The glycoform profile of a glycoprotein is non-templated, i.e., is not encoded within the genome or otherwise predetermined; however, it is estimated that ~50% of human genes having an open reading frame encode a -N-X-S/T- amino acid sequence, where X represents any amino acid other than proline, that comprises a potential site (sequon) for N-linked glycosylation of the translated protein. N-linked glycosylation is both a co- and post-translational modification. The complex oligosaccharide GlcNAc2Man9Glu3 may be added at a -N-X-S/T- sequon as the polypeptide chain emerges from the ribosome tunnel. Local secondary structure determines whether oligosaccharide is added and the extent of addition. Higher occupancy is observed for -N-X-T- sequons than at -N-X-S- sequons, and the efficiency of addition can be further influenced by adjacent amino acid residues.

The oligosaccharide can exercise a pivotal role, in conjunction with chaperones, in the folding of a glycoprotein within the endoplasmic reticulum. When quality control criteria have been met, transport vesicles facilitate transport of the nascent glycoprotein to the Golgi complex. Complete oligosaccharide processing may not be achieved for all molecules passing the Golgi, which results in a heterogeneous glycoform profile, and hence the structural heterogeneity of glycoproteins. Glycoproteins bearing O-linked sugars may also be generated as protein transits the Golgi apparatus; however, it is not possible currently to predict potential O-linked sites from DNA or protein sequences. The glycoform profile of a glycoprotein plays an essential role in “protein sorting,” e.g., directing the product to the cytoplasm, nucleus, cell surface or marking it for secretion, prior to it fulfilling an overt function.

The glycoform profile of an approved recombinant glycoprotein therapeutic is a Critical Quality Attribute (CQA) and constitutes a Quality by Design (QbD) parameter. The glycoform profile can vary between different cells within the same organism, between organisms or the sexes. These differences are extended to cell lines and provide a challenge for the commercial production of recombinant glycoprotein therapeutics; selection of a production platform able to deliver glycoform fidelity being a primary criterion. The success of recombinant glycoprotein therapeutics is manifest, and as the earliest approved therapeutics approach the end of patent protection, there is a compelling incentive for the production of biosimilar therapeutics. This constitutes another challenge as a candidate biosimilar must be comparable to the innovator product. The demonstration of glycoform heterogeneity and differences in biologic activity associated with defined glycoforms has demanded control over the production process. Increased understanding of the functional activities of individual glycoforms has resulted in glyco-engineering to produce individual glycoforms and the generation of “bio-better” therapeutics. This is illustrated in the foreword to this volume “Glyco-engineering of
monoclonal antibodies,” contributed by Janice Reichert.

The last decade has seen the development and introduction of increasingly sensitive and accessible techniques for the qualitative and quantitation analysis of the glycoform profile of glycoproteins. Biopharmaceutical companies have established “in-house” expertise or outsourced to a contract research organization (CRO) dedicated to the task. Given the size and possible heterogeneity of glycoprotein therapeutics, it is essential that characterization employs multiple orthogonal techniques, i.e., analytical protocols dependent on different physicochemical principles. As the title suggests, Glycosylation Engineering of Biopharmaceuticals, Methods and Protocols provides comprehensive “state-of-the-art” protocols delivered by practitioners in the field, with Editor Alain Beck being a co-author on four contributions.1

While most protocols are applicable to any given glycoprotein, recombinant antibody molecules are recurring targets. Under the heading “Glyco-engineering of Therapeutic Proteins,” Part I comprises five contributions on mammalian and non-mammalian production platforms. Part II, “Glycanalytics,” provides ten contributions covering equipment and protocols for the analysis of glycoprotein therapeutics, while Part III, “Glycoprotein Complexes Characterization,” focuses on physical parameters that may be relevant to mechanisms of action, both beneficial and adverse. Similarly, Part IV, “PK/PD Assays for Therapeutic Antibodies,” contributes bio-assays for defined mechanisms of action and clinical efficacy. Each chapter is composed of four sections, i.e., Introduction, Materials, Methods and Notes; the latter is a particularly valuable addition because each note succinctly summarizes experiences gained “at the bench.”

In Part I, case studies for glyco-engineering of O- and N-linked glycans in Chinese hamster ovary (CHO), Pichia, Saccharomyces cerevisiae and baculovirus are covered. The first two chapters cover glycoprotein production in CHO cells, glyco-engineering of O-glycans on recombinant mucin-type immunoglobulin fusion proteins and protocols to engineer human-like glycosylation of therapeutic glycoproteins, e.g., requiring the addition of sialic acid in α(2–6) linkage [CHO cells add sialic acid in α(2–3) linkage]. The following three chapters provide protocols for the humanization of N-glycosylation of antibodies in Pichia and Saccharomyces cerevisiae and baculovirus.

Characterization and quantitation of the glycoform profile of natural and recombinant glycoproteins requires application of “state-of-the-art” orthogonal methods. The ten chapters of Part II include methodologies for mass spectrometry (MS)/MS characterization of glycopeptides generated from trypsin digestion of monoclonal antibodies and liquid chromatography (LC)/MS characterization of N-glycans released from cetuximab following digestion with IdeS; the latter offering the challenge of being glycosylated within the heavy chain variable region, in addition to the Fc. Other contributions employ normal phase-HPLC and sedimentation velocity analytical ultracentrifugation in the analysis of mAbs. The chapter contributed by a CRO illustrates the application of multiple orthogonal analytical techniques to determination of the N-glycan structure of a single recombinant antibody therapeutic (trastuzumab); the methods/results sections are followed by 40 notes. Several modes of capillary electrophoresis are introduced to characterize glycan occupancy and charge heterogeneity of intact monoclonal antibodies, erythropoietin and granulocyte colony-stimulating factor, with the N-glycan components being characterized using capillary zone electrophoresis. One chapter is devoted to high throughput CE-SDS chip (LC90; Caliper) analysis of intact and deglycosylated monoclonal antibody, erythropoietin and a heavily glycosylated Fc fusion protein. It has been interesting to follow the development of lectin-based glyco-profiling protocols. Micro-plates are now available that are dedicated to determination of the glycoform profile of a selected glycoprotein, as illustrated by a methodology dedicated to study of the glycosylation profile of purified recombinant human IL-7 derived from CHO cells.

An essential parameter that receives too little study, in my opinion, is characterization of immune complexes formed following interaction of antibody with the target antigen. This, in turn, is dependent on the valency of both antigen and antibody, the affinity (avidity) of binding and the antigen/antibody ratio. The first chapter of Part III introduces non-covalent MS techniques for characterization of immune complexes formed between a monoclonal antibody and its 25 kDa antigen (recombinant soluble human functional adhesion molecule A) at differing antigen/antibody ratios. In addition to the expected 1:1 and 1:2 complexes, higher molecular weight complexes are detected, indicative of antibody or antigen aggregation. The epitope specificity of an antibody is an additional parameter that can influence immune complex structure and consequent functional activity. A contribution on epitope mapping provides both excision and extraction MS protocols. In the former, antigen bound to immobilized antibody is subject to enzyme digestion and, following washing, bound peptide is released for MS characterization. In the latter, a protein antigen is subject to enzyme digestion prior to exposure to immobilized antibody; following washing, captured peptides are released and characterized by MS. The extraction protocol is further extended to allow epitope identification following deuterium/hydrogen exchange with structural characterization of released deuterium-labeled peptide. The last chapter of Part III extends the application of deuterium/hydrogen exchange to include MS (H/DXMS) techniques to probe the conformation and dynamics of proteins in solution. This technique allows identification of sites of altered conformation through the generation of differentially-labeled peptides between different samples of the same glycoprotein, e.g., as a result of deamidation, deglycosylation.

While Part IV is entitled “PK/PD Assays for Therapeutic Antibodies,” only one contribution has direct relevance to events occurring in vivo. The first contribution presents a protocol for determination of natural killer cell-mediated antibody-dependent cell-mediated cytotoxicity by measurement of lactate dehydrogenase generation; in the second contribution, a protocol to measure complement-dependent cytotoxicity by ATP generation is discussed. The third contribution provides for surface plasmon...
resonance determination of the binding of soluble FcγR to antibody captured on a CM-SPA chip. Each of these assays is valuable and necessary for the development and routine monitoring of essential biologic activities; however, extrapolation to activity in vivo is problematic. The final chapter claims to provide an absolute quantitation of monoclonal antibody present in serum. The antibody is isolated by immunopurification from a target antigen column, digested with trypsin, and then key peptides specific for the antibody are quantitated by MS. The example given is cetuximab for which three peptides are selected, one unique for the light chain, one for the heavy chain and one common to all IgG-Fc regions. The latter allows monitoring of normal serum IgG that may be present as contaminant.

This volume maintains the high standard of the Methods in Molecular Biology series in providing a lucid text that will be a companion in the laboratory and at the bench.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Reference

1. Glycosylation Engineering of Biopharmaceuticals. Beck, Alan (Ed.) 2013. Series: Methods in Molecular Biology, Vol. 988. 355 pp. ISBN 978-1-62703-326-8.