

Trends in Glycosylation, Glycoanalysis and Glycoengineering of Therapeutic Antibodies and Fc-Fusion Proteins

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Abstract: Monoclonal antibodies (MAbs) are the fastest growing class of human pharmaceuticals. More than 20 MAbs have been approved and several hundreds are in clinical trials in various therapeutic indications including oncology, inflammatory diseases, organ transplantation, cardiology, viral infection, allergy, and tissue growth and repair. Most of the current therapeutic antibodies are humanized or human Immunoglobulins (IgGs) and are produced as recombinant glycoproteins in eukaryotic cells. Many alternative production systems and improved constructs are also being actively investigated. IgGs glycans represent only an average of around 3 % of the total mass of the molecule. Despite this low percentage, particular glycoforms are involved in essential immune effector functions. On the other hand, glycoforms that are not commonly biosynthesized in human may be allergenic, immunogenic and accelerate the plasmatic clearance of the linked antibody. These glyco-variants have to be identified, controlled and limited for therapeutic uses. Glycosylation depends on multiple factors like production system, selected clonal population, manufacturing process and may be genetically or chemically engineered.

The present account reviews the glycosylation patterns observed for the current approved therapeutic antibodies produced in mammalian cell lines, details classical and state-of-the-art analytical methods used for the characterization of glycoforms and discusses the expected benefits of manipulating the carbohydrate components of antibodies by bio- or chemical engineering as well as the expected advantages of alternative biotechnological production systems developed for new generation of therapeutic antibodies and Fc-fusion proteins.

Keywords: Therapeutic antibody, Fc-fusion protein, mass spectrometry, capillary electrophoresis, glycoengineering, glycomics, glycoanalytics.

INTRODUCTION

It is estimated that 30 % of new drugs, which will be licensed in the next decade will be based on antibodies and antibody derivatives [1,2,3]. Five classes of human antibodies or immunoglobulins are defined (IgG, IgM, IgA, IgD and IgE) differing from a functional and a structural point of view, including carbohydrates [4,5]. Glycosylation is one of the most common post-translational protein modifications and has essential roles in antibody effectors functions, immunogenicity, plasmatic clearance [6] and resistance towards proteases [7,8]. The size of the human glycome is much more larger than the genome and over 30 genetic diseases linked to glycan synthesis and structure alterations have been identified [9]. In glycoproteins, most of the oligosaccharides are attached via an N-glycosidic bond to asparagine residues (N-oligomannose, complex or hybrid oligosaccharides [10] or via an O-glycosidic bond to serine or threonine bonds [11].

All current approved therapeutic antibodies are IgGs or derivatives. Human G immunoglobulins are tetrameric glycoproteins (≈ 150 kDa) composed of two heavy chains (HC, ≈ 50 kDa) and two light chains (LC, ≈ 25 kDa) (Fig. 1A) at around 10 g/L plasmatic concentration. They are divided in four subclasses or isotypes defined by different heavy chains ($\gamma 1$, $\gamma 2$, $\gamma 3$ and $\gamma 4$ in a 66/23/7/4 ratio). Disulfide bridges (sixteen for IgG1 and IgG4; eighteen for IgG2) and non-covalent interactions maintain their 3-dimensional structure. The heavy and light chains are linked by one disulfide bond and the heavy chains by two (for IgG1 and IgG4) or three (for IgG2) disulfide bonds all located in the small hinge domain, which also contains a papain cleavage site yielding two Fabs and one Fc fragments (≈ 50 kDa each). The other twelve or fourteen cystine bridges are intramolecular and delimit six different globular domains: one variable (V_L) and one constant for the light chains (C_L); one variable (V_H) and three constant for the heavy chains (C_{H1} , C_{H2} and C_{H3}) [5]. IgG3 are characterized with a longer and more flexible hinge domain with eleven inter heavy chain disulfide bridges [12]; they are generally not selected for therapeutic antibody development mainly because the plasmatic half-life is shorter than for the 3 others isotypes (7 vs 21 days, respectively) [3,13].

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Like natural IgGs, all recombinant antibodies contain an Asn-X-Ser/ Thr (where X amino-acid except a Pro) consensus sequence for N-glycosylation in their heavy chain C_H2 constant domain. IgG glycans represent only an average of 2 to 3% of the total antibody mass, which is low compared for example to Erythropoietin (EPO, 265 aminoacids, MW= 32-45 kDa, 40 % carbohydrates, three N-glycosylation sites (Asn^{24,36,83}) composed by di- trisialylated, tri- and tetraantennary complexes; one O-glycosylation site (Ser¹²⁶) [14,15,16]. Despite the high complexity of EPO, three biosimilars have been recently approved in Europe (www.emea.europa.eu). IgGs N-glycans are located generally on residue Asn²⁹⁷ of each heavy chain (Fig. 1A) and particular glycoforms are involved in important immune cytotoxic effector functions [17]. Glycosylation plays also a role in the plasmatic half-life via binding to neonatal Fcγ Receptor (FcγRn) [18]. Glycoforms that are not commonly biosynthesized in human may potentially be immunogenic and must be identified, quantified and limited for clinical therapeutic uses, particularly in case of repeated administration. Glycans of normal polyclonal human IgGs belong to the biantennary complex type (Fig. 2a) [4]. A conserved heptasaccharide core is composed of 2 N-acetylglucosamine (GlcNAc), 3 mannose (Man) and 2 GlcNAc residues that are β-1,2 linked to α-6 Man and α-3 Man, forming two arms. Additional fucose (Fuc), galactose (Gal) and N-acetylneuraminic acid (NANA) residues, may be present or not. Glycans present considerable heterogeneity, with potentially more than 400 glycoforms due to the random pairing of Heavy Chains glycans of different structures as well documented by R. Jeffers and co-workers in many studies [6,19]. Mathematical model of N-linked Glycosylation are proposed to predict glycosylation patterns and to direct glycoengineering of antibodies [20].

GLYCOSYLATION OF CURRENT MARKETED THERAPEUTIC ANTIBODIES

Immunoglobulin Isotypes and Glycosylation

Monoclonal antibodies are a very successful class of therapeutics [2,21,22]. Since the first registration of a murine monoclonal antibody 22 years ago (Orthoclone/ muromonab), at least 28 further antibodies and antibody-derivatives (Fab fragments, immunoconjugates, radioimmunoconjugates, soluble Fc-fusion proteins) have been approved in different countries mainly in oncology (11) and in rheumatoid and autoimmune diseases (11; rituximab is indicated in both groups of diseases). MAbs gained also approval in many other therapeutic indications like organ transplantation (3), cardiology (1), viral infection (1), allergy (1), tissue growth and repair (1), and hemoglobinuria (1) Table 1. Interestingly, a decade after the registration of rituximab (anti-CD20), the first biosimilar antibody was launched in India in April 2007 (Reditux, Dr Reddy). Second and third generation of CD20 antibodies characterized by different mechanism of action and targeting different epitopes are actively investigated in pre-clinical and clinical trials including phase III. Rituximab polypeptide sequence is also often used as prototype for novel expression systems evaluation of glycolengineered variants.

Most of the chimeric, humanized and human IgGs belong to the IgG1 isotype. Nevertheless, two IgG4s (Mylotarg/ gemtuzumab and Tysabri/ natalizumab), one IgG2 (Vectibix/ panitumumab) and one engineered mixed IgG2/4 (Soliris/ eculizumab) also reached the market recently [23] and dozens more are in clinical development [4,21,24]. Despite their higher heterogeneity and their different oligomeric status

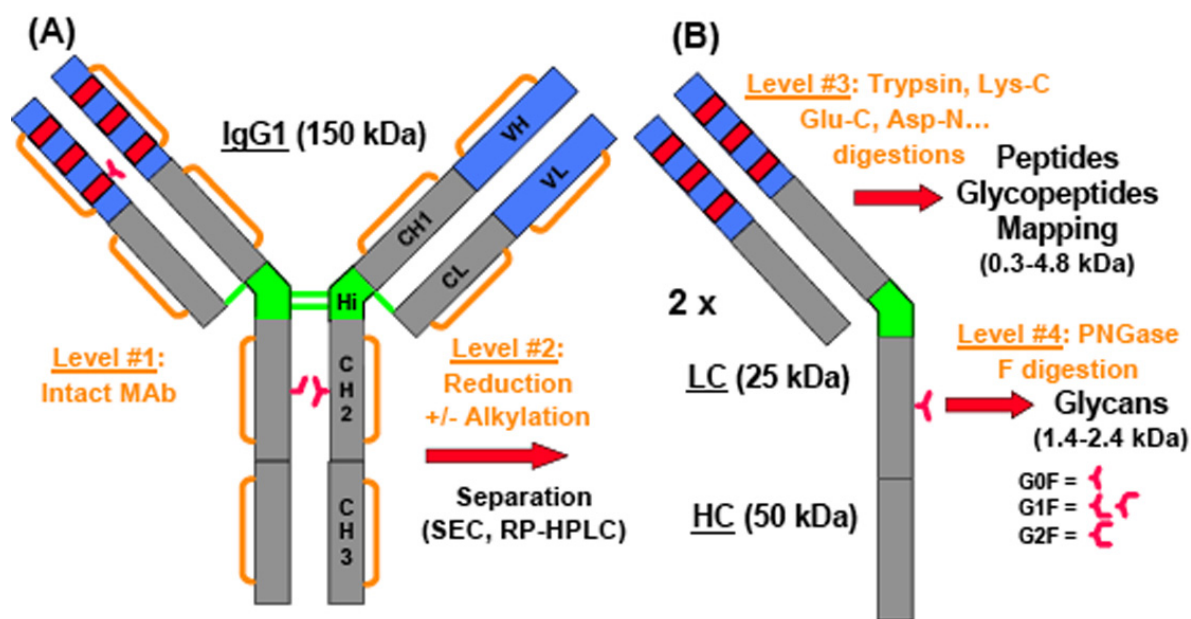


Fig. (1). IgG1k structural characterization at four different levels by a “Top-down” (level # 1 = intact IgG; level # 2 = light & heavy chains after reduction) and a “Bottom-up” analysis (level # 3 = peptide and glycopeptide mapping after enzymatic digestion; level # 4 glycan analysis after PNGase F release)

compared to IgG1 (half-antibodies and bispecific heterotetramers for IgG4s [13,25,26]; 2 vs 4 disulfide bridges linking the heavy chains and forming dimers for IgG2s [13,27], both isotypes are often selected and clinically developed to avoid cytotoxic effectors function, which are directly linked to Fc structure and to their glycosylation.

It is also estimated that in human plasma around 20% of IgGs have an additional N-glycosylation site in their variable domains both in light (kappa and lamda) and heavy chains (gamma)[24,28,29]. This was also observed for the recombinant chimeric Erbitux/ cetuximab antibody, which was shown to be hypogalactosylated in its constant domain but fully galactosylated in its variable domain on the heavy chain Asn⁸⁸ (-Asn-Asp-Thr-Ala- consensus sequence) [30,31] (Fig. 1). A similar observation was recently described for a fully human recombinant IgG2 with a fully galactosylated and partially sialylated Fab and low galactosylated Fc, explained by steric hindrance between the two inside facing CH2-glycan chains [32]. Cetuximab contains also a third potential N-glycosylation site on the light chain Asn⁴² (-Asn-Gly-Ser-Pro- consensus sequence), but which is in fact not glycosylated (R. Jefferis observation [18]). Dubois *et al.* proposed recently an explanation: the presence of a Pro at Y of -Asn-X-Ser/Thr-Y- consensus sequence is like in position X, unfavorable to glycoprocessing [33].

The removal of glycosylation sites from the variable domains by genetically engineering to get more homogeneous MABs is generally a preferred option for the more recent generation of antibodies. The parental mouse antibody 4D5 of Herceptin for example has a N-glycosylation site located on light-chain Asn⁶⁵ in the framework (-Asn-Arg-Ser-Gly- consensus sequence). These carbohydrates were not necessary for the pharmacological activity and Asn⁶⁵ was mutated into a Ser in the final humanized version of trastuzumab which reached the market [34]. In such a case, affinity maturation may be necessary to reconstruct framework portions and to accommodate with the CDR mutations, following glycosylation sites removal in the variable domains [35]. The resulting antibodies are then more homogeneous from a chemical point of view and easier to develop as biopharmaceuticals especially during scale-up, process changes and concomitant comparability studies.

A Serine O-linked mannosylation (+ 162 Da) was also recently described for the first time for a human IgG2 lamda light chain produced in two different cell lines (a stable CHO and a transient COS) indicating that the modification was production system-independent [36]. It is currently not known if this hexose addition can be found or not on natural IgGs and its biological significance. The involved Ser⁶⁶ residue is only present in lamda light chains, not in kappa. The kappa/ lambda ratio is 99/1 and 67/33 in mice and human, respectively. Currently there is only one lamda light chain containing antibody on the market (Bexxar/ tositumumab), probably because the first generation of MABs was obtained by immunizing mice followed by chimerization or humanization of the lead mouse antibody (Table 1). This may be change in the future: several fully human MABs containing lamda chain are now in clinical trials. They were generated by phage-display libraries encoding human single-chain variable antibody fragments (scFv) or by immunizing

transgenic mice bearing human IgG genes, with the target-antigen [2].

Beside IgGs only a few IgMs and IgAs were investigated to date in early clinical trials and only for particular indications (septic shock for IgMs and mucosal administration for IgAs). Both isotypes are complex multimeric glycoproteins with several glycosylation sites (7 to 11 % glycans and O-glycosylation in the hinge domain of dimeric IgA1s (360 kDa); 12 % glycans and multiple N-glycosylation in pentameric IgMs (935 kDa) [5]. IgMs and IgAs upstream and downstream processes are less developed and straightforward than those for IgGs, especially for large-scale pharmaceutical production [37].

Current Cell Production Systems and Glycosylation (Table 1)

Glycosylation is highly dependent on the production system, the selected clonal cell population and the culture process (i.e., feeding strategy [38,39]. Chinese Hamster Ovary cells (CHO) and mouse myeloma cells (NS0, SP2/0) have become the gold-standard mammalian host cells used for the production of therapeutic antibodies and Fc-fusion proteins, which have reached the market [40,41]. Statistically, 48 % of them are produced in CHO cells, 45 % in mouse-derived cells (21 % in NS0, 14 % in SP2/0 and 10 % in hybridomas) and 7 % in *E. coli* for (non-glycosylated Fab fragment) Table 1.

Most of these cell lines have been adapted to grow in suspension culture and are well suited for reactor culture, scale-up and large volume production up to 20,000 L and a productivity up to 5 g/L [37]. These are essential features for supplying chronically used antibodies [42,43]. Depending on the therapeutic indications, the design and the number of clinical trials, 0.5 to 1 kg is needed for clinical phase(s) I, 1 to 5 kg for phase(s) II and 10 to 60 kg for phase(s) III. Blockbuster antibodies are currently produced at a multiple-ton scale per year. The glycoforms of CHO produced IgGs (Fig. 2b) are close to human ones, except for the third N-acetylglucosamine bisecting arm which represent around 10 % of human IgGs glycoforms and very low amounts of terminal N-Acetyl Neuraminic Acid (NANA) [6]. Murine NS0 cells produced MABs show more differences (Fig. 2c). They exhibit small amounts of glycoforms with additional α -1,3-galactose and different sialic acids: N-Glycolyl Neuraminic acid (NGNA) vs N-Acetyl Neuraminic Acid (NANA). NGNA is the predominant sialic acid present in glycoproteins produced by mouse cells and only as traces when derived from CHO cells [44,45]. NGNA is reported as immunogenic in human [46] but from a practical point of view, the amount present in most of the NS0-produced MABs is generally very low in the Fc part (around 1-2 %). Moreover, no serious adverse events linked to these glycoforms were reported for the current marketed NS0 and SP2/0 produced MABs, including Synagis/ palivizumab anti-RSV MAB used in neonates since 1998. The same stands for the mouse α -1,3-galactose residue which is generally a very minor glycoform (2-4 %) on Asn²⁹⁷ [47].

One very particular and important exception is Mab C225 (Erbitux), characterized by a second N-glycosylation site in the Fab portion on heavy chain Asn⁸⁸. For the mar-

Table 1. Current Approved Therapeutic Antibodies and Derivatives: Isotypes or Formats, Expression Systems, Targets and Indications, Pharmacokinetic and Immunogenicity

MAb / INN (www.who.org)	Prod.	Format	Approval	Companies	Indications	Target Ag	1/2-Life*	Adm. Route	Immunogenicity**
Arcalyst/ rilonacept	CHO	IL1R-Fc	2008	Regeneron	CAPS (auto-inflammatory)	IL-1 trap	5.3-7.6	s.c.	/
Vectibix/ panitumumab	CHO	HuIgG2k	2006	Amgen	Colorectal Cancer	EGFR	3.6-10.9	i.v. (2 wks)	1-4 %
Actemra/ tocilizumab	CHO	HzIgG1k	2006 (Jpn)	Roche/ Chugai	Castelman's Disease	IL-6R	/	i.v.	/
Orencia/ abatacept	CHO	CTLA4-Fc	2005	BMS	Rheumatoid arthritis	CD80/CD86	/	/	/
Avastin/ bevacizumab	CHO	HzIgG1k	2004	Genentech/ Roche	CRC, H&N, Breast	VEGF-A	12-14	i.v.	0 %
Xolair/ omalizumab	CHO	HzIgG1k	2003	Genentech/ Novartis	Allergy	IgE-Fc	20-35	s.c.	0 %
Raptiva/ efalizumab	CHO	HzIgG1k	2003	Genentech/ Serono	Psoriasis	CD11a	5-10	s.c.	2-6 %
Amevive/ alefacept	CHO	LFA-3-Fc	2003	BiogenIdec	Psoriasis, RA, Transplant.	CD2	11.3	s.c.	/
Humira/ adalimumab	CHO	HuIgG1k	2002	Abbott	Rheumatoid arthritis	TNFalpha	14.7-19.3	s.c.	5-12 %
¹¹¹ In/ ⁹⁰ Y-Zevalin/ ibritumomab	CHO	MuIgG1k	2002	Biogen-dec	NHL	CD20	1.1	i.v.	30 %
MabCampath/ alemtuzumab	CHO	HzIgG1k	2001	Millenium	CLL	CD52	12	i.v.	50 %
Herceptin/ trastuzumab	CHO	HzIgG1k	1998	Genentech	Breast cancer	HER-2	2.7-10	i.v.	0.1 %
Enbrel/ etanercept	CHO	p75 TNFR-Fc	1998	Amgen/ Wyeth	Psoriasis, RA	TNFalpha	4	s.c.	/
Rituxan/ rituximab	CHO	HzIgG1k	1997	Genentech/ BiogenIdec	NHL, RA	CD20	9.4	i.v.	0 % (NHL); 67 % (RA)
Soliris/ eculizumab	NS0	HzIgG2/4k	2007	Alexion	PNH	C5	5-11	i.v.	/
TheraCIM/ nimotuzumab	NS0	HzIgG1k	2005 (Chi)	YM Biosciences	Head & Neck cancer	EGFR	/	i.v.	/
Tysabri/ natalizumab	NS0	HzIgG4k	2004	Biogen-Idec	Multiple sclerosis	alpha4 integrin	14	i.v.	7 %
Mylotarg/ gemtuzumab (Immunoconjugate)	NS0	HzIgG4k	2000	Wyeth	Acute Myeloid Leukemia	CD33	1.9-2.5	i.v.	0 %
Synagis/ palivizumab	NS0	HzIgG1k	1998	Medimmune/ Astra-Zen.	Respiratory Syncytial Virus	F protein	19-27	i.m.	0-1 %
Zenapax/ daclizumab	NS0	HzIgG1k	1997	PDL/ Roche	Transpl. rejection	CD25	20	i.v.	8-34 %
Erbix/ cetuximab	SP2/0	ChIgG1k	2004	Imclone/ Merck-Serono/BMS	CRC, H&N	EGFR	4.8	i.v. (1 wk)	5 %
Remicade/ infliximab	SP2/0	ChIgG1k	1998	Centocor/ Schering	Crohn's disease, Psoriasis	TNFalpha	9.5	i.v.	8-61 %
Simulect/ basiliximab	SP2/0	ChIgG1k	1998	Novartis	Transplantation rejection	CD25	4-16	i.v.	0-1 %
Reopro/ abciximab	SP2/0	ChFab	1994	Centocor/ Lilly	High-risk angioplasty	GPIIb	4.8	i.v. (1 wk)	4-21 %
¹³¹ I-Bexxar/ tositumomab	Hybr.	MuIgG2a	2003	GSK	NHL	CD20	2.7-2.8	i.v.	9 %
Panorex/ edrecolomab	Hybr.	MuIgG2ak	1995 (Ger)	Centocor/ GSK	CRC	EpCAM	/	i.v.	/
Orthoclone/ muromab	Hybr.	MuIgG2ak	1986	Johnson & Johnson	Transplantation rejection	CD3	0.73	i.v.	53 %
Cimzia/ certolizumab pegol	<i>E. coli</i>	H2Fab'-PEG	2008	UCB	Crohn's disease	TNFalpha	/	s.c.	low (PEG)

(Table 1) contd....

MAB / INN (www.who.org)	Prod.	Format	Approval	Companies	Indications	Target Ag	1/2-Life*	Adm. Route	Immuno-genicity**
Lucentis/ ranibizumab	<i>E. coli</i>	HzFab	2006	Genentech	Age-related macular deg.	VEGF-A	/	i.o.	/

*in days (PK data adapted from Tang L *et al.*, 2004; Lobo E *et al.*, 2004 and www.emea.europa.eu. ** Immunogenicity data adapted from van Walle *et al.*, 2007[159] and www.emea.europa.eu

AML = Acute Myeloid Leukemia; CAPS = Cryopyrin-Associated Periodic Syndromes; CLL = Chronic Lymphocytic Leukemia; CRC = ColoRectal Cancer; H&N = Head & Neck cancer; i.m. = intra-muscular; i.o. = intra-ocular; i.v. = intra-venous; NHL = Non-Hodgkin's Lymphoma; PNH = Paroxysmal Nocturnal Hemoglobinuria (Chronic red blood cell destruction); RA = Rheumatic Arthritis; Reditux = Rituximab biosimilar approved in 2007 in India; s.c. = subcutaneous; MABs international non-propriety names (www.who.int). A generic name is composed by a distinct prefix, followed by an infix representing the target disease (tu = tumor; cir = cardiovascular; vi = virus; lim = immune) and a suffix linked to the type of the MAb [Mouse = -omab; Chimera = -ximab (mouse, rat, non-human primate); Humanized = -zumab; Human = -umab]. The last consonant of the target/ disease syllable can be dropped to facilitate pronunciation (eg, palivizumab, trastuzumab, rituximab...).

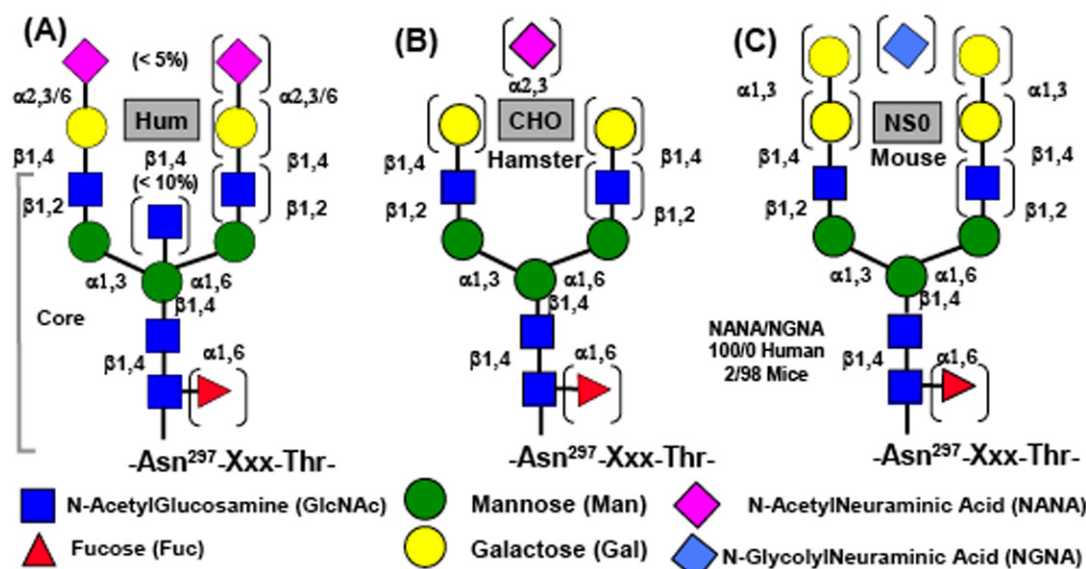


Fig. (2). (A) Human and recombinant antibody glycans produced in (B) hamster (CHO cell line) and (C) murine (NS0 or SP2/0 cell lines, mice hybridoma). These structures are used to calculate theoretical masses for mass spectrometry analysis (GlcNAc = 203.19 Da; Man, Gal = 162.14 Da; Fuc = 146.14 Da; NGNA = 291.09 Da; NANA = 275.00 Da; 2AB = 136.15 Da). The structures are represented according to the nomenclature outlined by the Consortium for Functional Glycomics (<http://www.functionalglycomics.org/>).

keted version of cetuximab produced in SP2/0 cells, 21 different glycoforms were recently identified with around 30 % capped by at least one α -1,3-galactose residue, 12 % capped by a NGNA residue and traces of oligomannose [31]. Importantly both α -1,3-galactose and NGNA were found only in the Fab moieties in contrast to the Fc fragment, for which only typical IgGs G0F, G1F and G2F glycoforms were identified. In a recent report on cetuximab-induced anaphylaxis, pre-existing IgEs specific for this galactose- α -1,3-galactose epitope were detected in patients treated with Erbitux [48]. Using a solid phase immunoassay (ImmunoCAP), these IgEs were found to bind to SP2/0 produced cetuximab and F(ab')₂ fragment and not to the Fc fragment. Interestingly, no IgE immunoreactivity was found against a version of a CHO-produced cetuximab (CHO-C225).

Like for Erbitux, extensive glycan profiles of marketed MABs produced in different cell lines can be found in the literature: MabCampath/ alemtuzumab [46], Rituxan/ rituximab and Herceptin/ trastuzumab [49] produced in CHO; TheraCIM/ nimotuzumab [50] and Synagis/ palivizumab

[51] produced in NS0 cells. It is of prime importance to have access to this kind of data, to gain more knowledge in glycans structure-activity relationships as well as for benchmarking purposes, for the next generation of improved Mabs or for comparability studies of biosimilars.

Alternative Cell Production Systems and Glycosylation

Antibodies produced in alternative systems to the current mammalian cells will certainly also reach the market in the future (Table 2). Nevertheless, one of the current limitations is the difference of glycosylation machinery, which yields immunogenic recombinant glycoproteins [52]. Yeast, fungi, insect cells and plants have restricted abilities to glycosylate [53]; this should be overcome by humanization of glycosylation pathways and will be discussed in the glycoengineering section.

Prokaryotes. Bacteria do not glycosylate proteins and are a production system of choice for naked (e.g. Lucentis/ ranibizumab) or PEGylated Fab' fragments (e.g. Cimzia/ cer-

Table 2. Current and Alternative Production Systems for Therapeutic Antibodies and Derivatives

• Cell cultures
○ Mammalian cell culture:
▪ CHO (Chinese Hamster Ovary)/ Dihydrofolate reductase + Methotrexate or Glutamine synthetase ⁽¹⁾
▪ NS0 (Mouse myeloma cells)/ Glutamine synthetase (GS) ⁽¹⁾
▪ SP2/0 (Mouse myeloma cells) ⁽¹⁾
▪ YB2/0 (Rat myeloma cells)
▪ BHK (Baby Hamster Kidney)
▪ COS (Kidney fibroblast cells from African Green Monkey)
▪ HEK293 (Human Embryonic Kidney)
▪ PER.C6 (Human retina cells)
▪ NM-F9 (Human, Leukemia-derived)
○ Avian cells (EBx)
○ Insect cells (Sf9)/ Baculovirus ⁽²⁾ , <i>Drosophila melanogaster</i>
○ Yeasts (<i>Pichia pastoris</i> , <i>Saccharomyces cerevisiae</i> ⁽²⁾); Filamentous fungi (<i>Aspergillus niger</i>)
○ Bacteria (<i>E. coli</i> , <i>Campilobacter jejuni</i>): scFv, Fab ⁽¹⁾ , (Fab') ₂ , IgG (a-glycosylated)
○ Plant cells (in bioreactors): <i>Lemna minor</i> (aquatic plant), <i>Physcomitrella patens</i> (moss), tobacco
• Transgenic organisms
○ Transgenic animals: goat ⁽²⁾ (<i>hu anti-thrombin</i> , EMEA 2006), rabbit, mouse
○ Transgenic chicken eggs
○ Transgenic plants (in open fields): tobacco, corn, potatoes, soy, rice, alfalfa

⁽¹⁾ Approved for marketed therapeutic MAbs; ⁽²⁾ Approved for marketed vaccines or biologicals

tilizumab pegol). Full-length antibodies may also be produced without glycan when expressed in *E. coli* [54] or in cell-free expression systems, based on *E. coli* lysate [55]. Another option to obtain non-glycosylated antibodies in non-prokaryotic systems consist to replace by bioengineering Asn²⁹⁷ by Asp, Gln or Ala residues for example [56] or by mutation of the Thr present in the -AsnXxxThr²⁹⁹- consensus sequence.

Human cells. PER.C6 human retinoblastoma derived cells were recently proposed as a new system to produce MAbs carrying an human glycosylation profile and a G0F/G1F/G2F ratio close to 1/2/1 [57]. In fact this ratio can also be observed for NS0-produced MAbs [58] and other glycoforms are essentially a minority as discussed below.

Transgenic animals. A first biopharmaceutical produced in the milk of transgenic goats gained approval in Europe in 2006 (Atryn/ GTC Biotherapeutics, recombinant human anti-thrombin) [59]. This recombinant glycoprotein contains 3 N-glycosylation sites and shows a different glycoprofile when compared with the human one (higher level of mannose, 50 % NGNA) [60].

ANALYTICAL CHARACTERIZATION OF GLYCOFORMS

All IgGs show common post-translational modifications: light and heavy chain pyroglutamic acid cyclization for MAbs containing N-terminal glutamine or glutamic acid residues, heavy chain C_H2 domain N-glycosylation and C-terminal lysine processing [19]. These micro-variants can be characterized by liquid chromatography (LC), electrophore-

sis and mass-spectrometry (MS) based methods [27,61,62]. Both “top-down” (direct mass measure of intact IgG and Light & Heavy Chains) and “bottom-up” (peptides, glycopeptides, glycans fingerprinting and structural analyses) approaches are necessary in a first-round antibody fine structural characterization (Fig. 1) [63]. In a second round when the glycoforms are identified after PNGase F release for a reference batch, then a direct mass-fingerprint of the intact IgG with the new generation of high-precision mass spectrometers may be sufficient for routine QC [64,65].

SDS-PAGE and IEF

Slab-gel electrophoresis (Sodium Docedyl sulfate – Polyacrylamide Gel Electrophoresis, SDS-PAGE, separation of mass variants) and IsoElectroFocusing (IEF, separation of charge variants) are two methods used routinely for the characterization of proteins and glycoproteins.

Fig. (3A) shows a SDS-PAGE gel of two IgGs (MAb 1 & 2) treated with and without PNGase F and with and without reducing agent. The slab gel is not really able to distinct the glycosylated whole MAb from the deglycosylated one (loss of only 3 out of 150 kDa). After reduction, a slight shift of mass is observed for the heavy chain (3 kDa out of 50 kDa) and no difference is observed for the light chain of both MAbs, as expected.

Fig. (3B) shows an Isoelectric focusing gel for the same antibodies also treated with and without PNGase F. In both cases “acidic” shifts are observed for all the bands, which are explained by the generation of a negatively charged aspartic

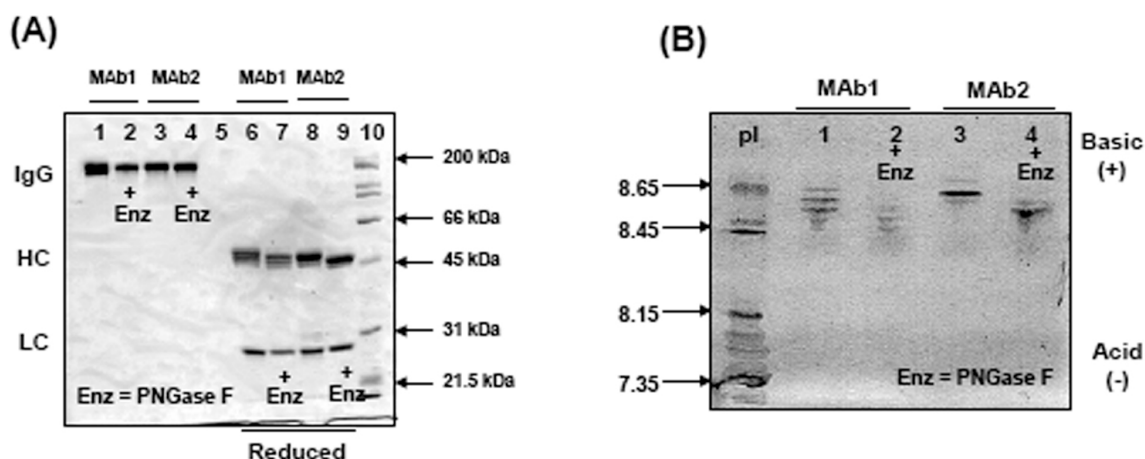


Fig. (3). (A) SDS-PAGE slab-gel of two different MAbs (line 1 and 3), digested with PNGase F (line 2 and 4), reduced (line 6 and 8), reduced and digested with PNGase F (line 7 and 9). (B) IEF gels of two different MAbs (line 1 and 3), digested with PNGase F (line 2 and 4). On the SDS-PAGE gel, a shift of mass is visible for the Heavy Chain after PNGase F treatment but not for the whole IgG because the method is not sensitive enough. On the IEF, shift of pI are observed for all bands, which appear more acid and indicate the generation of negative charges.

acid instead of the neutral N-glycan linked asparagine. The observed remaining heterogeneity in charge variants is attributable to C-terminal lysine variants.

Capillary Electrophoresis-Sodium Dodecyl Sulfate (CE-SDS)

Capillary Electrophoresis-Sodium Dodecyl Sulfate is a more resolutive and quantitative method (peak area surface integration), which tends to replace slab-gels [66]. Fig. (4) shows examples of electropherograms of an intact IgG (glyco-H2L2) treated or not with PNGase F under reducing and non-reducing conditions. In comparison to Fig. (3A), this last method is much more resolutive and allows a relative quantification of the main peak (glyco-H2L2), of a non-glycosylated variant (NG-H2L2) as well as of antibody fragments incompletely processed during the culture step (H2L, H2, H and L) [49,51].

The same remarks concerning resolution and relative quantification stand for capillary isoelectric focusing (cIEF) and imaged cIEF (iCE280) versus classical slab gel IEF [67,68].

Normal and Reverse Phase High-Performance Liquid Chromatography (NP- and RP-HPLC) of PNGase Released Glycans

NP-HPLC is a well-established analytical method to separate mixtures of oligosaccharides after enzymatic release (e.g. PNGase F) and fluorescent derivation (e.g. 2-aminobenzamide). Sub-picomolar levels can be detected and accurately quantified [69].

Fig. (5) shows a representative example of an NP-HPLC chromatogram obtained for oligosaccharides, which were released after PNGase F digestion of a NS0 produced Mab. The different peaks were isolated and submitted to off-line analysis by MALDI-TOF mass spectrometry. For seven of them the experimental masses were in excellent agreement

with the calculated masses from the glycan-2AB labeled structures.

For further confirmation by biochemical methods, the glycans can be subjected to sequential digestion with selective exoglycosidases as described by Guile *et al.* [69] (neuraminidase, α -galactosidase, β -galactosidase, β -hexosaminidase, α -fucosidase, α -mannosidase, β -mannosidase) and since, routinely used in many labs. An improved high-throughput version of this protocol was developed in P.M. Rudd and R.A. Dwek groups [70]. Alternatively an on-line RP-HPLC Mass Spectrometry method was recently reported [71], which is able to resolve high-mannose, hybrid and complex glycans released from IgG therapeutic candidates in late-stage development; using this glycoprofiling method through a screening process, the most heterogeneous antibodies were eliminated.

NP-HPLC can routinely be used for IgGs glycan fingerprinting as illustrated in (Fig. 6) for MAbs produced in different cell lines (eg. CHO or NS0), which clearly exhibit different pattern. NS0 cell produced MAbs exhibit small amounts of glycoforms with additional α -1,3-galactose and different sialic acids (NGNA vs NANA). The same stands for plant vs CHO produced MAbs [72], using PNGase A for the release of the glycans.

Alternative separation methods like zwitterionic type of hydrophilic-interaction chromatography (ZIC-HILIC) were also reported for better isomeric N-glycan separations [73].

Fluorophore-Assisted Carbohydrate Electrophoresis

DNA sequencer-assisted fluorophore-assisted carbohydrate electrophoresis (DSA-FACE) is based on polyacrylamide electrophoresis separation of 8-amino-1,3,6-pyrenetrisulfonic acid derived carbohydrates chains [74]. These method was adapted to a standard DNA sequencer and was for example recently used to characterize a mannosylated antibody-enzyme fusion protein [75].

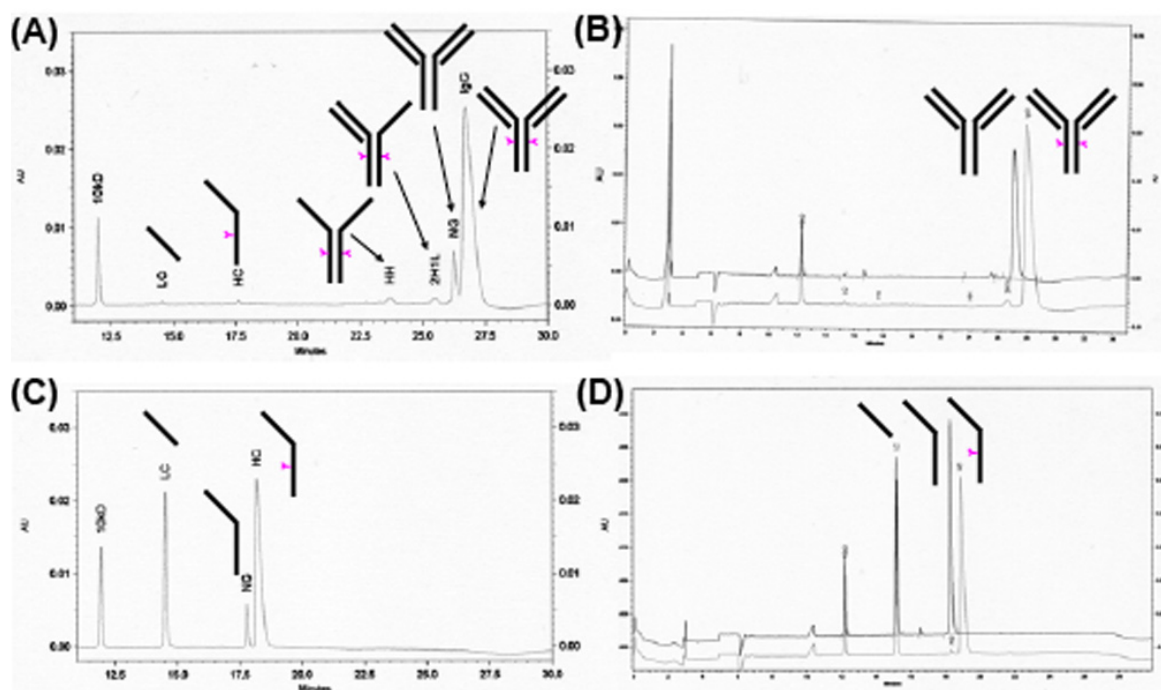


Fig. (4). Capillary Electrophoresis of (A) an intact recombinant IgG; (B) an intact and a PNGase F treated recombinant IgG; (C) a reduced IgG and (D) a reduced and PNGase F treated recombinant IgG.

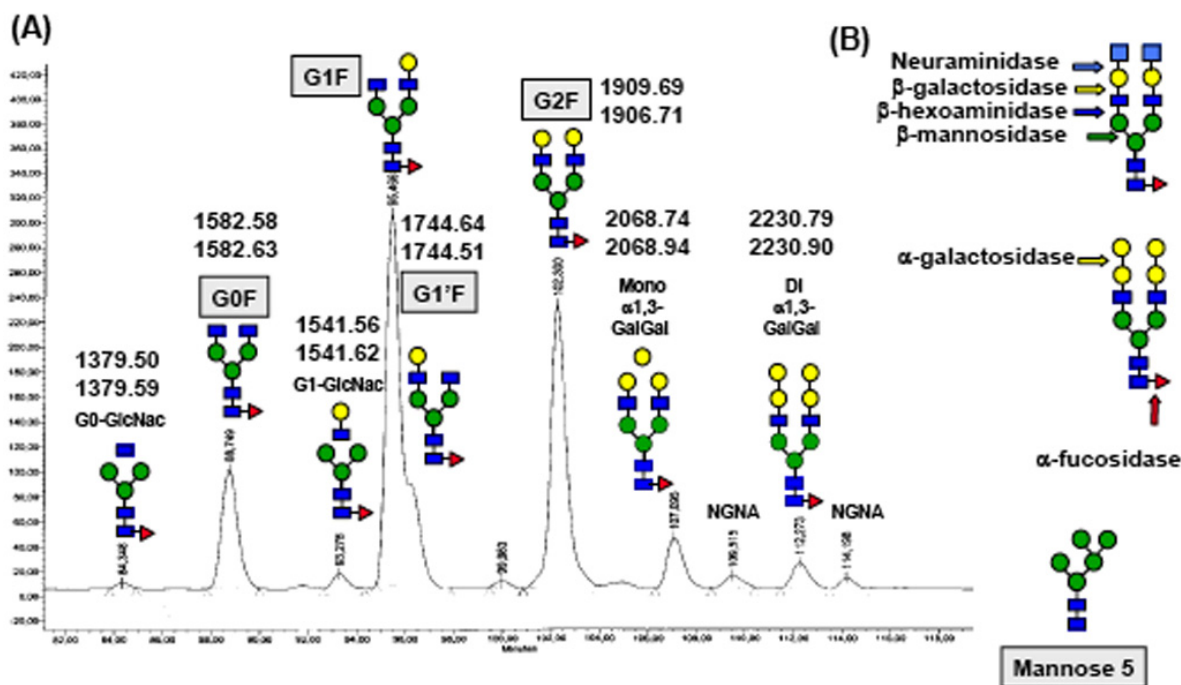


Fig. (5). (A) Normal-Phase High-Performance Liquid Chromatogram of oligosaccharides released by a PNGase treatment of a recombinant IgG and derivatized with a fluorescent dye (2-AB). The structure of each isolated peak can be submitted to MALDI-TOF MS (calculated mass/ experimental mass) and/or to sequential digestion with exoglycosidases for interpretation.

Sialic Acids of Released Glycans Analyses

In contrast to other glycoproteins, IgGs are poorly sialylated [6, 76]. Fig. (7) shows the glycoform profile of an

PNGase F treated recombinant MAb, derivatized (2-AB), analyzed by NP-HPLC and treated or not with Neuraminidase. Only two very small peaks were identified as capped by Neuraminic acid.

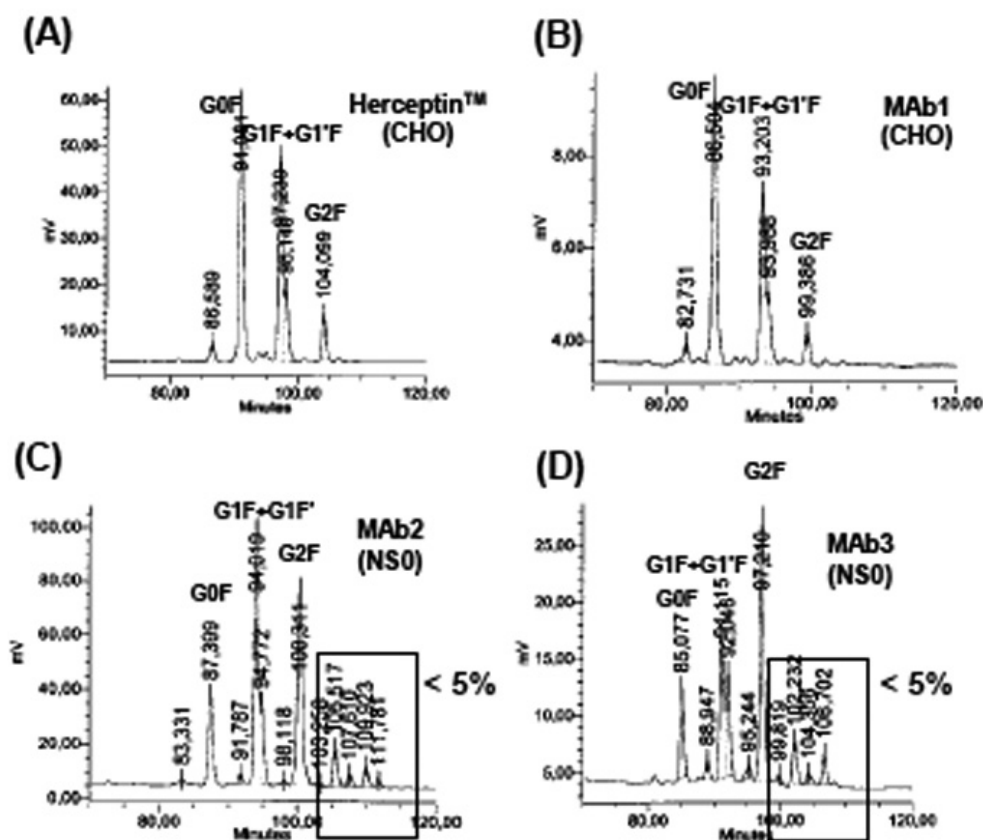


Fig. (6). NP-HPLC fingerprinting of two recombinant antibodies produced in CHO cells (A and B) and two in NS0 cells, respectively. For all four antibodies 4 mains glycoforms are observed in different ratios (G0F, G1F/G1'F, G2F). For the MAb's produced in the NS0 cell line additional glycoforms are present in low amount and are characteristic of mice (Mono and Di alpha-1, 3 GalGal are present as well as traces of mono and di-NGNA).

Sialic acid units (also called Neuraminic acids) are often described as more labile than other glycans and may be degraded during glycan release and sample preparation steps. In order to address this point specific methods have been set-up [77] for fine dosage of both N-Glycolyl Neuraminic acid (NGNA) vs N-Acetyl Neuraminic Acid (NANA) as illustrated in Fig. (7C & D), which is based on MAb hydrolysis, 1,2-diamino-4,5-methylenedioxybenzene (DMB) labeling and RP-HPLC analysis with a fluorescent detection. New carbohydrates dosage methods were also recently proposed by groups involved in high-throughput glycomics [78].

Capillary Electrophoresis (CE) of Released Glycans

Capillary electrophoresis with laser-induced fluorescence detection (LIF) is another powerful technique for oligosaccharide analysis due to its high resolution and compatibility with automatic operating systems. Applications of this new method was recently published by Kamoda *et al.* [51] also on IgG released oligosaccharides with 2-AB derivatization. As illustrated in Fig. (8) this method offer new advantages over the classical NP-HPLC: easier and faster sample preparation, shorter analytical runs (10 vs 120 minutes) and better resolution (e.g. the G1F/G1'F glycoforms are fully resolve in the case of CE analysis an may be better quantified for structure-activity relationship studies). In complement, structural analysis using capillary electrophoresis combined to mass-spectrometry (CE-MS) looks also promising [49, 79].

Mass Spectrometry (MS)

Mass spectrometry is a particularly fast and powerful technique to differentiate glycoform fingerprints of MAb's produced for instance in CHO and NS0 cells [58] and to estimate the relative proportion between the main glycoforms (G0F, G1F and G2F). Interestingly, with the latest generation of Electrospray Ionization – Time-of-Flight (ESI-TOF) mass spectrometers, the mass of intact antibodies can be measured with a precision reaching 40 ppm (± 6 Da), which speeds up considerably the screening and routine MAb characterization by a top-down approach [65,80]. Moreover, the current resolution of mass spectra allows also investigating the non-symmetry of N-linked biantennary oligosaccharides between the two heavy chains, as illustrated in Fig. 9 on intact and reduced antibodies. Such type of analysis was not feasible with classical carbohydrate analytical methods used after enzymatic glycans release or indirectly, by LC-MS analysis of Fc fragments retaining the interchain disulfide bridges following an endoprotease Lys-C proteolysis [81]. Since the extent of symmetric or non-symmetric structure is what biological systems encounter, this new type of information will certainly contribute in the future to the better knowledge of IgG/ Fc gamma receptor structure-activity relationships and the development of MAb's with improved Fc gamma R binding properties [82,83].

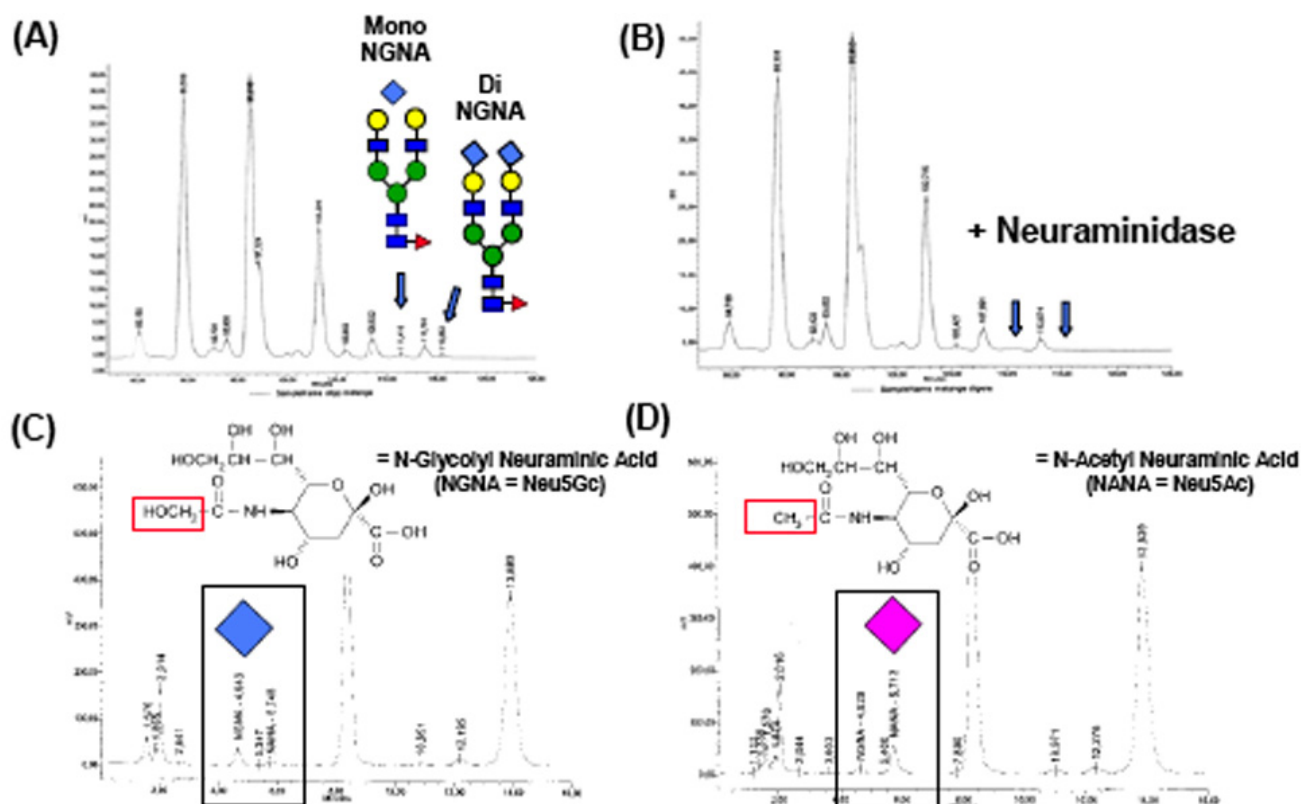


Fig. (7). Sialic acids. NP-HPLC chromatogram of oligosaccharides removed with PNGase F from and 2-AB derivatized from NS0 produced IgG (A) without and (B) with neuraminidase treatment: only two minors peaks are removed which indicates the presence of only trace of sialylated oligosaccharides. This is also confirmed by a specific dosage of NGNA and NANA by HPLC as shown for (C) the same antibody and for (D) Herceptin used as control (CHO produced).

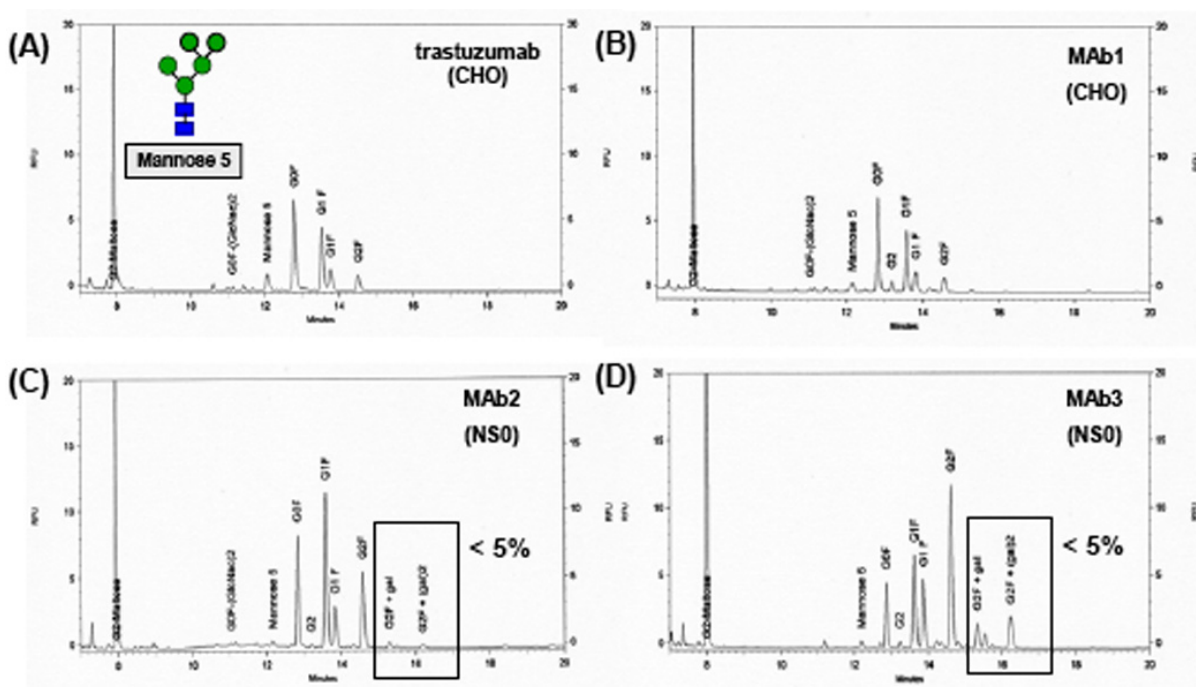


Fig. (8). CE-LIF fingerprinting of two recombinant antibodies produced in CHO cells (A and B) and two in NS0 cells, respectively. For all four antibodies 4 mains glycoforms are observed in different ratios (G0F, G1F/G1'F, G2F). For the MABs produced in the NS0 cell line additional glycoforms are present in low amount and are characteristic of mice (Mono and Di alpha-1, 3 GalGal are present as well as traces of mono and di NGNA). Capillary Electrophoresis (CE) vs NP-HPLC allows easier & faster sample preparation, shorter running time (16 min vs 115 min) and better resolution (e.g. G1F/G1'F resolution, G2...).

Fig. (9) shows ESI mass spectra of a humanized monoclonal antibody produced in NS0, either intact (A) or treated with PNGase F (B) as well as the corresponding Light (C) and Heavy Chains (D) analyzed after reduction by dithiothreitol (DTT) of the same antibody. The spectra consist of typical series of multiply charged ions with m/z values ranging from 1,500 to about 4,000 m/z . The associated deconvoluted spectra are shown below in each figure. Experimental masses were compared to calculated masses deduced from the cDNA-derived amino acid sequence, assuming 16 disulfide bridges, heavy chain N-terminal pyroglutamic acids formation and C-terminal lysine clipping (145 685 Da). The experimental mass of the PNGase F treated MAb was in good agreement with the calculated one (145 695 Da; + 10 Da mass difference). The spectrum of the non-deglycosylated MAb was characterized by 9 equidistant peaks (162 Da increment), which correspond to the mass of additional hexoses (e.g. galactose). Very close experimental masses were also measured for the light chains (exp. 24 063 Da/calc. 24 063 Da) and for the heavy chains (calc. 48 796 Da corrected by the masses of the main reported glycoforms (G0F: 50 240 Da/ + 1444 Da; G1F: 50 402 Da/ + 1606 Da; G2F: 50 563 Da/ + 1767 Da; G2FG: 50 726 Da/ + 1930 Da). Ultimately, to assess the fine molecular structure of the glycans, tandem mass spectrometry (LC-IT-MS/MS, MALDI-TOF/TOF) is performed on released N-Glycans [31,65]. A typical fragmentation pattern of the N-glycan G2F performed by IT-MS/MS is presented Fig. (9). Using a bottom-up approach the 9 previously mentioned peaks can be interpreted as the following pairs of glyco-variants: G0F/G0F, G0F/G1F, G1F/G1F or G0F/G2F (isobaric), G1F/G2F, G2F/G2F, G2F/G2F + G, G2F/G2F + 2G, G2F/G2F + 3G, G2F/G2F + 4G [64]. Using LC-ESI-TOF for a CHO produced MABs, the spectra would exhibit less glyco-variants [65]. Peptides and glycopeptides LC-MS mapping performed after enzymatic digestion of the whole antibody or Heavy Chain or Fab/Fc fragments is another way to gain information about glyco-variants [58]. Trypsin digested Asn²⁹⁷-containing nonapeptides (EEQYN²⁹⁷STYR vs EEQYD²⁹⁷STYR following PNGase digestion) were also recently used to quantify by LC-MS the level of non-glycosylated variants for IgG produced in transgenic plants [84] and in HEK293 cells [85], respectively.

Improved glycopeptides analysis from complex peptide mixture of MAB can also be achieved by enrichment of glycopeptides on nanoLC-chip packed with a graphitized carbon column [65]. Among other improved MS-methods recently described a new quadrupole ion-mobility time-of-flight (ESI-Q-IM-TOF) mass spectrometer was also reported [86]. The use of ion mobility combined with mass spectrometry was shown to ascertain conformational changes of MABs related to the glycosylation state of the heavy chain. In the future, impact of glycosylation on the conformation state of the intact MAB may be evaluated more precisely by a differential analysis with this approach of the molecule +/- deglycosylated in native conditions [65].

It should also be keeping in mind that a plus 162 Da (mass of an hexose) or multiple of 162 Da could be due to glycation on Lysine linked to the process or to the formulation [87] or to O-mannosylation [36]. Such type of glyco-variants are not sensitive to PNGase digestion and can be

finely mapped using for example boronate affinity chromatography and mass spectrometry in case of glycation [88,89,90].

X-Ray Diffraction and Nuclear Magnetic Resonance (NMR)

Fine structural glycans characterization of glycans was also obtained by Fc fragment crystallization and by Nuclear Magnetic Resonance analysis of released glycans [91,92,93]. Both structural methods are not routinely used for antibody carbohydrates, but were essential to demonstrate that glycan moieties are required for structural integrity of MABs [94].

Glycans NMR analyses are often difficult because the spectra are often overlapping and conformational restraints low. Fortunately, also in this area of analytical sciences, tremendous progress have been made. Ultra-high field NMR at 920 MHz for example, looks particularly promising for structural glycobiology [95].

High-Throughput Glycoanalysis

Several new-high throughput methods of glycan analysis are in development as illustrated by P. Rudd, G Kwek groups [70,96] and for Lectin-based array, recently proposed as screening methods during clone selection, process development and manufacturing of antibodies [97]. Lectins are a large family of glycan-recognizing proteins based on high-affinity of monosaccharides [98]. The assays are based on well-characterized plant lectins with overlapping specificities for the most abundant glycoforms (G0F; G1F; G2F; Gal (alpha 1-3) Gal; Sialic acids; high-mannose; bi-, tri- and tetra-antennary).

BENEFITS OF MANIPULATING THE CARBOHYDRATE COMPONENTS OF ANTIBODIES

In complement to classical humanized and human antibodies, engineered derivatives are actively investigated to optimize effector function and to minimize immunogenicity [2,35,99]. All the analytical methods described above are key-tools for the fine-tuning of the next this generation therapeutic antibodies.

ADCC and CDC Enhancement (Fig. 11a)

Antibody-Dependent Cellular Cytotoxicity (ADCC) and Complement-Dependent Cytotoxicity (CDC) are important effector functions especially for IgG1 MABs developed in Oncology, when the major goal is to selectively destroy tumor cells. The presence of a bisecting N-acetylglucosamine [100,101,102,103] associated with the depletion in Fucose residues (e.g. by genetic knockdown of alpha-1,6-fucosyltransferase) from oligosaccharides in the conserved attachment region to Fc receptors result in an increase of ADCC *in vitro* up to 100 fold [10,93,104,105,106]. ADCC was also showed to be enhanced for non-fucosylated IgG4 through improved Fc gammaRIII binding [107], for Fc-fusion proteins (TNFRII-Fc, LFA3-Fc, IL-1R-Rc) [108,109], for Fc-fusion peptides also called "peptibodies" [32], for single chain-Fc (scFv-Fc) and for bispecific antibodies (bsAbs) composed of two different single-chain Fv with dual specificity (TAG72 tumor associated antigen and MUC1 mucin), fused to an Fc moiety (scFv2-Fc) [110].

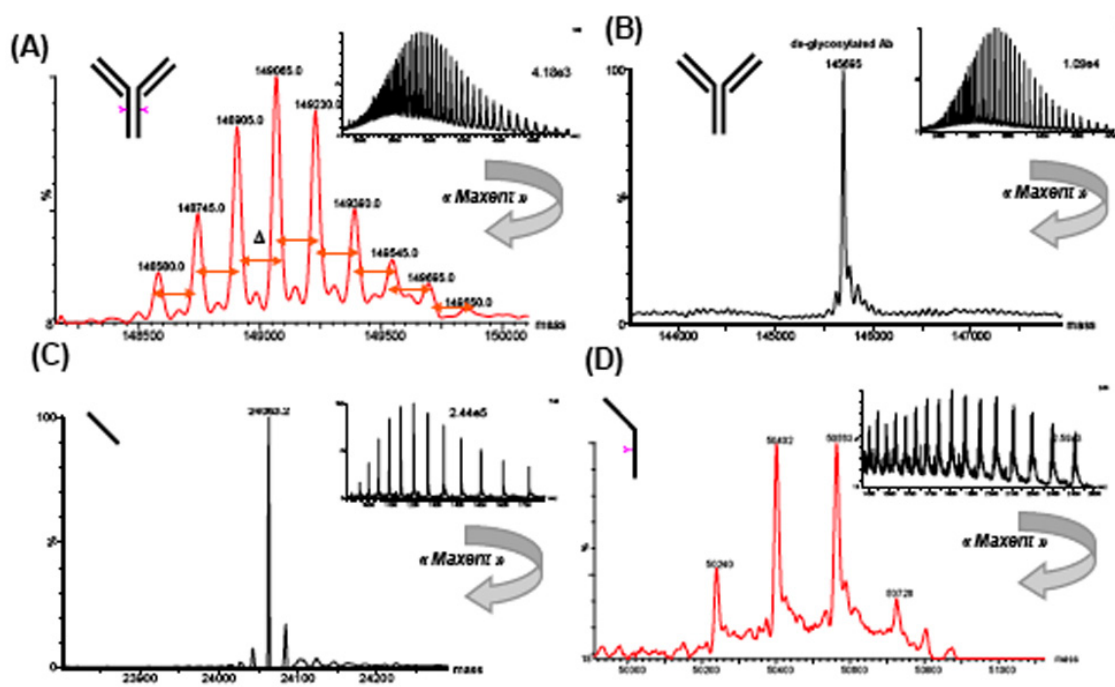


Fig. (9). LC-ES-TOF mass spectrometry of (A) a recombinant intact IgG produced in NS0, (B) the same antibody submitted to PNGase F digestion, (C) the light chain and the heavy chain obtained after reduction, alkylation and chromatographic separation.

Such specific glycosylation profiles can be favored by glycolengineering of the production systems thereby improving also significantly *ex vivo* ADCC functions. Suzuki *et al.* for example showed using peripheral blood mononuclear cells (PMBCs) from healthy donors and from breast cancer patients that ten-fold less antibody was needed to achieve comparable ADCC compared to Herceptin/ trastuzumab [111]. If confirmed in clinical trials that could allow a reduction of the cost of combination treatments (cytotoxic drugs and/ or monoclonal antibodies) directed against several targets or acting by different mechanism of action (growth factors, apoptosis, angiogenesis...), which are the most effective treatments to delay tumor escape e.g. HER1/ VEGFR, HER1/HER2, HER2/ HER3, HER1/ IGF-1R...[112,113]. For large-scale manufacturing of non-fucosylated therapeutic antibodies, a stable high-producing CHO glycoengineered cell line was developed (1.7 g/l), indicating that the technology is mature to produce pilot batches for clinical trials [104].

Recently, Q. Zhou also reported the production of non-fucosylated oligomannose containing antibodies [114] in CHO cells grown in the presence of kifunensine, an alpha-mannosidase I inhibitor. These antibodies showed an increase affinity for FcγRIIIA receptors and ADCC and a reduced C1q binding. The serum-half in mice was not altered but pharmacokinetic in primates remain to be conducted; high-mannose glycoprotein have generally a reduced half-life [37].

Cytotoxic enhancement for glycoengineered MAbs with a bisecting N-acetylglucosamine and/ or a depletion of Fucose was not only demonstrated for CHO cells but also for a plethora of alternative systems like baculovirus-infected insect cells [101], avian cells [115], YB2/0 rat cells [116], yeast [117], aquatic plant [72], moss [103,118] and tobacco [103].

To prevent allo-immunization, anti-Rhesus D antibodies produced in YB2/0 myeloma rat cell and characterized by low fucose and high galactose contents (FOG-1, R297) were shown to exhibit higher ADCC compared to the same antibody produced in a CHO cell line [116]. In a phase I study MAb R297 was shown to promote rapid and complete clearance of red blood cells in healthy male volunteers [119]. However clearance was extremely rapid and faster than for polyclonal anti-D IgGs from pooled plasma of donors [120]; altered glycosylation may increase the binding to innate immune system receptors. The same rat YB2/0 cell line was selected to produce EMAB-6, a anti-CD20 MAb with low fucose content and improved FcγRIIIA binding. In a recent *ex vivo* study based on peripheral blood mononuclear cells from chronic lymphocytic leukemia patients (CLL), EMAB-6 was shown to induce higher ADCC compared to rituximab [121].

Several other glycoengineered antibodies directed against GD3 (BioWa), CCR4 (KM2760, BioWa) [122], CD20 (GA101/R7159, Glycart-Roche), CD30 (MDX-1401, BioWa/Medarex), CD52 (Glycart/Roche [2], IL5R (BIW-8405, BioWa/ Medimmune) are currently investigated in early clinical trials in different indications (cancer, inflammation, asthma) Table 3. The outcome of these trials will be interesting to follow. Most of the encouraging ADCC data were recorded *in vitro*, *ex vivo* (patients' or volunteers' peripheral blood mononuclear cells) [111] or *in vivo* in transgenic mice models; the real clinical benefit for patients remain to be demonstrated and is still discussed. In the case of anti-EGFR MAbs for example, three MAbs gained approval in Colorectal and/ or Head and Neck cancers (Erbix/ cetuximab) [21] Table 1.

Table 3. Biotech Companies Specialized in Recombinant Glycoproteins Production, Glycoengineering and/ or Glycoanalyses

Companies (countries)	Glycoengineering Technologies	Glycoprofiling Methods	MAbs [Target/ Indication/ Clinical Status/ Partner(s)]	Web sites
Biolex Therapeutics (USA)	<i>L. minor</i> Expression System LEX® (-Fuc, - Xyl/ siRNA)	Mass Spectrometry	BLX-301 [CD20/ NHL/ Pre-clin.]	www.biolex.com
BioWa (Kiowa Hakko) (JPN)	YB2 rat cell, CHO cell (-Fuc) Pottelligent®	Mass Spectrometry	KW-0761 [CCR4/ oncol./ Ph I/ Amgen] KW-2871 [oncol./ Ph I/ LICR] MDX-1401 [CD30/ lymphomas / PhI/ Medarex] BIW-8405 [IL5R/ Astma/ Ph I/ Medimmune/ Astra]	www.biowa.com
Genzyme (USA)	CHO + antibiotics (- Fuc oligomannose)	Mass Spectrometry	/	www.genzyme.com
GlycArt (CH) (Taken-of by Roche in 2005, 190 million USD)	CHO cells (bisecting sugar)	Mass Spectrometry	R7159/GA101/RO5072759 [CD20/ NHL/ PhI/II] Roche/ Genentech	www.glycart.com
Glycode (FRA)	Yeast (Hz glycans)	qPCR/ glycotranscriptomics	/	www.glycode.fr
GlycoDiag (FRA)	/	Lectin-arrays	/	www.glycodiag.fr
GlycoFi (USA) (Taken-of by Merck in 2006, 400 million USD)	Yeast (P. pastoris, Hz glycans)	Mass Spectrometry	/	www.glycofi.com
Glycotope (GER)	Human Leukemia-derived NM cells (0 to 100 % Sialyl) GlycoExpress®	RT-PCR FACS (Lectins)	/	www.glycotope.com
Greenovation (GER)	<i>P. mitrella</i> moss (- Fuc, - Xyl complexe glycans)	Mass Spectrometry	/	www.greenovation.com
LFB (FRA)	YB2/0 (bisecting arm, -Fuc)/ EMABling®	Mass Spectrometry	R297 [RhD/ immunol./ Ph I] EMAB-6 [CD20/ NHL/ pre-clin.]	www.lfb.fr
M-Scan (UK/CH/USA)	/	Mass Spectrometry	/	www.m-scan.ch www.m-scan.uk
Neose (USA)	GlycoPEGylation	Mass Spectrometry	/	www.neose.com
Procogonia (ISR)	/	Lectin-arrays	/	
Proteodynamics (FRA)	/	Mass spectrometry	/	www.proteodynamics.com
Prozyme (GER)	Glyko tools	Enzymes/ Glycans	/	www.prozyme.com
Vivalis (FRA)	EbX duck cells (-Fuc)	Mass Spectrometry	/	www.vivalis.com

Two of them (Erbix/ cetuximab and TheraCIM/ nimotuzumab) are IgG1s and show *in vitro* ADCC. On the other hand Vectibix/ panitumumab the third one is an IgG2, acts as an EGF antagonist and is clinically efficacious on tumor regression without effector functions. HuMax-EGFR another anti-HER1 MAb in clinical development was demonstrated to block EGF binding, to interfere with cellular signaling and to recruit effector cells for ADCC independently of Fc fuco-

sylation. Another example concerns IGF-1R antagonist MAbs currently in cancer clinical trials. For these investigational antibodies three different isotypes were chosen able or not to trigger effector functions: on one hand IgG1s (Amgen, ImClone, Merck/Pierre Fabre, Roche/GenmAb, Sanofi-Aventis/Immunogen, Schering-Plough) and on the other hand IgG2 (Pfizer-Abgenix) [21] and a non-glycosylated IgG4 (BiogenIdec).

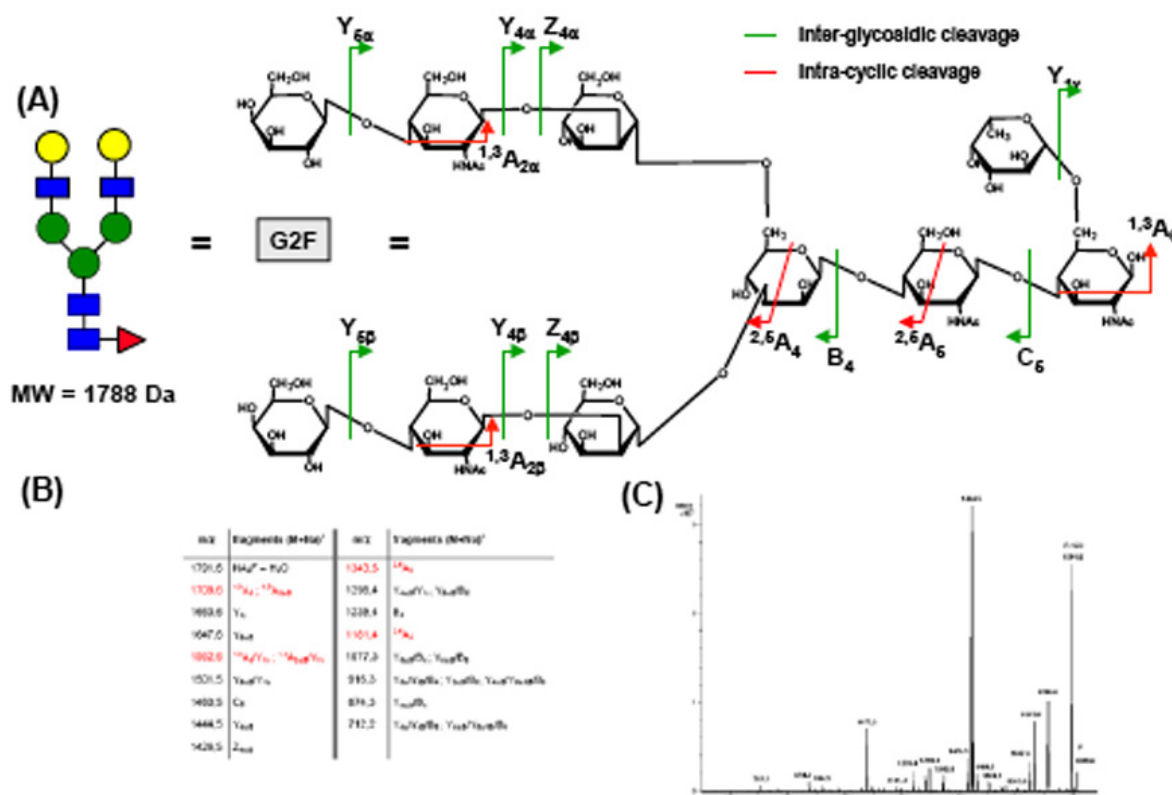


Fig. (10). N-glycan G2F structural characterization by tandem Mass Spectrometry (MS/MS).

Humanization of Glycosylation in Heterologous Expression Systems and Effector Function Enhancement

High mannose-type N-glycans contain from five to nine mannose residues and are found in yeast [16], insect cells [123] and plants [124] and not in normal human antibodies [6,125,126]. Clearance of high-mannose glyco-variants is increased by binding to macrophage mannose receptor in the liver [127]. More generally, immune responses are triggered against xenobiotic glycoforms, accelerate blood clearance and limit the therapeutic potential [128,129]. Tremendous research efforts are ongoing to produce proteins with humanized engineered glycoforms. These systems include transgenic animals [59], chicken [130,131], insect cells [131] and transgenic plant-derived antibodies for example that featured high level of galactose, undetectable levels of xylose and traces of fucose [132,133]. Yeast strains were also genetically transformed to produce MAbs with "humanized" glycoforms, with enhanced effectors functions (e.g. third bisecting arm, lack of fucose) [134] and the perspective to achieve higher proteins titers (> 2 g/l) or similar titers in shorter time than with the current mammalian cells systems at lower production costs (e.g. less expensive chemically-defined culture media, no-viral inactivation steps).

Major advances in the glycolengineering of *Pichia pastoris* was achieved for fully humanized sialylated glycoproteins [45]; yeast strains were engineered to produce anti-CD20 antibodies with a unique glycan structure for each antibody [117].

Filamentous fungi (Aspergillus niger) were also glycolengineered by deletion on genes coding for fungal glyco-

sylation enzymes and introduction of genes necessary to produced humanized complex N-glycans [135].

Plants are another attractive production system for recombinant proteins [124]. A major concern is the presence of beta-1,2 Xylose (unknown in human glycans) and alpha-1,3 Fucose sugars (instead of alpha-1,6 Fucose), which are allergenic epitopes in human [136]. The first generation of plant-derived antibodies ("plantibodies") were only investigated in early clinical trials a decade ago for topical applications and stopped later on (e.g. genital herpes [137], dental carries [138]).

More recently, controlled glycosylation of anti-rabies antibodies was achieved in tobacco plants by expression of human light and heavy chains genetically fused to a Lys-Asp-Glu-Leu ("KDEL") sequence at the C-terminal parts [139]. Interestingly, this signal peptide allows the retention of the glycoproteins in the Endoplasmic Reticulum (ER) and the biosynthesis of mainly oligomannose variants free of beta-1,2 Xylose and alpha-1,3 Fucose. Nevertheless, oligomannose are not found in normal human plasmatic IgGs and the persistence of additional "LEDKRS" or "LEDKESGRA AASGGGGDV" xenoepitopic sequences on both light and heavy chains C-term [140] limit the therapeutic applications for MAbs in human.

Moss (*Physcomitrella patens*) is alternatively proposed as a contained tissue culture system for production of MAbs in photo-bioreactors [118,141,142]. Non-immunogenic and ADCC-improved glycan patterns were obtained by targeted gene replacements to block the processing of two non-mammalian sugar moieties (xylosyltransferase and fucosyl-

mia-derived cell lines grown in controlled supplemented media [53]. Higher level of antibody sialylation is associated with reduced ADCC [147].

Increasing the Glycan Homogeneity

To limit the number of glycoforms and to improve antibodies homogeneity, enzymes involved in glycan synthesis can be used *in vitro* to add glycan units. For example full galactosylation of human IgGs using glycosyltransferase was achieved at 1 kg pilot scale for the G2F glycoform as a proof-of-concept of the feasibility (> 98% purity) [148].

Plasmatic Half-Life Improvement

Most glycoproteins are cleared from the circulation if they carry carbohydrates structures that are recognized by specific receptors (e.g. high-mannose and asialo complex oligosaccharides by mannose and asialoglycoprotein receptors, respectively [149,150]). The half-life and therapeutic potency of EPO for example was shown to be clearly dependent on the presence of terminal sialic acid [16]. Nevertheless, among the circulating glycoproteins, antibodies are considered as an exception [16]. The role of antibody glycosylation in the long plasmatic half-life via binding to neonatal FcγR remains discussed in the literature and in mice are difficult to extrapolate to humans [3,10,104,151, 152]. Serum half-life of human IgG1, 2 and 4 is remarkably long (21-23 days) when compared to IgG3 (7 days), IgA (6 days), IgM (5 days) or antibodies fragments like F(ab')₂, Fab, scFv (a couple of hours) when not derivitized with PEG (e.g. Cimzia/ certolizumab pegol). The half-life of the recombinant current marketed humanized and human antibodies can be around 20 days, close to natural human IgGs and independent of the CHO or NS0 production systems Table 1 [128, 129]. Engineered antibodies with longer half-life (e.g. 6 weeks) are designed to reduce the frequency of administration.

Sialylated glycans are known to be characteristic and essential components of glycoproteins in species as illustrated for example by the switch in specificity of avian influenza viruses hemagglutinins to human flu viruses (alpha 2-5 sialyl to alpha 2-6, [144]). Nevertheless, in contrast to other circulating glycoproteins (e.g. EPO [16], human IgGs are poorly sialylated [76]). The same observation was reported for recombinant antibodies produced in eukaryotic cells [145]. Interestingly, it was recently shown that antibody sialylation could suppress inflammation and reduce cytotoxicity through the engagement of its Fc fragment with different Fc gamma receptors [17,146]. For this purpose, *in vitro* desialylation was achieved by antibody incubation with neuraminidase and the anti-inflammatory properties of the IgGs were lost. On the other hand over-sialylated antibodies were obtained by affinity-chromatography purification with agarose-bound lectins (*Sambucus nigra* agglutinin) and shown to have enhanced anti-inflammatory activities. Alternatively terminally sialylated recombinant antibodies could be obtained in engineered yeast [4, 16] or in NM-F9 leuke-

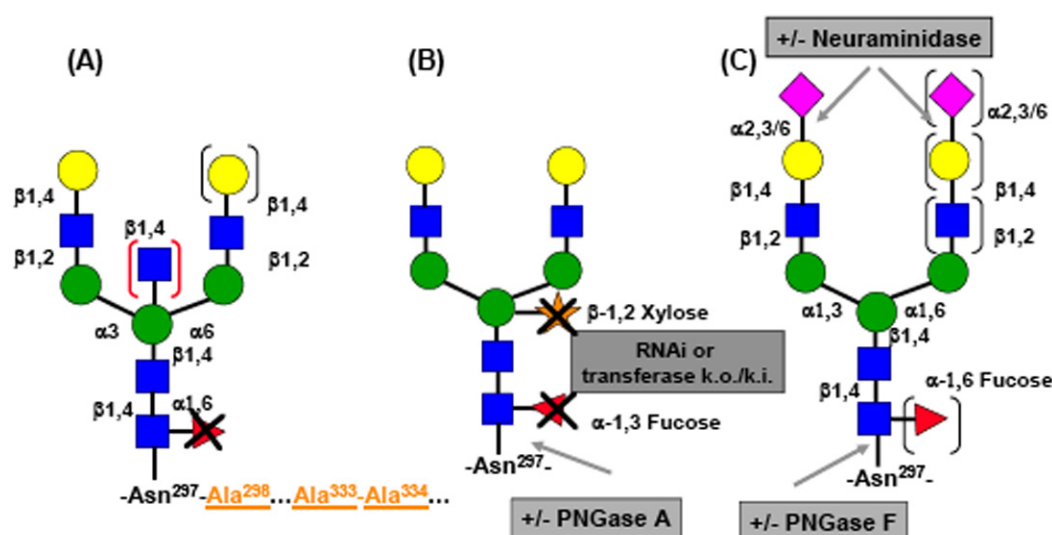


Fig. (11). Tailored glycans: glyco-engineering of an antibody (A) in CHO cells by removing Fucose or adding a bisecting Mannose, (B) in plant or moss cells by knocking down beta 1,4-Xylose, alpha 1,6-Fucose addition, (C) over-sialylated (lectin enrichment or in engineered yeast).

tion in patients undergoing long-term therapy and also to allow reduce doses in the future; such progresses have been reported with mutations in amino acid residues in the Fc region [151,153]. In two recent papers, Raju and Scallion reported data that indicate that terminal units of Fc glycans are also important for antibody stability (resistance to protease) and therefore pharmacokinetics [7,154].

Mannosylated Antibody-Enzyme Fusion Proteins

Antibody-directed enzyme prodrug therapy (ADEPT) is based on antitumor antibodies fused to enzymes able to selectively transform a prodrug into an active cytotoxic product in a tumor. A clinical grade mannosylated antibody-enzyme fusion protein was produced in *Pichia pastoris* and shown to be rapidly cleared which is required for ADEPT [75]. Kolgelberg *et al.* clearly demonstrated by flow cytometry and by immunofluorescence, that MFECP1, a fungi produced antibody fusion protein specific for carcinoembryonic antigen (CEA) was cleared by endocytic and mannose receptors. These receptors are known to bind mannose-type of glycans. The clearance of MFECP1 was inhibited *in vivo* by mannan.

Cytotoxic Immuno-Conjugates

Highly cytotoxic drugs may also be covalently attached to antibodies to improve their therapeutic efficacy [155,156], their selectivity (e.g. immunotargeting of tumor cells) or their ability to be delivered into the cytoplasm upon MAB internalization. Such drugs may be attached randomly to the exposed lysine residues distributed on Light and Heavy Chains. Though fine-tuning of the labeling condition could differentiate somewhat the lysine residues (80 to 95), the risk exists for coupling to lysine present in CDRs thereby losing target binding. Alternatively, site-specific-coupling can be achieved to one or several of the eight cysteine residues involved in inter-chain disulfide bridges after mild reduction or to the glycan moiety after mild oxidation [157] as though an

hydrazone linkage as illustrated in Fig. (12) for vinca-alkaloids. Chemoenzymatic methods based on beta 1,4-galactosyltransferase can be used to transfer C2 keto galactose to asialo GlcNAc residues on monoclonal antibodies for further selective glycoconjugation [158].

CONCLUSION

Glycans represent only a small fraction of an antibody structure (2-3 %) but add complexity, heterogeneity and play a unique role in effector functions. All the current recombinant MABs are produced in eukaryotic cells and show glyco-profiles close to the human ones. Conversely, non-mammalian glycosylation is one the major limitation for the development of alternative production system to generate therapeutic MABs used by systemic routes.

Development of human cell lines (HEK293, PER.C6), humanization of glycan biosynthesis in non-mammalian expression systems (yeast, fungi, insect cells, plants), selection of low-fucose glycoproteins and glyco-engineering to enhance cytotoxic effector function (third acetylglucosyl bisecting arm, low fucose) or anti-inflammatory properties represent major trends for the next generation of therapeutic antibodies and Fc-chimeric derivatives.

Beyond classical electrophoretic and chromatographic methods, mass spectrometry coupled to liquid chromatography or capillary electrophoresis plays an increasing role both during MAB screening and for fine carbohydrate structural characterization. New-high throughput methods of glycan analysis are in development. All these high-performance new analytical methods will also help to analyze the glycan patterns of the targeted antigens in structure-activity relationship studies.

Additional indications of the pharmaceutical growing interest in glycan tailoring and profiling of MABs is pinpointed by the recent acquisition of GlycArt by Roche (CH) and GlycoFi by Merck (USA) [96] as well by the creation

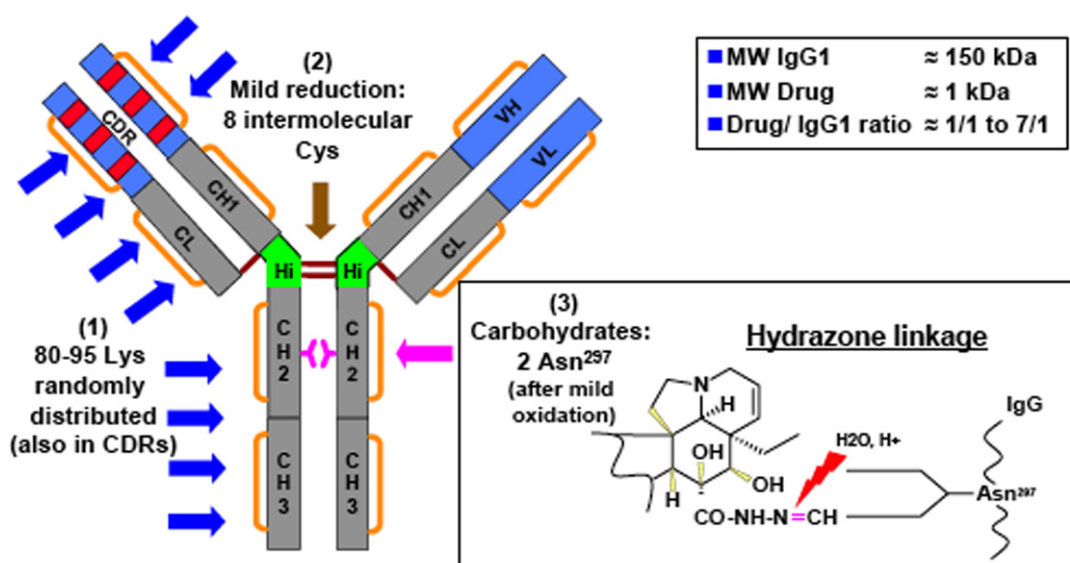


Fig. (12). Immunoconjugates: chemical-engineering of an antibody using the glycan arms for site-specific covalent grafting of cytotoxic drugs

and the development of biotech companies specialized in recombinant glycoproteins production, glycoengineering and/ or glycoanalyses [AviGen (USA), Biolex (USA), BioWa (JAP), GlyCode (FRA), GlycoDiag (FRA), GlycoForm (UK), GlycoTope (GER), Greenovation (GER), Glyence (JPN), LFB (FRA), M-Scan (UK/CH/USA), Neose (USA), ProBiogen (GER), Procogonia (ISR), ProteoDynamics (FRA), Prozyme (USA), Vivalis (FRA)] Table 3.

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ABBREVIATIONS

2-AB	= 2-Aminobenzamide
ADCC	= Antibody-dependent cellular cytotoxicity
ADEPT	= Antibody-directed enzyme prodrug therapy
CDC	= Complement-dependent cytotoxicity
CDR	= Complementary determining region
CE	= Capillary electrophoresis
CHO	= Chinese hamster ovary
cIEF	= Capillary electrophoresis isoelectric focalization
Da	= Dalton
DTT	= Dithiothreitol
EPO	= Erythropoietin
ESI	= Electrospray ionization
ESI-Q-IM-TOF	= Quadripole ion-mobility time-of-flight
FcγRn	= Neonatal Fc gamma receptor
Fuc	= Fucose
Gal	= Galactose
GlcNAc	= N-acetylglucosamine
HC	= Heavy chain
iCE280	= Imaged capillary electrophoresis isoelectric focalization

IEF	= Isoelectric focalization
IgG	= Immunoglobulin G
IT	= Ion trap
LC	= Liquid chromatography
LIF	= Laser-induced fluorescence
MAb	= Monoclonal antibody
MALDI-TOF	= Matrix-assisted laser desorption/ ionization
Man	= Mannose
MS	= Mass spectrometry
MS/MS	= Tandem mass spectrometry
NANA	= N-acetylneuraminic acid
NGNA	= N-glycolylneuraminic acid
NMR	= Nuclear magnetic resonance
NP-HPLC	= Normal-phase high performance liquid chromatography
NS0	= Mouse myeloma cells
PBMC	= Peripheral blood mononuclear cells
PEG	= Polyethylene glycol
PK	= Pharmacokinetic
RP-HPLC	= Reverse-phase high performance liquid chromatography
scFv	= Single chain Fv
SDS-PAGE	= Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
UPLC	= Ultra high performance liquid chromatography
VH	= Heavy chain variable domain
VL	= Light chain variable domain
ZIC-HILIC	= Zwitterionic type of hydrophilic-interaction chromatography

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