University of Natural Resources and Life Sciences, BOKU Vienna

Department of Biotechnology

Recombinant production of the complex human IgM - Evaluation of variations in quality attributes

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BioToP Biomolecular Technology of Proteins

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Abstract

IgM antibodies are large molecules with complex posttranslational modifications, which possess a broad therapeutic potential. However, variations in product quality impede fundamental research as well as therapeutic development. In the herein presented thesis, the quality attributes glycosylation and polymer distribution were characterized in detail. The site-specific glycosylation pattern attached by CHO DG44 cells was analyzed. The glycan pattern was not influenced by environmental cultivation conditions, but slightly differed when the IgM was produced in another host cell line, the HEK293E cells. However, these differences in glycosylation did not affect the interaction with the antigen or the effector protein C1q. The reasons for appearance of other polymers than pentamers during recombinant production were not identified so far. In this thesis, the impact of external conditions, as well as the dissociation of pentamers after secretion could be excluded as possible factors influencing IgM polymer distribution. The formation of incomplete polymers was not influenced by the expression system and the host cell line. More likely, the interactions of the variable regions of the model antibodies have a huge impact and could strongly interfere during the intracellular polymer assembly. Incorrectly assembled pentamers contributed to Russell body formation and therefore the probability for dimer secretion is increased. Residual DNA in IgM preparations represents a serious challenge for the subsequent downstream purification process. Herein, it is shown that the content of nucleic acid contamination does not depend on the cultivation conditions, but increase with prolonged cultivation time. In addition, an efficient purification strategy was developed to remove nucleic acids and host cell proteins. Transient expression in HEK cells was identified as useful alternative to stable expression in CHO cell lines for fast production of IgMs.

Kurzfassung

IgM Antikörper sind große Moleküle mit komplizierten postranslationalen Proteinmodifikationen, die ein großes therapeutisches Potenzial besitzen. Abweichungen in der Produktqualität erschweren jedoch die Grundlagenforschung und Entwicklung von Therapeutika. In der vorliegenden Doktorarbeit wurden die Qualitätsmerkmale Polymerverteilung und Glykosylierung detailliert charakterisiert. Das positionsspezifische Glykosylierungsmuster, welches von CHO DG44 Zellen produziert wurde, wurde analysiert. Dieses Muster wurde nicht durch Kultivierungsbedingungen beeinflusst, aber veränderte sich leicht als die IgMs von einer anderen Wirtszelllinie, den HEK293-E Zellen, produziert wurde. Diese Veränderungen beeinflussten die Interaktion mit dem Antigen und dem Effektorprotein C1q jedoch nicht. Bisher wurden die Gründe für das Auftreten von anderen Polymeren als Pentameren während der rekombinanten Herstellung nicht entdeckt. In dieser Arbeit konnte der Einfluss von äußeren Bedingungen, sowie die Dissoziation der Pentamere nach der Sekretion ausgeschlossen werden. Die Entstehung von unvollständigen Polymeren wurde nicht durch das Expressionssystem und die Hostzelllinie beeinflusst. Es ist wahrscheinlicher, dass die Interaktion der variablen Bereiche der Modelantiköper einen großen Einfluss besitzt und das Zusammenfügen der Polymere in den Zellen stark beeinträchtigen kann. Fehlerhaft zusammengesetzte Pentamere aggregierten in *Russell bodies* und daher ist die Wahrscheinlichkeit Dimere zu sekretieren erhöht. Die DNA-Kontamination in IgM-Präparationen stellt eine Herausforderung für die Weiterverarbeitung dar. In dieser Doktorarbeit wurde gezeigt, dass Nukleinsäurekontaminationen nicht von Kultivierungsbedingungen abhängen, aber mit der Kultivierungsdauer zunehmen. Weiterhin wurde eine effiziente Reinigungsstrategie entwickelt um Nukleinsäuren und Wirtszellproteine zu entfernen. Als nützliche Alternative zur stabilen Expression in CHO-Zelllinien wurde die transiente Expression in HEK-Zellen für die schnelle Herstellung von verschiedenen IgMs aufgezeigt.

Table of Contents

1. Introduction

1.1. The IgM molecule - a unique immunoglobulin

Immunoglobulin M (IgM) is a member of the immunoglobulin superfamily. The immunoglobulin isotypes differ in their heavy chain (HC) and effector functions. The IgM µ-heavy chain consists of one variable (V_H) and four constant domains (Cµ1-Cµ4, Fig. 1). Two µ-chains and two light chains (LC) assemble to an IgM monomer. IgM monomers that contain an additional C-terminal peptide are incorporated into the B cell membrane (Fig. 1A) and are part of the B cell receptor [1]. Instead of this peptide, the secretory IgM has an 18 amino acid tail piece containing a cysteine (Cys575), which is important for polymerization [2]. In humans, polymerization leads predominantly to formation of pentamers (five monomeric subunits), which comprise an additional joining (J)-chain attached to Cys575 via disulfide bonds (Fig. 1B) [3]. However, IgM pentamer assembly can occur in the absence of the J-chain, but the J-chain influences which polymer is formed [4].

The fraction of pentamers is reduced in the absence of J-chain, because other polymers lacking J-chain like hexamers or low molecular weight IgM are produced [5]. Increased concentrations of hexamers or monomers in the human serum were correlated with several autoimmune diseases [6,7]. Secretory IgMs are found in all vertebrates, but they differ in their polymeric status. The tetrameric IgM is the predominant immunoglobulin in teleost fish [8], whereas frog (*Xenopus*) secrete hexameric and monomeric IgM only [9]. Hence, this suggests that there is a species specific polymer assembly control system that ensures the immune homeostasis within the organism. Due to its potential immunogenicity and reduced biological activity, the polymer distribution was considered as a quality attribute in this study.

IgM polymers are heavily glycosylated with a total carbohydrate content of up to 10 %. Each heavy chain has five conserved N-glycosylation sites that are site specifically glycosylated (Fig. 1) [10]. The glycan sites Asn171 (GS1), Asn332 (GS2) and Asn395 (GS3) are mainly occupied by complex type glycans, whereas exclusively oligomannose type is attached to Asn402 (GS4) and Asn563 (GS5) [10]. An additional complex glycosylated glycan site is present in the J-chain (Asn43). It was reported that very often, the GS5 is very often not fully glycosylated [10,11], although it is a quality check point in the polymer assembly control in the endoplasmic reticulum (ER) [12]. With respect to glycosylation, only minor differences at

GS5 between pentamer and hexamer could be detected [11]. The GS4 is considered to be important for the binding to effector proteins like the complement component C1q and is therefore believed to be crucial for its biological function [13,14]. Due to its presumed effect on biological functions, the glycosylation was regarded as quality attribute for recombinant IgM production in this study.

Many IgMs have a low affinity for their antigen, which is compensated by a high avidity because of the high number of antigen binding fragments (Fab). IgM's structure additionally enables efficient agglutination of cells, which is considered as its main defense reaction. Most IgMs are polyreactive to fulfill its major role in the immune system.

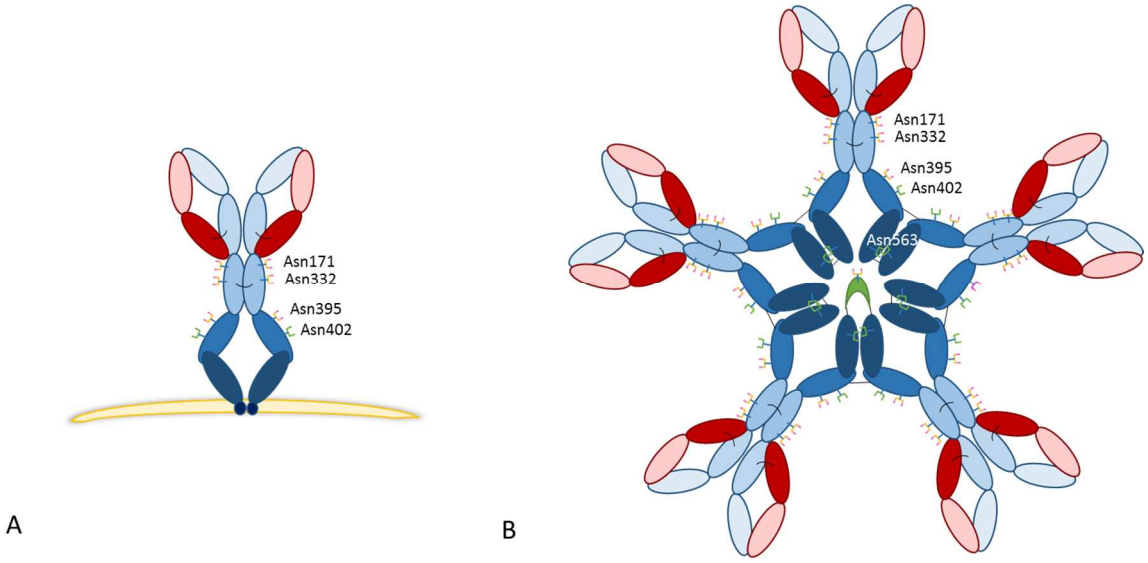

Figure 1: Schematic model of the IgM molecules. A membrane bound IgM and **B** secreted pentameric form of IgM. Displayed are the two light chain domains in red and the five heavy chain domains (V_H, Cμ1-Cμ4) in blue. Joining chain is displayed in green. The five glycan sites per IgM heavy chain are labeled.

1.2. IgM's broad function in the immune system

IgM plays an important role in innate and adaptive humoral immune response. Phylogenetically, IgM is the earliest immunoglobulin that evolved in vertebrates [15]. Additionally, it is the first antibody isotype that can be detected in human fetus [16]. Serum of unimmunized mice already contains natural IgM (but not IgG) against several viruses [17]. Concordantly, IgM deficient patients suffer from recurrent infections with bacteria, virus, fungi or protozoa and increased frequency of allergic and autoimmune diseases [18]. IgM is

also suggested to regulate the B cell development and thereby exhibit immunoregulatory functions [19].

Their immunological function is often triggered via the interaction of IgM with one of the three Fc-receptors or the complement system. The polymeric immunoglobulin receptor (pIgR) is able to bind to IgA dimers and IgM pentamers containing J-chain and thereby triggers the transcytosis across epithelial surfaces into mucosal tissues, where they can detect invading pathogens [20]. The Fcα/μ receptor also binds polymeric IgM and IgA [21]. It induced the endocytose of particles and staphylococcus bound by IgM [21]. Cells presenting the Fcμ receptor on their surface, which is uniquely specific for IgM, were able to internalize IgM-conjugated antigens [22]. The most studied effector function of IgM is the complement activation through the classical pathway, which will be described in more detail in chapter 3.3.

Besides the monomeric surface bound IgM on B cells, two different types of secretory IgMs are known: the natural (antigen-independent) IgM and immune-induced IgM (Fig. 2). The natural IgMs are mainly produced by B1 cells, which lack the mechanisms of V-gene arrangement, isotype switching and somatic hypermutation [23]. Therefore, natural IgMs are mainly germline encoded and have low affinity and specificity. The natural IgM binds to phylogenetically conserved structures, e.g. nucleic acids, heat shock proteins, carbohydrates and phospholipids [24]. These antigens are often self-antigens or found on the surface of pathogens and their detection prevents infectious diseases and fulfills housekeeping functions [17,25,26]. Natural IgM are produced without antigen stimuli and are present in unimmunized mice [17].

In contrast, follicular (FO) B2 cells switch from membrane bound IgM production to secretory IgM due to alternative splicing of the μ-chain mRNA upon interaction with the antigen [27]. Therefore, IgM is the first Ig isotype that is produced after antigen stimulation. The further B cell development depends on the type of antigen: T-dependent antigens, which need co-stimulation by helper T cells to induce class switch recombination and somatic hypermutation, lead to the variety of known Ig isotypes [28]. Those cells represent the majority of the adaptive humoral immunity. Repetitive epitopes (e.g. bacterial cell wall components) can stimulate B cells without involvement of helper T cells (T-independent antigens) [29]. B cells rarely undergo class switch recombination and hypermutation upon

T-independent activation and therefore the major response to such an activation is IgM production [30].

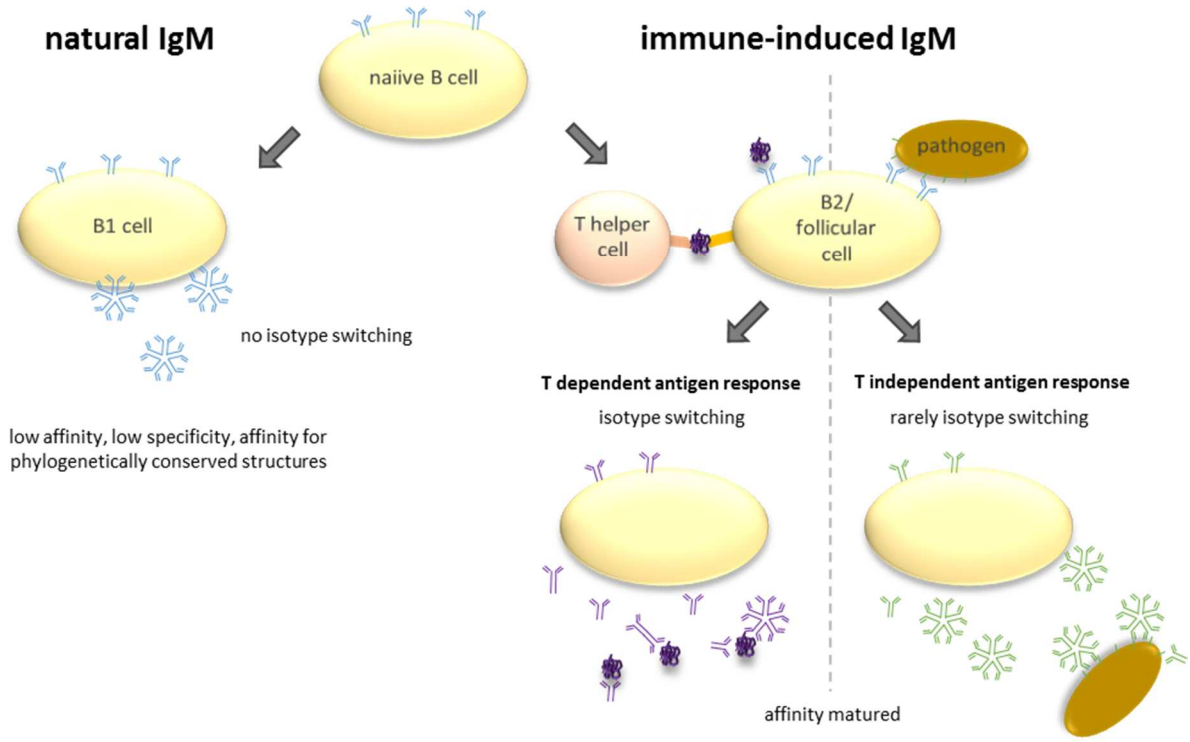

Figure 2: IgM production in different types of B cells. Natural (IgM) antibodies are produced without antigen stimulation by B1 cells. In contrast, B2 (or follicular) cells are stimulated by antigens. The produced isotype of the immune-induced antibody response is determined by the type of antigen (T-dependent or T-independent).

1.3. IgM - an underestimated weapon for various therapeutic applications

The importance of IgM in the immune system becomes clear in patients with selective IgM deficiencies, who have a high susceptibility for autoimmune diseases such as arthritis and systemic lupus erythematosus (SLE) and/or recurrent infections with pathogens [31]. Injection of polyclonal IgM rescued abnormal B cell development in IgM deficient mice and reduced the titers of harmful IgG autoantibodies [32]. IgM enriched Ig preparations (Pentaglobin®) were successfully used to treat infectious diseases in patients [33] or inflammations in rats [34]. These results already lead to the assumption that IgM exhibits a high therapeutic potential for infectious and non-infectious diseases. Below, monoclonal IgMs that offered promising results in clinical or preclinical studies for various types of diseases were summarized.

One of IgMs natural functions is the detection of foreign pathogens and prevention of infection. Recently, an anti-human immunodeficiency virus (HIV) IgM was shown to protect against mucosal virus transmission in rhesus macaques and thereby providing a basis for vaccine development [35]. An anti-phosphorylcholine IgM (T15) triggered protection against *Streptococcus pneumoniae* in a mouse model [36]. Panubacumab, an IgM targeting an O-polysaccharide on the surface of *Pseudomonas aeruginosa* completed a phase IIa study for treatment of nosocomial pneumonia [37].

Furthermore, IgMs were investigated with respect to their housekeeping function. Via binding to and removing of oxidized low-density lipoprotein (LDL), the IgM T15 reduced atherogenesis in an atherosclerosis mouse model [38]. In humans, an inverse correlation of anti- oxLDL IgM titers and disease degree was found [39]. The T15 IgM was also shown to mediate clearance of apoptotic cells by dendritic cells and macrophages and thereby inhibited inflammatory arthritis [40]. Anti-dsDNA antibodies of the IgM isotype were able to inhibit systemic lupus erythematosus (SLE) in a mouse model [41]. Also in humans, natural anti-dsDNA IgMs, anti-PC IgMs and anti-ß2 glycoprotein IgMs were suggested to play a protective role in systemic lupus erythematosus and renal damage and thus possess therapeutic potential [42,43]. Recently, IgMs' potential for the treatment of neurological diseases was explored. The 15B3 IgM is able to bind specifically to the oligomeric form of amyloid ß, which is implicated in pathogenesis of Alzheimer's disease [44]. It showed promising preclinical results *in vitro* and *in vivo* and is therefore regarded as a potential research, diagnostic and therapeutic tool for Alzheimer's disease. The rHIgM22 is a promising therapeutic for multiple sclerosis (MS) because it induced remyelination of oligodendrocytes in animal models and recently completed phase I clinical trial with MS patients [45,46]. Alternatively, IgM12, which interacts with gangliosides on the surface of neurons, preserved neurological functions in mouse models for MS and amyotrophic lateral sclerosis (ALS) [47,48].

Gangliosides are also important targets for anti-cancer immunotherapy. An anti-GM3 IgM showed promising results in preclinical studies for treatment of melanomas [49]. Mulens *et al.* injected gangliosides on proteoliposomes into breast cancer patients as a cancer vaccine to boost natural IgM response *in vivo* [50]. This idiotypic vaccination strategy already entered phase III of clinical trials [50]. Often, natural IgMs react with abnormal carbohydrate

moieties on the surface of altered tumorigenic cells representing the natural immunosurveillance mechanism against tumors [51]. Some of them, such as PAT-SC1, PAT-SM6 and mAb216 were isolated with human hybridoma technology and tested in clinical trials for treatment of gastric cancer, multiple myeloma and acute lymphoblastic leukemia (ALL), respectively [52–54]. In the 1990's, the murine FC-2.15 IgM led to a huge reduction of metastases in an phase I clinical trial, however, the patients suffered from side effects related to the murine origin of the antibody [55].

Concluding, although no therapeutic IgM has been approved yet, IgM comprise a large potential for the treatment of a broad range of diseases because of their natural function as a guardian of homeostasis within the immune system. They could become particularly important for diseases for which an efficient therapeutic solution has not been developed so far such as HIV infection, atherosclerosis, SLE, Alzheimer's disease, MS, ALS and different types of cancers.

1.4. Challenges in recombinant IgM production

A prerequisite for clinical trials and developing a therapeutic product is an economic and consistent production process. The first IgMs were isolated from serum, B lymphoma cultures or hybridoma cultures [56,57]. Since that time, other recombinant expression hosts like plants, NS0 cells, Per.C6 cells and Chinese hamster ovary (CHO) cells were developed [2,58–61]. Nevertheless, some cases were reported, in which clinical trials involving promising IgMs needed to be interrupted or terminated due to lack of availability of the IgM or high variation in IgM quality during the manufacturing process (ClinicalTrials.gov Identifier: NCT01123304, www.patrys.com/pat-sm6). The reasons of the production failure can only be speculated. However, it is known that IgM is produced in lower yields than IgG, leading to a less economic process. Furthermore, production of secretory IgM can lead to lower molecular weight polymeric side products in the supernatant [49,62,63]. In addition, the published purification methods are quite inefficient and suffer from the high susceptibility to low ionic strength and hydrophobicity leading to protein unfolding and aggregation.

Until now, the molecular mechanisms behind the role of IgMs within the immune system are not fully understood. Therefore, the investigation of biophysical properties and

characteristics of the IgM molecule during the production process will build a basis for further progress in fundamental research and the development of therapeutic IgMs.

2. Aim of the thesis

This thesis aimed to address the challenges in recombinant IgM production and manufacturing. Better understanding of the IgM production and biochemical behavior will help to find solutions for the high variation during production and to increase the yields during manufacturing.

The definition of quality attributes that are generated by the host cell should be the focus of this thesis. Furthermore, methods needed to be established for quality characterization. Recombinant production and quality criteria of the human model proteins IgM617, IgM012 and IgM012_GL had to be characterized. IgM617 is a natural IgM that recognizes gangliosides which are enriched on the surface of tumor cells. IgM012 was originally developed by hybridoma technology using human peripheral blood mononuclear cells and class switched for recombinant human IgM expression in CHO cells [61]. IgM012 and its mutant IgM012_GL bind to the surface protein gp160 of the HIV. CHO cell lines producing the three model IgMs have previously been generated [64]. The cell lines producing IgM617 and IgM012 had been characterized with respect to cell culture performance, genomic and transcript level and ER stress factors [64]. This previous study had revealed that CHO cells producing IgM012 exhibit a lower productivity and generate higher amounts of dimers [64]. To improve the stability and the producibility of the protein, 17 mutations have been introduced in the variable regions of the HC and LC of the IgM012 leading to IgM012_GL. The stability, but not the fraction of dimeric IgM could be slightly improved by this approach [65].

The herein presented PhD project should encounter relevant quality criteria of IgM and investigate the impact of process conditions on the quality of IgM012_GL. The results of this study were published in Hennicke *et al.* [66]. Additionally, it had to be studied if the quality is provided by the host cell line. Therefore, the model IgMs should be produced with different expression systems and host cell lines. As some of the quality analyses require pure proteins, an efficient purification strategy needed to be developed leading to a further publication in the context of this thesis [67]. Moreover, it had to be studied if the protein sequence and structure determine intracellular folding and polymer assembly. Finally, the interaction of recombinant produced IgM with natural partners should be characterized and visualized with electron microscopy.

3. Human recombinant IgM and its quality attributes

3.1. The impact of cultivation

Process conditions and their effect on IgM quality

Process conditions such as temperature, pH, dissolved oxygen and CO_2, nutrient concentrations and trace elements are parameters that are described to influence the host cell growth, metabolism and cell cycle [68]. Mild hypothermia can influence the number and variety of HCP [69] or decrease aggregation level of the product [70]. Glycosylation can be affected by cultivation temperature, bioreactor pH or dissolved oxygen [71,72]. Especially for difficult-to-express proteins, folding and aggregation can be improved by carefully selected process conditions leading to increased product quality [73]. Optimization studies to increase product quantity and quality were performed for a variety of proteins, such as EPO-Fc, IgG and human granulocyte macrophage colony stimulating factor [70,74,75].

The complex structure of IgM suggests challenges in production regarding expression rate as well as product quality. Different cultivation temperatures and pH values were analyzed in the following systematic design of experiment (DoE) study with respect to their impact on IgM quality criteria [66]. It was assumed that lower temperatures might have a beneficial impact on folding and assembly. The CHO DG44 cell line producing the IgM012_GL was used as a model since it produces a mixture of pentameric and dimeric IgM molecules. Additionally, the complex glycosylation pattern might be influenced by the environmental process conditions. Therefore, the total glycan pool was analyzed for the IgM012_GL produced at different pH and temperature. It was reported that IgM tends to interact with chromatin in the culture supernatant, which would impede downstream processing [76]. In this study, nucleic acid content after an initial purification step was an additional quality criterion [66].

Summarizing the results of the study, the reduction of cultivation temperature severely reduced IgM titers, while the pH had no impact. In contrast, IgM quality was not significantly influenced by the investigated bioprocess parameters. Concluding these results, variation in IgM quality during manufacturing is not induced by cultivation temperature and pH. The herein defined quality attributes should still be considered for recombinant production of therapeutic IgMs in the future.

Impact of temperature and pH on recombinant human IgM quality attributes and productivity

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Impact of temperature and pH on recombinant human IgM quality attributes and productivity

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ABSTRACT

IgM antibodies are arousing considerable interest as biopharmaceuticals. Despite their immunotherapeutic potential, little is known about the impact of environmental conditions on product quantity and quality of these complex molecules. Process conditions influence the critical quality attributes (CQAs) of therapeutic proteins and thus are important parameters for biological safety and efficacy. Here, the results of a systematic study are presented that characterized the influence of temperature and pH on cell-specific productivity and IgM quality attributes. Biphasic temperature and pH shift experiments were performed as batch cultures in DASGIP® bioreactors under controlled conditions and defined by a specific design of experiment (DOE) approach. An internally-developed recombinant IgM producing CHO cell line was used. With respect to product quality, after an initial purification step efforts were focused on pentamer content, nucleic acid (NA) impurities and the glycosylation profile after an initial purification step. All quality attributes were evaluated by densitometric and chromatographic methods. The reduction of cultivation temperature severely reduced IgM titers, while pH variation had no impact. In contrast, IgM quality was not significantly influenced by bioprocessing parameters. Data revealed that an additional purification step is required to reduce the presence of NAs for *in vivo* applications. In conclusion, the results showed that for the chosen IgM model, IgM012_GL, variation in quality attributes is not caused by the environmental conditions of temperature and pH.

1. Introduction

Immunoglobulin M (IgM) antibodies are promising therapeutic candidates and several have already been investigated in preclinical studies [1–3] and clinical trials [4,5] for therapy of cancer, autoimmune diseases or atherosclerosis. As a first line immune response following antigen stimulation, naïve B cells differentiate into plasma cells producing secretory IgM pentamers containing ten heavy and light chains each and one joining (J) chain [6]. The molecular weight of IgMs is approximately 1 MDa of which up to 10% can be attributed to N-glycans. Recombinant IgMs have been successfully produced in plants [7], hybridomas [8], Per.C6 cells [9], NS0 cells [10] and Chinese hamster ovary (CHO) cells [11].

The influence of process-related changes on product quality is a pre-requisite in the manufacture of biopharmaceuticals. The most critical quality attributes (CQAs), i.e. glycosylation, protein aggregation, charge variants, host cell protein or DNA content, are strongly dependent on both the product and the entire process. With respect to glycosylation, it has been reported that the cultivation of CHO cells at lower temperatures reduced the EPO (erythropoetin)-Fc sialylation [12], while others found a reduction of galactosylation of an IgG when produced at reduced temperature [13] and that IgG glycosylation was not affected by pH, pCO_2 or pO_2, whereas pH had an influence on the IgG charge variants [14]. In another study, an impact of pH on the glycosylation of a humanized camelid antibody was described [15]. In short, the influence of environmental conditions on CQAs is protein-dependent and it is necessary therefore to investigate each case individually. This is particularly true for IgM antibodies as, to date, no such characterization studies have been performed.

Abbreviations: IgM, immunoglobulin; CQA, critical quality attributes; DOE, design of experiment; CHO, Chinese hamster ovary; NA, nucleic acid; EPO, erythropoetin; ELISA, enzyme –linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SEC-HPLC, size-exclusion high-performance liquid chromatography; MALS, multiangle light scattering; VCCD, viable cumulative cell days; HMWA, high molecular weight aggregates

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For several reasons, the complexity of IgMs emphasizes the importance of process parameter control and monitoring of product quality. First, each IgM heavy chain contains five conserved N-linked glycosylation sites [16], compared with only one site in IgG heavy chains, so that the probability of altered glycosylation is increased. Secondly, incomplete pentamer formation is a known issue in recombinant IgM production [17–20]. Thirdly, nucleic acids (NA) are known as potential contaminants in therapeutic drugs. Indeed, free DNA in the culture supernatant interacts with IgMs to form complexes that are difficult to separate during downstream processing [21]. Therefore, the aim of this study was to investigate whether carefully selected process conditions could beneficially influence product quality of IgM.

Here, a systematic and controlled cultivation of one IgM producing CHO cell line is presented, to reveal how the process conditions affect quality attributes of the IgM012_GL. Moreover, important quality criteria for IgM antibodies were defined and evaluated, which should be taken into consideration in establishing a (large-scale) manufacturing process for therapeutically-relevant IgM antibodies. A biphasic cultivation strategy with an initial growth phase at 37 °C and pH 7.0 was used. After a switch to a subsequent production phase at different temperatures and pH values, the impact on yield, assembly, glycosylation and NA content were investigated. All experiments were defined by a specific design of experiment (DOE) that was previously implemented to successfully improve the quality of EPO-Fc [22].

2. Materials and methods

2.1. Design of experiment

A central composite design was used in this study to analyze the influence of two process factors, temperature and pH, on product quantity and quality. Five temperature levels (38.5 °C, 37.0 °C, 33.5 °C, 30.0 °C and 28.5 °C) and three pH (7.05, 6.90, 6.75) levels were combined in 12 biphasic batch fermentations. All cultivations were performed in three consecutive runs from four bioreactors. The center point conditions were performed in triplicate.

2.2. Process parameters and sample preparation

A monoclonal CHO DG 44 cell line producing the IgM012_GL was established as previously described [23] and cultivated in chemically-defined media (MV3-2, a gift of Polymun Scientific, Austria) supplemented with 6 mM glutamine (Roth, Germany, #9183.1). Medium composition included 42 mM glucose, 2.6 mM glutamate, 2 mM ammonium ions (measured with BioProfile 100 Plus, Nova Biomedical). As an alternative, commercial media such as ProCHO5 (Lonza, #12-766Q) may be used for reproduction of the experiments. In a previous study, we have observed a similar growth behavior for cells grown in ProCHO5 compared to the MV3-2 medium (**Table S1**). The precultures were propagated in Erlenmeyer shake flasks at 37 °C, initial pH of 7.05, 150 rpm, 80% humidity and 7% CO_2. The DASGIP® bioreactors (Eppendorf, Germany) were inoculated with exponentially-growing cells at a concentration of 0.5×10^6 cells/mL in a final working volume of 0.6 L. In the initial growth phase, cells were grown at 37 °C, pH 7, 30% dissolved oxygen (DO), 80 rpm stirring speed and 1 L/h gas volumetric flow rate. The temperature and pH shift to a production phase was performed at a cell density of 1×10^6 cells/mL (day 2). The cultures were harvested at a viability of 60% or after a maxiumum process duration of 13 d. Cell concentration, viability and IgM concentration were measured every 24 h. If the viability decreased below 90%, additional samples were taken to analyze the intracellular product content and product quality. Cell concentration and viability were determined using the Vi-CELL XR™ Cell Viability Analyzer (Beckman Coulter, Germany). IgM012_GL concentration was quantified with a standard μ-κ-ELISA using purified IgM012_GL as reference material.

Purification of IgM012_GL was performed as previously described [24]. Briefly, POROS CaptureSelect™ IgM Affinity Matrix (Thermo Fisher Scientific, Netherlands, #195289005) was used for affinity chromatography. IgM012_GL was eluted with 1 M Arginine pH 3.5, 2 M $MgCl_2$, and dialyzed against 0.1 M NaH_2PO_4 pH 5.5, 0.2 M NaCl.

2.3. Flow cytometry

Cells were fixed in 70% EtOH and stored at 4 °C until analysis. After washing with Tris buffer (0.1 M Tris-HCl pH 7.4, 2 mM $MgCl_2$, 0.1% Triton-X100) cells were blocked with 20% FCS in Tris buffer for 30 min at 37 °C. Cells were either incubated with anti-human IgM (μ-chain specific) − FITC antibody (Sigma Aldrich, Germany, #F5384) or anti-human kappa light chain − FITC antibody (Sigma Aldrich, Germany, #F3761) for 30 min at 37 °C. Cells were washed with Tris buffer and resuspended in Tris buffer with 50 ng mL^{-1} 4′,6-Diamidin-2-phenylindol (DAPI, Sigma Aldrich, Germany, # 10234276001). In each experiment, 10.000 cells were analyzed with a Gallios Flow Cytometer (Beckman Coulter, Germany) at the wavelength of 525 nm (FL1 channel). Non-producing CHO K1 cells were used as a negative control.

2.4. Product characterization

Polymer distribution was characterized from the cell culture supernatant using soudium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) separation and densitometric band quantitation as described [17]. Sypro® Ruby gel staining was used in order to obtain linear quantitation over three orders of magnitude [25]. The cell culture supernatant was separated with NativePAGE™ Novex™ 3–12% Bis-Tris Protein Gels (Thermo Fisher Scientific, USA, #BN1001BOX) and stained with SyproRuby™ protein gel stain (Thermo Fisher Scientific, USA, #S12001) before visualization with a Typhoon FLA 9500 (GE Healthcare, USA) at an excitation wavelength of 450 nm and an emission wavelength of 610 nm. Image analyses were performed with ImageQuant TL (GE Healthcare, USA). Alternatively, the affinity-purified product was separated using size exclusion high performance liquid chromatography (SEC-HPLC) and the IgM012_GL isoforms were detected using a diode array detector (SPD-M20 A, Shimadzu, Austria), a refractive index detector (RID-10 A, Shimadzu, Austria) and a multi-angle light scattering (MALS) detector (WYATT Heleos Dawn8+ plus QELS, software ASTRA 6).

Polymer distribution and NA impurity of the purified IgM012_GL were identified and quantified by comparing the UV signals at 254 nm and 280 nm after analytical SEC-HPLC separation performed using an Acquity UPLC Protein BEH SEC column (450 Å, Waters, USA, #186006851) on a Shimadzu prominence LC20 HPLC system. The column was equilibrated with 0.1 M NaH_2PO_4 pH 5.5, 0.2 M NaCl before 20 μL of the purified IgM012_GL were separated at 0.3 mL/min. A gel filtration standard (Bio-Rad Laboratories, USA, #1511901) containing thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobulin (17 kDa) and vitamin B12 (1.35 kDa) was used to estimate the hydrodynamic radii of the separated protein peaks. Peak integration was performed with LabSolutions (Shimadzu, Austria) at 280 nm and at 254 nm for protein and NA peaks, respectively.

For glycan analysis, the purified IgM012_GL samples were enzymatically deglycosylated by PNGase F. Released glycans were labeled with procainamide as described [26], with the modification that excess procainamide was removed using Chromabond CN cartridges (Macherey-Nagel, Germany, #730061). Eluted glycans were analyzed by hydrophilic liquid interaction chromatography (HILIC) using an AdvanceBio Glycan Mapping column (2.1 × 150 mm, 1.8 μm, Agilent, Germany, #859700-913) on a Nexera X2 HPLC system with a RF-20Axs fluorescence detector equipped with a semi-micro flow cell (Shimadzu, Austria). An acetonitrile gradient was applied and peaks were detected by fluorescence at 310/370 nm. Peaks were integrated with LabSolutions (Shimadzu, Austria).

2.5. Data evaluation & statistics

Cell specific productivity (qP) was calculated as in (1):

$$qP = \frac{\Delta cIgM}{\Delta VCCD} \quad (1)$$

where ΔcIgM represents the increase in IgM012_GL concentration between two time points and ΔVCCD (viable cumulative cell days) represents the accumulation of cells within this time period:

$$\Delta VCCD = \sum_{i=1}^{n} \frac{(x_{i+1} - x_i) \cdot (t_{i+1} - t_i)}{\ln x_{i+1} - \ln x_i} \quad (2)$$

where x_{i+1} and x_i are the viable cell concentrations at the time points t_{i+1} and t_i.

Percentage of IgM012_GL pentamer or dimer was calculated using either the determined pixels of densitometric SyproRuby™ gel stain evaluation or peak areas of UV signal at 280 nm in SEC-HPLC chromatograms.

Relative quantities of NA were calculated with the corrected peak areas (A) of the SEC-HPLC chromatogram as in Eq. (3)

$$c(NA) = \frac{A_{NA}/\varepsilon_{mass}}{A_{NA}/\varepsilon_{mass} + A_{Pentamer}/\varepsilon_{mass} + A_{Dimer/\varepsilon_{mass}}} \quad (3)$$

The absorbance of nucleobases at 254 nm and the absorbance of aromatic amino acids at 280 nm was used for calculation of peak areas. The peak areas were corrected using the mass extinction coefficient (ε_{mass} = 20 $Lg^{-1}\,cm^{-1}$) for NAs.

The mass extinction coefficient of IgMs was calculated according to (4):

$$\varepsilon_{mass} = \frac{\varepsilon_{molar}}{M} \quad (4)$$

where ε_{molar} was calculated with the ExPASy tool [27] (pentamers: 1,099,415 $M^{-1}\,cm^{-1}$ and dimers: 433,790 $M^{-1}\,cm^{-1}$). The molar mass (M) was detected by MALS according to the software ASTRA 6. Correct molar mass calculation by MALS was verified by the determination of a sample of bovine serum albumin.

A *t*-test for method comparison of pentamer quantification and a Pearson correlation to analyze the influence of temperature and pH were performed with Sigma Plot 12.5.

3. Results

3.1. Cell growth and productivity

During the growth phase (up to d 2), the process conditions were identical for all cultures. The conditions were altered at a cell concentration of 1 × 10^6cells/mL (d 2) in the exponential growth phase. At higher temperatures (37.0 °C and 38.5 °C, Fig. 1A), the cells grew to 35–70% higher peak cell densities (1.6–2.1 × 10^6 cells/mL) compared to lower temperatures (30 °C and 28.5 °C, 1.2–1.4 × 10^6 cells/mL, Fig. 1A). The exponential growth phase ceased immediately after switching to 28.5 °C d 2. Peak concentrations were reached at d 3 or d 4 for most cultures (Fig. 1A). After the applied alteration, the specific growth rate was directly correlated with temperature (data not shown, $p < 0.0001$). Due to the slow growth, the process duration was prolonged in cultures grown at sub-physiological (≤33.5 °C) temperatures (Fig. 1A) and the cultures showed a weaker increase in viable cumulative cell days (VCCD) with time (Fig. 1B). The pH value had no impact on peak cell concentrations or on specific growth rates ($p > 0.05$).

The cultures accumulated more IgM012_GL at higher temperatures (Fig. 1C) due to the higher cell concentrations and higher cell specific productivities. This was also observed in Fig. 1C, where cells at similar VCCDs produced more IgM012_GL if cultivated at higher temperatures. At harvest, IgM012_GL concentrations of 91–108 mg/L (38.5 °C and 37.0 °C), 61–72 mg/L (33.5 °C) and 46–52 mg/L (30.0 °C and 28.5 °C) were reached. These data indicated a positive influence of temperature on IgM012_GL titer ($p < 0.0001$). No influence of pH was observed ($p > 0.05$, Fig. 1C). Note that for the cultures that were incubated at lower temperatures, the IgM012_GL titer reached ~50% of the final titer during the growth phase (up to d 2, Fig. 1C). In this case, the harvested culture supernatant was composed of a mixture of IgM012_GL produced at 37 °C and lower temperatures. Thus, the quality analysis was representative of a mixture of IgM012_GL produced under initial and production conditions.

To compare productivities during the entire process, the individual processes were divided into two stages. The first stage consisted of the data calculated from d 0 to d 4, which marked the end of the exponential phase for most processes. The second stage corresponded to the stationary phase, generally from d 4 to harvest. During the first 4 days, cell-specific productivity was 50–440% higher than in the stationary stage ranging from ~8-10 pg/(cell*day) for all conditions (d 4 to harvest, Fig. 1D) with only a minor influence of temperature ($p = 0.04$) or pH ($p = 0.18$) evident. This is the result of a rather slow switch in cellular metabolism of mammalian cells (d 2–4). In contrast, a clear influence of temperature on productivity was observed during the stationary stage. Cultures incubated at low temperatures produced only 1–2 pg/(cell*day) while those incubated at higher temperatures produced 4–6 pg/(cell*day) (Fig. 1D). Thus, a direct correlation of productivity with temperature was observed during the stationary stage ($p < 0.0001$); whereas the pH did not have any influence ($p > 0.05$).

To exclude intracellular aggregation of the IgM or spontaneous silencing of IgM expression in the cell lines during the batch process, the intracellular product accumulation or the formation of non-producing sub-populations was determined by flow cytometric analysis of the intracellular product content. To avoid the impact of viability on the measurement, the samples for flow cytometry were taken at a viability of ~90%. Homogeneous fluorescence peaks were obtained for all conditions, thus indicating an absence of sub-populations (Fig. S1). Mean fluorescence intensity (MFI) did not show a trend for intracellular heavy or light chain aggregation at each of the distinct conditions (data not shown).

3.2. IgM012_GL quality

Quality attributes must be defined according to the unique properties of the respective protein group. Some, such as incomplete pentamer formation [17,18] or DNA interaction [21], have been previously described for IgM antibodies. Glycosylation is important for several glycoproteins including IgG, erythropoietin or colony stimulating factors, and was also analyzed as an additional quality criterion. It is also known that IgM glycosylation can affect the cytolytic activity and has immunomodulatory effects [28,29]. To avoid changes in product quality as a consequence of cell viability, samples for IgM012_GL quality attributes were harvested at a viability of ~90%.

The polymer distribution was analyzed by two complementary methods and two different IgM sample formats; either from the cell culture supernatant or from purified IgMs. Cell culture supernatant was analyzed by densitometry. Purified IgM012_GL containing only pentamers and dimers (estimated molecular weight of ~900 kDa and ~340 kDa, respectively) was loaded onto a gel (Lane C, Fig. 2A) to identify the IgM polymers. The pentamer content ranged from 80 to 85% (Fig. 2C) with no significant impact of temperature ($p > 0.05$) or pH ($p > 0.05$). SEC-HPLC was used to analyze the polymer distribution of the affinity-purified product. Baseline separation of such large proteins is not possible with commercially-available columns. Therefore, the peak area between 8.8 min and 11.45 min and between 11.5 min and 12.55 min was used to estimate pentamer and dimer content, respectively. A gel filtration standard (Bio-Rad) was also separated (dashed line, Fig. 2B) to assign the peaks to the correct polymer. The IgM012_GL dimer eluted between thyroglobulin (670 kDa) and IgG (158 kDa), whereas the IgM012_GL pentamer eluted earlier than thyroglobulin due to its greater hydrodynamic radius (Fig. 2B). The peak

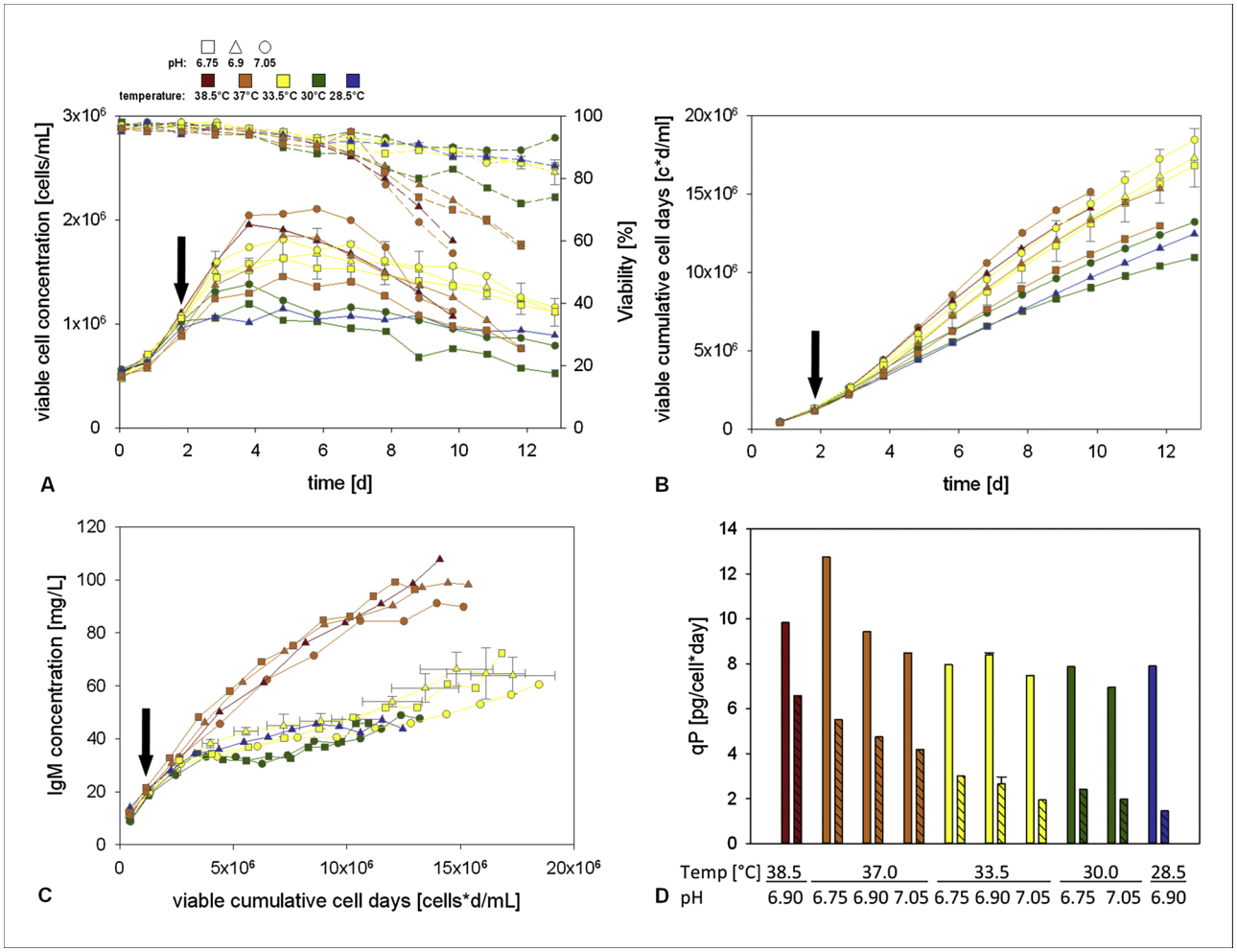

Fig. 1. Biphasic IgM012_GL cultivation. Red 38.5 °C, orange 37.0 °C, yellow 33.5 °C, green 30.0 °C, blue 28.5 °C, rectangle pH 6.75, triangle pH 6.90, circle pH 7.05. Arrow indicates shift, the center point experiment at 33.5 °C pH 6.90 was performed in triplicate. (**A**) Viable cell concentration (continuous line) and viability (dashed line) during the process; (**B**) viable cumulative cell days during the process; (**C**) IgM concentration and viable cumulative cell days; (**D**) specific productivity (d 0-4: full bars, d 4-harvest: dashed bars).

with the shortest retention time is indicated as a high molecular weight aggregate (HMWA) and was later identified as NA. Comparison of the peak areas of the two IgM012_GL peaks enabled calculation of a pentamer content of 75–88 %. The remaining fraction was assigned to dimers (Fig. 2C). Similar to densitometric analysis, SEC-HPLC did not reveal any effect on pentamer content of temperature ($p > 0.05$) or pH ($p > 0.05$). The difference in the mean values of polymer distribution obtained by SDS-PAGE and SEC-HPLC was not sufficient to exclude random variability ($p > 0.05$).

The NA that co-eluted with the IgM012_GL from the affinity column was separated by SE-chromatography. This resulted in an additional peak at a retention time of 6–8 min (continuous line, Fig. 2B). HMWAs of NA were identified by comparison of the UV absorbance at 254 nm and 280 nm. The intensity of the A_{254nm} signal was approximately twice as high as the A_{280nm} signal (Fig. 3A, NAs having a A_{254}/A_{280} of ˜2 [30]) in contrast to the two IgM peaks with an A_{254}/A_{280} ratio of ˜0.5. In addition, the UV-spectra at the peak maxima showed clear NA or protein spectra, respectively (Fig. S2). Multi-angle light scattering (MALS) revealed a molecular weight of 2,000–50,000 kDa for the HMWA (Fig. S3). As NAs have a much higher extinction coefficient, corrected peak areas were used to calculate NA and protein content (Fig. 3B). The amount of NA in the purified IgM012_GL increased with decreasing cultivation temperature (Fig. 3B), indicating a significant impact of temperature on NA content ($p < 0.05$). Nevertheless, the NA content at the lowest cultivation temperature (28.5 °C) was as low as for higher cultivation conditions, which was not in accord with the general trend. It should be noted, however, that the samples for analysis were harvested at a cell viability of ˜90%, so IgM produced at sub-physiological conditions was obtained at a later time point in the process. The pH did not have an impact on NA content in the IgM012_GL preparations ($p > 0.05$).

Another quality attribute of IgMs is the glycosylation profile. IgMs are heavily- and site-specifically glycosylated. To investigate if environmental conditions impact the glycan structure of IgM012_GL total glycan content was analyzed, showing that approximately 30% of the total glycan structures were comprised of oligomannosidic structures (Table 1). These increased slightly at a production temperature of 38.5 °C (36% of total glycans). Hybrid type glycans represented only a minor portion of the overall glycans and no difference was observed under the different process conditions (Table 1), while complex structures were approximately 70% of the whole glycans and some were sialylated. Almost all complex glycans were fucosylated (Table 1). For IgM012_GL, the lowest quantity of complex and fucosylated glycans was produced at 38.5 °C (62% complex glycans and 59% fucosylated). Despite these observations, no statistically-significant trend was observed for the impact of temperature or pH on glycosylation pattern.

4. Discussion

The effect of temperature and pH on cell growth and IgM quantity and quality was investigated in order to characterize their impact on

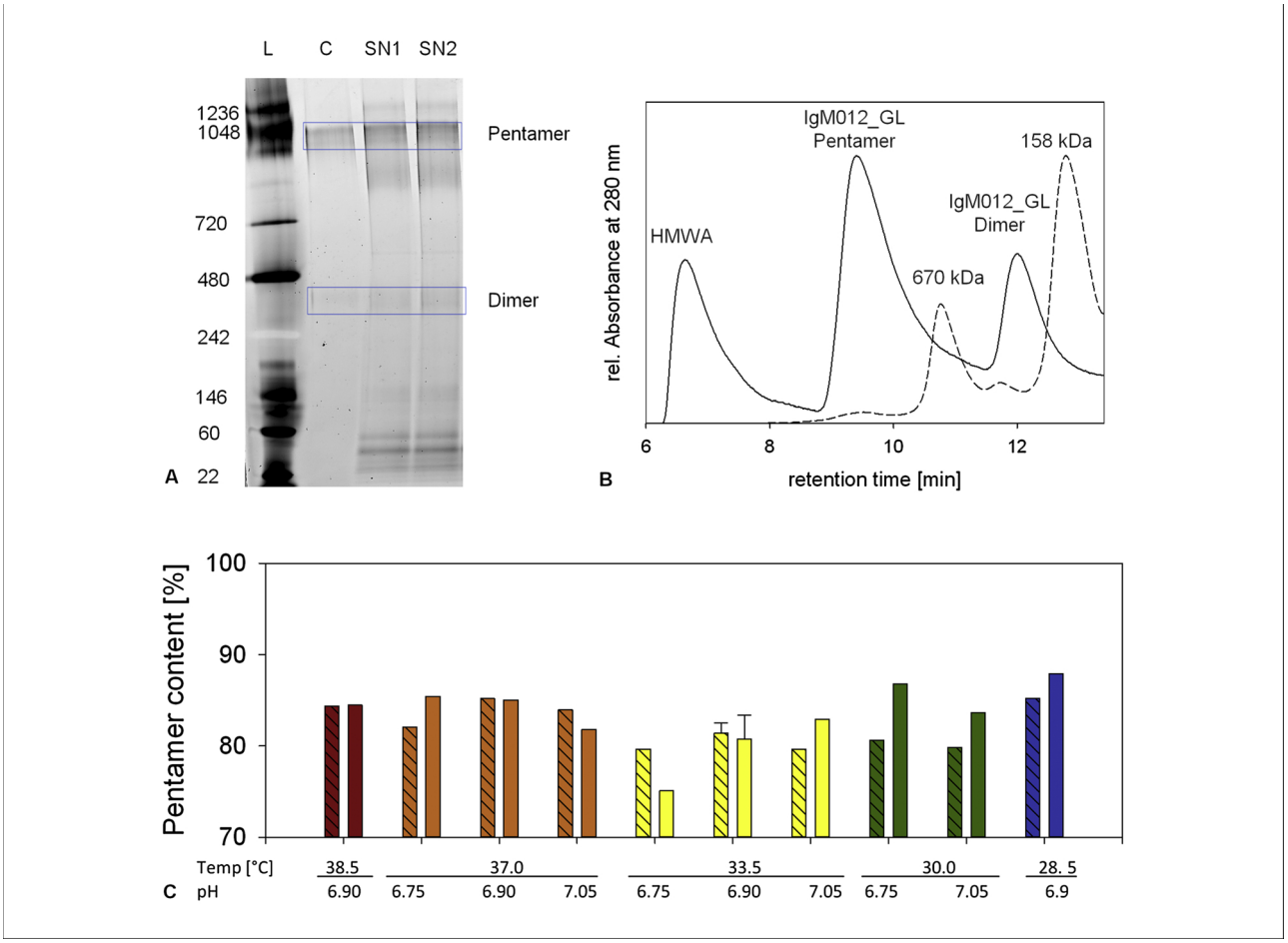

Fig. 2. Polymer distribution / Pentamer content. (**A**) SDS-Gel used for densitometry. The gel was stained with SyproRuby and scanned using the Typhoon FLA biomolecular imager. L-Molecular weight marker, C-control IgM012_GL, SN1 + SN2- duplicate of one representative cell culture supernatant cultivated at 37.0 °C, pH 7.05. (**B**) SEC Chromatogram at 280 nm of purified IgM012_GL cultivated at 37.0 °C, pH 7.05 (continuous line) and BioRad Gel filtration standard (dashed line). **C** Comparison of results for pentamer content obtained by densitometry (striped) and SEC-HPLC (filled). The center point experiment at 33.5 °C pH 6.90 was performed in triplicates.

the bioprocess of IgM production. Growth of recombinant CHO cells was reduced at sub-physiological temperatures (≤ 33.5 °C). This may be due to cell cycle arrest in the G0/G1 phase and reduced metabolism [22,31,32]. A shift to lower temperatures has frequently been used to optimize (specific) productivity [12,13,33]. In contrast to these previous studies, a decrease in volumetric and cell-specific productivity at sub-physiological temperatures was observed (Table 2), which has also been reported for other model proteins such as IgG antibodies [32,34]. Due to cultivation at reduced temperatures, the cells required a longer time period to reach a similar VCCD. Such prolongation of the batch process can lead to an accumulation of host cell proteins and NA in the culture supernatant [35]. The data revealed that a temperature shift is

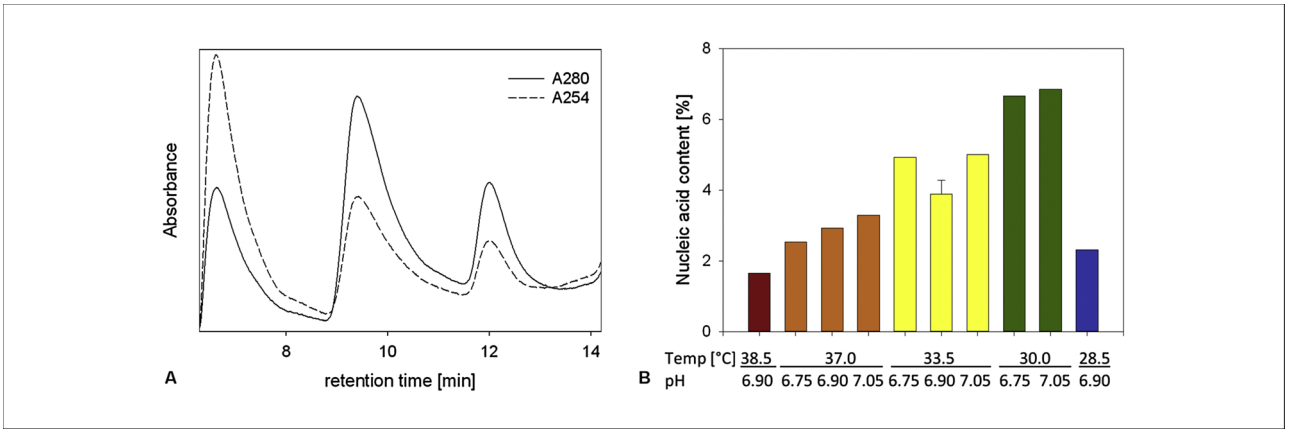

Fig. 3. Nucleic acid content. (**A**) SEC-Chromatogram at 280 nm (solid) and 254 nm (dashed) of purified IgM012_GL cultivated at 37.0 °C, pH 7.05. (**B**) Nucleic acid content was calculated according to Eq. (3). Red 38.5 °C, orange 37.0 °C, yellow 33.5 °C, green 30.0 °C, blue 28.5 °C. The center point experiment at 33.5 °C pH 6.90 was performed in triplicate.

Table 1
Glycan analysis: Relative abundance of grouped glycan structures for IgM012_GL produced at different conditions. Center point (33.5 °C, pH 6.90) was analyzed in triplicates.

Set point		Glycan structures [%]				
Temperature	pH	Oligomannose	Hybrid	Complex	Sialylated	Fucosylated
38.5	6.90	36.4	1.8	61.8	27.2	58.8
37.0	6.75	28.1	1.7	70.3	37.9	65.8
37.0	6.90	30.9	1.6	67.5	32.4	62.0
37.0	7.05	29.1	1.8	69.1	28.4	64.3
33.5	6.75	29.9	1.9	68.2	29.4	62.8
33.5	6.90	30.4 ± 2.2	1.9 ± 0.1	67.7 ± 2.2	25.9 ± 1.3	62.8 ± 2.1
33.5	7.05	27.0	1.8	71.2	28.2	64.2
30.0	6.75	25.3	1.8	72.9	30.6	68.8
30.0	7.05	27.7	1.9	70.4	21.8	65.7
28.5	6.90	31.3	1.4	67.3	36.4	62.0

not always beneficial for recombinant protein production and strongly depends on the cell line and the expressed product. Here, higher culture temperatures (37.0 °C and 38.5 °C) significantly increased IgM012_GL production. In contrast to other studies [14,15], an impact of culture pH on cell growth or antibody production was not observed.

Due to the complex structure of IgMs, folding and assembly can be a bottleneck in recombinant production. A reduction in cultivation temperature is sometimes beneficial in the production of 'difficult-to-express' proteins because the protein folding capacity and expression of chaperones from the endoplasmic reticulum is increased [36,37]. We hypothesized that the quantity of correctly-folded protein in the supernatant and intracellular aggregated IgM012_GL would be affected by cultivation temperature. However, this parameter had no impact on pentamer content (Table 2) or intracellular IgM012_GL content. Hence, these results suggest that incomplete pentamer formation may be driven by factors other than environmental conditions, such as structural instability of IgM012_GL or intracellular lack of the correct assembly machinery in CHO cells. Non-pentameric multimers have also been observed for other recombinant monoclonal IgMs [11,17,18].

Particularly for downstream processing, another critical point to consider is the NA content. Samples harvested at a culture viability of ˜90% were investigated and, indeed, 1.6–5% NAs were shown to co-purify with IgM012_GL by affinity chromatography and quantified after release by analytical SEC-HPLC. The NA content was highest (7% NA) in cultivations at 30 °C, indicating an impact of temperature. In contrast, IgM012_GL produced at 28.5 °C contained only 2.3% NA (Table 2). It was not expected that IgM012_GL produced at 30 °C and 28.5 °C would differ to such a degree with respect to protein byproducts. This might be derived from the fact that at 28.5 °C half of the protein analyzed was already produced at the time point of the temperature shift. Nevertheless, statistical analysis revealed a significant correlation between temperature and NA content. The prolonged culture time at sub-physiological temperatures increased NA contamination and should therefore be omitted. Ideally, a second purification step would be necessary to reduce the NA content to the required purity (≤10 ng of recombinant DNA per dose) [38]. Size exclusion chromatography could be used as an additional purification step, as the NA was separated from the IgM012_GL in the analytical SEC-experiments.

Glycosylation of IgM antibodies is fundamentally important for their biological role [28,29], thus the effect of the process conditions on glycan distribution for IgM012_GL was also assessed. Using a total glycan analysis, it was observed that at all conditions ˜30% of the glycan structures were oligomannosidic. According to known literature [24], these should primarily occupy glycosylation sites 4 and 5. The hybrid structures that are usually found at glycosylation site 1, had only a minor contribution to the overall glycan structures [24]. Approximately 70% of the total glycans were complex and occupied glycosylation sites 1-3. Overall, no significant change in glycan distribution as a consequence of the environmental conditions was observed (Table 2). This is in agreement with others [14,33]. The latter analyzed the influence of temperature on glycosylation of granulocyte macrophage colony stimulating factor; while the former studied the impact of pH on glycosylation of IgG [14,33]. Nevertheless, there are studies that show an impact of temperature or pH on the glycan patterns of IgG antibodies or EPO-Fc [12,13,15].

Product accumulation during processing must be considered for all product-related analyses. Thus, part of the analyzed product was generated during the first phase of the biphasic process at 37 °C and pH 7 and only 41–50% (at viability of 90%) was produced at lower temperatures during the production phase. Thus minor changes caused by the decrease in temperature may have been obscured.

The conclusion to the results in Table 2 is that reduced cultivation temperature significantly decreased the harvest titer and the specific productivity. A significant improvement or change in product quality, however, could not be observed by altering the environmental

Table 2
Experimental set-up and summary of results. Center point (33.5 °C, pH 6.90) was analyzed in triplicates.

Experimental set-up			IgM quantity		IgM quality		
Experiment number	T [°C]	pH	IgM Titer [µg/mL]	$qP_{d4\text{-}harvest}$ [pg/c/d]	Ø Pentamers [%]	Nucleic acid content [%]	complex type glycans [%]
5	38.5	6.90	108	6.6	84	1.6	61.8
10	37.0	6.75	99	5.5	84	2.5	70.3
12	37.0	6.90	99	4.7	85	2.9	67.5
2	37.0	7.05	91	4.2	83	3.3	69.1
8	33.5	6.75	72	3.01	77	4.9	68.2
1,6,11	33.5	6.90	69 ± 8.0	2.7 ± 0.32	81 ± 1.9	3.9 ± 0.41	67.7 ± 2.2
7	33.5	7.05	61	2.0	81	5.0	71.2
4	30.0	6.75	46	2.4	84	6.7	72.9
3	30.0	7.05	52	2.0	82	6.9	70.4
9	28.5	6.90	47	1.5	87	2.3	67.3
P-Value Temperature			****	****	n.s	*	n.s

****p-value ≤0.0001, i.e.highy significant; n.s. p-value > 0.05, i.e. not significant; *p-value ≤ 0.05, i.e. significant.

conditions. With respect to product quality, the IgM012_GL production process in CHO cells appears to be reasonably robust and insensitive to culture temperature or pH.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.nbt.2019.01.001.

References

[1] Binder CJ, Hörkkö S, Dewan A, Chang M-K, Kieu EP, Goodyear CS, et al. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. Nat Med 2003;9:736–43. https://doi.org/10.1038/nm876.

[2] Grönwall C, Akhter E, Oh C, Burlingame RW, Petri M, Silverman GJ. IgM autoantibodies to distinct apoptosis-associated antigens correlate with protection from cardiovascular events and renal disease in patients with SLE. Clin Immunol 2012;142:390–8. https://doi.org/10.1016/j.clim.2012.01.002.

[3] Xu X, Denic A, Warrington A, Bieber AJ, Rodriguez M. Therapeutics to promote CNS repair: a natural human neuron- binding IgM regulates membrane-raft dynamics and improves motility in a mouse model of multiple sclerosis. J Clin Immunol 2013;33:50–6. https://doi.org/10.1007/s10875-012-9795-8.

[4] Rasche L, Duell J, Castro IC, Dubljevic V, Chatterjee M, Knop S, et al. GRP78-directed immunotherapy in relapsed or refractory multiple myeloma - results from a phase 1 trial with the monoclonal immunoglobulin M antibody PAT-SM6. Haematologica 2015;100:377–84. https://doi.org/10.3324/haematol.2014.117945.

[5] Hensel F, Timmermann W, Von Rahden BHA, Rosenwald A, Brändlein S, Illert B. Ten-year follow-up of a prospective trial for the targeted therapy of gastric cancer with the human monoclonal antibody PAT-SC1. Oncol Rep 2014;31:1059–66. https://doi.org/10.3892/or.2014.2987.

[6] Sitia R, Neuberger M, Alberini C, Bet P, Fra A, Valetti C, et al. Developmental regulation of IgM secretion: the role of the carboxy-terminal cysteine. Cell 1990;60:781–90. https://doi.org/10.1016/0092-8674(90)90092-S.

[7] Loos A, Gruber C, Altmann F, Mehofer U, Hensel F, Grandits M, et al. Expression and glycoengineering of functionally active heteromultimeric IgM in plants. Proc Natl Acad Sci 2014;111:6263–8. https://doi.org/10.1073/pnas.1408639111.

[8] Sugahara T, Yano S, Sasaki T. High IgM production by human-human hybridoma HB4C5 cells cultured in microtubes. Biosci Biotechnol Biochem 2003;67:393–5. https://doi.org/10.1271/bbb.67.393.

[9] Tchoudakova A, Hensel F, Murillo A, Eng B, Foley M, Smith L, et al. High level expression of functional human IgMs in human PER.C6® cells. MAbs 2009;1:163–71. https://doi.org/10.4161/mabs.1.2.7945.

[10] Gilmour JEM, Pittman S, Nesbitt R, Scott ML. Effect of the presence or absence of J chain on expression of recombinant anti-Kell immunoglobulin M. Transfus Med 2008;18:167–74. https://doi.org/10.1111/j.1365-3148.2008.00853.x.

[11] Wolbank S, Kunert R, Stiegler G, Katinger H. Characterization of Human Class-Switched Polymeric (Immunoglobulin M [IgM] and IgA) Anti-Human Immunodeficiency Virus Type 1 Antibodies 2F5 and 2G12. J Virol 2003;77:4095–103. https://doi.org/10.1128/JVI.77.7.4095.

[12] Trummer E, Fauland K, Seidinger S, Schriebl K, Lattenmayer C, Kunert R, et al. Process parameter shifting: part I. Effect of DOT, pH, and temperature on the performance of epo-fc expressing CHO cells cultivated in controlled batch bioreactors. Biotechnol Bioeng 2006;94:1033–44. https://doi.org/10.1002/bit.21013.

[13] Sou SN, Sellick C, Lee K, Mason A, Kyriakopoulos S, Polizzi KM, et al. How does mild hypothermia affect monoclonal antibody glycosylation? Biotechnol Bioeng 2015;112:1165–76. https://doi.org/10.1002/bit.25524.

[14] Brunner M, Fricke J, Kroll P, Herwig C. Investigation of the interactions of critical scale-up parameters (pH, pO2and pCO2) on CHO batch performance and critical quality attributes. Bioprocess Biosyst Eng 2017;40:251–63. https://doi.org/10.1007/s00449-016-1693-7.

[15] Aghamohseni H, Ohadi K, Spearman M, Krahn N, Moo-Young M, Scharer JM, et al. Effects of nutrient levels and average culture pH on the glycosylation pattern of camelid-humanized monoclonal antibody. J Biotechnol 2014;186:98–109. https://doi.org/10.1016/j.jbiotec.2014.05.024.

[16] Arnold JN, Wormald MR, Suter DM, Radcliffe CM, Harvey DJ, Dwek RA, et al. Human serum IgM glycosylation: identification of glycoforms that can bind to Mannan-binding lectin. J Biol Chem 2005;280:29080–7. https://doi.org/10.1074/jbc.M504528200.

[17] Vorauer-Uhl K, Wallner J, Lhota G, Katinger H, Kunert R. IgM characterization directly performed in crude culture supernatants by a new simple electrophoretic method. J Immunol Methods 2010;359:21–7. https://doi.org/10.1016/j.jim.2010.05.003.

[18] Azuma Y, Ishikawa Y, Kawai S, Tsunenari T, Tsunoda H, Igawa T, et al. Recombinant human hexamer-dominant IgM monoclonal antibody to ganglioside GM3 for treatment of melanoma. Clin Cancer Res 2007;13:2745–50. https://doi.org/10.1158/1078-0432.CCR-06-2919.

[19] Brewer JW, Randall TD, Parkhouse RME, Corley RB. Mechanism and subcellular localization of secretory IgM polymer assembly. J Biol Chem 1994;269:17338–48.

[20] Brewer JW, Corley RB. Quality control in protein biogenesis: thiol-mediated retention monitors the redox state of proteins in the endoplasmic reticulum. J Cell Sci 1996;109:2383–92.

[21] Gan HT, Lee J, Latiff SMA, Chuah C, Toh P, Lee WY, et al. Characterization and removal of aggregates formed by nonspecific interaction of IgM monoclonal antibodies with chromatin catabolites during cell culture production. J Chromatogr A 2013;1291:33–40. https://doi.org/10.1016/j.chroma.2013.03.028.

[22] Kaisermayer C, Reinhart D, Gili A, Chang M, Aberg PM, Castan A, et al. Biphasic cultivation strategy to avoid Epo-Fc aggregation and optimize protein expression. J Biotechnol 2016;227:3–9. https://doi.org/10.1016/j.jbiotec.2016.03.054.

[23] Chromikova V, Mader A, Steinfellner W, Kunert R. Evaluating the bottlenecks of recombinant IgM production in mammalian cells. Cytotechnology 2014;67:343–56. https://doi.org/10.1007/s10616-014-9693-4.

[24] Hennicke J, Lastin AM, Reinhart D, Grünwald-Gruber C, Altmann F, Kunert R. Glycan profile of CHO derived IgM purified by highly efficient single step affinity chromatography. Anal Biochem 2017;539:162–6. https://doi.org/10.1016/j.ab.2017.10.020.

[25] Berggren K, Chernokalskaya E, Steinberg TH, Kemper C, Lopez MF, Diwu Z, et al. Background-free, high sensitivity staining of proteins in one- and two-dimensional sodium dodecyl sulfate-polyacrylamide gels using a luminescent ruthenium complex. Electrophoresis 2000;21:2509–21. https://doi.org/10.1002/1522-2683(20000701)21:12<2509::AID-ELPS2509>3.0.CO;2-9.

[26] Pabst M, Kolarich D, Pöltl G, Dalik T, Lubec G, Hofinger A, et al. Comparison of fluorescent labels for oligosaccharides and introduction of a new postlabeling purification method. Anal Biochem 2009;384:263–73. https://doi.org/10.1016/j.ab.2008.09.041.

[27] Gasteiger E, Hoogland A, Gattiker MR, Wilkins RD, Bairoch A. Protein identification and analysis tools on the ExPASY server. Proteomics Protoc Handb 2005:571–607.

[28] Muraoka S, Shulman MJ. Structural requirements for IgM assembly and cytolytic activity. Effects of mutations in the oligosaccharide acceptor site at Asn402. J Immunol 1989;142:695–701.

[29] Colucci M, Stöckmann H, Butera A, Masotti A, Baldassarre A, Giorda E, et al. Sialylation of N-linked glycans influences the immunomodulatory effects of IgM on T cells. J Immunol 2015;194:151–7. https://doi.org/10.4049/jimmunol.1402025.

[30] Glasel JA. Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios. Biotechniques 1995.

[31] Moore A, Mercer J, Dutina G, Donahue CJ, Bauer KD, Mather JP, et al. Effects of temperature shift on cell cycle, apoptosis and nucleotide pools in CHO cell batch cultues. Cytotechnology 1997;23:47–54. https://doi.org/10.1023/A:1007919921991.

[32] Goey CH, Tsang JMH, Bell D, Kontoravdi C. Cascading effect in bioprocessing—the impact of mild hypothermia on CHO cell behavior and host cell protein composition. Biotechnol Bioeng 2017;114:2771–81. https://doi.org/10.1002/bit.26437.

[33] Bollati-Fogolín M, Forno G, Nimtz M, Conradt HS, Etcheverrigaray M, Kratje R. Temperature reduction in cultures of hGM-CSF-expressing CHO cells: effect on productivity and product quality. Biotechnol Prog 2005;21:17–21. https://doi.org/10.1021/bp049825t.

[34] Yoon SK, Kim SH, Lee GM. Effect of low culture temperature on specific antibody productivity and transcription level of Anti-4-1BB Antibody in recombinant Chinese hamster ovary cells. Biotechnol Prog 2003;19:1383–6. https://doi.org/10.1021/bp034051m.

[35] Tait AS, Tarrant RDR, Velez-Suberbie ML, Spencer DIR, Bracewell DG. Differential response in downstream processing of CHO cells grown under mild hypothermic conditions. Biotechnol Prog 2013;29:688–96. https://doi.org/10.1002/btpr.1726.

[36] Baik JY, Lee MS, An SR, Yoon SK, Joo EJ, Kim YH, et al. Initial transcriptome and proteome analyses of low culture temperature-induced expression in CHO cells producing erythropoietin. Biotechnol Bioeng 2006;93:361–71. https://doi.org/10.1002/bit.20717.

[37] Mason M, Sweeney B, Cain K, Stephens P, Sharfstein S. Reduced culture temperature differentially affects expression and biophysical properties of monoclonal antibody variants. Antibodies 2014;3:253–71. https://doi.org/10.3390/antib3030253.

[38] WHO. Guidelines on the quality, safety, and efficacy of biotherapeutic protein products prepared by recombinant DNA technology. WHO Tech Rep Ser 2013.

Supplementary data to

Impact of temperature and pH on recombinant human IgM quality attributes and productivity

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Table S1: Comparison of cultivation of CHO IgM012_GL in ProCHO5 (Lonza) and the MV3-2 medium (Polymun Scientific, Austria) in a batch culture in MiniBioreactor tubes (Corning). Cells were seeded at $0.3*10^6$ cells/mL and cultivated until the viability of the cells decreased below 60%.

	ProCHO5	**MV3-2**
Peak viable cell concentration [10^6 cells/mL]	5.40	4.40
Max growth rate [d^{-1}]	0.55	0.54
Max IgM concentration [mg/L]	85.00	80.00
Specific productivity [pg/(cell d)]	2.70	2.40

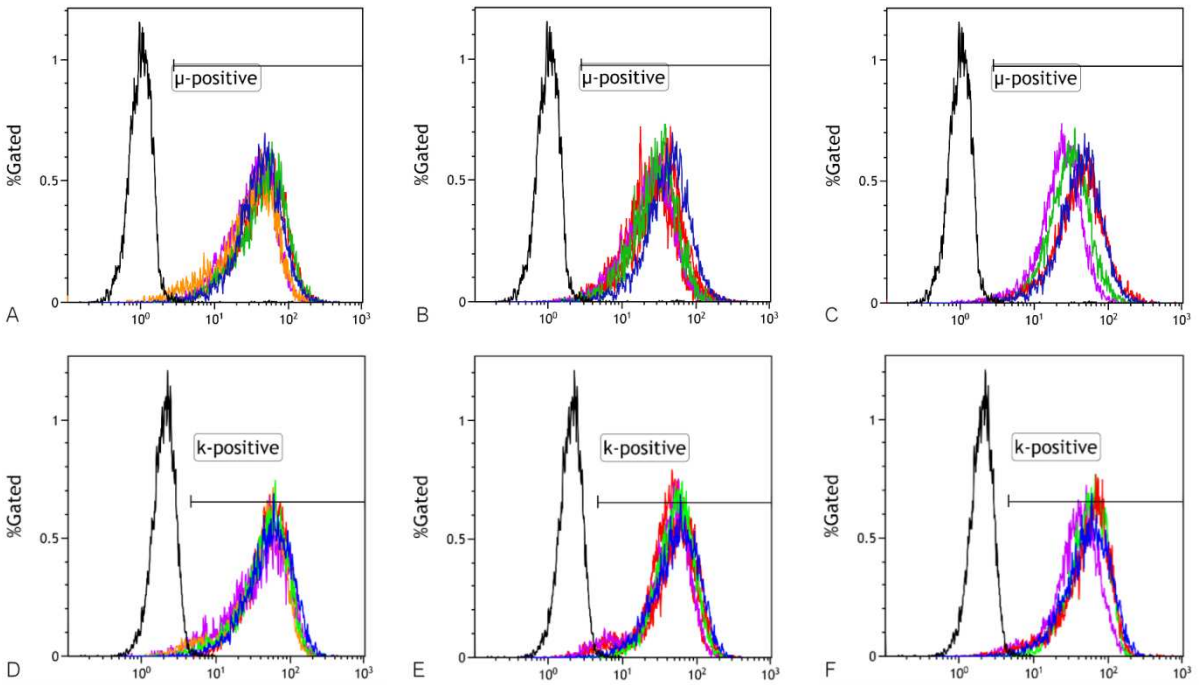

Figure S1: Intracellular light chain and heavy chain content. Cells were fixed in ethanol and stained with anti-μ-chain-FITC conjugate (**A-C**) or anti-κ-chain-FITC conjugate (**D-F**). As negative control (black) a non-producing CHO cell line was used. For comparison of homogeneity, cells at day 2 (day of shift) were shown in blue. Green: pH 6.75, red: pH 6.90, magenta pH 7.05, **A** and **D** 37.0 °C (38.5 °C are shown in yellow), **B** and **E** 33.5 °C, **C** and **F** 30.0 °C or 28.5 °C.

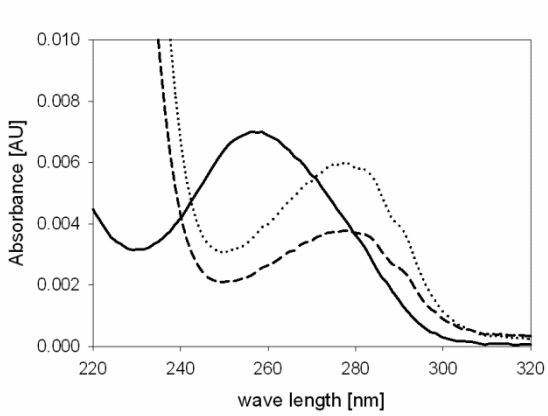

Figure S2: Peak spectra for each peak after SEC-HPLC for identification of nucleic acid and protein fractions. IgM012_GL produced at 37 °C, pH 7.05 is displayed as an example. Solid line - peak spectrum at retention time 6.63 min identified as HMWA NA, dotted line – peak spectrum at retention time 9.39 min identified as pentamer, dashed line – peak spectrum at retention time 11.98 min identified as dimer.

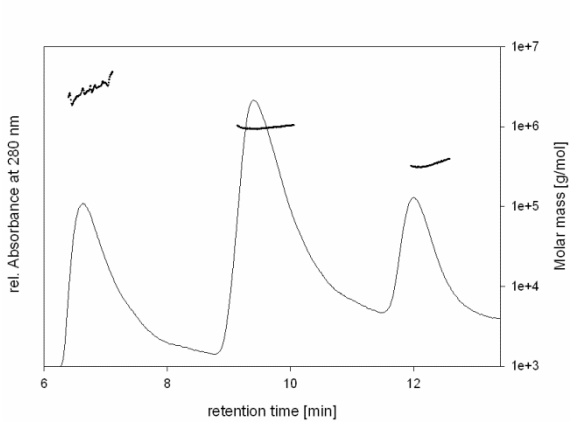

Figure S3: Molar mass determination using SEC-HPLC coupled to MALS detector. IgM012_GL produced at 37 °C, pH 7.05 is displayed as an example. The continuous line represents the absorbance at 280 nm. The molar mass was determined for each absorbance peak with MALS using the ASTRA software (upper lines). The top left line represents the molecular weight calculation of the nucleic acid high molecular weight aggregates, which is more variable as for homogenous protein solutions due to their heterogeneous shape, size and scatter behavior.

Influence of the expression system on IgM quality

Mammalian expression systems enable the synthesis of proteins with similar molecular structure and biochemical properties compared to their naturally occurring counterparts [77]. Mammalian expression hosts have the basic machinery to express and secrete the product and to attach glycan structures with high similarity to the human glycan pattern, which enables them for production of glycosylated multimeric proteins, such as IgM. A good host cell line harbors different properties: 1) A non-human glycosylation would be highly immunogenic or rapidly cleared from the circulation in the human body in case the protein of interest has a therapeutic application [78,79]. Therefore, the host-specific attached glycan pattern is of particular interest. 2) The cells need to be amenable to genetic modification, which allows easy introduction of foreign DNA for recombinant protein production. 3) The host cell line should easily adapt to growth in suspension and in serum-free media, which is necessary for the volumetric scalability in large scale bioprocessing. 4) The expression machinery should not be strongly regulated in order to allow heterologous gene expression.

CHO cells are well-studied host cell lines and were isolated from ovary tissue of a female Chinese hamster (*Cricetulus griseus*) [80]. They produce glycoforms that are compatible and bioactive in humans, which makes CHO produced products less immunogenic compared to products from other non-human expression hosts. CHO cells are resistant to many human-pathogenic viruses as important genes for viral entry and recognition are absent in the genome or lacking expression [81]. In the last decades, safety issues were addressed to demonstrate that CHO cells are safe hosts, which increased the chance of acceptance by the regulatory agencies and led to an intensive use of CHO cells for production of biopharmaceuticals in industry [82]. An advantage of CHO is the easy introduction of genetic modifications, which enabled the isolation of glutamine synthetase (GS) or dihydrofolate reductase (DHFR) deficient cell lines. The DHFR enzyme catalyzes the production of tetrahydrofolate, which is a cofactor required in various biosynthetic reactions (e.g. synthesis of amino acids and nucleobases). DHFR deficiency can be resolved by transfection of the *DHFR* gene genetically linked to the gene of interest. The addition of DHFR inhibitors like methotrexate (MTX) to the cultivation medium allows the selection of cell clones that comprise multiple copies of the construct or integrated the genetic cassette into highly transcriptionally active loci. The DHFR system dramatically increased the specific

productivities of the cells and is nowadays a widely used strategy in industry to generate stable recombinant cell lines [83].

However, generation, isolation and characterization of stable cell lines are long and tedious processes. A rapid, efficient and high-throughput alternative is transient gene expression [84]. The most widely used cell line for transient expression is the HEK293 cell line, which is derived from human embryonic kidney cells and thereby performs human-like post-translational modifications [85]. The genetic variant HEK293E cell line constitutively expresses the Epstein-Barr virus nuclear antigen (EBNA), which allows episomal amplification of plasmids containing the viral EBV origin of replication and thereby enhancing the productivity. The HEK293E cell line grows easily in suspension culture in serum-free media and offers high transfection yields, which is a prerequisite for high production efficiency.

In the herein presented study, a DHFR deficient CHO DG44 cell line and the HEK293E cell line were compared regarding their suitability as IgM producing host cells. Our three model antibodies were produced in CHO DG44 cells representing a stably producing cell line and in the HEK293E cells representing the transient system. Both systems have their individual application in IgM production. Large-scale production of therapeutic IgM could be performed in stable CHO cell lines. The transient expression in HEK is more suitable in the first stage of therapeutic development for screening of different therapeutic candidates or in research for characterization of IgM mutants.

The stable IgM producing CHO DG44 cell lines were generated as described in Chromikova *et al.* by using random gene integration and the pIRES vector for co-transfection of two genes of interest per plasmid [64]. The host cell line was transfected with two pIRES constructs containing either IgM heavy chain and *DHFR* genes or light chain and J-chain genes (Fig. 3A). The IgM heavy chain and *DHFR* genes were connected by an internal ribosome entry site (IRES) sequence and expressed under the SV40 promoter. The light chain is expressed under the CMV promoter and followed by the IRES and J-chain sequence.

The transient expression was performed with HEK293E cells and two pCEP4 constructs containing either IgM heavy chain gene or light chain and J-chain genes (Fig. 3B). The pCEP4 vector encodes the *EBNA1* gene for episomal plasmid amplification and the CMV promoter upstream of the gen of interest (Fig. 3B). The DNA sequences for the IgM chains were

identical to the constructs used for stable transfection. The stable expression of the adenovirus 13 SE1a in the HEK293E cells enhances transcription of CMV promoter [86].

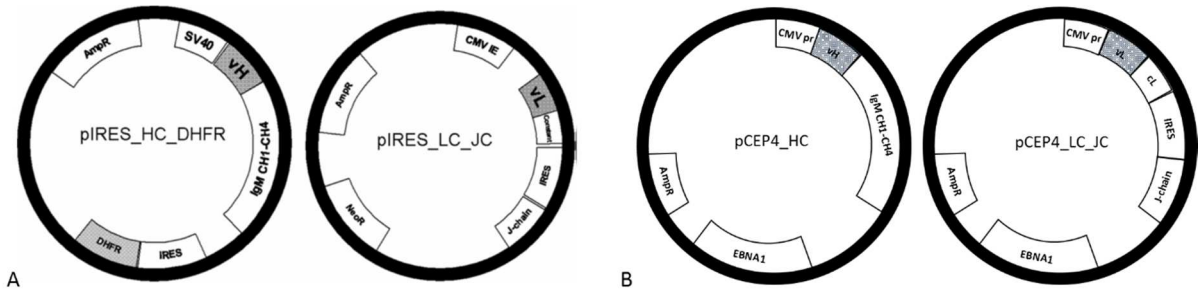

Figure 3: Schematic plasmid maps of the genetic constructs. A pIRES plasmids for stable transfection into the CHO DG44 host cell line. Image was reprinted from Chromikova *et al.* [64]. **B** pCEP4 plasmids for transient transfection into the HEK293E host cell line. **Abbreviations:** AmpR- ampicillin-resistance gene, SV40 – SV40 promoter, CMV – CMV promoter, vH/vL – variable domain of heavy chain/ light chain, IgM CH1-CH4- IgM constant domains, cL – kappa light chain constant domain, IRES - internal ribosome entry site, DHFR - dihydrofolate reductase, EBNA1 - Epstein-Barr virus nuclear antigen 1.

The stably producing CHO cell lines and the HEK293E host cell line were seeded at a similar cell density (10^6 cells/mL) to compare cultivation and productivity of both systems. The transiently expressing HEK293E cell lines were fed with valproic acid (VPA) and tryptone N1 (TN1) 48 h post transfection following the established protocol of our working group. The addition of VPA and TN1 is known to increase the protein synthesis in transient expression up to the yields of stable expression systems [87,88]. Stable IgM producing CHO DG44 cell lines were cultivated in batch cultures. A high variability in viable cell concentration was observed in different independent transient transfections. Therefore, only the results of the best transient transfection for each IgM antibody are shown in Table 1. The IgM production with CHO DG44 cells was much more reproducible, which is displayed in the low standard deviation of the triplicates. When the transfected HEK293E cells grew to similar maximum viable cell concentrations as the CHO DG44 cell lines, comparable amounts of the respective IgM antibody were produced (Tab. 1). The three model IgMs were produced with different yields as described previously in Chromikova *et al.* [64]. In detail, the IgM617 was produced much better than IgM012_GL and IgM012. Finally, all IgMs were successfully produced in both cell lines. The low reproducibility in the transient system can be explained by a high variation in transfection efficiency due to aging of the HEK293E host cell line [89]. If these variations in transient transfection can be eliminated, it could become a quite useful tool in

fast IgM production. However, the transient expression system was slightly lacking behind in this experiment with regard to product concentration.

Table 1: Comparison of cell culture parameters for stable and transient expression of IgM

	max. viable cell concentration		max. IgM concentration	
	CHO DG44	HEK293E	CHO DG44	HEK293E
IgM012	6.6±0.3·10^6c/mL	8.0·10^6c/mL	32±4 µg/mL	26 µg/mL
IgM012_GL	8.4±0.1·10^6c/mL	8.1·10^6c/mL	36±3 µg/mL	30 µg/mL
IgM617	13.0±0.4·10^6c/mL	3.2·10^6c/mL	295±13 µg/mL	133 µg/mL

Stable CHO DG44 cell lines and HEK293E host cell lines were seeded at 10^6cells/mL. Three cultures of the IgM producing CHO DG44 cells were cultivated in a batch for each model IgM. HEK293E were transfected with the constructs described in Fig. 3 and polyethlyenimine. The HEK293E cells were fed with 5 mM valproic acid and 0.5 % tryptone N1 48 hours post transfection. Due to the high variability of the transient transfection, only the best result is shown.

IgM polymer distribution and glycosylation were analyzed as quality criteria. The polymer distribution of each model IgM was different (Fig. 4). All model IgMs contained predominantly pentamers. However, IgM012 and IgM012_GL were additionally produced as dimers independently of the expression system. In general, neither the host cell line nor the expression system affected the polymer distribution. Nevertheless, a lower molecular weight could be observed for all protein bands, particularly for the dimeric portions of IgM012 and IgM012_GL, if they were produced in HEK293E compared to CHO DG44. This was already a hint for microheterogeneity in the glycosylation pattern attached by the two host cell lines.

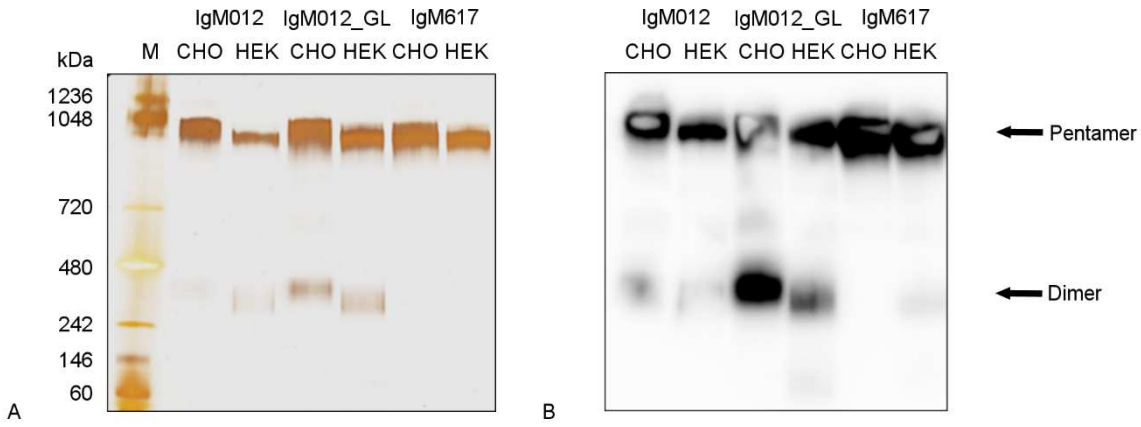

Figure 4: Polymer distribution of IgM produced by stably transfected CHO DG44 or transiently transfected HEK293E. SDS-PAGE, Western blot analysis and silver staining of the purified IgMs were conducted as described in [64,90]. **A** silver stained SDS-gel **B** anti-µ-chain western blot. Arrows indicate the polymer fractions.

Glycosylation is an important quality attribute of IgMs as it can affect its secretion, cytolytic activity, immunogenicity and pharmacokinetics [13,78,79,91,92]. IgMs have five potential N-glycosylation sites (GS) which can be divided into the mainly complex glycosylated GS1-3 and the GS4 and GS5 which are only occupied by the oligomannose type. The GS1 can be occupied by oligomannosidic or hybrid glycans, particularly in recombinant IgM [67].

This general classification was found for recombinant IgM produced by stably transfected CHO DG44 cells and transiently transfected HEK293E cells. However, a closer view revealed differences between the two host cell lines. IgM produced in the CHO DG44 cell line comprised mainly complex type glycans, 10-16 % hybrid type glycans and 20-35 % oligomannose type glycans at GS1. In contrast, the glycan structures found on IgMs produced in HEK293E cells showed a minimum of 50 % oligomannose type glycans and a maximum of approximately 30 % complex type glycans at GS1 (Tab. 2). Hence, the portion of less processed glycans attached to GS1 is higher in IgMs produced in HEK293E. This trend is even more pronounced when the IgM012 was produced in HEK, which is nearly completely occupied by oligomannose type glycans. At GS2, the CHO DG44 cells attached almost only complex type glycans, which are mainly sialylated. In contrast, the HEK293E cells produced only 40% sialylated complex glycans, but an increased fraction of oligomannosidic glycans which is the highest for IgM012 (Tab. 2). The GS3 of IgM produced by both expression hosts is fully occupied by complex type glycans, except for IgM012. Nevertheless, the HEK293E cells produced a higher portion of truncated glycan structures

with terminal N-acetylglucosamins, which represent the less processed complex type. Oligomannose type is the predominant glycan structure found on GS4 and GS5 of IgM produced by both expression hosts (Tab. 3). However, the GS5 remained unglycosylated to a great extent and the oligomannose signals were too low for quantitative comparison. The HEK293E cells tend to attach more mannose molecules to GS4 compared to CHO DG44 cells. In a previous study, the glycosylation of 12 transiently produced proteins was compared with respect to the impact of the host cell line [93]. Although posttranslational modifications of recombinant proteins produced in mammalian cells are frequently regarded as identical, the author observed significant differences in the produced glycan pattern [93]. Similar to the herein presented results, the HEK293E cell line secreted less processed glycoproteins, which could result from different expression levels of the enzymes participating in glycosylation by the two host cell lines.

Overall, the transient transfection is a good alternative for fast and efficient protein production. The HEK293E cells are able to produce complex multimeric proteins such as IgMs. Although the expression efficiency varied to a great extent, the amounts and polymer distribution of the IgM produced in HEK were similar to CHO produced IgM. However, the glycan profile was less processed, which could change the biological properties or pharmacokinetics. Therefore the transient expression should only be used for applications in which the glycan profile is not of special interest or minor changes in the glycan profile have no impact.

Table 2: Comparison of site specific glycosylation profile at GS1-3 of IgM produced by stable (CHO DG44) and transient expression (HEK293E)

	Glycan type	complex type			hybrid type	oligomannose
	model IgM	MGn/ GnGnF	galactosylated	sialylated		type
Glycosylation site 1						
CHO	IgM012	1	9	43	13	35
	IgM012_GL	1	9	59	10	20
	IgM617	0	14	43	16	27
HEK	IgM012	0	1	5	1	93
	IgM012_GL	3	5	24	17	50
	IgM617	3	7	24	21	45
Glycosylation site 2						
CHO	IgM012	47	10	32	0	1
	IgM012_GL	6	17	75	0	2
	IgM617	2	13	84	0	1
HEK	IgM012	15	2	5	0	77
	IgM012_GL	19	7	36	0	36
	IgM617	27	17	42	0	14
Glycosylation site 3						
CHO	IgM012	15	31	51	0	3
	IgM012_GL	10	21	68	0	0
	IgM617	1	18	81	0	0
HEK	IgM012	4	4	2	0	90
	IgM012_GL	39	34	27	0	0
	IgM617	37	34	29	0	0

Glycan analysis was performed as described in [67]. Mass spectra of the glycan structures can be found in chapter 6.1, Fig. S1 and Fig. S2.

Table 3: Comparison of oligomannose structures at GS4 of IgM produced by stable (CHO DG44) and transient expression (HEK293E)

		Man4/Man5	Man6/Man7	≥Man8
Glycosylation site 4				
CHO	IgM012	33	43	24
	IgM012_GL	24	47	29
	IgM617	21	49	30
HEK	IgM012	7	10	83
	IgM012_GL	26	32	41
	IgM617	20	39	41

Glycan analysis was performed as described in [67]. Mass spectra of the glycan structures can be found in chapter 6.1, Fig. S2.

3.2. Purification of a pH sensitive glycoprotein

The formerly published IgM purification strategies combined precipitation, hydroxyapatite, ion exchange and/or size exclusion chromatography with yields of 40-80 % and only limited purity [94–96]. An efficient and gentle purification strategy was necessary to reduce the volume of IgM containing cell culture supernatant and to characterize the unaltered IgM molecule. Affinity chromatography was a very promising choice as it relies on a very specific interaction of the affinity ligand with the protein of interest. The POROS CaptureSelect™ IgM Affinity Matrix (Thermo Fisher Scientific) was used in this study under application of a low pH elution strategy according to the manufacturer's instruction. Nevertheless, the herein presented publication shows that this method results only in low elution yields and aggregation was observed because of the sensitivity of the IgM at the elution conditions [67]. Finally, a fivefold improvement of the elution yield was achieved by screening of different elution buffers. The optimized elution buffer combined a moderate pH reduction and an increase in ionic strength for IgM elution. The best elution conditions were verified with five different IgMs. After purification, the site specific glycan pattern of the IgM012 could be analyzed leading to the first publication about the glycosylation pattern of recombinant IgM produced in CHO cells.

Glycan profile of CHO derived IgM purified by highly efficient single step affinity chromatography

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Glycan profile of CHO derived IgM purified by highly efficient single step affinity chromatography

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ABSTRACT

Immunoglobulin M (IgM) antibodies are reckoned as promising tools for therapy and diagnostic approaches. Nevertheless, the commercial success of IgMs is hampered due to bottlenecks in recombinant production and downstream processing. IgMs are large, complex and highly glycosylated proteins that are only stable in a limited range of conditions. To investigate these sensitive IgM antibodies we optimized the elution conditions for a commercially available IgM affinity matrix (CaptureSelect™). Applying a small-scale screening system, we optimized our single step purification strategy for high purity, high yield and retained antigen binding capacity. Here we show that IgMs are sensitive to aggregation at very acidic conditions (pH ≤ 3.0) despite often being used for affinity chromatography. We combined pH 3.5 with a high salt concentration to prevent aggregation during elution. The elution strategy presented in this paper will improve IgM processes for further applications. The herein used IgMs were produced in Chinese hamster ovary (CHO) cells. We present the first detailed glycan analysis of IgM produced in CHO cells with predominantly complex type structures at Asn171, Asn332 and Asn395 and oligomannosidic structures at Asn402 and Asn563 similar to human serum-IgM.

Introduction

Immunoglobulin M (IgM) is one of the most complex molecules in the human body, produced by a small subset of B lymphocytes [1]. Considering therapeutic application, monoclonal IgMs PAT-SM6 and PAT-SC1 are promising anti-cancer therapeutics already investigated in clinical trials [2,3]. Another potent IgM was developed against oxidation-specific epitopes to reduce atherosclerotic plaque formation or cardiovascular diseases [4,5]. Furthermore, neuron-binding IgMs improved the motility in a multiple sclerosis mouse model [6]. Until now, three monoclonal IgMs gained the orphan drug designation by the FDA or EMA for rare diseases [7,8], which reflects their quite challenging (and therefore uneconomical) production. The large size and complex physico-chemical properties render the purification of IgMs difficult. In early stage of IgM research, IgMs were isolated from serum using size exclusion chromatography or precipitation [9–11]. Unfortunately, neither yield nor purity was satisfactory. Immunoaffinity chromatography did not seem suitable because of the harsh elution conditions that are harmful to the labile IgM molecule [12]. Hydrophobic interaction chromatography can only be used with mild ligands as IgMs are prone to denaturation on contact to hydrophobic surfaces [13]. In the last 20 years a few multistep purifications were published, mainly using ion exchange chromatography coupled to a hydroxyapatite chromatography, size exclusion chromatography or precipitation [13–15]. Such complex downstream strategies are less efficient and time consuming and therefore different affinity ligands like protein L were developed [16].

Here we present an optimization study for the commercially available CaptureSelect™ affinity column matrix, which can be used for human IgMs. Additionally we present the glycosylation pattern of a purified monoclonal IgM antibody that was produced in Chinese hamster ovary (CHO) cells in chemically defined medium. Approximately 10% of the molecular weight of IgM is attributed to N-glycans contributing to the biological function, specifically its secretion [17] and complement activation [18]. The human serum derived μ-chain contains five conserved N-glycosylation sites, three carry complex type sialylated structures (Asn171, Asn332 and Asn395), while Asn402 and Asn563 are oligomannose type [19]. Until today, no publication on the glycosylation pattern of a recombinant monoclonal IgM antibody produced by CHO cells has appeared, even though CHO cells are the

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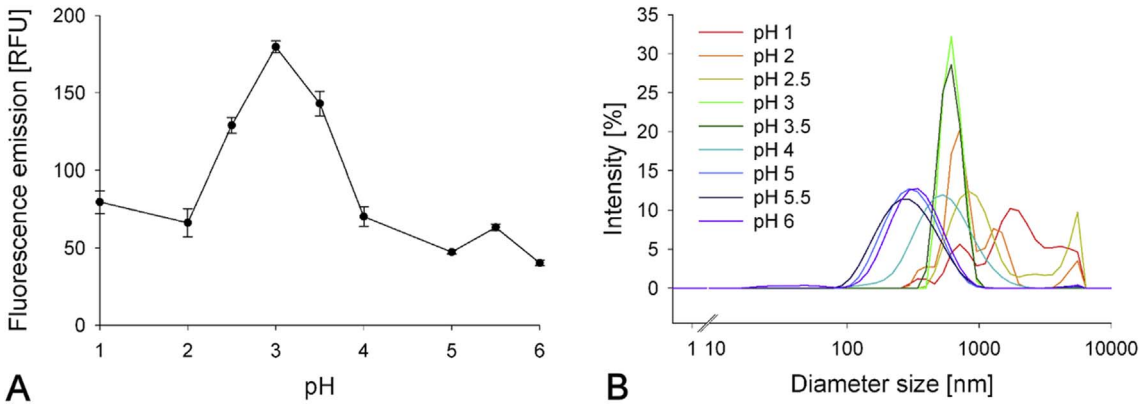

Fig. 1. Stability analyses of IgM012_GL. IgM012_GL was incubated at indicated pH values for 45 min. **A** Sypro®Orange was added to the samples, excited at 495 nm and FE was measured at 595 nm. Error bars show the standard deviation of three measurements. **B** Presence of high molecular weight aggregates was analyzed using DLS. Curves of five technical replicates were averaged.

main expression system used for therapeutic protein production [20].

Materials and methods

Antibodies and cell culture

IgM012 and IgM012_GL were expressed by mammalian cell culture in CHO DG44 cells as described in Chromikova et al. (2015) [21]. Purified IgM103, IgM104 and IgM617 were obtained from Polymun Scientific Immunbiologische Forschung GmbH and diluted in conditioned cell culture supernatant to verify the purification strategy.

pH stability of IgM

IgM012_GL (396 µg/mL) in 100 mM phosphate, 200 mM NaCl buffer pH 5.5 was titrated to pH 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0 with 32% HCl or pH 6.0 with 2 M NaOH and readjusted to pH 5.5 after 45 min incubation. Unfolding of IgM012_GL was measured using fluorescence emission (FE) after incubation with Sypro®Orange Protein Gel Stain (Thermo Fisher Scientific). Sypro®Orange was excited at 495 nm and FE was detected from 550 nm to 650 nm with an Infinite M1000 microplate reader (Tecan). The presence of high molecular weight species was monitored with dynamic light scattering (DLS) using Zetasizer Nano ZS (Malvern instruments).

Buffer screening for affinity matrix elution optimization

The POROS CaptureSelect™ IgM Affinity Matrix (Thermo Fisher Scientific, V = 20 µL) was equilibrated with PBS in a centrifuge tube. Afterwards it was incubated with 200 µL concentrated cell supernatant (cIgM = 174 µg/mL). Unbound protein was washed with PBS and elution was performed with different screening buffers. Elution efficacy was determined with a standard µ-µ sandwich ELISA.

Lab-scale purification with CaptureSelect IgM affinity matrix

Following the manufactures instruction POROS CaptureSelect™ IgM Affinity Matrix (Thermo Fisher Scientific) was equilibrated with PBS and loaded with 3 mg IgM in concentrated cell culture supernatant at a flow rate of 1 mL/min on an ÄKTA lab-scale protein purification systems (GE Healthcare). The column was washed at least with 15 column volumes PBS. Bound protein was eluted with different pH 3.5 buffer compositions or manufacturers' recommended buffer at a flow rate of 0.5 mL/min. After elution, the IgM solution was neutralized immediately with 1 M Tris-HCl (pH 8.5). Elution efficacy was determined with a standard µ-µ sandwich ELISA.

Glycopeptide analyses (LC/ESI-MS)

IgM012 was S-alkylated with iodoacetamide and digested with Trypsin (Promega) and/or GluC (Roche) in a 100 mM ammonium bicarbonate buffer. The digested samples were loaded on a BioBasic C18 column (BioBasic-18, 150 × 0.32 mm, 5 µm, Thermo Scientific) using 80 mM ammonium formiate buffer as. A gradient from 5 % to 32% acetonitrile (AcCN) was applied in 35 min, followed by a 15 min gradient from 32 % to 75% AcCN at a flow rate of 6 µL/min. Detection was performed with a QTOF MS (Bruker maXis 4G) equipped with the standard ESI source in positive ion, DDA mode. MS-scans were recorded (range: 150–2200 Da) and the 3 highest peaks were selected for fragmentation. Manual glycopeptide searches were made using DataAnalysis 4.0 (Bruker). The abbreviation system used in the result section is explained in http://www.proglycan.com/sites/default/public/pdf/nomen_2007.pdf [22].

Results

Stability of IgM decreases at acidic pH

Acidic conditions are recommended and widely used elution conditions for affinity purification of diverse proteins. Our model IgM012_GL was incubated at different acidic pH values for 45 min and subsequently screened for instability. Unfolding of the molecule was detected with Sypro®Orange fluorescence. At pH 4.0–6.0 FE was not increased compared to non-treated IgM (pH 5.5), which represents the native protein (Fig. 1A). During denaturation hydrophobic patches, which are hidden in the native protein in the core, get exposed at the protein surface, causing a higher FE at lower pH. At pH 3.0 the most IgM seems to be unfolded, but not aggregated indicated by the highest FE. At a pH ≤ 2.5, aggregation of the IgM started and reduced the Sypro®Orange fluorescence (Fig. 1A). Aggregation was proven by DLS. IgM incubated at pH 4.0–6.0 showed a broad distribution of similar sizes (Fig. 1B), but when incubated at pH 3.0 and 3.5 a monodisperse peak, shifted to larger diameters was detected. At pH < 3, IgM stability decreased and a less defined (polydisperse), shifted signal caused by intensive product aggregation, was observed.

These results indicated that at pH 3.0–3.5 the protein is already partly unfolded, but not irreversible aggregated (as it is at pH lower that 3.0). Thus, we decided to treat our IgMs with a pH > 3.0. At a pH ≥ 3.5 no aggregation of IgM012_GL occurred.

Small scale screening enabled improvement of elution conditions

The POROS CaptureSelect™ IgM Affinity Matrix is a commercially available product for the purification of IgMs with an immobilized

Table 1
Screened elution conditions at small scale and elution yields of IgM012_GL from POROS CaptureSelect™ IgM Affinity Matrix using indicated buffers in optimization screening at small scale. Conditions later used for lab-scale purifications are marked with an asterisk.

Elution buffer	pH	Abbreviation	Yield [%]
0.1 M glycine	3.5		0
0.1 M glycine	3.0		2
0.1 M glycine	2.5		4
0.1 M glycine	2.0	G	20
0.1 M glycine	1.5		24
0.1 M HAc/NaAc, 4 M NaCl	3.5		1
0.1 M HAc/NaAc, 4 M $MgCl_2$*	3.5	A	58*
2 M arginine	3.5		38
1 M arginine, 2 M $MgCl_2$*	3.5	R	76*
2 M arginine, 4 M $MgCl_2$	3.5		36

camelid-derived single domain [VHH] antibody fragment directed against μ-heavy chain of IgM. According to the specificity of the ligand only human, mouse or rat IgM will interact with the column matrix, whereas the impurities elute with the flow through.

The manufacturer recommended elution of IgM at 0.1 M glycine, pH 3.0–2.0 for complete IgM elution. We tried to elute the IgM012_GL at pH 3.0 and 2.0, but only little to no IgM012_GL was recovered (Table 1). Similar results were generated during purification of the IgM012 with 0.1 M glycine, pH 2.0 (Fig. 2, buffer G). Higher pH of the recommended elution buffer did not improve the recovery yields (Table 1). To prevent aggregation and to enable elution of IgM, different buffer salts and concentrations were combined with relatively moderate pH 3.5. Using the small scale screening system two promising buffer candidates, 0.1 M NaAc 4 M $MgCl_2$ pH 3.5 (buffer A, 58% recovery) and 1 M Arg 2 M $MgCl_2$ pH 3.5 (buffer R, 76% recovery), were identified (Table 1).

Purification of IgMs

Best performing buffers, buffer A and buffer R, were tested at column scale using an ÄKTA lab-scale protein purification system together with the standard glycine buffer (G). Both optimized elution buffers were suitable to elute IgM012_GL almost completely from the POROS CaptureSelect™ IgM Affinity Matrix, which corresponded to a six fold improvement compared to the manufacturers' conditions (Fig. 2). For IgM012 we found also a strong elution capacity with buffer R, but the achieved elution yields for buffer A were not satisfying for IgM012 (55% recovery), although elution was increased by at least 2.7 fold compared to buffer G. The elution of other IgMs from the POROS CaptureSelect™ IgM Affinity Matrix was tested with buffer R for verification. Indeed, the buffer R achieved a high recovery yield for all tested IgMs (80 %–97%, Fig. 2). The purity of the eluate was analyzed

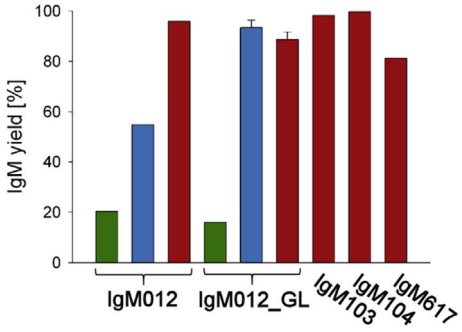

Fig. 2. Purification of different IgMs at column scale. POROS CaptureSelect™ IgM Affinity Matrix was used for purification. Indicated IgMs were eluted with buffer G (green), buffer A (blue) or buffer R (red). Purification of IgM012_GL with optimized buffer was performed at least in duplicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Relative abundances of major site specific glycan structures of IgM012 produced by CHO.

Glycan structure	Glycosylation site				
	GS1 [%]	GS2 [%]	GS3 [%]	GS4 [%]	GS5 [%]
Complex type	**38.4**	**100.3**	**100.1**		
NaM	6.3				
AAF	7.3	30.8	39.4		
NaA	6.7				
NaAF	12.1	36.9	29.3		
NaNaF		9.2	29.0		
NaAAF		6.6			
NaNaAF		8.8	2.4		
Other complex[a]	6.0	8.0			
Hybrid type	**21.6**				
Man5Gn + Hex	8.3				
Man5Na	9.9				
Other hybrid*	3.4				
Oligomannose type	**40.4**			**100**	**100**
Man5	40.4			17.7	11.2
Man6				29.5	88.8
Man7				28.0	
Man8				14.0	
Man9				5.8	
Other oligoman[a]				5.0	
Fucosylated	24	100	100		
Non-fucosylated	76			100	100
Na	39.5	61.4	60.7		
Non-Na	60.5	38.6	39.4	100	100

GS1-5 = glycosylation site 1-5, Abbreviations for glycan structures are visualized in Fig. 3.

[a] Glycan structures with occurrence < 5% were summarized in "other".

by SDS-PAGE, indicating that all impurities including free light chain were separated (Fig. S1A). Additionally, the initial polymeric status was sustained and the integrity of the complex molecules comprising μ-chains, κ-chains and j-chains was intact after elution with the new elution buffer (Figs. S1B–D). IgM012 and IgM012_GL were checked for retained biological specificity after IgM elution. Non-purified and purified IgMs showed a similar antigen binding capacity (Fig. S2).

Glycosylation

Until now, no site-specific glycosylation pattern of monoclonal IgM produced by CHO cells has been reported. We found that IgM produced by CHO cells showed the same pattern as human serum IgM (Tables 2 and 3). In detail, complex glycans at positions Asn171, Asn332 and Asn395 (glycosylation site [GS] 1-3) and oligomannose type structures at positions Asn402 (GS4) and Asn563 (GS5) were observed with carbohydrate structures as indicated in Fig. 3. GS2 and GS3 showed the highest degree of sialylation (61% sialylated glycans) and complete fucosylation (Table 2). Glycans at GS1 exhibited complex type but also hybrid type and oligomannose type structures (Table 2). More diverse oligomannosidic glycan structures were found at GS4 whereas only Man5 and Man6 were identified for GS5. Additionally we observed that GS5 remained non-glycosylated to a high extend.

Discussion

In this study the pH stability of monoclonal IgM was investigated to optimize the elution conditions for affinity purification of IgM, resulting in a fast and efficient single step downstream strategy. Additionally, we present the first analysis of site specific glycosylation of IgM produced

Table 3
Relative abundances of major glycan structures at G1-3 and GS4-5 of IgMs produced by different hosts.

Glycosylation site	GS 1-3 [%]			GS 4-5 [%]		
Origin	Serum[b]	PER.C6[b]	CHO	Serum[b]	PER.C6[b]	CHO
Complex	**99**	**86**	**80**	**0**	**4**	**0**
AGnF		7	2			
AAF		10	26			
NaAF	35	30	26			
NaAF + F		10				
NaAFbi	33					
NaNaF	10		13			
NaNaFbi	8					
Other complex[a]	13	29	13		4	
Hybrid			**7**			
Oligomannose	**1**	**14**	**13**	**100**	**96**	**100**
Na	≥86	≥40	54			
Non-Na	≤14	≤60	46	100	≤100	100

[a] Glycan structures with occurrence < 5% were summarized in "other".
[b] Data for serum IgM and PER.C6® were obtained from Loos et al. [22].

by CHO cells.

Early purification strategies of IgMs were often inefficient with a high risk of inactivation of the IgM antibodies [9–12]. More recent publications dealt with multi-step purification systems which lead to a higher purity, but simultaneously reduced recovery yields (40–80%) and longer process duration due to the increased number of steps [13–15].

Nowadays IgM purification by affinity chromatography is often recommended at low pH (2-3). Our results showed that incubation at such acidic pH result in denaturation and aggregation of IgMs and thus reduce the purification yield. In order to prevent aggregation but retain a high elution efficiency the pH needs to be increased to the minimal compatible pH, which was 3.5 in our study, and combined with a high ionic strength. Additionally, the eluted IgM should rapidly be adjusted to a neutral pH to avoid unnecessary long incubation times at low pH that may cause a partial unfolding (Fig. 1). Arginine and Mg^{2+} as buffer supplements decrease protein-protein interactions and thus promote elution from the matrix and prevent aggregation [23]. According to the Hofmeister series, Mg^{2+} has a higher capacity to alleviate interactions between IgM and affinity ligand ("salting in" effect) than e.g. Na^{+} [24]. This makes Mg^{2+} more suitable as elution buffer salt compared to Na^{+}, which was shown in our study. Arginine is commonly used in protein refolding as aggregation suppressor [23], but was also applied for purification of sensitive IgG antibodies [25,26]. Our screening experiment showed that highest arginine or Mg^{2+} concentrations did not correspond to the highest elution yields. This indicated that excess amounts of arginine or ionic strength might also destabilize protein structure.

The optimized elution conditions may also be suitable for other affinity chromatography resins for IgM purification such as Protein L- but were not investigated in this study in order to prevent co-purification of free light chains.

Additionally we present the first glycoproteomic analysis by LC/ESI-MS of IgM012 produced by CHO cells. The general site specific glycan profile of complex type glycans at GS1-3 and oligomannose type glycans at GS4-5 were similar as for serum derived IgM. Exclusively oligomannosidic structures with incomplete site occupancy were found on GS4 and GS5, which perfectly matches with serum derived IgM (Table 3) [19,22,27].

GS1-3 profiles of human serum are differently described in literature. Bisecting structures were identified in human serum IgM with a high degree of sialylation at GS1-3 [22,28] whereas we found 46% non-sialylated glycans in CHO derived IgM012 (Table 3). Nevertheless, Pabst et al. identified also a minor portion of oligomannosidic and hybrid-type glycans on GS1 in human serum IgM and complex type glycans with varying sialic acid residues, bisecting GlcNAc and a fucose residue on GS3 [28]. GS3 of CHO derived IgM012 was fully fucosylated like the serum IgM but non-sialylated structures were also found for IgM012 (Table 2). An incomplete sialylation was reported for IgM produced by PER.C6® where less processed precursor forms like non-sialylated complex type, hybrid type or oligomannosidic structures at GS1-3 are described (Table 3) [22,27]. These findings could be explained by a massive protein overload during recombinant protein production leading to a low substrate availability and high workload for the processing enzymes. Another reason of heterogeneity are the varying substrate conditions for the glycosyltransferases during cultivation [29], which can be controlled by the manufacturing mode (batch, fed-batch, perfusion) and process strategy (environmental control parameters).

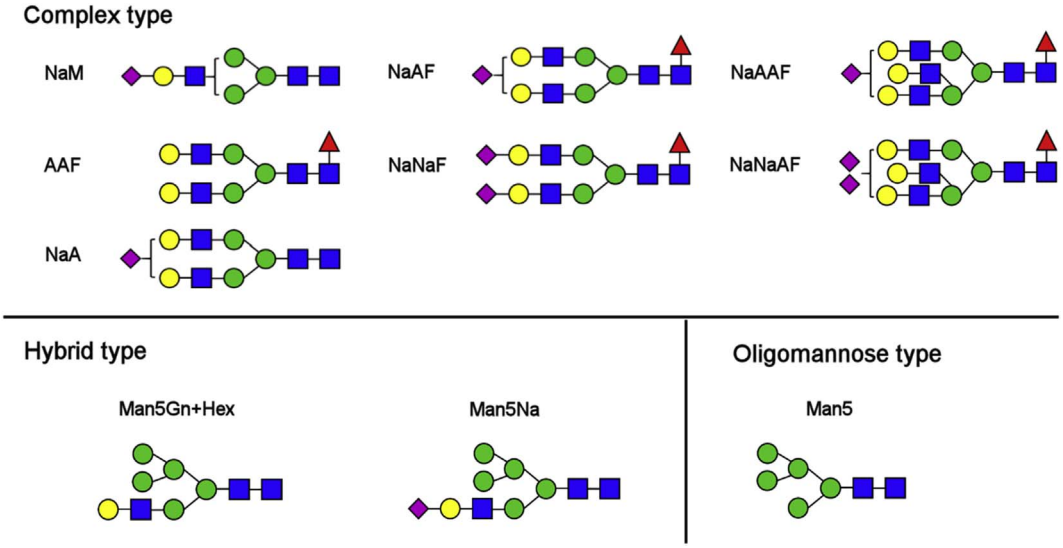

Fig. 3. Structures of N-glycans found for CHO produced IgM. Monosaccharide symbols visualize the used proglycan terminology (www.proglycan.com) and were applied according to the symbol nomenclature of the Consortium of Functional Glycomics (CFG). Note that only one representative isomer is shown, but more combinations are possible.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

We thank Polymun Scientific Immunbiologische Forschung GmbH for providing the antibodies IgM103, IgM104 and IgM617 as a kind gift. This work was supported by the PhD program BioToP (Biomolecular Technology of Proteins) funded by the Austrian Science Fund (FWF Project W1224).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2017.10.020.

References

[1] N. Baumgarth, J.W. Tung, L.A. Herzenberg, Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion, Springer Semin. Immunopathol. 26 (2005) 347–362, http://dx.doi.org/10.1007/s00281-004-0182-2.

[2] L. Rasche, J. Duell, I.C. Castro, V. Dubljevic, M. Chatterjee, S. Knop, F. Hensel, A. Rosenwald, H. Einsele, M.S. Topp, S. Brändlein, GRP78-directed immunotherapy in relapsed or refractory multiple myeloma - results from a phase 1 trial with the monoclonal immunoglobulin M antibody PAT-SM6, Haematologica 100 (2015) 377–384, http://dx.doi.org/10.3324/haematol.2014.117945.

[3] F. Hensel, W. Timmermann, B.H.A. Von Rahden, A. Rosenwald, S. Brändlein, B. Illert, Ten-year follow-up of a prospective trial for the targeted therapy of gastric cancer with the human monoclonal antibody PAT-SC1, Oncol. Rep. 31 (2014) 1059–1066, http://dx.doi.org/10.3892/or.2014.2987.

[4] C.J. Binder, S. Hörkkö, A. Dewan, M.-K. Chang, E.P. Kieu, C.S. Goodyear, P.X. Shaw, W. Palinski, J.L. Witztum, G.J. Silverman, Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL, Nat. Med. 9 (2003) 736–743, http://dx.doi.org/10.1038/nm876.

[5] C. Grönwall, E. Akhter, C. Oh, R.W. Burlingame, M. Petri, G.J. Silverman, IgM autoantibodies to distinct apoptosis-associated antigens correlate with protection from cardiovascular events and renal disease in patients with SLE, Clin. Immunol. 142 (2012) 390–398, http://dx.doi.org/10.1016/j.clim.2012.01.002.

[6] X. Xu, A. Denic, A. Warrington, A.J. Bieber, M. Rodriguez, Therapeutics to promote CNS repair: a natural human neuron- binding IgM regulates membrane-raft dynamics and improves motility in a mouse model of multiple sclerosis, J. Clin. Immunol. 33 (2013) 50–56, http://dx.doi.org/10.1007/s10875-012-9795-8. Therapeutics.

[7] orpha.net, (n.d.). http://www.orpha.net/consor/cgi-bin/Drugs_Search_SubstanceTradename.php?lng=EN (accessed April 3, 2017).

[8] Aridis Pharmaceuticals, AR-101: Fully Human mAb Against Pseudomonas aeruginosa LPS serotype O11, (n.d.). http://www.aridispharma.com/ar101.html (accessed April 3, 2017).

[9] J.-P. Bouvet, R. Pires, J. Pillot, A modified gel filtration technique producing an unusual exclusion volume of IgM: a simple way of preparing monoclonal IgM, J. Immunol. Methods 66 (1984) 299–305, http://dx.doi.org/10.1016/0022-1759(84)90341-7.

[10] F. Steindl, A. Jungbauer, E. Wenisch, G. Himmler, H. Katinger, Isoelectric precipitation and gel chromatography for purification of monoclonal IgM, Enzyme Microb. Technol. 9 (1987) 361–364, http://dx.doi.org/10.1016/0141-0229(87)90060-3.

[11] M. García-González, S. Bettinger, S. Ott, P. Olivier, J. Kadouche, P. Pouletty, Purification of murine IgG3 and IgM monoclonal antibodies by euglobulin precipitation, J. Immunol. Methods 111 (1988) 17–23, http://dx.doi.org/10.1016/0022-1759(88)90054-3.

[12] P.-L. Lim, Isolation of specific IgM monoclonal antibodies by affinity chromatography using alkaline buffers, Mol. Immunol. 24 (1987) 11–15, http://dx.doi.org/10.1016/0161-5890(87)90106-4.

[13] P. Gagnon, F. Hensel, R. Richieri, Purification of IgM monoclonal antibodies, Bio Pharm. Int. 2008 Supplement (3) (2008) RP0038.

[14] I. Tornøe, I.L. Titlestad, K. Kejling, K. Erb, H.J. Ditzel, J.C. Jensenius, Pilot scale purification of human monoclonal IgM (COU-1) for clinical trials, J. Immunol. Methods 205 (1997) 11–17, http://dx.doi.org/10.1016/S0022-1759(97)00051-3.

[15] J. Lee, A. Tscheliessnig, A. Chen, Y.Y. Lee, G. Adduci, A. Choo, A. Jungbauer, Adaptation of hybridomas to protein-free media results in a simplified two-step immunoglobulin M purification process, J. Chromatogr. A 1216 (2009) 2683–2688, http://dx.doi.org/10.1016/j.chroma.2008.10.067.

[16] B.H.K. Nilson, L. Lögdberg, W. Kastern, L. Björck, B. Akerström, Purification of antibodies using protein L-binding framework structures in the light chain variable domain, J. Immunol. Methods 164 (1993) 33–40, http://dx.doi.org/10.1016/0022-1759(93)90273-A.

[17] R. Sitia, A. Rubartelli, U. Hämmerling, Rich, the role of glycosylation in secretion and membrane expression of immunoglobulins M and A, Mol. Immunol. 21 (1984) 709–719 https://doi.org/10.1016/0161-5890(84)90023-3.

[18] S. Muraoka, M.J. Shulman, Structural requirements for IgM assembly and cytolytic activity. Effects of mutations in the oligosaccharide acceptor site at Asn402, J. Immunol. 142 (1989) 695–701.

[19] J.N. Arnold, M.R. Wormald, D.M. Suter, C.M. Radcliffe, D.J. Harvey, R.A. Dwek, P.M. Rudd, R.B. Sim, Human serum IgM glycosylation: identification of glycoforms that can bind to Mannan-binding lectin, J. Biol. Chem. 280 (2005) 29080–29087, http://dx.doi.org/10.1074/jbc.M504528200.

[20] J.Y. Kim, Y.G. Kim, G.M. Lee, CHO cells in biotechnology for production of recombinant proteins: current state and further potential, Appl. Microbiol. Biotechnol. 93 (2012) 917–930, http://dx.doi.org/10.1007/s00253-011-3758-5.

[21] V. Chromikova, A. Mader, W. Steinfellner, R. Kunert, Evaluating the bottlenecks of recombinant IgM production in mammalian cells, Cytotechnology 67 (2014) 343–356, http://dx.doi.org/10.1007/s10616-014-9693-4.

[22] A. Loos, C. Gruber, F. Altmann, U. Mehofer, F. Hensel, M. Grandits, C. Oostenbrink, G. Stadlmayr, P.G. Furtmüller, H. Steinkellner, Expression and glycoengineering of functionally active heteromultimeric IgM in plants, Proc. Natl. Acad. Sci. 111 (2014) 6263–6268, http://dx.doi.org/10.1073/pnas.1408639111.

[23] E. Bernardez Clark, E. Schwarz, R. Rudolph, Inhibition of aggregation side reactions during in vitro protein folding, Methods Enzymol. 309 (1999) 217–236, http://dx.doi.org/10.1016/S0076-6879(99)09017-5.

[24] F. Hofmeister, Zur Lehre von der Wirkung der Salze - zweite Mittheilung, Arch. Für Exp. Pathol. Und Pharmakol. 24 (1888) 247–260, http://dx.doi.org/10.1007/BF01918191.

[25] T. Arakawa, J.S. Philo, K. Tsumoto, R. Yumioka, D. Ejima, Elution of antibodies from a Protein-A column by aqueous arginine solutions, Protein Expr. Purif. 36 (2004) 244–248, http://dx.doi.org/10.1016/j.pep.2004.04.009.

[26] D. Ejima, R. Yumioka, K. Tsumoto, T. Arakawa, Effective elution of antibodies by arginine and arginine derivatives in affinity column chromatography, Anal. Biochem. 345 (2005) 250–257, http://dx.doi.org/10.1016/j.ab.2005.07.004.

[27] E.S.X. Moh, C.-H. Lin, M. Thaysen-Andersen, N.H. Packer, Site-specific N-Glycosylation of recombinant pentameric and hexameric human IgM, J. Am. Soc. Mass Spectrom. 27 (2016) 1143–1155, http://dx.doi.org/10.1007/s13361-016-1378-0.

[28] M. Pabst, S.K. Küster, F. Wahl, J. Krismer, P.S. Dittrich, R. Zenobi, A microarray-matrix-assisted laser desorption/ionization-mass spectrometry approach for site-specific protein N-glycosylation analysis, as demonstrated for human serum immunoglobulin M (IgM), Mol. Cell. Proteomics 14 (2015) 1645–1656, http://dx.doi.org/10.1074/mcp.O114.046748.

[29] B. Liu, M. Spearman, J. Doering, E. Lattová, H. Perreault, M. Butler, The availability of glucose to CHO cells affects the intracellular lipid-linked oligosaccharide distribution, site occupancy and the N-glycosylation profile of a monoclonal antibody, J. Biotechnol. 170 (2014) 17–27, http://dx.doi.org/10.1016/j.jbiotec.2013.11.007.

Supplementary Data

Glycan profile of CHO derived IgM purified by highly efficient single step affinity chromatography

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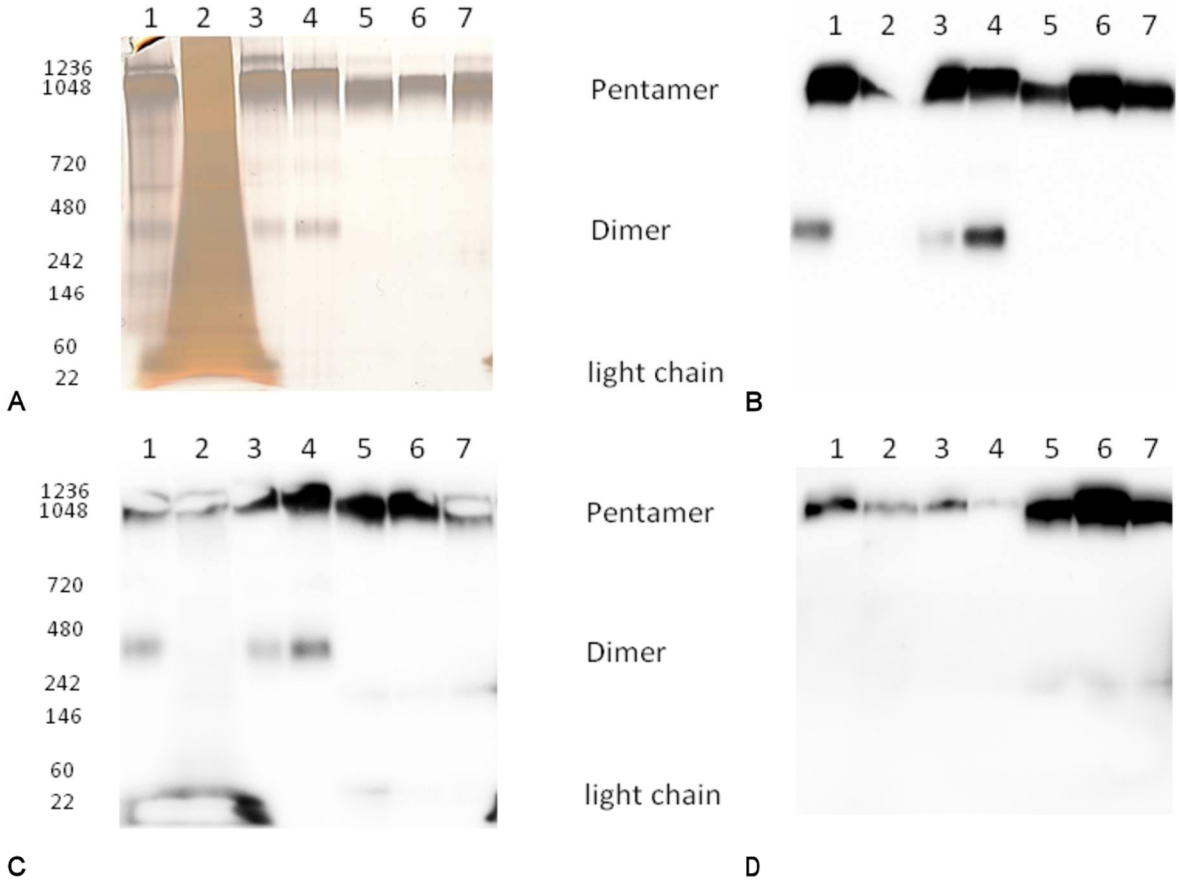


Figure. S1: Purity analyses of eluted IgM by SDS-PAGE and western blot. 300 ng IgM was loaded on Native PAGE 3-12 % Bis-Tris gels. (A) Silver stained gel. Western blot stained for (B) µ-chain, (C) κ-chain and (D) j-chain. Lane 1: concentrated supernatant IgM012, lane 2: flow-through of IgM012 purification, lane 3, 4, 5, 6, 7: purified IgMs: IgM012, IgM012_GL, IgM103, IgM104, and IgM617.

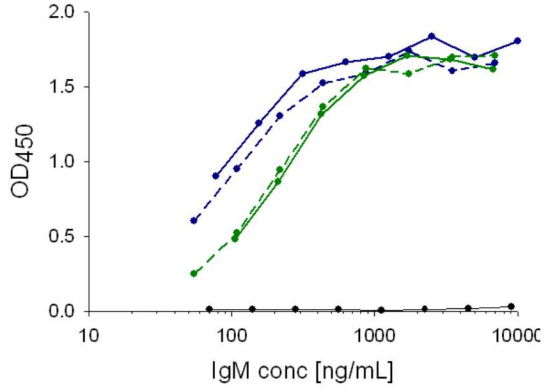


Figure S2: Comparison of antigen (UG37gp140) binding by an ELISA assay. Supernatants containing IgM are indicated by a continuous line, purified IgMs are indicated by a dashed line. IgM012 samples are shown in blue and IgM012_GL samples in green**.** Negative control was a non-binding IgM (black line). Approximately 10 µg/ml pure IgM and IgM in culture supernatant were serially diluted and incubated on the plate at room temperature for 1 h. Antigen binding was detected with a peroxidase-conjugated goat anti-human κ-chain specific antibody and tetramethylbenzidine (Thermo Fisher Scientific).All binding analyses were performed as duplicates.

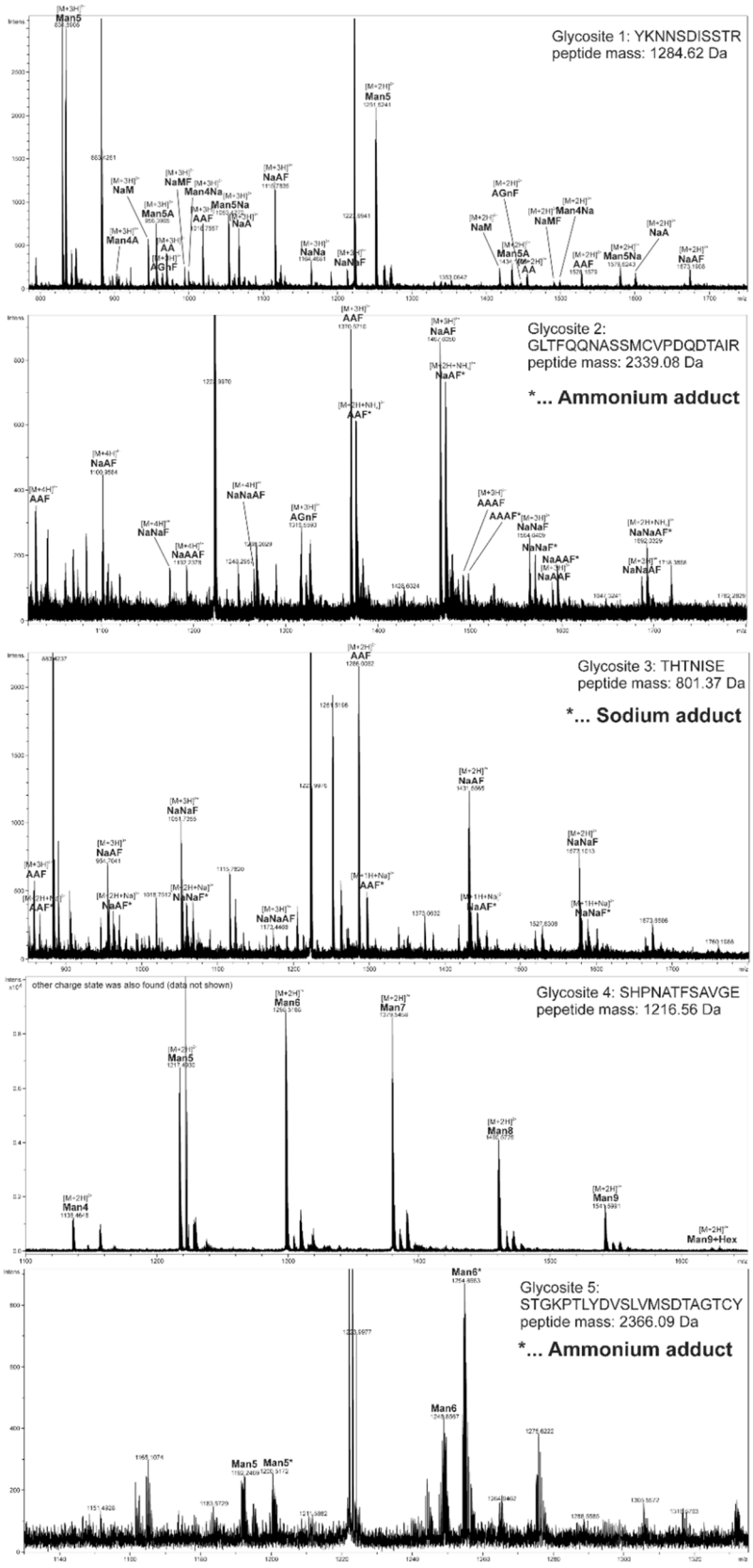


Figure S3: Mass spectra of the different N-glycosylation sites of µ-heavy chain of IgM012. Spectra were obtained by LC-ESI-MS after trypsin and/or GluC digestions. Glycoforms are labeled in accordance with the ProGlycAn system (www.proglycan.com).

Using the published single-step purification strategy, already a very high purity was obtained, which was verified with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. S1) and size-exclusion high performance liquid chromatography (SE-HPLC, data not published). Nevertheless, nucleic acids (NA) could be identified as remaining impurities with UV-Vis spectroscopy (Fig. 5) as they were not detected with the formerly used methods.

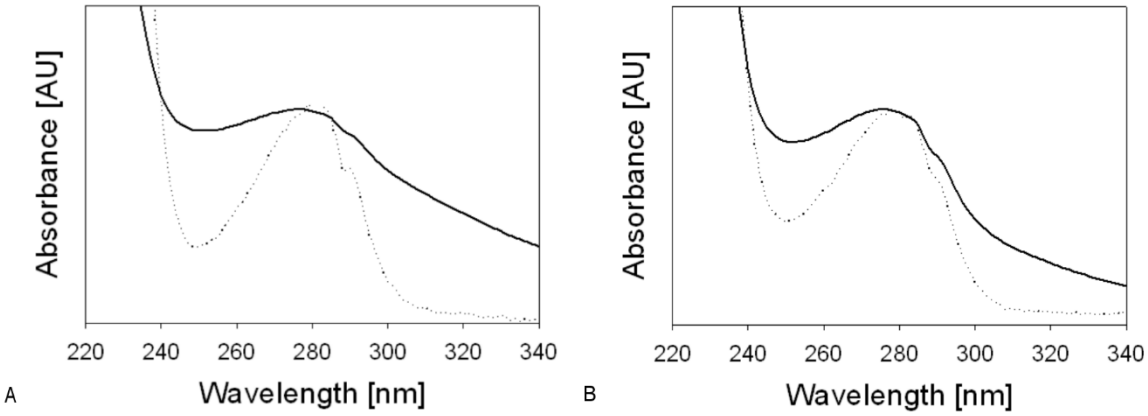


Figure 5: Verification of purity with UV-Vis spectroscopy. Continuous line: NA containing IgM preparation after affinity chromatography; dotted line: pure IgM preparation after affinity chromatography and size-exclusion chromatography. **A** IgM012_GL; **B** IgM012.

The non-specific interaction of IgMs with chromatin was already described to interfere with the efficiency of the downstream process and to survive various commonly used purification steps [76,97,98]. Besides the obvious concerns about a therapeutic application, the remaining NA could also cause large background interferences in biological assays. Although the non-specific interaction of IgM and DNA was reported to be quite stable, the dissociation of NA from the IgM purified with affinity chromatography could be observed during SE-HPLC. Therefore, preparative size exclusion chromatography (SEC) was included as a second purification step. The separation of two peaks could be observed in the SE-Chromatogram, in which the first peak contained the IgM molecules and the second contained the NA fraction (Fig. 6). The contaminating NA appeared as fragments because the retention time of IgM is lower than the one of the NA. The pure IgM-fractions were pooled according to the $A_{260/280}$ ratio of ≤0.6, which separates the NA-fractions. Additionally, this data shows that low amounts of remaining DNA have a high absorption at 280 nm in the

UV-Vis spectrum and could interfere with concentration determination of proteins. Finally, the obtained IgM preparation could be verified with a characteristic UV-Vis spectrum of proteins (Fig. 5).

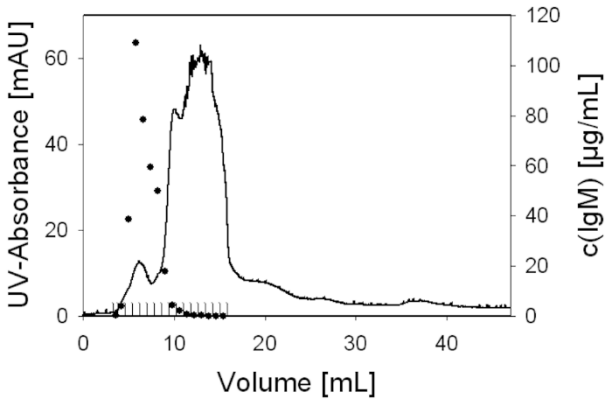


Figure 6: Size-exclusion chromatography. A Superose™ 6 prep grade (GE Healthcare) column was equilibrated in 0.1 M sodium phosphate buffer pH 5.5, 0.2 M NaCl. Affinity purified IgM was injected and separated at a flow rate of 0.5 mL/min. Continuous line: UV-Vis chromatogram at 280 nm; dots: IgM012_GL concentration determined with standard µ-κ-sandwich ELISA.

3.3. Characterization

Structure

The first structural information about IgM pentamers was gained with negative stain transmission electron microscopy (TEM) and small angle X-ray scattering (SAXS). These studies revealed a planar, star-shaped structure with the Fab domains located at the extremity of each radial arm [99,100]. A homology modeling approach based on the IgE-Fc crystallography structure combined with Cryo-atomic force microscopy (AFM) disclosed that the pentamer structure is not planar, but adopt a mushroom shape with radially-directed Fab units around a central circular region and a protruding part formed by the Cμ4 domains [101]. Additionally, it was presumed that the Fab domains were very flexible and can be bent in the direction of the protruding Fc part [101]. The high flexibility of the molecule, especially of the Fab arms, was supposed to cause the unavailability of a crystal structure of IgM monomers or pentamers. Therefore, Müller *et al.* crystallized the Fc-domains of IgM separately and analyzed the Cμ4 oligomers with SAXS to gain a better understanding about the hexamer assembly [102]. They confirmed a Cμ4 inner core projecting out of the plane defined by Cμ2 and Cμ3 of hexamers, which are flexibly arranged in a star-shaped manner [102]. A more recent study using room temperature and cryo-TEM showed that due to the high flexibility of the Fab regions, various conformations of the randomly oriented Fab units around the dome-like core are possible [103]. The authors tried to identify three main conformations (extended, turtle shaped and bell shaped), which differ in bending of the pentamer, diameter and appearance of the Fab units. The most recent study revealed a new model of the IgM pentamer [104]. Single particle EM and reference-free 2D class averaging showed a pentamer shaped asymmetric pentagon with a large gap, like a symmetric hexagon with one monomer missing. This model is questioning the previously accepted model of a planar symmetric pentamer. However, this study agrees with previous studies in terms of the high flexibility of the Fab portions [104].

In the herein presented work, structural studies of the different recombinant pentameric model IgMs were performed with negative stain TEM. Pentameric IgM was found for all model antibodies; dimeric IgM012 and IgM012_GL were not clearly identified (Fig. 7). Presumably, IgM dimers are too small and too flexible to have a distinct appearance. The pentameric IgMs had a diameter of approximately 30-40 nm, as previously reported by

Czajkowsky and Shao or Akhouri *et al.* [101,103]. The size of the pentamer particles differed depending on the resolution of the Fab units, but independent on the model antibody. The pentameric IgM contained a central circular core with projecting Fab units in a star-shaped manner (Fig. 7). In accordance to several previous studies, less than five visible Fab-arms were detected for some molecules which indicates that the projecting arms are not in the same plane and very flexible [99,101,103,104]. In addition, many orientations of the Fab units were found (Fig. 7, zoomed images). The variety of possible conformations were grouped into three classes by Akhouri *et al.* [103]. However, it is more likely that the different conformations represent snap-shots of the continuous movements of the Fab units and therefore cannot be strictly classified. Symmetric and asymmetric shapes were found, which supports both the conventional model and the results of the study by Hiramoto *et al.* [104]. The herein presented results lead to the assumption that also the regions around the Cμ2- and Cμ3-domains exhibit flexibility. The Cμ2-domain is an extra domain in the μ-heavy chain that is thought to replace the very flexible hinge region found between the CH1 and CH2 domains in IgG molecules [102]. Different studies localized the origin of flexibility in the connecting loops between Cμ1 and Cμ2 or Cμ2 and Cμ3, respectively [101,105]. Further investigations of high resolution structures of the full-length IgM are necessary to confirm these assumptions. Overall, the conventional star-shaped image of IgM pentamers could be confirmed. The structure of IgM dimers is not determined yet and remains still an open question.

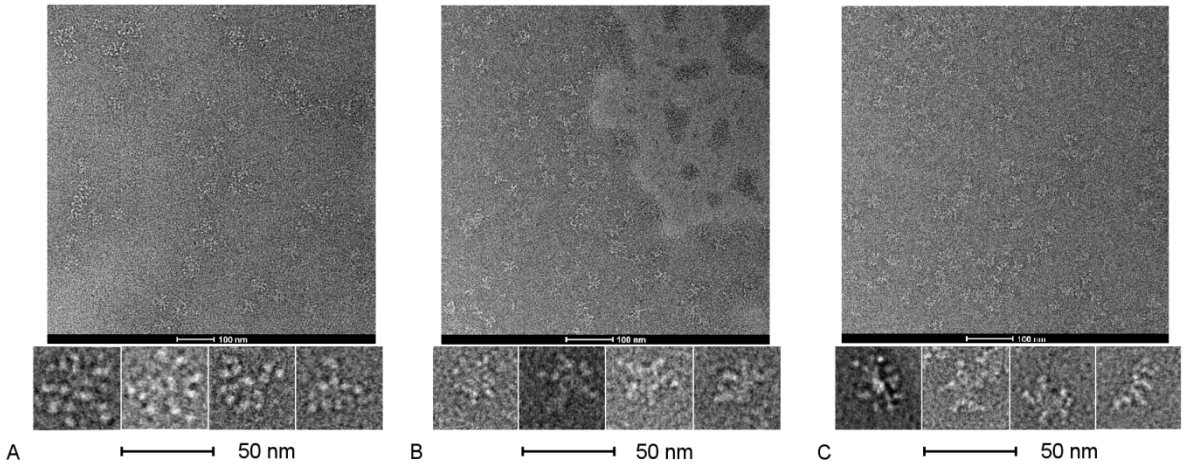


Figure 7: Non-processed images of the negative stain TEM. 4µL of IgMs at 15-20 µg/mL were applied to a mica sheet covered with evaporated carbon film. The film was floated off in ~100 µL 2 % sodium silicotungstate (SST) and fished onto a 300mesh Cu TEM grid. Images were taken with a Tecnai F20 TEM microscope at 200 keV. Upper pictures: the scale bar represents 100 nm. Lower pictures: Zooms of individual particles, the scale bar represents 50 nm. **A** IgM012, **B** IgM012_GL, **C** IgM617.

Formation of incomplete polymers and polymer distribution

The human IgM is produced by B1 cells or plasma cells that resulted from differentiation of B cells after antigen stimulation. In such IgM secreting cells, folding and assembly is accompanied by quality control mechanisms. First of all, the IgM HC is co-translationally translocated into the ER-lumen. Free HC or HC dimers are associated with the ER chaperone BiP/GRP78 that is replaced upon LC attachment [106]. The HC/LC or HC_2/LC_2 intermediates are then stepwise incorporated by non-covalent interaction followed by intersubunit disulfide bond formation [62]. ERp44, ERGIC-53 and pERp1 are chaperones that are strongly upregulated after B cell differentiation into plasma cells and were implicated in IgM assembly and secretion [12,107]. The IgM quality is controlled at a minimum of four checkpoints: 1) Free HC and IgM polymers lacking LC interact with molecular chaperones such as BiP/GRP78 that contain an ER retention motif and are not secreted to the Golgi apparatus [106]. 2) Incomplete IgM polymers exposure a non-oxidized Cys575 that is recognized by ERp44, which is part of the thiol retention mechanism [12,57]. 3) Incorrectly folded or assembled polymers non-specifically aggregate intracellularly preventing its secretion. 4) Lectins like ERGIC-53 capture the GS5 at monomers, which is not accessible in

higher polymers, and rearrange the monomers for correct polymerization [12]. Pentameric or hexameric IgM fulfilling all quality checkpoints of the ER are immediately secreted into the Golgi indicating that polymerization is the rate limiting step in IgM production [62]. The glycan sites one to three are processed in the Golgi before secretion [10,72]. However, under certain conditions the assembly intermediates become oxidized at the Cys575 and escape from the thiol retention mechanism resulting in secretion of lower polymers [108]. Approximately 1 % of such incompletely assembled polymers are secreted by IgM-producing plasma cells [109]. In recombinant IgM production the amount and polymerization degree of incomplete polymers varied for different antibodies and production systems. Until now, it is not fully understood if the polymer distribution in recombinant production is influenced by the intrinsic nature of the specific antibody (primary sequence), the production system and cultivation conditions or whether it is a result of a lack of chaperones for checkpoint control in the host cells or some kind of unknown degradation process of an instable protein.

Concluding the results explained in chapter 3.1, the impact of the process conditions or the production system can be excluded. To shed light into the intracellular polymer distribution and assembly of recombinant IgM, CHO cell lysates were characterized by western blot and µ-heavy chain detection. In total, three model CHO cell lines secreting IgM in different quantity and polymeric quality were investigated. The genetic constructs and cell culture parameters are described in chapter 3.1 (Fig. 3 and Tab. 1). Pentameric IgM is the predominant form secreted by all three cell lines due to co-expression of the J-chain and no hexameric IgM occurred (Fig. 8). However, protein bands with a molecular weight of dimers were produced by all cell lines as additional polymer, even though with different quantity (Fig. 8). The amino acid composition and structure of the variable regions might weaken the non-covalent interaction between assembly intermediates prior to disulfide bond formation and affect the capacity of IgM assembly to a different degree. IgM617 was secreted to a minor extent as dimers to the supernatant, which was close to the detection limit when analyzed by western blotting (Fig. 8). Whole cells were solubilized with RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.25 % deoxycholic acid, 1% NP-40, 1 mM EDTA) for 15 min at 4°C. To gain more information about intracellular IgM assembly and distribution within the organelles, CHO cells were consecutively lysed into subcellular fractions according to the protocol established by Holden et al [110]. The protein bands of the

dimeric IgMs were more dominant and additional monomers appeared in the whole cell lysates for all three cell lines (Fig. 8). This result suggests that the intracellular quality control mechanisms described for differentiated B-cells are also present in CHO cells to recognize and retain the dimers and monomers. Compared to secreted IgM, the protein bands of intracellular pentamers and dimers appear slightly shifted to lower molecular weights as post-translational modification were attached shortly before secretion (Fig. 8). The whole cell lysates of CHO cells producing IgM012 and IgM012_GL showed only a thin protein band with the molecular weight of pentamers as these are secreted immediately after polymerization [62]. In comparison to IgM012 and IgM012_GL, the whole lysate of IgM617 contained a higher pentamer/dimer-ratio and therefore a larger intracellular pool of correctly assembled polymers for secretion, which could explain the better productivity of the IgM617-producing CHO cells (Fig. 8).

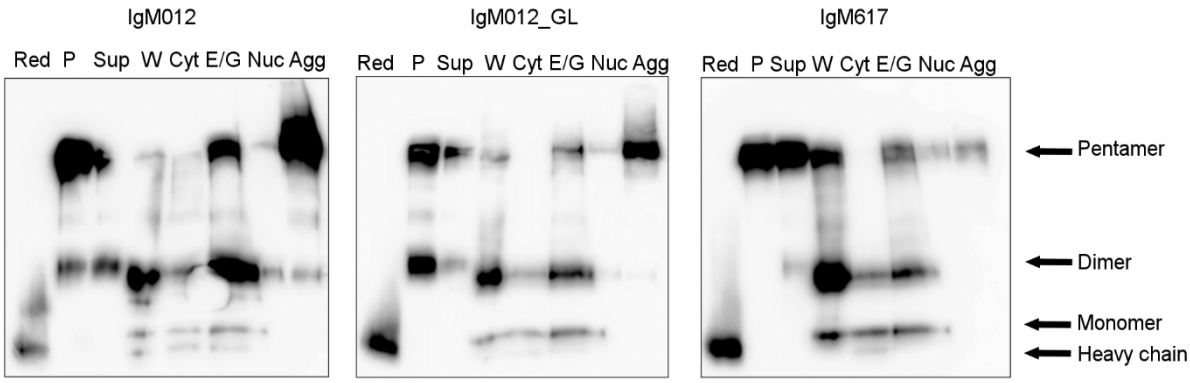


Figure 8: Intracellular IgM polymer formation and distribution. Anti-HC western blot analysis of secreted product or cell lysates of the cell lines CHO IgM012 (left), CHO IgM012_GL (middle), CHO IgM617 (right). Western blotting was performed as described in [64]. Except for subsequent lysis, 100 ng (IgM012) or 200 ng (IgM012_GL and IgM617) IgM were loaded on the gel. Samples: **Red** – purified IgMs were reduced with 50 mM DTT, **P** – purified IgMs (non-reduced), **Sup** – secreted IgMs in supernatant, **W** – whole cell lysate was obtained by incubating cell pellets in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.25 % deoxycholic acid, 1% NP-40, 1 mM EDTA) for 15 min at 4°C. Cell debris were separated by centrifugation at 8 000xg for 10 min. Subsequent lysis was performed according to [110] and equal volumes were loaded: **Cyt** – cytosolic fraction, **E/G** – ER and Golgi fraction, **N** – nuclear fraction, **Agg** – insoluble aggregates.

Subcellular cell lysis revealed the highest IgM amount in the ER and Golgi fraction (Fig. 8), which are the compartments of translation, assembly and posttranslational modifications. In the ER/Golgi fraction, complete IgM pentamers, but also dimers and monomers were found.

This suggests that dimers and monomers are acting as a stable assembly intermediate pool from which pentamers are subsequently built up. As intermediates like trimers and tetramers were not detected for all three cell lines, it is likely that the association to trimers (dimer and monomer) or tetramers (dimer and dimer) is the rate limiting step before completing and secreting pentamers. As dimers are accumulating in the stable assembly intermediate pool, they somehow seem to escape the ER-retention mechanism and proceed to secretion (Fig. 8) leading to a reduced IgM quality in the supernatant. Usually, incomplete IgM cannot be secreted as it does not fulfill all quality criteria and it is translocated into the cytosol for proteasomal degradation [111,112]. Indeed, in this study dimeric and monomeric IgMs were found in the cytosolic fraction (Fig. 8), indicating that the chaperone machinery in CHO cells recognizes most of the incomplete assembled polymers and triggers their removal via the proteasome pathway. However, it was already reported that B cells secrete incomplete polymers to a low extent [108]. In the cytosol, the disulfide bridges were cleaved as single HC could be detected for IgM012 and IgM617 (Fig. 8). The further degradation process in the proteasome is too fast to be monitored with the method used as no HC fragments were detected. In the nuclear fraction, a faint protein band was found (Fig. 8). However, it is rather unlikely that IgM was transported to the nucleus. A cross-contamination, presumably from the “insoluble” fraction, is assumed. Interestingly, mainly pentameric IgM was found to aggregate intracellularly in Russell bodies for all three cell lines (Fig. 8). Only IgM012 additionally aggregated as dimers. The formation of Russell bodies is thought to rely on inability to secrete and degrade misfolded proteins that form stable aggregates [59]. By accumulating such secretion-deficient proteins into Russell bodies, the normal secretory pathway is not blocked. Mutagenesis studies identified the reason for polymeric IgM accumulation as a combination of an abnormal HC-LC stoichiometry and incorrect disulfide bond formation [59]. Additionally, the specific structure of the variable regions of HC and LC were suspected to influence the formation of Russell bodies. The results of this study definitely confirmed this theory as the model IgM antibodies were accumulated in varying degree. The different aggregation tendencies for observed in this study correlated with productivity of the cell lines (Fig. 8 and Tab. 1). Strategies to increase the yield of pentameric IgM should target the ER quality control mechanism, cytosolic degradation and aggregation into Russell bodies. Overexpression of IgM specific B cell chaperones like pERp1 could improve assembly [107]. In general,

overexpression of the ER chaperones responsible for quality control could improve detection of incomplete polymers leading to proteasomal degradation and lower metabolic burden of the host cells. Addition of reducing agents into the cultivation media could be a non-host-cell engineering approach and prevents Russell body formation by improving secretion [59]. Finally, as it has been shown previously for IgM012, antibody engineering of the variable region could also affect IgM yield and polymer assembly [65].

Intracellular deficiency of retention mechanisms for incomplete polymers is only one source of poor IgM quality. Additionally, structural instability of the large complexes might lead to disassembly of IgM pentamers after secretion into the culture supernatant. To study if pentamer-dimer mixtures were a result of an assembly equilibrium or time-dependent dissociation, the affinity purified IgM012_GL pentamers and dimers were separated by preparative SEC (Superose^{TM} 6 prep grade column) as described in 3.2 and stored for 45 days. In the preparative SEC, pentameric IgM eluted in the early fractions due to its higher molecular weight, with no contamination of dimers (Fig. 9A). Nevertheless, the column length (15 cm) and resolution was not high enough to completely separate pentameric and dimeric polymers in this experiment. All dimer containing fractions were contaminated with pentamers (Fig. 9A). However, SDS-PAGE analysis was repeated and expanded by western blot after 45 days for the same fractions (Fig. 9B). Although the polymer distribution was different for the three investigated fractions, it did not change within the 45 days storage period. It should be noted, that for the fraction F1 no dissociation of pentamers into dimers could be detected (Fig. 9B). Concluding these results, the disassembly of IgM pentamers into dimers after secretion can be excluded. Furthermore, monomeric IgM, which would be an accompanying dissociation side product, was never observed outside the cells during cultivation or long-term storage.

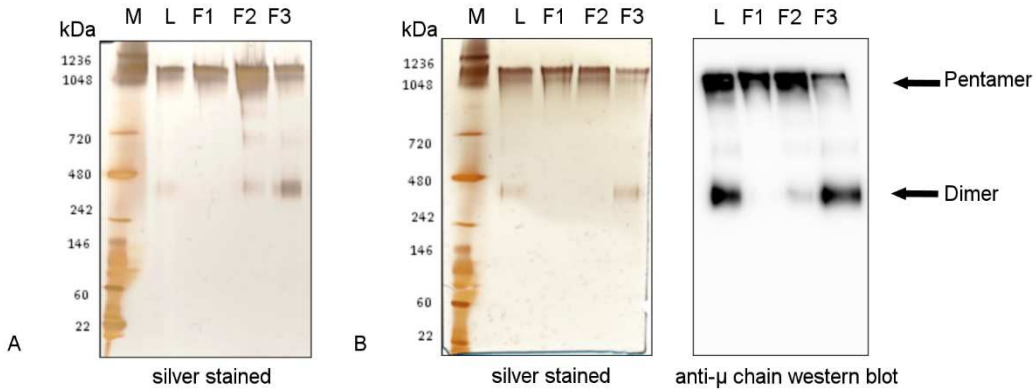


Figure 9: Separation of IgM012_GL pentamers and dimers with SEC. SEC was performed as described in 3.2. SDS-PAGE, Western blot analysis and silver staining of collected fractions was conducted as described in [64,90]. **A** SDS-PAGE analysis after separation, 600 ng sample load, F1-F3 undiluted sample was loaded. **B** SDS-PAGE (left, 1200 ng IgM each lane) and anti-HC western blot (right, 600 ng IgM each lane) 45 days after separation. Samples: **M** – NativeMark^{TM} Unstained Protein Standard, **L** – sample load (affinity-purified IgM012_GL), **F1-F3**, IgM containing sequentially collected fractions.

IgM and its interaction partners

The aim of recombinant production of biopharmaceuticals is to increase the availability of proteins that mimic their biological counterparts. Therefore, it is necessary to prove if the recombinant proteins execute their biological functions. In case of immunoglobulins, the most important biological role is the binding to their antigens and the interaction with the effector proteins to trigger an immune response. To ensure that recombinant IgMs are functional, their binding to the natural interaction partners was studied.

The antigen of the IgM012 and IgM012_GL is the gp160 envelope glycoprotein of the Human Immunodeficiency Virus (HIV).The gp160 envelope protein is a heterodimer of a transmembrane glycoprotein (gp41) and a surface glycoprotein (gp120), which forms trimers on the surface of the virus and triggers the fusion of the viral and cell membranes during infection [113,114]. A recombinant truncated version of the envelope protein (gp140), which is composed of the entire gp120 component and an approximately 20 kDa peptide of gp41, was developed to circumvent the formation of aggregates as often described for the full length g120/gp41 trimers [115,116]. Due to the high viral diversity of HIV, several groups and isotypes were identified so far [117]. The isolate used for the antigen binding experiments, UG37, belongs to clade A.

First of all, it was investigated if the established elution conditions during the purification reduce the binding affinity to the antigen due to partial unfolding. Therefore, binding of IgM in culture supernatants of the CHO cell lines producing IgM012 and IgM012_GL were compared to the respective affinity purified IgM in an enzyme-linked immunosorbent assay (ELISA) set-up. The antigen gp140 was coated on the microtiter plate and incubated with serial dilutions of the supernatant or purified IgM. It could be shown that both recombinant IgMs were able to bind their antigen and that the developed purification strategy had no influence on the antigen binding affinity (Fig. 10A), although quite harsh conditions (1 M Arginine pH 3.5, 2M $MgCl_2$) were applied to the labile protein. Next, the impact of the recombinant expression host on antigen binding was studied. As described in chapter 3.1, the post-translational modifications generated by HEK or CHO cells could differ. The effect of minor changes in the glycosylation profile on the antigen binding affinity should be analyzed in this experiment. The binding curves of purified IgM012_GL produced by the different expression hosts (CHO versus HEK) and systems (stable expression in CHO cells versus transient expression in HEK cells) overlapped (Fig. 10B) showing that minor changes in glycosylation did not affect the binding affinity. A more pronounced impact of the expression system would have been expected if the polymer distribution changed dramatically, since this would change the number of binding sites per IgM molecule. However, both expression host cell lines produced a similar polymer distribution.

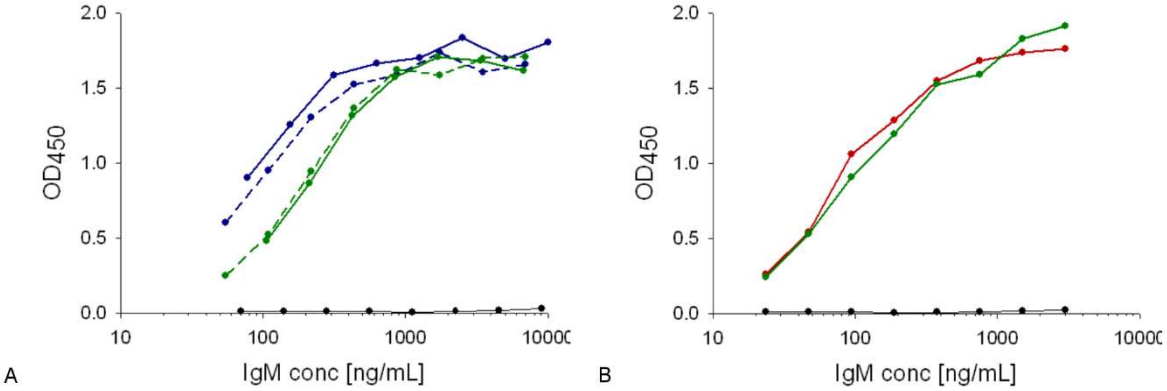


Figure 10: Binding to the antigen (UG37) of anti-gp140 IgMs. UG37 was coated on a microtiter plate. The washed wells were incubated with 50 µL/well of the IgM sample. Binding of the IgM to UG37 was detected with anti-kappa-HRP conjugate and TMB. All samples were analyzed in duplicates, except for the negative control, which was the IgM617 (black lines). **A** Impact of the purification conditions on antigen binding of IgM012 (blue) and IgM012_GL (green). Supernatant (dashed lines) and affinity purified product (continuous lines) produced in CHO cells were compared. **B** Impact of the expression system and accompanying post-translational differences on antigen binding of purified IgM012_GL. HEK (red) and CHO (green).

The interaction of IgM with C1q was studied as representative for the interaction of recombinant IgM with an effector protein. The complement cascade is activated by IgM through the classical pathway (Fig. 11). This pathway requires the interaction of C1q, which is one subunit of the C1 complex, with IgM or another activator. Upon IgM recognition, the collagenous regions of C1q underlies a structural change, which is transmitted to another C1 subunit, called C1r [118]. The serine protease domain of C1r thereby becomes active, leading to a cascade of proteolytic activation of the C1s, C2 and C4 molecules (Fig. 11, reviewed in [119]). The C4b and C2a fragments assemble into the C3 convertase, which cleaves C3 into fragments C3a and C3b to generate C5 convertase (C4b2a3b). The released C5b fragments form together with C6-9 molecules the membrane attack complex (MAC) which creates pores on the target cell membranes for lysis (Fig 11). The peptides C3a and C5a have chemotactic properties and recruit immune effector cells to the site of infection.

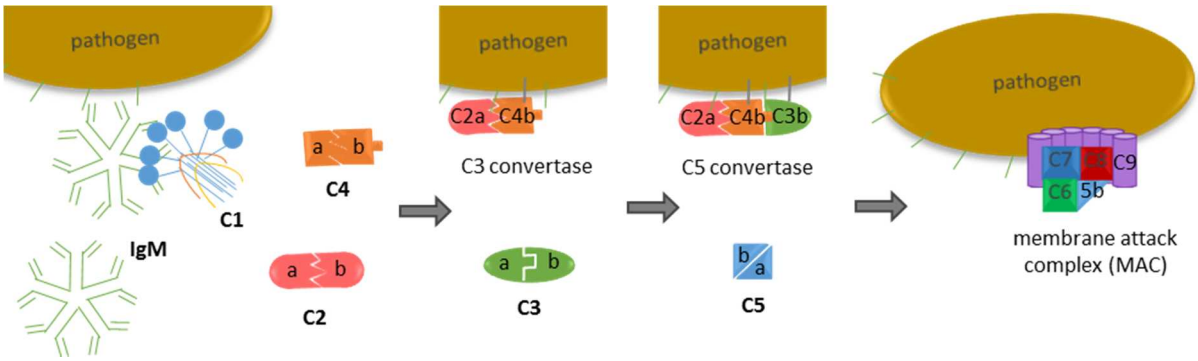


Figure 11: Classical complement pathway. Interaction of IgM with C1 complex activates a proteolytic cascade involving C2, C3, C4 and C5. Finally, the membrane attack complex is formed leading to cell lysis.

Binding of all recombinant IgMs to C1q was confirmed with surface plasmon resonance (data not shown). To study complement activation and the compounds of the complement cascade, an ELISA based method was developed in the 1980s [120]. IgM is thereby adsorbed to microtiter plates in the published set-up to mimic an immune lattice [120]. After saturation and incubation with human serum, the deposition of activated complement proteins is detected with monospecific antibodies.

The deposition of the C4b fragment, which is a marker for the classical pathway, was detected in the herein presented work to compare the recombinant IgMs and the human polyclonal serum IgM (Sigma) regarding their ability to activate the complement system. Additionally, the impact of the glycosylation (different expression hosts) and polymeric status (different recombinant IgM antibodies) was investigated. The coated IgM was incubated with normal human serum (NHS), NHS depleted for C1q (NHSD) as negative control or NHSD with reconstituted C1q. IgMs that were incubated with NHSD showed no cleavage of the C4 into C4a and C4b (Fig. 12), which could be reconstituted by the addition of 4 µg/mL C1q. These results showed that the interaction between IgM and C1q initiate the classical complement pathway. The polyclonal IgM isolated from human serum (pIgM) was used as control to estimate the basal level of activation. No difference between the pIgM and the recombinant IgMs could be detected in this experiment (Fig. 12), which leads to the assumption that the recombinant IgMs were as active as the serum derived IgM. However, it cannot be excluded that a few active pentameric IgMs in each sample are sufficient to activate the cascade due to the high sensitivity of the ELISA assay. The glycosylation, particularly GS4 (Asn402), was reported to be important for the C1q binding and

complement-dependent cytolysis [13,14]. The IgMs produced by HEK and CHO exhibited only minor changes in the oligomannose composition attached at GS4 and activated the complement cascade to a similar extent (Fig. 12). This result suggested that the glycan type at the GS4 is more important than the specific composition indicated by microheterogeneity. Furthermore, it is known that IgM monomers are not able to activate the complement-mediated cytolysis [121]. Hence, it was of interest, if the IgMs produced with different polymer patterns possess different complement activation potentials because of the different dimer portions. However, a decreased activation level was not observed for the IgM012 or IgM012_GL, which produced ~10-20 % dimers. Therefore, it cannot be excluded that also dimers activate the complement cascade. In contrast, it is possible that the coated pentameric IgMs are sufficient for complement activation as it is the predominant polymer in all model IgMs.

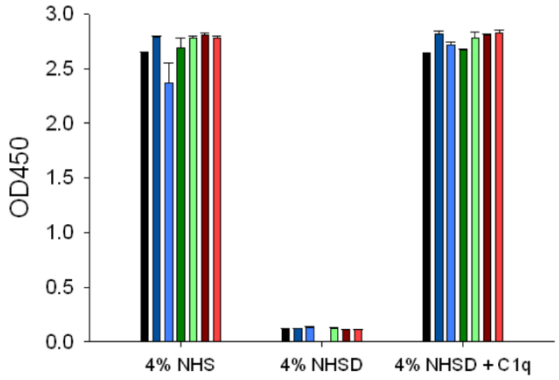


Figure 12: Complement activation via IgM C1q interaction. The following IgMs were coated on a 96-well plate: polyclonal IgM from human serum (black), IgM012 produced in CHO (dark blue), IgM012 produced in HEK (light blue), IgM012_GL produced in CHO (dark green), IgM012_GL produced in HEK (light green), IgM617 produced in CHO (dark red), IgM617 produced in HEK (light red). To prevent non-specific binding to the plate, the surface was saturated with 2 % BSA. Each IgM sample was incubated with 4 % normal human serum (NHS), 4 % NHS depleted by C1q (NHSD) or 4 % NHSD with reconstituted C1q (4 µg/mL). Cleavage of C4 into C4a and C4b was detected with an antiserum to human C4 specific for C4b and an anti-rabbit-HRP antibody conjugate. Visualization was performed with TMB and a plate reader. Samples were performed in duplicates.

The classical pathway was further studied by visualization of the interaction between IgM and the C1q subunit with TEM. As described in a previous chapter, the IgM pentamers exhibited a star-shaped structure (Fig. 7). The C1q itself was investigated to distinguish IgM

from C1q in the complexes (Fig. 13A). C1q assembles from 18 polypeptide chains, which have a characteristic shape that resembles a bouquet of tulips [122]. The six peripheral globular regions as well as the central bundle of fibers could be identified with TEM (Fig. 13A). IgM and C1q were mixed in a 1:1 ratio and incubated overnight prior to staining. Only the characteristic star-shape of the IgM012 could be detected in the mixture of IgM012 and C1q, but no C1q molecule alone or within the complexes (Fig. 13B). One explanation is that the C1q is covered by the IgM as mainly the top view was observed. In this case, C1q and IgM would interact with a 1:1 binding. However, the absence of free C1q remains an open question. Therefore this interaction pair needs further investigation. Individual IgM617 and C1q particles as well as large complexes comprising several molecules could be found in the mixture of IgM617 and C1q (Fig. 13C). Such a clustering was not reported before. More precisely, a cluster of IgMs was surrounded by the globular regions of C1q (Fig. 13C, enlarged image), which contain the recognition site of the IgM Fc part [123]. In this conformation, the bundle of fibers of C1q, to which the proteases C1s and C1r are associated, protrude into the periphery and can transmit the activation signal to the proteases. However, these results should be verified in further analysis to obtain a better understanding in IgM and C1q complexation as these two mixtures contained different types of complexes.

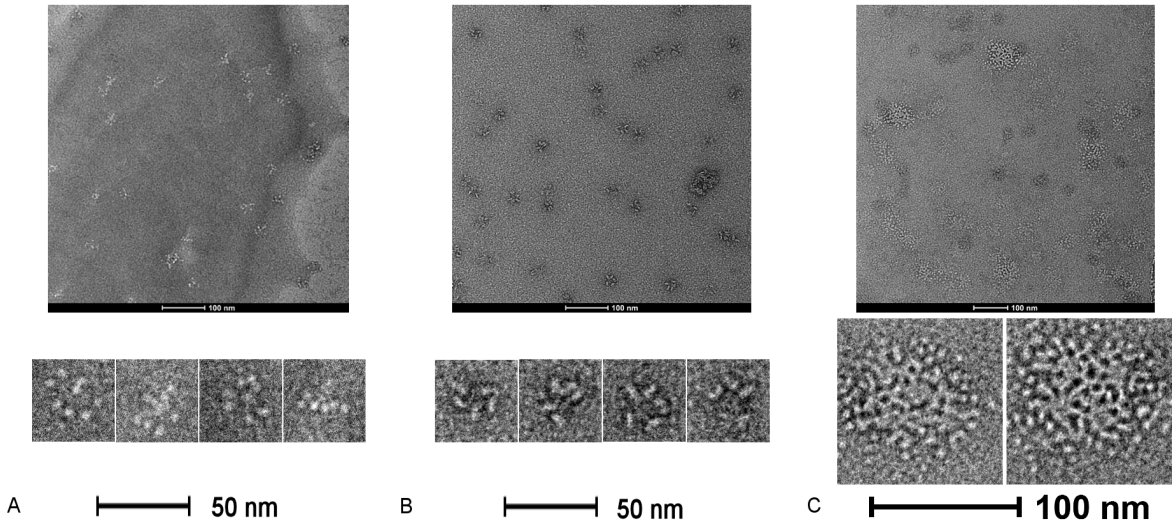


Figure 13: Non-processed images of the negative stain EM of C1q-IgM complexes. Protein solutions were adsorbed onto carbon grids and stained with 2 % SST. Images were taken with a FEI Tecnai F20 microscope at 200 keV. Upper pictures: scale bar represents 100 nm. Lower pictures zoomed into protein particles or complexes, scale bar represents 50 nm (A,B) and 100 nm (C) respectively. **A** 20 ng/mL C1q; **B** 10 ng/mL C1q + 10 ng/mL IgM012; **C** 10 ng/mL C1q + 10 ng/mL IgM617.

4. Conclusion

Although IgM antibodies harbor a huge therapeutic potential, often clinical investigation is impeded by challenges in manufacturing. Incomplete polymer formation, interaction with nucleic acids and site specific glycosylation provide the basis for reduced biological activity and complex downstream processing.

This thesis aimed to encounter the complexity in recombinant IgM production. First, the impact of the cultivation was investigated. Thereby, the process conditions as well as the host cell line were taken into account. A design of experiment study was performed to analyze if the factors process temperature and pH have an impact on IgM012_GL quantity and quality. In this experiment, the cultivation of the CHO cells was beneficial at higher temperatures to improve the IgM titer. The quality, however, was not affected by cultivation temperature or pH and can therefore be excluded as factor responsible for quality variation. The rationale behind the pH and temperature reduction was to slow down the speed of expression in order to decrease intracellular product accumulation and improve polymerization. This strategy was already successfully used for other aggregation prone proteins such as Epo-Fc or IgG to decrease the amount of aggregates and to increase the product concentration in the culture supernatant [70,124]. Intracellular product accumulation was measured with flow cytometry and did not reveal an improvement with temperature or pH reduction. In addition, the fraction of dimers could not be decreased by pH or temperature reduction. Concluding, intracellular aggregation and incomplete polymer formation is not induced by an enhanced expression rate. Another effect of pH and temperature reduction is a slowed metabolism, which keeps the cells viable for a prolonged time [70]. Thus, we thought that the amount of contaminating nucleic acids could be minimized. Although a prolonged viability was observed in our study, the nucleic acid contamination could not be reduced. In contrast, accumulation of nucleic acids was observed with prolonged cultivation time. Process conditions are known to influence the glycosylation of different glycoproteins [125]. In case of IgM, no impact of cultivation temperature or pH on the total glycan content could be observed. Concluding, results obtained with “simpler” model proteins are rarely transferable to the complex IgM molecule, which has to be investigated individually.

Another factor of the cultivation is the host cell line and the expression system. IgM produced by stable CHO cell lines and transient HEK cells were compared regarding productivity, polymer distribution and the attached glycosylation pattern. The transient expression of all model IgMs in HEK cells was successful, however, the cell growth and product yield was inconsistent to a distinct degree from one transfection to another. This variation can most likely be contributed to the transient expression system rather than to the host cell line. The IgM titers were only slightly lower in the transient system at comparable viable cell densities. The polymer distribution of the individual model IgMs was independent of the expression system and host cell line, but differed amongst the three IgM model proteins. Glycosylation is known to depend on the host cell line [125]. The glycosylation pattern of the IgM012 produced in CHO was compared with IgM produced in another mammalian host cell line (PER.C6) and isolated from the human serum [67]. The two mammalian cell lines attached a similar glycan pattern as described for human IgM isolated from serum, namely a predominantly complex glycosylation at glycosylation sites 1-3 and oligomannose type at GS4 and GS5 [67]. In a further study, the three model IgMs were produced by HEK and CHO cells. The transient HEK cells tended to secrete less processed IgMs as the fraction of oligomannose type glycans and terminal N-acetylglucosamins was higher compared to the CHO produced counterpart. At GS1, the transient HEK cells attached a minimum of 50% of the oligomannose type. Such differences could be observed for a range of transiently expressed proteins in another study and presumably rely on an inefficient glycan processing machinery of the HEK host cell line rather than on the transient expression system [93]. Therefore, the host cell line should be carefully selected by considering the effect of an altered glycosylation on the application. The transient expression system, however, can be useful for fast product generation or screening of different mutants which not necessarily need consistent production of high amounts. In the latter case, stable cell lines should be generated.

Some quality analysis and biological assays require a high purity of the investigated sample material. Therefore, a suitable purification strategy needed to be established. Previously published methods for IgM purification were inefficient and time consuming. During the establishment of an affinity chromatography purification with the POROS CaptureSelect™ IgM Affinity Matrix (Thermo Fisher Scientific), it became clear that the frequently used very acidic elution conditions, lead to unfolding and aggregation of the IgM012_GL. A gentler pH

in combination with an increased ionic strength improved the elution yields by six fold and was verified with other IgMs. The optimization of the purification yields enabled to reduce the amount of protein in the load and thereby the volume of crude supernatant, which decreased production costs and time. Quality analyses revealed that nucleic acids co-elute with the IgMs during affinity chromatography. Nucleic acid contamination interferes with biological assays such as the complement activation assay. Therefore, conditions to separate nucleic acids and IgM in preparative SE chromatography were set up. Finally, the purity could be verified with UV-Vis spectroscopy. This gentle purification strategy allowed further characterization of structure, quality attributes and biological activity of the complex molecules.

The structure was analyzed with negative stain TEM. The three recombinant model IgMs had a star-like assembly of monomers into pentamers with projecting flexible Fab units that were rarely resolved. As various conformations were observed, the common model of a ridged star conformation should be replaced by a model of symmetric and asymmetric pentamers that are very flexible and able to perform continuous movements. Dimeric IgM could not be identified as the structures were too small and flexible for negative stain TEM.

The formation of correctly assembled pentamers and incomplete polymers was characterized with the comparison of the intracellular polymer distribution and investigation of the stability of purified pentamers. CHO cells secreting the three model IgMs in different quality were compared to identify if the intracellular assembly is the origin of incomplete polymer formation. All cell lines had a higher intracellular dimer fraction compared to the supernatant, which suggests that incomplete pentamers were recognized as not correctly folded by the quality control mechanisms and retained within the host cells. The incomplete IgM is most likely removed via the proteasome degradation pathway as only dimers and monomers were found in the cytosolic fraction. The lower producing cell lines IgM012 and IgM012_GL contained less pentamers in the ER/Golgi fraction compared to IgM617, which leads to the assumption that the pentamer formation rate is slower for IgM012 and IgM012_GL than for IgM617 and that the pentamers are secreted immediately. As no trimers and tetramers were found in the ER/Golgi fraction, the monomers and dimers seem to represent an intracellular pool of assembly intermediates from which higher polymers are built up. The assembly of trimers and tetramers seemed to be the rate limiting step, which could be influenced by the interactions between the variable regions of the

subunits. In addition, the aggregation of pentamers into insoluble Russell bodies correlated inversely with the amount of pentamers in the ER/Golgi fraction and the productivity of the cell lines. The difficult-to-express IgMs might have a higher tendency to abnormal HC-LC stoichiometry and incorrect disulfide bond connection leading to the formation of Russell bodies and the removal of pentamers from the ER/Golgi pathway. This would explain why IgM012 and IgM012_GL appeared in a lower pentamer/dimer ratio in the ER. Due to the high dimer concentration in the ER/Golgi, not all dimers were retained by the quality control and some were accidentally secreted. In case of IgM617, the intracellular polymer pool contained more correctly assembled pentamers, which decreased the chance of dimer secretion. Dimer secretion in combination with slow pentamer formation and secretion led to the reduction of IgM quality in the supernatant.

Another factor that could reduce the IgM quality in the supernatant is the disassembly of pentamers after secretion, because of the instability of the IgM structure. Therefore, pentamer and dimer of IgM012_GL were separated with SE chromatography and analyzed 45 days after separation. This approach showed that the IgM dimer is not a product of pentamer dissociation, but originated from the incomplete assembly in the host cell line.

Finally, the interaction of recombinant IgM with its natural partners was studied as it can be affected by the manufacturing conditions or variations in polymer distribution and post-translational modifications. By comparing the antigen binding affinity of IgM012 and IgM012_GL before and after the purification, it was confirmed that the acidic pH and high ionic strength of the elution buffer did not lead to a partial denaturation e.g. in the paratope, which would have decreased the binding affinity. Hence, characterization of the intact IgM was ensured. Furthermore, the impact of the expression host was analyzed by comparing the antigen binding of purified IgM012_GL produced by CHO and HEK cells. As described previously, both host cell lines slightly differ in their attached glycan profile. However, the antigen binding of IgM012_GL did not depend on the heterogeneity of the Fc glycosylation.

Compared to antigen binding, the Fc glycosylation is more important for the antibody effector functions, at least in case of IgG [126]. One effector protein of IgM is C1q, which is part of the complement cascade. It was shown in the herein presented thesis that recombinant human IgM activated the complement cascade via C1q interaction with similar efficiency to IgM isolated from human serum. The glycosylation at position GS4 was

previously reported to be important for the activation [13,14]. In addition, monomeric IgM was not able to activate the complement cascade [121]. Therefore, the activation capability of IgMs produced by two host cell lines and with different polymer distribution was investigated. No difference in activation level was detected for the recombinant IgMs with variable pentamer content (three model IgMs) or glycosylation (two host cell lines). This unexpected result could be caused by a very sensitive detection assay which required only a few correctly folded and glycosylated pentamers for activation. Another explanation could be that the oligomannose type at GS4, which is attached by both host cell lines, is more important than the specific glycan structure. In addition, dimers seemed to activate the cascade since pentamer/dimer mixtures (IgM012 and IgM012_GL) reached similar activation levels as pentamers (IgM617). Further investigations are necessary to certainly explain the reasons for the similar activation level.

The interaction of IgM and C1q was further studied by visualization of the complexes. The structure of such complexes could not be clearly defined as two analyzed model IgMs reveals different complexes with C1q. Again, further investigations should clarify if IgM-C1q-interaction lead to a complex of one IgM molecule and one C1q molecule or a clustering of several IgM and C1q molecules.

All in all, the results of this thesis demonstrate the complexity of the IgM molecule and shed light into challenges of recombinant protein production. During this work, important aspects of quality attributes were investigated. The optimization of the purification process enabled us to produce sufficient amounts of the protein in high purity to analyze the interaction of IgM with natural binding partners. However, there is still a high demand on further investigation, which could take steps forward in fundamental research by studying the biological role of IgM and its complex interaction network. Acquiring such knowledge would provide the basis to develop new therapeutic IgM antibodies.

5. References

[1] J. Rogers, P. Early, C. Carter, K. Calame, M. Bond, L. Hood, R. Wall, Two mRNAs with different 3' ends encode membrane-bound and secreted forms of immunoglobulin μ chain, Cell. 20 (1980) 303–312. doi:10.1016/0092-8674(80)90616-9.

[2] R. Sitia, M. Neuberger, C. Alberini, P. Bet, A. Fra, C. Valetti, G. Williams, C. Milstein, Developmental regulation of IgM secretion: The role of the carboxy-terminal cysteine, Cell. 60 (1990) 781–790. doi:10.1016/0092-8674(90)90092-S.

[3] J. Mestecky, R.E. Schrohenloher, Site of attachment of J chain to human immunoglobulin M, Nature. 249 (1974) 650–652. doi:10.1038/249650a0.

[4] V. Sørensen, I.B. Rasmussen, V. Sunvold, T.E. Michaelsen, I. Sandlie, Structural requirements for incorporation of J chain into human IgM and IgA, Int. Immunol. 12 (2000) 19–27. doi:10.1093/intimm/12.1.19.

[5] E.J. Wiersma, C. Collins, S. Fazel, M.J. Shulman, Structural and functional analysis of J chain-deficient IgM., J. Immunol. 160 (1998) 5979–5989.

[6] C.T. Hughey, J.W. Brewer, A.D. Colosia, W.F. Rosse, R.B. Corley, Production of IgM hexamers by normal and autoimmune B cells: implications for the physiologic role of hexameric IgM., J. Immunol. 161 (1998) 4091–4097.

[7] P.J. Roberts-Thomson, K. Shepherd, Molecular size heterogeneity of immunoglobulins in health and disease., Clin. Exp. Immunol. 79 (1990) 328–334.

[8] S. Fillatreau, A. Six, S. Magadan, R. Castro, J.O. Sunyer, P. Boudinot, The astonishing diversity of Ig classes and B cell repertoires in teleost fish, Front. Immunol. 4 (2013) 1–14. doi:10.3389/fimmu.2013.00028.

[9] J. Schwager, I. Hadji-Azlmi, Mitogen-induced B-cell differentiation in Xenopus laevis, Differentiation. 27 (1984) 182–188. doi:10.1111/j.1432-0436.1984.tb01426.x.

[10] J.N. Arnold, M.R. Wormald, D.M. Suter, C.M. Radcliffe, D.J. Harvey, R.A. Dwek, P.M. Rudd, R.B. Sim, Human serum IgM glycosylation: Identification of glycoforms that can bind to Mannan-binding lectin, J. Biol. Chem. 280 (2005) 29080–29087. doi:10.1074/jbc.M504528200.

[11] E.S.X. Moh, C.-H. Lin, M. Thaysen-Andersen, N.H. Packer, Site-Specific N-Glycosylation of Recombinant Pentameric and Hexameric Human IgM, J. Am. Soc. Mass Spectrom. 27 (2016) 1143–1155. doi:10.1007/s13361-016-1378-0.

[12] T. Anelli, S. Ceppi, L. Bergamelli, M. Cortini, S. Masciarelli, C. Valetti, R. Sitia, Sequential steps and checkpoints in the early exocytic compartment during secretory IgM biogenesis, EMBO J. 26 (2007) 4177–4188. doi:10.1038/sj.emboj.7601844.

[13] S. Muraoka, M.J. Shulman, Structural requirements for IgM assembly and cytolytic activity. Effects of mutations in the oligosaccharide acceptor site at Asn402., J. Immunol. 142 (1989) 695–701.

[14] J.F. Wright, M.J. Shulman, D.E. Isenman, R.H. Painter, C1 binding by mouse IgM. The effect of abnormal glycosylation at position 402 resulting from a serine to asparagine exchange at residue 406 of the μ-chain, J. Biol. Chem. 265 (1990) 10506–10513. doi:10.18865/ed.26.1.37.

[15] L.W. Clem, Phylogeny of Immunoglobulin Function, J. Biol. Chem. 246 (1971) 9–15.

[16] B. van Furth, H.R.E. Schuit, M.D. Hijmans, The immunological development of the human fetus, J. Exp. Med. 122 (1965) 1173–1188.

[17] A.F. Ochsenbein, T. Fehr, C. Lutz, M. Suter, F. Brombacher, H. Hengartner, R.M. Zinkernagel, Control of early viral and bacterial distribution and disease by natural antibodies, Science (80-.). 286 (1999) 2156–2159. doi:10.1126/science.286.5447.2156.

[18] S. Gupta, A. Gupta, Selective IgM deficiency-An underestimated primary immunodeficiency, Front. Immunol. 8 (2017) 1–7. doi:10.3389/fimmu.2017.01056.

[19] M. Boes, C. Esau, M.B. Fischer, T. Schmidt, M. Carroll, J. Chen, Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM., J. Immunol. 160 (1998) 4776–87.

[20] P. Brandtzaeg, H. Prydz, Direct evidence for an integrated function of J chain and secretory component in epithelial transport of immunoglobulins, Nature. 311 (1984) 71–73.

[21] A. Shibuya, N. Sakamoto, Y. Shimizu, K. Shibuya, M. Osawa, T. Hiroyama, H.J. Eyre, G.R. Sutherland, Y. Endo, T. Fujita, T. Miyabayashi, S. Sakano, T. Tsuji, E. Nakayama, J.H. Phillips, L.L. Lanier, H. Nakauchi, Fcα/μ receptor mediates endocytosis of IgM-coated microbes, Nat. Immunol. 1 (2000) 441–446. doi:10.1038/80886.

[22] H. Shima, H. Takatsu, S. Fukuda, M. Ohmae, K. Hase, H. Kubagawa, J.Y. Wang, H. Ohno, Identification of TOSO/FAIM3 as an Fc receptor for IgM, Int. Immunol. 22 (2009) 149–156. doi:10.1093/intimm/dxp121.

[23] N. Baumgarth, O.C. Herman, G.C. Jager, L. Brown, L. a. Herzenberg, L. a. Herzenberg, Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system., Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 2250–2255. doi:10.1073/pnas.96.5.2250.

[24] R.R. Hardy, K. Hayakawa, CD5 B cells, a fetal B cell lineage, Adv. Immunol. 55 (1994) 297–338. doi:10.1111/myc.12331.

[25] M. Boes, A.P. Prodeus, T. Schmidt, M.C. Carroll, J. Chen, A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection., J. Exp. Med. 188 (1998) 2381–2386. doi:10.1084/jem.188.12.2381.

[26] S. Avrameas, Natural autoantibodies: from “horror autotoxicus” to “gnothi seauton,” Immunol. Today. (1991). doi:10.1016/S0167-5699(05)80045-3.

[27] S.R. Bruce, R.W.C. Dingle, M.L. Peterson, B-cell and plasma-cell splicing differences: A potential role in regulated immunoglobulin RNA processing, Rna. 9 (2003) 1264–1273. doi:10.1261/rna.5820103.

[28] S. Lederman, M.J. Yellin, A.M. Cleary, A. Pernis, G. Inghirami, L.E. Cohn, L.R. Covey, J.J. Lee, P. Rothman, L. Chess, T-BAM/CD40-L on helper T lymphocytes augments lymphokine-induced B cell Ig isotype switch recombination and rescues B cells from programmed cell death., J. Immunol. 152 (1994) 2163–2171.

[29] T. V. Obukhanych, M.C. Nussenzweig, T-independent type II immune responses generate memory B cells, J. Exp. Med. 203 (2006) 305–310. doi:10.1084/jem.20052036.

[30] K.-M. Toellner, W.E. Jenkinson, D.R. Taylor, M. Khan, D.M.-Y. Sze, D.M. Sansom, C.G. Vinuesa, I.C.M. MacLennan, Low-level hypermutation in T cell-independent germinal centers compared with high mutation rates associated with T cell-dependent germinal centers., J. Exp. Med. 195 (2002) 383–389. doi:10.1084/jem.20011112.

[31] M.F. Goldstein, A.L. Goldstein, E.H. Dunsky, D.J. Dvorin, G.A. Belecanech, K. Shamir, Selective IgM immunodeficiency: Retrospective analysis of 36 adult patients with review of the literature, Ann. Allergy, Asthma Immunol. (2006). doi:10.1016/S1081-1206(10)60962-3.

[32] T.T.T. Nguyen, E.R. A, N. Baumgarth, Natural IgM prevents autoimmunity by enforcing B cell central tolerance induction, J. Immunol. 194 (2015) 1489–1502. doi:10.4049/jimmunol.1401880.

[33] A. Norrby-Teglund, K.N. Haque, L. Hammarström, Intravenous polyclonal IgM-enriched immunoglobulin therapy in sepsis: A review of clinical efficacy in relation to microbiological aetiology and severity of sepsis, J. Intern. Med. 260 (2006) 509–516. doi:10.1111/j.1365-2796.2006.01726.x.

[34] R. Rieben, A. Roos, Y. Muizert, C. Tinguely, A. Gerritsen, M. Daha, Immunoglobulin M-enriched human intravenous immunoglobulin prevents complement activation in vitro and in vivo in a rat model of acute inflammation, Blood. 93 (1999) 942–951.

[35] S. Gong, K. Tomusange, V. Kulkarni, O.S. Adeniji, S.K. Lakhashe, D. Hariraju, A. Strickland, E. Plake, P.A. Frost, S.J. Ratcliffe, L. Wang, E.M. Lafer, R.M. Ruprecht, Anti-HIV IgM protects against mucosal SHIV transmission, Aids. 32 (2018) F5–F13. doi:10.1097/QAD.0000000000001857.

[36] D.E. Briles, C. Forman, S. Hudak, L.J. Claflin, Anti-phosphorylcholine antibodies of the T15 idiotype are optimally protective against Streptococcus pneumoniae, J. Exp. Med. 156 (1982) 1177–1185.

[37] Y. Que, H. Lazar, M. Wolff, B. François, P. Laterre, E. Mercier, J. Garbino, J.-L. Pagani, J.-P. Revelly, E. Mus, A. Perez, M. Tamm, J.-J. Rouby, Q. Lu, J. Chastre, P. Eggimann, Assessment of panobacumab as adjunctive immunotherapy for the treatment of nosocomial Pseudomonas aeruginosa pneumonia, Eur. J. Clin. Microbiol. Infect. Dis. 33 (2014) 1861–1867. doi:10.1007/s10096-014-2156-1.

[38] C.J. Binder, S. Hörkkö, A. Dewan, M.-K. Chang, E.P. Kieu, C.S. Goodyear, P.X. Shaw, W. Palinski, J.L. Witztum, G.J. Silverman, Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL, Nat. Med. 9 (2003) 736–743. doi:10.1038/nm876.

[39] J. Karvonen, M. Päivänsalo, A. Kesäniemi, S. Hörkkö, Immunoglobulin M Type of Autoantibodies to Oxidized Low-Density Lipoprotein Has an Inverse Relation to, Circulation. 108 (2003) 2107–2112. doi:10.1161/01.CIR.0000092891.55157.A7.

[40] Y. Chen, S. Khanna, C.S. Goodyear, Y.B. Park, E. Raz, S. Thiel, C. Grönwall, J. Vas, D.L. Boyle, M. Corr, D.H. Kono, G.J. Silvermann, Regulation of Dendritic Cells and Macrophages by an Anti-Apoptotic Cell Natural Antibody that Suppresses TLR Responses and Inhibits Inflammatory Arthritis, J. Immunol. 183 (2009) 1346–1359. doi:10.4049/jimmunol.0900948.

[41] S. Werwitzke, D. Trick, K. Kamino, T. Matthias, K. Kniesch, B. Schlegelberger, R.E. Schmidt, T. Witte, Inhibition of Lupus Disease by Anti – Double-Stranded DNA Antibodies of the IgM Isotype in the (NZB x NZW) F 1 Mouse, Arthritis Rheum. 52 (2005) 3629–3638. doi:10.1002/art.21379.

[42] C. Grönwall, E. Akhter, C. Oh, R.W. Burlingame, M. Petri, G.J. Silverman, IgM autoantibodies to distinct apoptosis-associated antigens correlate with protection from cardiovascular events and renal disease in patients with SLE, Clin. Immunol. 142 (2012) 390–398. doi:10.1016/j.clim.2012.01.002.

[43] T. Mehrani, M. Petri, IgM anti-β 2 glycoprotein I is protective against lupus nephritis and renal damage in systemic lupus erythematosus, J. Rheumatol. (2011). doi:10.3899/jrheum.100650.

[44] M. Stravalaci, L. Tapella, M. Beeg, A. Rossi, P. Joshi, E. Pizzi, M. Mazzanti, C. Balducci, G. Forloni, E. Biasini, M. Salmona, L. DIomede, R. Chiesa, M. Gobbi, The Anti-Prion Antibody 15B3 Detects Toxic Amyloid-β Oligomers, J. Alzheimer's Dis. 53 (2016) 1485–1497. doi:10.3233/JAD-150882.

[45] B. Wootla, A. Denic, A.E. Warrington, M. Rodriguez, A monoclonal natural human IgM protects axons in the absence of remyelination, J. Neuroinflammation. 13 (2016) 1–9. doi:10.1186/s12974-016-0561-3.

[46] A. Eisen, B.M. Greenberg, J.D. Bowen, D.L. Arnold, A.O. Caggiano, A double-blind, placebo-controlled, single ascending-dose study of remyelinating antibody rHIgM22 in people with multiple sclerosis, Mult. Scler. J. – Exp. Transl. Clin. 3 (2017) 1–11. doi:10.1177/2055217317743097.

[47] X. Xu, A. Denic, A. Warrington, A.J. Bieber, M. Rodriguez, Therapeutics to Promote CNS Repair: A Natural Human Neuron- Binding IgM Regulates Membrane-Raft Dynamics and Improves Motility in a Mouse Model of Multiple Sclerosis, J. Clin. Immunol. 33 (2013) 50–56. doi:10.1007/s10875-012-9795-8.

[48] X. Xu, A. Denic, L.R. Jordan, N.J. Wittenberg, A.E. Warrington, B. Wootla, L.M. Papke, L.J. Zoecklein, D. Yoo, J. Shaver, S.-H. Oh, L.R. Pease, M. Rodriguez, A natural human IgM that binds to gangliosides is therapeutic in murine models of amyotrophic lateral sclerosis, Dis. Model. Mech. 8 (2015) 831–842. doi:10.1242/dmm.020727.

[49] Y. Azuma, Y. Ishikawa, S. Kawai, T. Tsunenari, H. Tsunoda, T. Igawa, S.I. Iida, M. Nanami, M. Suzuki, R.F. Irie, M. Tsuchiya, H. Yamada-Okabe, Recombinant human hexamer-dominant IgM monoclonal antibody to ganglioside GM3 for treatment of melanoma, Clin. Cancer Res. 13 (2007) 2745–2750. doi:10.1158/1078-0432.CCR-06-2919.

[50] V. Mulens, A. De La Torre, P. Marinello, R. Rodríguez, J. Cardoso, R. Díaz, M. O'Farrill, A. Macias, C. Viada, G. Saurez, A. Carr, T. Crombet, Z. Mazorra, R. Perez, L.E. Fernandez, Immunogenicity and safety of a NeuGcGM3 based cancer vaccine: Results from a controlled study in metastatic breast cancer patients, Hum. Vaccin. 6 (2010) 736–744. doi:10.4161/hv.6.9.12571.

[51] S. Brändlein, T. Pohle, N. Ruoff, E. Wozniak, H. Mu, H.P. Vollmers, S. Brändlein, T. Pohle, N. Ruoff, E. Wozniak, H.K. Müller-Hermelink, H.P. Vollmers, Natural IgM Antibodies and Immunosurveillance Mechanisms against Epithelial Cancer Cells in Humans, Cancer Res. 63 (2003) 7995–8005.

[52] F. Hensel, W. Timmermann, B.H.A. Von Rahden, A. Rosenwald, S. Brändlein, B. Illert, Ten-year follow-up of a prospective trial for the targeted therapy of gastric cancer with the human monoclonal antibody PAT-SC1, Oncol. Rep. 31 (2014) 1059–1066. doi:10.3892/or.2014.2987.

[53] L. Rasche, J. Duell, I.C. Castro, V. Dubljevic, M. Chatterjee, S. Knop, F. Hensel, A. Rosenwald, H. Einsele, M.S. Topp, S. Brändlein, GRP78-directed immunotherapy in relapsed or refractory multiple myeloma - results from a phase 1 trial with the monoclonal immunoglobulin M antibody PAT-SM6, Haematologica. 100 (2015) 377–384. doi:10.3324/haematol.2014.117945.

[54] M. Liedtke, C.J. Twist, B.C. Medeiros, J.R. Gotlib, C. Berube, M.M. Bieber, N.M. Bhat, N.N. Teng, S.E. Coutre, Phase I trial of a novel human monoclonal antibody mab216 in patients with relapsed or refractory B-cell acute lymphoblastic leukemia, Haematologica. 97 (2012) 30–37. doi:10.3324/haematol.2011.045997.

[55] J. Mordoh, C. Silva, M. Albarellos, A.I. Bravo, C. Kairiyama, Phase I clinical trial in cancer patients of a new monoclonal antibody FC-2.15 reacting with tumor proliferating cells., J. Immunother. with Emphas. Tumor Immunol. 17 3 (1995) 151–160.

[56] R. DiPauli, W.C. Raschke, A Hybridoma Secreting IgM Anti-Iglb Allotype, in: F. Melchers, M. Potter (Eds.), Lymph. Hybridomas, Springer Berlin Heidelberg, Berlin, Heidelberg, 1979: pp. 37–39. doi:10.1007/978-3-642-67448-8_5.

[57] C.M. Alberini, P. Bet, C. Milstein, R. Sitia, Secretion of immunoglobulin M assembly intermediates in the presence of reducing agents, Nature. 374 (1990) 485–487. doi:10.1038/347485a0.

[58] A. Loos, C. Gruber, F. Altmann, U. Mehofer, F. Hensel, M. Grandits, C. Oostenbrink, G. Stadlmayr, P.G. Furtmüller, H. Steinkellner, Expression and glycoengineering of functionally active heteromultimeric IgM in plants, Proc. Natl. Acad. Sci. 111 (2014) 6263–6268. doi:10.1073/pnas.1408639111.

[59] C. Valetti, C.E. Grossi, C. Milstein, R. Sitia, Russell bodies: A general response of secretory cells to synthesis of a mutant immunoglobulin which can neither exit from, nor be degraded in, the endoplasmic reticulum, J. Cell Biol. 115 (1991) 983–994. doi:10.1083/jcb.115.4.983.

[60] A. Tchoudakova, F. Hensel, A. Murillo, B. Eng, M. Foley, L. Smith, F. Schoenen, A. Hildebrand, A.R. Kelter, L.L. Ilag, H.P. Vollmers, S. Brandlein, J. McIninch, J. Chon, G. Lee, M. Cacciuttolo, High level expression of functional human IgMs in human PER.C6® cells, MAbs. 1 (2009) 163–171. doi:10.4161/mabs.1.2.7945.

[61] S. Wolbank, R. Kunert, G. Stiegler, H. Katinger, Characterization of Human Class-Switched Polymeric (Immunoglobulin M [IgM] and IgA) Anti-Human Immunodeficiency Virus Type 1 Antibodies 2F5 and 2G12, J. Virol. 77 (2003) 4095–4103. doi:10.1128/JVI.77.7.4095.

[62] J.W. Brewer, T.D. Randall, R.M.E. Parkhouse, R.B. Corley, Mechanism and subcellular localization of secretory IgM polymer assembly, J. Biol. Chem. 269 (1994) 17338–17348.

[63] K. Vorauer-Uhl, J. Wallner, G. Lhota, H. Katinger, R. Kunert, IgM characterization directly performed in crude culture supernatants by a new simple electrophoretic method, J. Immunol. Methods. 359 (2010) 21–27. doi:10.1016/j.jim.2010.05.003.

[64] V. Chromikova, A. Mader, W. Steinfellner, R. Kunert, Evaluating the bottlenecks of recombinant IgM production in mammalian cells, Cytotechnology. 67 (2014) 343–356. doi:10.1007/s10616-014-9693-4.

[65] V. Chromikova, A. Mader, S. Hofbauer, C. Göbl, T. Madl, J.S. Gach, S. Bauernfried, P.G. Furtmüller, D.N. Forthal, L. Mach, C. Obinger, R. Kunert, Introduction of germline residues improves the stability of anti-HIV mAb 2G12-IgM, Biochim. Biophys. Acta - Proteins Proteomics. 1854 (2015) 1536–1544. doi:10.1016/j.bbapap.2015.02.018.

[66] J. Hennicke, D. Reinhart, F. Altmann, R. Kunert, Impact of temperature and pH on recombinant human IgM quality attributes and productivity, N. Biotechnol. 50 (2019) 20–26. doi:10.1016/j.nbt.2019.01.001.

[67] J. Hennicke, A.M. Lastin, D. Reinhart, C. Grünwald-Gruber, F. Altmann, R. Kunert, Glycan profile of CHO derived IgM purified by highly efficient single step affinity chromatography, Anal. Biochem. 539 (2017) 162–166. doi:10.1016/j.ab.2017.10.020.

[68] A. Moore, J. Mercer, G. Dutina, C.J. Donahue, K.D. Bauer, J.P. Mather, T. Etcheverry, T. Ryll, Effects of temperature shift on cell cycle, apoptosis and nucleotide pools in CHO cell batch cultues., Cytotechnology. 23 (1997) 47–54. doi:10.1023/A:1007919921991.

[69] C.H. Goey, J.M.H. Tsang, D. Bell, C. Kontoravdi, Cascading effect in bioprocessing—The impact of mild hypothermia on CHO cell behavior and host cell protein composition, Biotechnol. Bioeng. 114 (2017) 2771–2781. doi:10.1002/bit.26437.

[70] C. Kaisermayer, D. Reinhart, A. Gili, M. Chang, P.M. Aberg, A. Castan, R. Kunert, Biphasic cultivation strategy to avoid Epo-Fc aggregation and optimize protein expression, J. Biotechnol. 227 (2016) 3–9. doi:10.1016/j.jbiotec.2016.03.054.

[71] E. Trummer, K. Fauland, S. Seidinger, K. Schriebl, C. Lattenmayer, R. Kunert, K. Vorauer-Uhl, R. Weik, N. Borth, H. Katinger, D. Müller, Process Parameter Shifting: Part I. Effect of DOT, pH, and Temperature on the Performance of Epo-Fc Expressing CHO Cells Cultivated in Controlled Batch Bioreactors, Biotechnol. Bioeng. 94 (2006) 1033–1044. doi:10.1002/bit.21013.

[72] M. Butler, Optimisation of the cellular metabolism of glycosylation for recombinant proteins produced by mammalian cell systems, Cytotechnology. 50 (2006) 57–76. doi:10.1007/s10616-005-4537-x.

[73] M. Mason, B. Sweeney, K. Cain, P. Stephens, S. Sharfstein, Reduced Culture Temperature Differentially Affects Expression and Biophysical Properties of Monoclonal Antibody Variants, Antibodies. 3 (2014) 253–271. doi:10.3390/antib3030253.

[74] M. Brunner, J. Fricke, P. Kroll, C. Herwig, Investigation of the interactions of critical scale-up parameters (pH, pO2and pCO2) on CHO batch performance and critical quality attributes, Bioprocess Biosyst. Eng. 40 (2017) 251–263. doi:10.1007/s00449-016-1693-7.

[75] M. Bollati-Fogolín, G. Forno, M. Nimtz, H.S. Conradt, M. Etcheverrigaray, R. Kratje, Temperature Reduction in Cultures of hGM-CSF-expressing CHO Cells: Effect on Productivity and Product Quality, Biotechnol. Prog. 21 (2005) 17–21. doi:10.1021/bp049825t.

[76] H.T. Gan, J. Lee, S.M.A. Latiff, C. Chuah, P. Toh, W.Y. Lee, P. Gagnon, Characterization and removal of aggregates formed by nonspecific interaction of IgM monoclonal antibodies with chromatin catabolites during cell culture production, J. Chromatogr. A. 1291 (2013) 33–40. doi:10.1016/j.chroma.2013.03.028.

[77] J. Zhu, Mammalian cell protein expression for biopharmaceutical production, Biotechnol. Adv. (2012). doi:10.1016/j.biotechadv.2011.08.022.

[78] C.H. Chung, B. Mirakhur, E. Chan, Q.-T. Le, J. Berlin, M. Morse, B.A. Murphy, S.M. Satinover, J. Hosen, D. Mauro, R.J. Slebos, Q. Zhou, D. Gold, T. Hatley, D.J. Hicklin, T.A.E. Platts-Mills, Cetuximab-Induced Anaphylaxis and IgE Specific for Galactose-α-1,3-Galactose, N. Engl. J. Med. 358 (2008) 1109–1117. doi:10.1056/NEJMoa074943.

[79] P. Tangvoranuntakul, P. Gagneux, S. Diaz, M. Bardor, N. Varki, A. Varki, E. Muchmore, Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid, Proc. Natl. Acad. Sci. 100 (2003) 12045–12050. doi:10.1073/pnas.2131556100.

[80] T. Puck, S.J. Cieciura, A. Robinson, Genetics of somatic mammalian cells III. Long-term cultivation of euploid cells from human and animal subjects, J. Exp. Med. 108 (1958) 945–956. doi:10.1084/jem.108.6.945.

[81] X. Xu, H. Nagarajan, N.E. Lewis, S. Pan, Z. Cai, X. Lui, W. Chen, M. Xie, W. Wang, S. Hammond, M.R. Andersen, N. Neff, B. Passarelli, W. Koh, H.C. Fan, J. Wang, Y. Gui, K.H. Lee, M.J. Betenbaugh, S.R. Quake, I. Famili, B.O. Palsson, J. Wang, The genomic Sequence of the Chinese Hamster Ovary (CHO) K1 cell line, Nat. Biotechnol. 29 (2012) 735–741. doi:doi:10.1038/nbt.1932.

[82] G. Walsh, Biopharmaceutical benchmarks 2014, Nat Biotech. 32 (2014) 992–1000.

[83] K.P. Jayapal, K.F. Wlaschin, W.-S. Hu, M.G.S. Yap, Recombinant protein therapeutics from CHO cells-20 years and counting, Chem. Eng. Prog. 103 (2007) 40–47. doi:10.1017/CBO9781107415324.004.

[84] S.D.J. Chapple, A.M. Crofts, S.P. Shadbolt, J. McCafferty, M.R. Dyson, Multiplexed expression and screening for recombinant protein production in mammalian cells, BMC Biotechnol. 6 (2006) 1–15. doi:10.1186/1472-6750-6-49.

[85] F.L. Graham, J. Smiley, W.C. Russel, R. Nairn, Characteristics of a Human Cell Line Transformed by DNA from Human Adenovirus Type 5, J. Gen. Virol. 36 (1977) 59–66. doi:10.1099/0022-1317-36-1-59.

[86] C.M. Gorman, D. Gies, G. McCray, M. Huang, The human cytomegalovirus major immediate early promoter can be trans-activated by adenovirus early proteins, Virology. 171 (1989) 377–385. doi:10.1016/0042-6822(89)90605-3.

[87] G. Backliwal, M. Hildinger, I. Kuettel, F. Delegrange, D.L. Hacker, F.M. Wurm, Valproic acid: A viable alternative to sodium butyrate for enhancing protein expression in mammalian cell cultures, Biotechnol. Bioeng. 101 (2008) 182–189. doi:10.1002/bit.21882.

[88] P.L. Pham, S. Perret, B. Cass, E. Carpentier, G. St.-Laurent, L. Bisson, A. Kamen, Y. Durocher, Transient gene expression in HEK293 cells: Peptone addition posttransfection improves recombinant protein synthesis, Biotechnol. Bioeng. 90 (2005) 332–344. doi:10.1002/bit.20428.

[89] M. De Los Milagros Bassani Molinas, C. Beer, F. Hesse, M. Wirth, R. Wagner, Optimizing the transient transfection process of HEK-293 suspension cells for protein production by nucleotide ratio monitoring, Cytotechnology. 66 (2014) 493–514. doi:10.1007/s10616-013-9601-3.

[90] R. Heukeshoven, J. ; Dernick, Simplified Method for Silver Staining of Proteins in Polyacrylamide Gels and the Mechanism of Silver Staining, Electrophoresis. 6 (1985) 103–112. doi:10.1002/elps.1150060302.

[91] R. Sitia, A. Rubartelli, U. Hämmerling, Rich, The role of glycosylation in secretion and membrane expression of immunoglobulins M and A, Mol. Immunol. 21 (1984) 709–719. doi:https://doi.org/10.1016/0161-5890(84)90023-3.

[92] F. Higel, A. Seidl, F. Sörgel, W. Friess, N-glycosylation heterogeneity and the influence on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins, Eur. J. Pharm. Biopharm. 100 (2016) 94–100. doi:10.1016/j.ejpb.2016.01.005.

[93] A. Croset, L. Delafosse, J.P. Gaudry, C. Arod, L. Glez, C. Losberger, D. Begue, A. Krstanovic, F. Robert, F. Vilbois, L. Chevalet, B. Antonsson, Differences in the glycosylation of recombinant proteins expressed in HEK and CHO cells, J. Biotechnol. 161 (2012) 336–348. doi:10.1016/j.jbiotec.2012.06.038.

[94] P. Gagnon, F. Hensel, R. Richieri, Purification of IgM monoclonal antibodies, Bio Pharm Int. (2008) RP0038.

[95] I. Tornøe, I.L. Titlestad, K. Kejling, K. Erb, H.J. Ditzel, J.C. Jensenius, Pilot scale purification of human monoclonal IgM (COU-1) for clinical trials, J. Immunol. Methods. 205 (1997) 11–17. doi:10.1016/S0022-1759(97)00051-3.

[96] J. Lee, A. Tscheliessnig, A. Chen, Y.Y. Lee, G. Adduci, A. Choo, A. Jungbauer, Adaptation of hybridomas to protein-free media results in a simplified two-step immunoglobulin M purification process, J. Chromatogr. A. 1216 (2009) 2683–2688. doi:10.1016/j.chroma.2008.10.067.

[97] P. Gagnon, F. Hensel, S. Lee, S. Zaidi, Chromatographic behavior of IgM:DNA complexes, J. Chromatogr. A. 1218 (2011) 2405–2412. doi:10.1016/j.chroma.2010.12.066.

[98] A. Tscheliessnig, D. Ong, J. Lee, S. Pan, G. Satianegara, K. Schriebl, A. Choo, A. Jungbauer, Engineering of a two-step purification strategy for a panel of monoclonal immunoglobulin M directed against undifferentiated human embryonic stem cells, J. Chromatogr. A. 1216 (2009) 7851–7864. doi:10.1016/j.chroma.2009.09.059.

[99] A. Feinstein, E.A. Munn, Conformation of the free and antigen-bound IgM antibody molecules, Nature. 224 (1969) 1307–1309. doi:10.1038/224488a0.

[100] S.J. Perkins, A.S. Nealis, B.J. Sutton, A. Feinstein, Solution structure of human and mouse immunoglobulin M by synchrotron X-ray scattering and molecular graphics modelling, J. Mol. Biol. 221 (1991) 1345–1366. doi:http://dx.doi.org/10.1016/0022-2836(91)90937-2.

[101] D.M. Czajkowsky, Z. Shao, The human IgM pentamer is a mushroom-shaped molecule with a flexural bias., Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 14960–14965. doi:10.1073/pnas.0903805106.

[102] R. Müller, M.A. Gräwert, T. Kern, T. Madl, J. Peschek, M. Sattler, M. Groll, J. Buchner, High-resolution structures of the IgM Fc domains reveal principles of its hexamer formation, Proc. Natl. Acad. Sci. 110 (2013) 10183–10188. doi:10.1073/pnas.1300547110.

[103] R.R. Akhouri, S. Goel, H. Furusho, U. Skoglund, M. Wahlgren, Architecture of Human IgM in Complex with P. falciparum Erythrocyte Membrane Protein 1, Cell Rep. 14 (2016) 723–736. doi:10.1016/j.celrep.2015.12.067.

[104] E. Hiramoto, A. Tsutsumi, R. Suzuki, S. Matsuoka, S. Arai, M. Kikkawa, T. Miyazaki, The IgM pentamer is an asymmetric pentagon with an open groove that binds the AIM protein, Sci. Adv. 4 (2018) eaau1199. doi:10.1126/sciadv.aau1199.

[105] A. Feinstein, N. Richardson, M.I. Taussig, Immunoglobulin flexibility in complement activation, Immunol. Today. 7 (1986) 169–174. doi:10.1016/0167-5699(86)90168-4.

[106] Y.-K. Lee, J.W. Brewer, R. Hellman, L.M. Hendershot, BiP and Immunoglobulin Light Chain Cooperate to Control the Folding of Heavy Chain and Ensure the Fidelity of Immunoglobulin Assembly, Mol. Biol. Cell. 10 (1999) 2209–2219. doi:10.1091/mbc.10.7.2209.

[107] E. van Anken, F. Pena, N. Hafkemeijer, C. Christis, E.P. Romijn, U. Grauschopf, V.M.J. Oorschot, T. Pertel, S. Engels, A. Ora, V. Lástun, R. Glockshuber, J. Klumperman, A.J.R. Heck, J. Luban, I. Braakman, Efficient IgM assembly and secretion require the plasma cell induced endoplasmic reticulum protein pERp1., Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 17019–17024. doi:10.1073/pnas.0903036106.

[108] J.W. Brewer, R.B. Corley, Quality control in protein biogenesis: thiol-mediated retention monitors the redox state of proteins in the endoplasmic reticulum., J. Cell Sci. 109 (1996) 2383–92.

[109] P.S. Reddy, R.B. Corley, The contribution of ER quality control to the biologic functions of secretory IgM, Immunol. Today. 20 (1999) 582–588. doi:10.1016/S0167-5699(99)01542-X.

[110] P. Holden, W. Horton, Crude subcellular fractionation of cultured mammalian cell lines, BMC Res. Notes. 2 (2009). doi:10.1186/1756-0500-2-243.

[111] T. O'Hare, G.D. Wiens, E.A. Whitcomb, C.A. Enns, M.B. Rittenberg, Cutting edge: proteasome involvement in the degradation of unassembled Ig light chains, J Immunol. 163 (1999) 11–14.

[112] C. Fagioli, A. Mezghrani, R. Sitia, Reduction of Interchain Disulfide Bonds Precedes the Dislocation of Ig-μ Chains from the Endoplasmic Reticulum to the Cytosol for Proteasomal Degradation, J. Biol. Chem. 276 (2001) 40962–40967. doi:10.1074/jbc.M107456200.

[113] J. Liu, A. Bartesaghi, M.J. Borgnia, G. Sapiro, S. Subramaniam, Molecular architecture of native HIV-1 gp120 trimers, Nature. 455 (2008) 109–113. doi:10.1038/nature07159.

[114] S.A. Gallo, C.M. Finnegan, M. Viard, Y. Raviv, A. Dimitrov, S.S. Rawat, A. Puri, S. Durell, R. Blumenthal, The HIV Env-mediated fusion reaction, Biochim. Biophys. Acta - Biomembr. 1614 (2003) 36–50. doi:10.1016/S0005-2736(03)00161-5.

[115] P.L. Earl, C.C. Broder, D. LONG, S.A. Lee, J. Peterson, S. Chakrabarti, R.W. Doms, B. Moss, Native oligomeric human immunodeficiency virus type 1 envelope glycoprotein elicits diverse monoclonal antibody reactivities, J. Vi. 68 (1994) 3015–3026.

[116] P.J. Klasse, R.S. Depetris, R. Pejchal, J.-P. Julien, R. Khayat, J.H. Lee, A.J. Marozsan, A. Cupo, N. Cocco, J. Korzun, A. Yasmeen, A.B. Ward, I.A. Wilson, R.W. Sanders, J.P. Moore, Influences on Trimerization and Aggregation of Soluble, Cleaved HIV-1 SOSIP Envelope Glycoprotein, J. Virol. 87 (2013) 9873–9885. doi:10.1128/JVI.01226-13.

[117] B.S. Taylor, M.E. Sobieszczyk, F.E. McCutchan, S.M. Hammer, The Challenge of HIV Subtype Diversity, N. Engl. J. Med. 358 (2008) 1590–1602. doi:10.1056/NEJMra0706737.

[118] J. Kardos, V. Harmat, A. Palló, O. Barabás, K. Szilágyi, L. Gráf, G. Náray-Szabó, Y. Goto, P. Závodszky, P. Gál, Revisiting the mechanism of the autoactivation of the complement protease C1r in the C1 complex: Structure of the active catalytic region of C1r, Mol. Immunol. 45 (2008) 1752–1760. doi:10.1016/j.molimm.2007.09.031.

[119] N.M. Thielens, F. Tedesco, S.S. Bohlson, C. Gaboriaud, A.J. Tenner, C1q: A fresh look upon an old molecule, Mol. Immunol. 89 (2017) 73–83. doi:10.1016/j.molimm.2017.05.025.

[120] J. Zwirner, E. Felber, C. Reiter, G. Riethmüller, H.E. Feucht, Deposition of complement activation products on plastic-adsorbed immunoglobulins. A simple ELISA technique for the detection of defined complement deficiencies, J. Immunol. Methods. 124 (1989) 121–129. doi:10.1016/0022-1759(89)90193-2.

[121] B. Taylor, J.F. Wright, S. Arya, D.E. Isenman, M.J. Shulman, R.H. Painter, C1q binding properties of monomer and polymer forms of mouse IgM mu-chain variants. Pro544Gly and Pro434Ala, J. Immunol. 153 (1994) 5303–5313.

[122] E. Shelton, K. Yonemasu, R.M. Stroud, Ultrastructure of the Human Complement Component, Clq, Proc. Natl. Acad. Sci. 69 (1972) 65–68. doi:10.1073/pnas.69.1.65.

[123] M.S. Kojouharova, I.G. Tsacheva, M.I. Tchorbadjieva, K.B.M. Reid, U. Kishore, Localization of ligand-binding sites on human C1q globular head region using recombinant globular head fragments and single-chain antibodies, Biochim. Biophys. Acta. 1652 (2003) 64–74. doi:10.1016/j.bbapap.2003.08.003.

[124] A.J. Paul, R. Handrick, S. Ebert, F. Hesse, Identification of process conditions influencing protein aggregation in Chinese hamster ovary cell culture, Biotechnol. Bioeng. 115 (2018) 1173–1185. doi:10.1002/bit.26534.

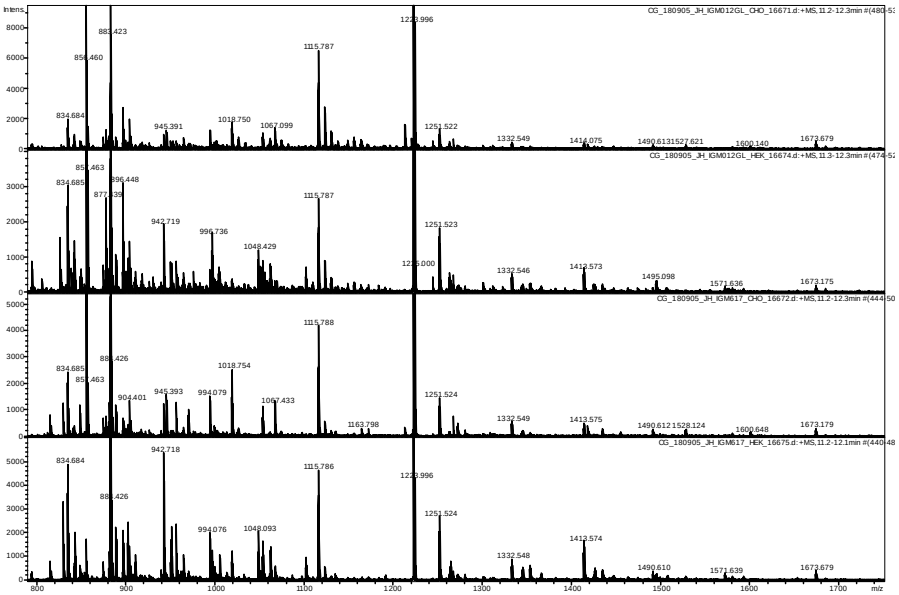
[125] P. Hossler, S.F. Khattak, Z.J. Li, Optimal and consistent protein glycosylation in mammalian cell culture, Glycobiology. 19 (2009) 936–949. doi:10.1093/glycob/cwp079.

[126] T.S. Raju, Terminal sugars of Fc glycans influence antibody effector functions of IgGs, Curr. Opin. Immunol. 20 (2008) 471–478. doi:10.1016/j.coi.2008.06.007.

6. Appendix

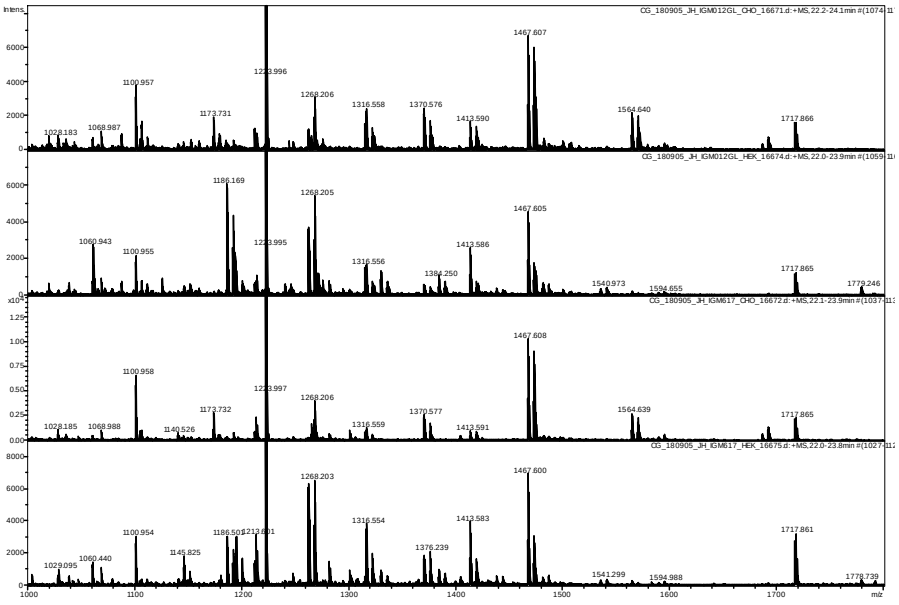
6.1. Supplementary data for glycan analysis

GS1



A

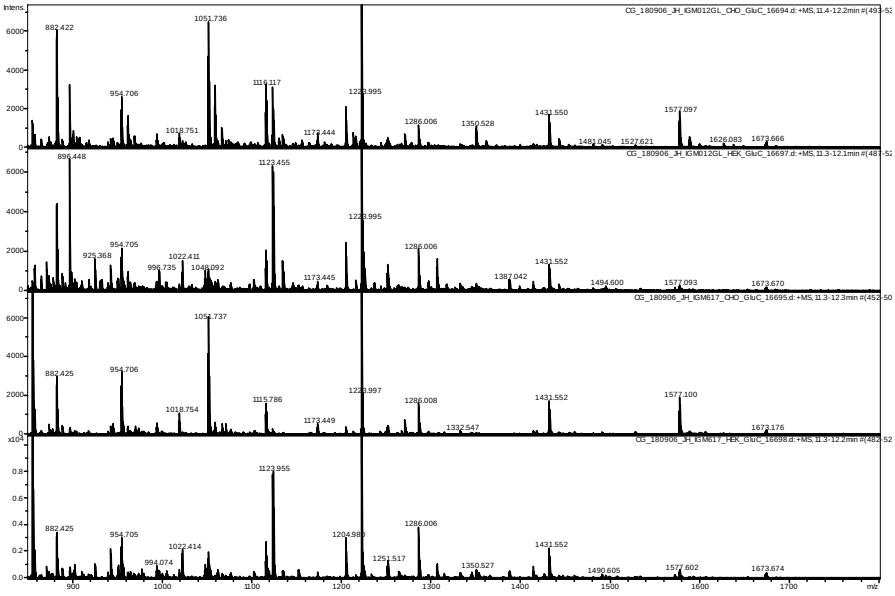
GS2



B

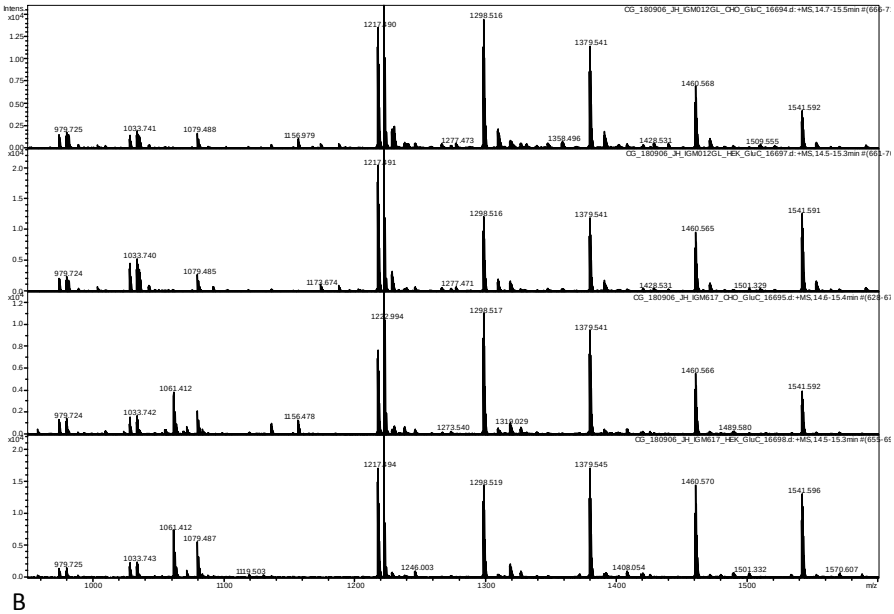
Fig. S1: Mass spectra of the site specific N-glycosylation at GS1 (A) and GS2 (B). Top to bottom: IgM012_GL produced in CHO, IgM012_GL produced in HEK, IgM617 produced in CHO and IgM617 produced in HEK. Spectra were obtained by LC-ESI-MS after trypsin and/or GluC digestions. Glycoforms are labeled in accordance with the ProGlycAn system (www.proglycan.com).

GS3



A

GS4



B

Fig. S2: Mass spectra of the site specific N-glycosylation at GS3 (A) and GS4 (B). Top to bottom: IgM012_GL produced in CHO, IgM012_GL produced in HEK, IgM617 produced in CHO and IgM617 produced in HEK. Spectra were obtained by LC-ESI-MS after trypsin and/or GluC digestions. Glycoforms are labeled in accordance with the ProGlycAn system (www.proglycan.com).

6.2. Curriculum vitae

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Education

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2012-2015	**Master of Science** in Biochemistry (Martin-Luther-University Halle-Wittenberg, Scil Proteins GmbH) Title : « Production and characterization of bispecific Affilin®-Fab-Fusions » Supervisor : Dr. Markus Liebscher
2009-2012	**Bachelor of Science** in Biochemistry Martin-Luther-University Halle-Wittenberg Title : « Analyses of conformational changes within the RNP-domain of the protein PABPN1 » Supervisor : Prof. Dr. habil. Elisabeth Schwarz

Practical experiences	
05/2018-06/2018	Research stay at the Institut de Biologie Structural in Grenoble
04-08/2015 and 10/2012-07/2013	Student research assistant at Scil Proteins GmbH
09/2013-10/2013	Research internship at Scil Proteins GmbH
08/2011-10/2011	Internship at Leibniz-Institute of plant biochemistry
Languages	
	German (native), English (fluent), French (basic)
Publications	
SCI-Publications	Hennicke *et al.* (2019) Impact of temperature and pH on recombinant human IgM quality attributes and productivity. *N Biotechnol.* 50:20-26. Hennicke *et al.* (2017) Glycan profile of CHO derived IgM purified by highly efficient single step affinity chromatography. *Anal Biochem.* 539:162–166.
Other publications	Hennicke and Kunert (2019) Analysis of product quality of complex polymeric IgM produced by CHO cells. *Animal Cell Biotechnology 4th Edition* (Ed. Ralf Pörtner). (under review) Hennicke and Kunert (2018) Quality matters - Is IgM production influenced by external process conditions? *N Biotechnol.* 44:S144. Hennicke *et al.* (2018) Analyses of product quality of complex polymeric IgM produced by CHO cells. *BMC Proceedings* 12, 26-27.

Conference presentations	
08/2018	10th Annual The Bioprocessing Summit, Boston, USA « Critical quality attributes in recombinant IgM production and how they are influenced by external process conditions» [Poster]
07/2018	18th European Congress on biotechnology, Geneva, Switzerland « Quality matters - Is IgM production influenced by external process conditions?» [Poster]
03/2018	6th Halle Conference on Recombinant Proteins, Halle (Saale), Germany « Quality matters: Analysis of recombinant polymeric IgM produced by CHO cells » [Poster]
05/2017	25th ESACT Meeting, Lausanne, Switzerland « Analyses of product quality of complex polymeric IgM produced by CHO cells. » [Poster]
04/2017	9th Conference on Recombinant Protein Production (RPP9), Dubrovnik, Croatia « Complex polymeric IgM produced in Chinese hamster ovary (CHO) cells. » [Talk, Poster]
02/2017	GlycoBioTec, Berlin, Germany « Production of recombinant glycosylated polymeric IgM antibodies in CHO cells. » [Poster]
10/2016	PEGS Europe, Lisbon, Portugal, « Purification of an aggregation prone protein: challenges and solutions for polymeric IgM production. » [Talk, Poster]