European Journal of Pharmaceutics and Biopharmaceutics xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Short review (expert opinion)

N-glycosylation heterogeneity and the influence on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins

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ARTICLE INFO

Article history:
Received 18 September 2015
Revised 7 January 2016
Accepted in revised form 8 January 2016
Available online xxxx

Keywords:
Monoclonal antibody
Fusion protein
N-glycosylation
Pharmacokinetics
Glyco-engineering

ABSTRACT

Monoclonal antibody and Fc fusion protein drugs are complex heterogeneous mixtures of numerous different protein variants and modifications. N-glycosylation as one of the most complex post-translational modifications influences the structural characteristics of the antibodies Fc part thereby potentially modulating effector function and pharmacokinetics. Several investigations on the relationship between N-glycosylation and pharmacokinetics have been published. However, this structure–function relationship is not fully understood. In this review potential alterations with focus on N-glycosylation of mAbs and Fc fusion proteins and the possible effects on the pharmacokinetics are reviewed and the current understandings of the underlying mechanisms are described.

1. Introduction

The fusion of murine myeloma cells with B-cells was a groundbreaking experiment of Köhler and Milstein and made production of antibodies in cell culture possible [1]. It was the beginning of immunoassays and therapeutic antibodies. In 2012, more than 35 years later, 34 recombinant monoclonal antibodies (mAbs) were marketed in the EU and US, mostly produced in CHO and SP2/0 cells [2]. The vast majority of marketed mAbs belongs to the IgG class or more precisely to the subclasses IgG1 and to some extent IgG2 and IgG4. Two-thirds of the marketed mAbs are either human or humanized and a small percentage is chimeric or murine [2]. With more than 1300 amino acids resulting in a mass of approximately 150 kDa mAbs are large molecules built from two heavy chains with 50 kDa each and two light chains with 25 kDa each (Fig. 1). The different heavy chains (γ1, γ2, γ3 and γ4) divide the IgGs into their subclasses 1–4. The light chains are the κ-type and λ-type. Heavy and light chains are connected by disulfide bridges giving the antibody its Y-shaped structure (Fig. 1). Intrachain disulfide bridges further stabilize the folding, 16 disulfide bridges per IgG1 and 7 per chain. The heavy and light chains consist of different domains. The variable domains variable light (VL) and variable heavy (VH) contain hypervariable regions that are responsible for antigen binding. The remaining domains are conserved sequences named constant domains constant light (CL) and constant heavy (CH1–3). The CH2 domain of each heavy chain contains one N-glycosylation site at approximately Asn297 and about one-fifth of human IgGs carry a N-glycosylation motif in the variable region [3,4]. The size and structure of IgGs give rise to a large number of possible alterations and modifications (Table 1) turning IgG drugs and in conclusion recombinant mAb drugs to heterogenic protein mixtures. Several of these alterations can have tremendous influence on the structure, PK and function of monoclonal antibodies. The complex post-translational modification of antibodies, N-glycosylation, will be discussed in detail in the following.

2. N-glycosylation and its influence on mAb structure and mAb effector function

Like most extracellular glycoproteins, therapeutic proteins and specifically also mAbs undergo glycosylation in the ER and Golgi network of cells. The glycan structures of therapeutic mAbs can be of importance for the efficacy and safety of the drug [21]. Monoclonal antibodies have one conserved N-linked glycosylation at the Fc part at position N297. Approximately 20% contain a second N-linked glycosylation site in their variable region. Both sites are

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http://dx.doi.org/10.1016/j.ejpb.2016.01.005
0939-6411/

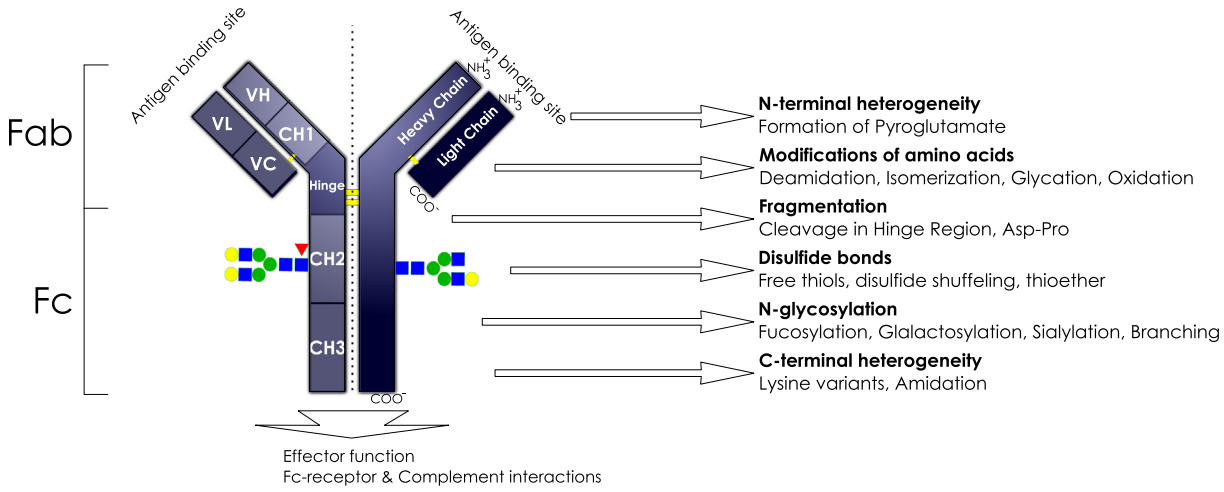

Fig. 1. Schematic structure of an IgG1 molecule. An IgG consists of two heavy and two light chains that contain several domains. The variable domains variable light (VL) and variable heavy (VH) that form the antigen binding site and the constant domains CL (constant light) and CH1–3 (constant heavy) building the framework. The IgG can be furthermore divided into the Fab (fragment antigen binding) and Fc (fragment crystallizable) which induce the effector functions. On the right possible modifications and alterations of the IgG are listed.

Table 1
Common modifications of biopharmaceuticals and their impact on structure and function.

Modification	Effect	Reference(s)
Glu or Gln cyclization at N-terminus	Loss of positive N-terminal charge	[5–7]
Lys variants at C-terminus	Introduction of a positive charge	[7,8]
Deamidation (Asn to Asp)	Introduction of a negative charge (Asn to Asp)	[5,7,9–11]
Isomerization (Asp to isoAsp)	Introduction of an additional CH2 group into the protein backbone (Asp to iAsp)	[5,7,12]
Deamidation (Gln to Glu)	Introduction of a negative charge	[13,14]
Glycation	Loss of a positive charge	[7,15,16]
Oxidation (Cys, His, Met, Tyr, Trp)	Increase of polarity	[5,7]
Disulfide bond heterogeneity (Shuffling, thioether and trisulfide formation)	Changes in the protein conformation	[17–20]
Glycosylation (N-linked, O-linked)	Influence on the structure and function	See next section

located on the heavy chain [3]. Glycosylation of biopharmaceuticals shows a high grade of heterogeneity and N-glycans belong to the most complex and diverse structures in nature due to the high number of different sugar moieties and the multitude of possible linkages. Fig. 2 shows the three different N-glycan types high mannose, complex and hybrid that are found on IgGs with their respective linkage. Complex and hybrid types exist with core fucosylation, addition of a fucose residue to the innermost N-acetylglucosamine, and without core fucosylation. mAbs represent a special group of glycoproteins as their N-glycans are of limited size. mAbs are usually free of N-glycans with more than two antennae and furthermore the sialic acid content is low compared with other glycoproteins [22]. This circumstance can be explained by the fact that the glycosylation site in the CH2 domain at Asn297 is buried in the protein structure. Typically, antibodies contain a high percentage of complex bi-antennary glycans with core-fucosylation [22–24].

N-glycans have important structural functions. They stabilize the CH2 domain of IgGs and deglycosylation makes mAbs thermally less stable and more susceptible to unfolding. In addition deglycosylated mAbs are more prone to aggregation [25]. Not only thermal and colloidal stability, but also functionality of the IgG is influenced by the attached N-glycans and their size [26]. In addition to the stabilization of the CH2 domain the attached N-glycans greatly influence the folding of the Fc part. Krapp et al. investigated crystalized Fc parts of IgG molecules with different homogenous glycosylations and could demonstrate that the conformation of the CH2 domain depends on the attached N-glycans [27]. Larger N-glycans, e.g. bi-antennary complex type with terminal galactosylation, open up the Fc part in the CH2 region to a horseshoe like structure, whereas smaller attached N-glycans like the core structure favor a more "closed" Fc conformation. This open and closed formation can greatly influence the effector functions induced by interactions of the Fc part with Fc receptor molecules. The fact that crystallization of deglycosylated IgG was not possible due to the high flexibility of the CH2 domain shows the importance of N-glycan protein interactions [27]. Hydrogen/Deuterium exchange MS experiments resulted in similar findings. Houde et al. showed that terminal galactosylation has major impact on the conformation of the Fc part and that fucosylation alone does not impact the conformation [28]. NMR analysis of G2F glycosylated mAbs revealed that the terminal galactose residues are exposed and accessible for protein binding and that there are differences between the 1,3 and the 1,6 arm concerning flexibility and accessibility [29]. These findings led to the assumption that Fc N-glycosylation influences the effector function of proteins interacting with the CH2 domain. Upon antigen binding mAbs are able to induce effector functions mediated by their Fc-part. By binding to Fc-receptors or complement proteins mAbs induce antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC), respectively. ADCC is induced after binding of Fc-γ receptors (FcγR) to the Fc part [30]. The affinity of FcγRs to the Fc part is influenced by N-glycosylation in the CH2 domain [28,31–34]. As a consequence

Please cite this article in press as: F. Higel et al., N-glycosylation heterogeneity and the influence on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins, Eur. J. Pharm. Biopharm. (2016), http://dx.doi.org/10.1016/j.ejpb.2016.01.005