interest in the development of biodegradable polymers continues to grow. Just a few examples are polyglutamic acid [4], dextrin [5], hyaluronic acid [6], hydroxyethyl starch [7] and polysialic acid [8].

This chapter, which focuses on hyaluronic acid and its conjugates, aims to highlight recent advances in the study of a polymer that can be the carrier for drugs, proteins, and peptides.

General Introduction of Hyaluronic Acid

Hyaluronic acid hyaloid (vitreous) + uronic acid (HA) was isolated for the first time in 1934 from the vitreous humor of cows' eyes by Karl Meyer and John

Palmer [9]. The naturally occurring linear polysaccharide has repeating units of D-glucuronic acid and *N*-acetyl-D-glucosamine disaccharide (Fig. **1**).

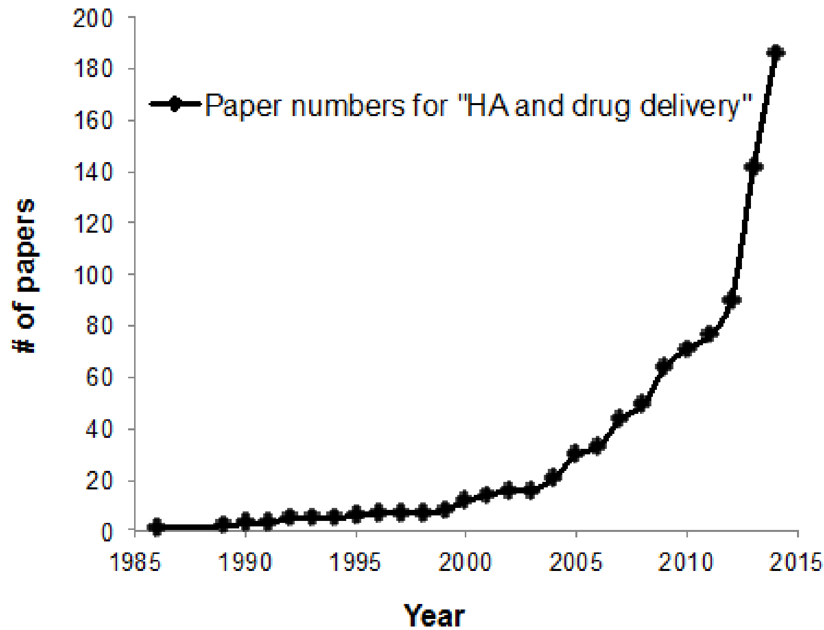

Fig. (1). Publications and citations concerning 'Hyaluronic acid AND drug delivery'. Source: PubMed.

The pK_a of HA's carboxyl groups is therefore 3–4; at pH = 7, the carboxyl groups are predominantly ionized and the polymer is a polyanion that has associated cations (the counterions) and thus referred to as hyaluronan. Found in nature in a wide range of molecular weights (from 20kDa to 4000kDa), HA is a highly hydrophilic polymer that can absorb great quantities of water and expand up to 1000 times its solid volume, forming a loose hydrated network [10].

Hyaluronic acid is present in all vertebrates and in several districts of the human body. It is estimated that the adult human body contains about 12-15 g of hyaluronan, almost half of which is located in the skin [11]. It is also found in the synovial fluid, in the vitreous body of the eye, and it plays an important role in a variety of biological processes: it helps to maintain the viscoelasticity of liquid connective tissues and is involved in controlling tissue hydration, water transport, the organization of the extracellular matrix (ECM) and in tissue repair.

CHAPTER 5

Utilization of Glycosylation Engineering in the Development of Therapeutic Proteins

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Abstract: Biologics are drugs made from complex molecules manufactured using living microorganisms, plant or animal cells. Many biologics are produced using recombinant DNA technology. Glycoproteins represent a major portion of biologics, including monoclonal antibodies, Fc-fusion proteins and other therapeutic proteins or enzymes. A thorough understanding of the nature and function of the carbohydrate moiety and its impact on pharmacology properties is essential in discovering, developing and manufacturing safe and efficacious glycoprotein biopharmaceuticals. This review summarizes the recent development in glycosylation engineering and characterization methodology. Examples of *N*-linked and/or *O*-linked glycosylation impacting drug pharmacology properties (including activity, pharmacokinetics, clearance, and immunogenicity) of marketed and developing therapeutic proteins are presented.

Keywords: ADCC, CHO, Fc-fusion, Fluorescence, Fucose, Glycan release, Glycoengineering, Glycoprotein, Glycosylation, Half-life, HILIC, HPLC, IgG, mAbs, Mass spectrometry, PAD, Pharmacodynamics, Pharmacokinetics, Recombinant human erythropoietin, Sialic acids.

INTRODUCTION

Achieving optimum therapeutic efficacy is dependent on maintaining a proper

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balance between drug exposure and its pharmacological effects. Therefore, the PK and PD parameters for therapeutics are often tuned through the drug design in a manner assuring that desired *in vivo* responses are achieved. PK refers to “what body does to the drug” and is influenced by drug absorption, distribution, metabolism and excretion, represented primarily by clearance rates, circulatory half-lives, volumes of distribution, and bioavailability. PD examines “what drug does to the body” and is influenced by the drug exposure and the drug pharmacological activities upon binding to the targets.

As a result of the intrinsic susceptibility of proteins to clearance mechanisms, protein drugs generally display limited plasma persistence. This can be overcome with higher protein concentrations and increased dosing frequencies. However, frequent treatment schedules coupled with the high target specificities and potencies can lead to unsuitably sharp dose/response profiles. The overstimulation of a targeted pathway can trigger autoregulatory feedback inhibition mechanisms that can lead to loss of *in vivo* efficacy. To overcome these limitations it has become routine practice to engineer the physicochemical and pharmacological properties of protein drugs in the early development.

Established technologies, such as targeted mutations, generation of fusion proteins and conjugates, glycosylation engineering, and pegylation, have been shown to significantly improve the efficacy of protein drugs by increasing their molecular stabilities and plasma persistence and consequently improving overall *in vivo* PD responses. Of these, engineered glycosylation is one of the most promising due to the fact that it has been shown to simultaneously improve the parameters necessary for therapeutic efficacy while maintaining target specificity.

The Processes of *N*- and *O*-linked Glycosylation

N-linked glycosylation is a post-translational modification that is conserved across eukaryotes. *N*-glycosylation begins in the endoplasmic reticulum (ER) with the transfer of a preassembled dolichol phosphate-linked fourteen sugar oligosaccharide to targeted asparagine residues in a protein [1, 2]. The preferred site for *N*-glycosylation contains the three amino acid sequence, Asn-Xaa-Ser/Thr where the second position can be any amino acid except Pro. This initial glycan

transfer reaction is catalyzed by the heteromeric oligosaccharyltransferase (OST) complex and is supposed to precede folding of the protein in the ER [3]. Immediately after the oligosaccharide transfer, the two terminal glucose residues are cleaved off by α-glucosidase I and II and the resulting polypeptide with mono-glucosylated glycan structures ($Glc_1Man_9GlcNAc_2$) can interact with the ER-resident membrane-bound lectin calnexin or its soluble homolog calreticulin. These lectins support protein folding in a glycan-dependent protein quality control cycle. Secretory glycoproteins that have acquired their native conformation are released from the calnexin/calreticulin cycle and exit the ER to the Golgi apparatus. In the Golgi, the ER-derived oligomannosidic *N*-glycans on maturely folded glycoproteins are subjected to further *N*-glycan processing which generates the highly diverse complex N-glycans with different functional properties.

In contrast, *O*-linked glycosylation involves the covalent attachment of a monosaccharide to a Ser or Thr residue. Sequence specificity for guiding *O*-glycosylation has not been determined although it is presumed that folding and solubility of the protein play important roles in the position and number of *O*-linked glycans that occur on a given protein. Mammalian *O*-glycosylation is complex due to a variety of enzymes involved in multiple pathways resulting in a broad range of possible *O*-linked glycoforms. The most common form of human *O*-glycosylation occurs in the Golgi compartment and involves the transfer of a GalNAc residue from UDP-GalNAc to a Ser or Thr through the action of a GalNAc transferase followed by the subsequent transfer of any number of different sugars in different linkages including galactose, GalNAc and GlcNAc. Branching of the *O*-GalNAc chain, as well as the addition of lactosamine, also occurs providing additional oligosaccharide diversity [4]. The *O*-linked glycan chains can also be capped with sialic acid [5]. Different types of *O*-glycosylation (*e.g.*, *O*-linked fucose, glucose, mannose, xylose, or GalNAc) have been described on secretory proteins in mammals [6]. A class of *O*-linked glycosylation in mammals involving *O*-linked mannosyl species has been observed in brain, peripheral-nerve, and muscle glycoproteins [7]. The most well-studied of these, α-dystroglycan, is a heavily glycosylated extracellular membrane glycoprotein that functions to regulate important interactions with extracellular matrix proteins [8]. *O*-mannosylation on α-dystroglycan plays a critical role in normal physiology as

CHAPTER 6

Antibody Engineering through Fc Glycans for Improved Therapeutic Index

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Abstract: The N-glycans from Asn-297 site (or N297 glycans) of antibodies play important roles in antibody function. There is enhanced antibody-dependent cellular cytotoxicity (ADCC) with antibodies containing non-fucosylated glycans, while high anti-inflammatory activity was observed with the antibodies having highly sialylated biantennary structure. Many different approaches have been applied to engineer the N297 glycans with various structures to improve therapeutic efficacy by increasing antibody function, including pro-inflammatory effector function for cancer or antiviral therapy and anti-inflammatory activity for autoimmune diseases. Furthermore, the involvement of various N297 glycan forms in antibody structure-function relationship has also been recently elucidated. The crystal structures of FcγRIIIA complexed with non-fucosylated Fc demonstrated a more favorable carbohydrate-carbohydrate interaction, which is required for high affinity binding and enhanced ADCC activity. The strong anti-inflammatory activity of highly sialylated antibody is related to its interaction with dendritic cell specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN). In addition, the N297 glycans were also remodeled for site-specific antibody conjugation. Thus, antibody glycoengineering provides valuable approaches for modulating antibody function, leading to increased therapeutic index.

Keywords: ADCC, Anti-inflammatory activity, Antibody-dependent cellular cytotoxicity, Autoimmune diseases, Bisecting GlcNAc containing glycans, Cancer, CDC, Complement-dependent cytotoxicity, Dendritic cell specific intercellular adhesion molecule-3-grabbing nonintegrin, FcγRIIIA, Glycoengineering, High mannose-type glycans, Highly galactosylated glycans,

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Highly sialylated glycans, Homogeneous and site-specific ADC, N-glycans, N297 glycans, Non-fucosylated glycans, Site-specific antibody conjugation, Therapeutic antibodies.

INTRODUCTION

The antibody (or immunoglobulin) belongs to a family of major serum glycoproteins produced by humoral immune system. Its function includes recognition of specific antigens (pathogens) to facilitate their neutralization or killing by other effector cells or serum complements. Among antibodies, IgG is the main isotype. It contains two identical heavy (H) and two identical light (L) chains in a Y-shape, which are held together by a combination of non-covalent interactions and covalent interchain disulfide bonds. Each IgG has Fc and Fab regions, in which two Fab arms contain complementarity-determining regions critical for high affinity interaction with the antigens. The Fc region containing C_H2 and C_H3 is important in binding to either Fcγ receptors (FcγRs) on effector cells or complements for killing pathogens, as well as in the interaction with FcRn for long serum half-life.

The antibody IgG also contains the highly conserved Asn-297 (N297) site with attached N-glycans located in central cavity. In contrast to those found in most glycoproteins, the N297 glycans in C_H2 domains of recombinant therapeutic antibody are only partially processed with core fucosylated biantennary complex-type structures containing zero or one galactose at non-reducing terminus as major species (G0F or G1F). Other glycans, such as the processed sialylated biantennary complex-type, or less processed high mannose-type forms are also present, but at much lower levels. This unique feature is likely due to the low accessibility of N297 glycans inside the C_H2 domains toward galactosyltransferase or sialyltransferase in Golgi apparatus of the cells even when the IgG Fc molecule is in "open" conformation [1].

Although relatively simple compared to those found in other glycoproteins, the N297 glycans play a critical role in antibody structure and function. When the glycans are absent, there are significant structural changes in Fc region, which switches from "open" to "closed" conformation [1]. The binding of aglycosylated

antibodies to FcγRs and complements was abolished with reduced antibody effector function, including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [2]. Antibody glycosylation also plays a critical role in stability [2]. Reduction in thermostability of antibodies was observed through sequential deglycosylation.

In addition, there were changes in N297 glycans under certain physiological or pathological conditions [3 - 12]. The glycan profiles vary with age and pregnancy [13]. The glycosylation of antibody is often altered in multiple diseases, especially the autoimmune diseases (Table **1**).

Table 1. The autoimmune disease associated alteration in N-glycans in antibody IgG in serum except those noted in multiple sclerosis.

Disease	Fucosylation	Galactosylation	Sialylation	Bisecting GlcNAc
Rheumatoid arthritis		↓ [9, 14 - 17]		
Osteoarthritis		↓ [17]		
Systemic lupus erythematosus	↓ [18]	↓ [18]	↓ [18]	↑ [18]
Ulcerative colitis		↓ [2]		↑ [2]
Crohn's disease		↓ [2]	↓ [2]	
Kawasaki disease			↓ [19]	
Guillain-Barre syndrom		↓ [12]	↓ [12]	
Multiple sclerosis	↑* [3, 11]	↓* [3, 11]		↑* [3, 11]
Lambert-Eaton myasthenic syndrome and myasthenia gravis		↓ [20]		
Coeliac disease		↓ [21]		
Granulomatosis with polyangiitis		↓↓** [11, 22]	↓** [11, 22]	↓** [22]
Microscopic polyangiitis		↓ [22]		

*: IgG in cerebrospinal fluid.

**: Disease associated autoantibody, anti-proteinase 3.

There are lower galactosylation and sialylation in the patients with those diseases than the control. However, it is unknown whether these changes are related to the mechanism of the disease. With significant progress made during last two decades in developing therapeutic antibodies for treatment of multiple diseases, including cancer and autoimmune diseases, the role of N297 glycans in recombinant

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CHAPTER 7

Engineering of Therapeutic Proteins through Hyperglycosylation

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Abstract: One of the major challenges associated with the development of protein-based biotherapeutics lies in achieving persistent concentrations of the active molecule in circulation. Most human proteins are rapidly cleared from circulation, mainly by renal filtration, and so increasing their *in vivo* residence time to reach appropriate therapeutic doses has been a matter of extensive investigation. The majority of therapeutic proteins exhibit post-translational modifications (PTMs). Among them, N- and O-glycosylation are the most abundant and complex modifications that proteins can undergo, affecting diverse biological properties, including solubility, protease and thermal stability, antigenicity, immunogenicity, bioactivity and pharmacokinetics. Thus, glycosylation represents one of the most relevant attributes of many therapeutic proteins, defining their potency and effectiveness. Also, both size and charge of proteins are completely modified by the presence of glycans, so that manipulation of this PTM represents a valuable tool to alter the pharmacokinetics and pharmacodynamics of biotherapeutics. This chapter deals with different glycoengineering strategies developed with the aim of increasing the plasma half-life of proteins, as well as other properties. Specifically, engineering of proteins through the addition of new glycosyl moieties is addressed. A thorough description of the properties conferred to proteins by glycans is first presented, followed by a description of the strategies developed for the rational manipulation of glycosylation parameters to improve such properties. Different approaches to incorporate new N- and O-glycans

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into proteins are described and exemplified. Finally, the application of N- and O-hyperglycosylation engineering to an emblematic protein such as recombinant human IFN-α2b is presented.

Keywords: Antigenicity, Bioactivity, Biotherapeutics, CTP, Efficacy, Glycoengineering, Glycosylation prediction, hCG, hEPO, hFSH, hIFN-α2b, Immunogenicity, N-glycosylation, O-glycosylation, Peptide fusion, Pharmacokinetics, Plasma clearance, Site-directed mutagenesis, Solubility, Stability.

INTRODUCTION

In the last decades there has been an accelerated development of protein-based biotherapeutics which have revolutionized the treatment of numerous diseases due to their high specificity and affinity towards its clinical target. Unfortunately, most proteins do not behave as ideal drugs, since their efficacy is compromised by several intrinsic limitations. These include the low *in vivo* activity displayed due to their low stability and short serum half-life, which represent the main obstacles to overcome in order to achieve a successful application as therapeutics [1 - 3]. Thus, many approaches have been developed with the aim of improving the residence time of proteins in circulation to reach appropriate therapeutic doses, as well as to improve other physicochemical and pharmacological properties [4, 5].

The mechanisms involved in plasma clearance of proteins are diverse [6]; however, the most commonly employed route is renal filtration. The structural and physicochemical characteristics of the glomerular barrier are responsible, to a great extent, of the elimination mechanisms. Small molecules are rapidly filtrated through the numerous small 4-10 nm diameter pores, while negatively charged glycosaminoglycans form an anionic barrier which selectively prevents the passage of macromolecules according to their charge. Another site of metabolism of biologics is the liver, where hepatocytes participate in their metabolism through receptor-mediated membrane transport and lysosomal degradation. In addition, peripheral blood-mediated elimination by proteolysis contributes to a great extent to the catabolism of administered proteins [7]. Thus, the structure and size of a protein, represented by its hydrodynamic ratio, as well as its physicochemical

properties constitute starting points for improvement of its plasma half-life.

The majority of proteins derived from eukaryotes undergo covalent modifications either during or after their ribosomal synthesis. This gives rise to the concept of co-translational and post-translational modifications (PTMs). Glycosylation is the most abundant and complex PTM among eukaryotic cells. It constitutes an enzymatic process in which oligosaccharide chains are covalently attached to the amide nitrogen of an asparagine side chain (N-glycosylation) or to oxygen of a hydroxyl group of serine or threonine residues (O-glycosylation), although other types of glycosylation have been described [8, 9]. More than 50% of human proteins are glycoproteins and it is estimated that 1-2% of genome includes genes related to glycan synthesis. Also, glycosylation represents one of the most relevant attributes of many therapeutic proteins, defining their potency and effectiveness [10, 11]. Both size and charge of proteins are completely modified by the presence of glycans, so that manipulation of this PTM represents a valuable tool to alter the pharmacokinetics and pharmacodynamics of biotherapeutics [12]. Thus, engineering of proteins through the addition of new glycosyl moieties has emerged as an area of great technological interest.

PROPERTIES CONFERRED TO PROTEINS BY GLYCANS

There are many theories about the roles of carbohydrates in different types of glycoconjugates, but none of them has demonstrated to be of universal application [13]. Oligosaccharides play a marked effect over diverse biological properties of the glycoproteins, including solubility, stability (regarding protease resistance and thermal degradation), antigenicity, immunogenicity, bioactivity, cell interaction, pharmacokinetics and plasma half-life [14].

Effects of Glycosylation on Solubility and Stability of Glycoproteins

Oligosaccharides often promote protein solubility and prevent protein aggregation. For example, complete removal of O-glycans of human granulocyte colony-stimulating factor (hG-CSF) resulted in a high level of autoaggregation, which led to its biological inactivation [15]. Besides, glycans could contribute to the increment of solubility of proteins through masking exposed hydrophobic amino acid residues.

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