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Research paper

Glycation during storage and administration of monoclonal antibody formulations

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Abstract

Covalent modifications of therapeutic proteins are of interest for the biotech industry as they potentially impact the quality of the material. This study focuses on covalent protein modifications by the reducing monosaccharide glucose via the glycation reaction. In particular, we examined (i) to which extent different therapeutic monoclonal antibodies are glycated, (ii) the glycation during storage in sucrose-containing formulation buffers where non-reducing sucrose potentially could hydrolyze into its reducing constituent monosaccharides and (iii) the risk of glycation in the course of short-term incubation in Dextrose infusion bags in simulated administration testing. A boronate affinity chromatography method was employed to detect and quantify glycation products in different antibody formulations. For confirmation and to determine the degree of glycation per molecule, selected samples were analyzed via LC–ESI-MS. We could demonstrate that different antibodies differed drastically regarding the degree of glycation, probably a result of their respective fermentation conditions and protein glycation susceptibility. We also demonstrated that sucrose is a non-critical excipient with respect to glycation when stored long-term at intended storage conditions (2–8 °C). Finally, we could show that short-term incubation of antibodies in Dextrose infusion bags might lead to huMAb glycation, suggesting to test on glycated products when considering diluting protein drug products in infusion media containing reducing sugars.

keywords: Antibody; Formulation; Administration; Glycation; Glucose; Sucrose; Compatibility

1. Introduction

Due to the tremendous progress in biotechnology since the early 1970s, the number of potential therapeutic recombinant proteins – especially monoclonal antibodies – entering the market increased dramatically [1]. One of the major challenges in the development of biotech therapeutics is protein stability [2,3], which has to be maintained during multiple process steps involved on their way to market. Furthermore, protein stability has to be maintained during storage as well as during administration to the patient.

Protein instability reactions are often divided into chemical and physical degradation. Physical degradation mostly describes mechanisms resulting in the alteration of the protein structure without necessarily changing or destroying covalent peptide bonds. This includes (i) denaturation [4,5], (ii) aggregation [6–9] or association [10], (iii) precipitation and surface adsorption of proteins [11–13]. Especially aggregation, association and/or precipitation are a major challenge for the formulation scientist [6,14].

Chemical degradation describes the change or loss of covalent bonds as in oxidation [15–17], hydrolysis [18,19], deamidation [8,20], isomerisation and disulfide rearrangement [21]. In addition, condensation reactions can occur, while the protein forms a covalent adduct with another reactive compound. An example is the reaction of proteins with reducing sugars such as glucose, the so-called glycation [22]. More precisely, the term glycation describes the

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Maillard condensation reaction between the aldehyde groups of reducing sugars and the primary amino groups of proteins – or more generally, the reaction with reducing sugars. Upon formation of a Schiff's base, the reaction proceeds to a ketoamine (Amadori product) [23] (Fig. 1).

Glycated physiological proteins have been related – to some extent – to severe chronic diseases. Such implications encompass (i) diabetes [24–30], (ii) vascular diseases [25,31–33], (iii) immune responses [34] and (iv) others [35,36]. In case of a glycation product observed in a therapeutic drug product, the modification may have an influence on stability, efficacy and safety [37].

So far, only limited research has been published on the glycation of biotech products. Glycation of proteins in dry-powder state has been described for formulations containing reducing sugars like lactose [38]. In addition, sucrose-containing formulations have been described to undergo browning with time – likely considered to be a result of Maillard-like reactions [39]. In a recent study, the glycation of IgG molecules has been described for sucrose-containing formulations [40] after extended storage under accelerated stress conditions. Notably, sucrose is a non-reducing disaccharide. However, it can undergo hydrolysis – e.g. at low pH – to α-glucose and fructose [40,41], which then potentially could lead to glycation.

Importantly, glycation of therapeutic proteins is not exclusively linked to their formulations. During its lifetime, a protein encounters other sources of glucose, e.g. during fermentation. The sugar feeds during upstream processes have been reported to potentially result in glycated proteins [42]. In addition, glucose is an ingredient of commonly used solutions for infusion, i.e. 5% Dextrose, which is used as a carrier for the dilution/infusion of liquid or freeze-dried therapeutic proteins.

The goal of the present work was to find out to which extent different therapeutic huMAb's are glycated and to assess batch-to-batch variability, to assess the extent of glycation during storage in sucrose-containing formulation buffers and in the course of short-term incubation in Dextrose infusion bags, commonly used in clinical practice for some therapeutic protein products. We aimed both for detection and also quantification of glycation levels and employed LC–ESI-MS and boronate affinity chromatography.

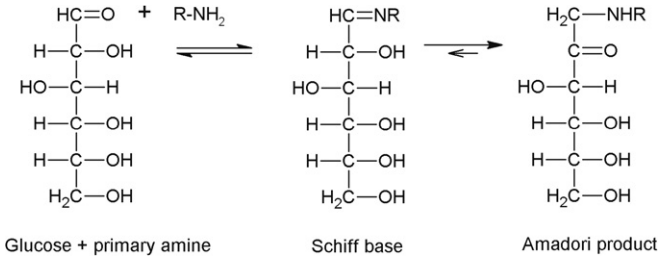

Fig. 1. Proposed reaction mechanism of reducing saccharides (e.g. glucose) with primary amines (e.g. in proteins). Upon the formation of a Schiff base, a rearrangement can lead to the formation of stable ketoamines (Amadori product).

In detail, we analyzed (i) various unstressed antibodies in terms of glycation level, (ii) liquid and freeze-dried formulations of therapeutic monoclonal antibodies containing 240 mM sucrose at pH 5.5 after storage at different temperatures for 52 weeks. Finally, we assessed (iii) glycation during simulated administration in 5% Dextrose infusion bags, where two antibody formulations were diluted in 5% Dextrose infusion bags and stored up to 14 days at ambient temperature.

2. Materials and methods

2.1. Materials

Different monoclonal human IgG antibodies of the IgG1 (MAb 1, 2, 3, 4, 5 and 7) and IgG4 subclass (MAb 6) (F. Hoffmann-La Roche Ltd., Basel, Switzerland) were used. The monoclonal antibodies in the study were produced by cell culture of different recombinant CHO cell lines and purified by a series of chromatographic steps and buffer exchange and final filtration at Roche Penzberg, Germany or Hoffmann-La Roche Ltd., Basel, Switzerland. For boronate affinity chromatography, only highly pure materials were used. Ultrapure (≥99.5%) 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) and ultrapure (≥99.5%) D-sorbitol were purchased from Fluka, Buchs, Switzerland. Ultrapure Tris-(hydroxymethyl)-aminomethane (Tris) (>99.9%) has been purchased by AppliChem GmbH, Darmstadt, Germany. D(+)-Glucose (99.5%) used for the glycation reaction was from Sigma, Steinheim, Germany. All other chemicals used were of analytical or pharmaceutical grade.

2.2. Preparation and characterization of glycated MAb

As a positive control for the glycation reaction, we prepared an artificially glycated MAb. For this purpose, MAb 1 (15 mg/ml in formulation buffer containing 240 mM sucrose at pH 5.5) was diluted 1:1 with 1 M glucose (in formulation buffer) to obtain 7.5 mg/ml MAb 1 in 500 mM glucose-containing formulation buffer and incubated for 7 days at 40 °C to allow for the glycation reaction. As a negative control for the glycation reaction, the respective antibody was diluted to the same concentration in formulation buffer without the addition of any glucose. Both standard solutions were aliquoted and frozen at −80 °C for further analysis. The positive and negative controls were further analyzed by size-exclusion chromatography and ESI-MS prior to their use for boronate affinity chromatography.

2.3. Size-exclusion chromatography (SEC)

The monomer, soluble aggregates (high molecular weight) (total HMW%) and protein fragments (low molecular weight) (total LMW%) percentages of MAb 1 (non-glycated and artificially glycated species) were analyzed by SEC. The HPLC consisted of a L-7200 autosampler, a

L-7100 pump, a L-7400 UV detector (Merck Hitachi, Tokyo, Japan) and a SEC column, TSK G3000 SWXL, 7.8 × 300 mm (Tosoh Bioscience, Stuttgart, Germany). The separation was performed using a mobile phase of 200 mM K_2HPO_4/KH_2PO_4 and 250 mM KCl pH 7.0 at flow rate of 0.5 ml/min at 25 °C. Undiluted samples were injected to obtain a total loading between 50 and 150 μg. Detection performed at a wavelength of 280 nm. The area % of monomer and total aggregation products were calculated using the Empower 2 software (Waters Corporation, Massachusetts, USA).

2.4. SDS–PAGE

Formation of aggregates and clipping products was analyzed by SDS–PAGE under both non-reducing and reducing conditions. Gel electrophoresis was carried out in a XCell SureLock Mini-Cell electrophoresis chamber (Invitrogen GmbH, Karlsruhe, Germany) with a 3000xi power supply (Bio-Rad, Basel, Switzerland) using Novex® 4–20% Tris–Glycine pre-cast gels (1.0 mm, 12 wells) and Novex® Tris–Glycine SDS Running Buffer (all Invitrogen GmbH, Karlsruhe, Germany). For reduced conditions, the samples were incubated using NuPAGE® Sample reducing Agent 10× (Invitrogen GmbH, Karlsruhe, Germany). For non-reduced conditions, sample was diluted 2-fold in Novex® Tris–Glycine SDS Sample Buffer 2× (Invitrogen GmbH, Karlsruhe, Germany). After centrifugation, samples were heated for 10 min at 70 °C, centrifuged again and loaded on the gel. As marker, Mark 12™ Unstained Standard (Invitrogen GmbH, Karlsruhe, Germany) was used. Gels were stained using a Coomassie staining protocol (Simply Blue™ Safe Stain, Invitrogen GmbH, Karlsruhe, Germany) including a washing, fixing, staining, destaining and drying step.

2.5. Boronate affinity chromatography

Separation was performed employing an Alliance 2795 HPLC system equipped with an 2487 UV detector (Waters Corporation, Massachusetts, USA). Samples were injected onto a TSK Boronate-5PW column (7.5 × 75 mm, Tosoh Bioscience, Stuttgart, Germany) equilibrated with Buffer A (100 mM Hepes, 200 mM NaCl, 70 mM Tris, pH 8.6). After injection, initial conditions were maintained for 10 min followed by a linear gradient of Buffer B (500 mM sorbitol in Buffer A) from 0% to 100% in 5 min. The column was washed with 100% Buffer B for 3 min. Then, within 1 min, the flow was changed to initial conditions (100% Buffer A). These conditions were kept until a total run duration of 35 min. The flow rate was constantly kept at 1 ml/min, and the column temperature was 40 °C. UV absorbance was monitored at 280 nm.

2.6. Determination of the degree of glycation

The degree of glycation was calculated based on the UV data assessed by boronate affinity chromatography. The degree of glycation (%) was calculated as the ratio of the AUC of the glycated protein peaks to the AUC of the non-glycated protein peaks multiplied by 100.

2.7. Liquid chromatography electrospray ionization mass spectrometry (LC–ESI-MS)

LC–ESI-MS was used to confirm and to measure the degree of glycation per molecule in MAb samples. Prior to analysis, samples were derivatized (via reduction and subsequent carboxyamidomethylation). To the sample aliquots (56 μg each), 256 μl reducing agent (0.4 M Tris, 8 M Guanidinhydrochloride) and 10 μl 650 mM DTT were added and incubated for 1 h at 50 °C. Subsequently, the preparations were further diluted with a solution of iodoacetamide in acetonitrile (to a final concentration of 75 mM) and incubated for 1 h under light protection. Finally, free non-reacted alkylation reagent was quenched with 5 μl of 500 mM DTT in water and the samples were desalted via Protein Desalting Spin Columns (Pierce).

Sample separation was performed using a capillary HPLC system (1100 Series, Agilent) on an Agilent Poroshell© C8 reversed-phase column (0.5 × 75 mm, pore size 5 μm). A column temperature of 70 °C and a flow rate of 15 μl/min were used throughout the gradient with Eluent A (0.1% formic acid in water) and Eluent B (0.1% formic acid in acetonitrile). Mass spectrometry was performed on a Micromass Q-TOF Ultima Mass Spectrometer (Waters) with electrospray ionization source. Data analysis was performed with the MassLynx software (Ver. 4.1, Waters); the deconvoluted singly charged spectra were generated by using the MaxEnt1 software.

2.8. (Simulated) administration testing

MAb 2 and 7 were diluted in 5% Dextrose infusion bags (Ecobag® G-5, B. Braun Medical AG, Emmenbrücke, Switzerland) to a final protein concentration of 2 mg/ml each. As a control, both antibody formulations were also diluted in 0.9% NaCl infusion bags (Ecobag® NaCl, B. Braun Medical AG, Emmenbrücke, Switzerland). In addition, protein-free formulation buffers (placebo) were injected into both infusion bags to serve as negative controls. At different time points (0 h, 3 h, 24 h, 48 h, 72 h and 13 days) during storage at ambient temperature, samples (1.0 ml) were withdrawn and analyzed via boronate affinity chromatography as described above.

2.9. Statistical analysis

Statistical differences between the means were analyzed by pairwise Student's *t*-tests. Differences were considered significant for $p \leqslant 0.05$.

3. Results

In a first step, an artificially glycated MAb (using MAb 1) was prepared as follows: respective MAb was incubated

for 1 week at 40 °C in formulation buffer containing 240 mM sucrose at pH 5.5 which was further supplemented with 500 mM glucose to allow for glycation.

For the demonstration of successful glycation, LC–ESI-MS was employed. The mass spectra revealed that the glycation reaction has been successfully conducted, as MAb 1 exhibited a mass shift of +162 Da (Fig. 2). In terms of mass change, the covalent addition of glucose to primary amines results in a mass increase of +180 Da due to the addition of glucose and a mass loss of −18 Da due to the loss of water. The glycation of MAb 1 was observed for both heavy and light chain (see Fig. 2) in MS analysis, upon deglycosylation, reduction and alkylation. For the light chain (theoretical mass 23,859 Da), three glycated species could be detected with 24,020, 24,182 and 24,344 Da. For the heavy chain, up to four glycated species were detected (masses correspond well with the theoretical masses of 49,909, 50,071, 50,232 and 50,394 Da). The SDS–PAGE analysis of both non-glycated and artificially glycated MAb 1 demonstrated that the glucose treatment did not induce major aggregation or clipping products. Aggregation, fragmentation products and main bands on SDS–PAGE were comparable for both treated (glycated) and non-treated materials (Fig. 3). This finding was further confirmed by size-exclusion-chromatography (SEC). For both non-glycated and glycated MAb 1, the amount of soluble high molecular weight (HMW) aggregates, mostly being dimers, was comparable as well as the percentage of low molecular weight (LMW) fragments (Table 1).

Subsequently, the non-glycated and glycated samples of MAb 1 were used to set up a boronate affinity chromatography to detect and quantify glycation products. A typical chromatogram is shown in Fig. 4, where Panel A corresponds to the non-glycated species and Panel B to the glycated species of MAb 1. To assess the performance of this method, linearity and specificity were tested. The method provided good linearity over a broad range of protein concentrations (total protein mass from 5 up to 150 μg) for both non-glycated and glycated MAb 1 peak areas (data not shown). In addition, when spiking in glycated to non-glycated, this glycated material could nicely be re-detected and it exhibited excellent linearity (data not shown). Using the boronate affinity chromatography, we examined different unstressed MAb formulations with respect to their degree of glycation. For this purpose, six

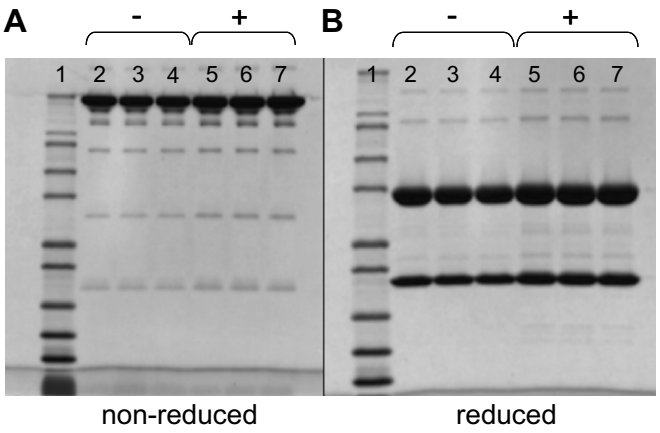

Fig. 3. SDS–PAGE of non-glycated (−) and glycated (+) MAb 1 under non-reducing (A) and reducing conditions (B). Lane 1 represents a standard protein ladder; lanes 2–4 and 5–7, respectively, represent sample triplicates.

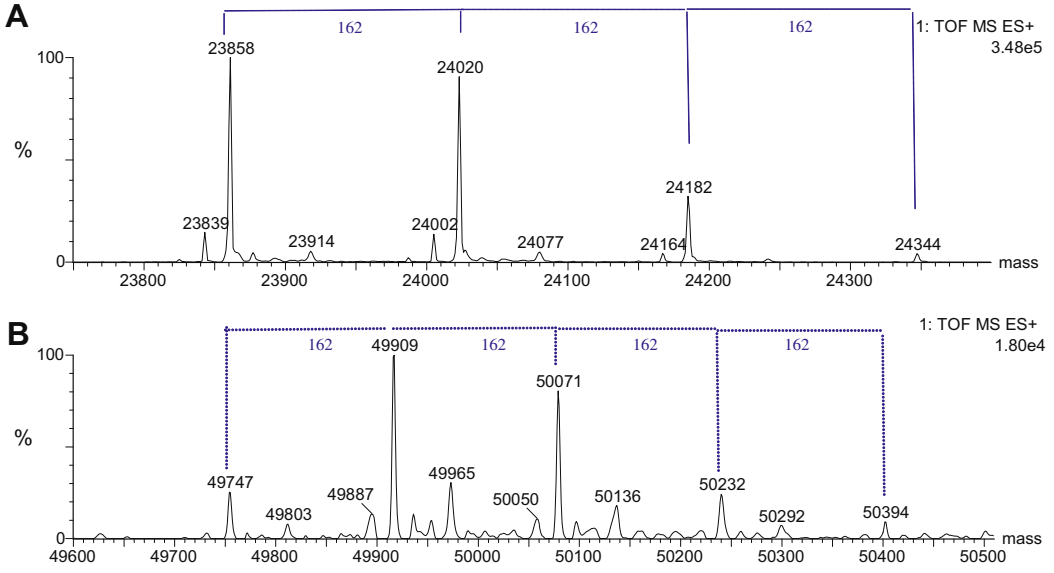

Fig. 2. LC–ESI-MS spectra (deconvoluted) of the reduced and alkylated light (A) and heavy chain (B) of the artificially glycated MAb 1. A: theoretical mass of light chain 23,859 Da. The signal at 24,020 Da can be related to a single glycation (theoretical mass 24,021 Da). Two further glycated species are indicated by typical mass changes (+162). B: theoretical mass of heavy chain 49,747 Da. Further mass changes (+162) are indicated and correspond to different glycated species (theoretical mass 49,909, 50,071, 50,233 and 50,395 Da).

Table 1
Size-exclusion analysis of non-glycated and the artificially glycated MAb 1

	Non-glycated MAb 1	Glycated MAb 1
Monomer (%)	98.89 ± 0.03	98.88 ± 0.02
Soluble aggregates (% HMW)(= % dimer)[a]	0.45 ± 0.01	0.45 ± 0.01
Fragments (% LMW)	0.66 ± 0.03	0.67 ± 0.02

Data represents means from duplicate injections.
HMW, high molecular weight; LMW, low molecular weight.
[a] For soluble aggregates of HMW, only dimer was found.

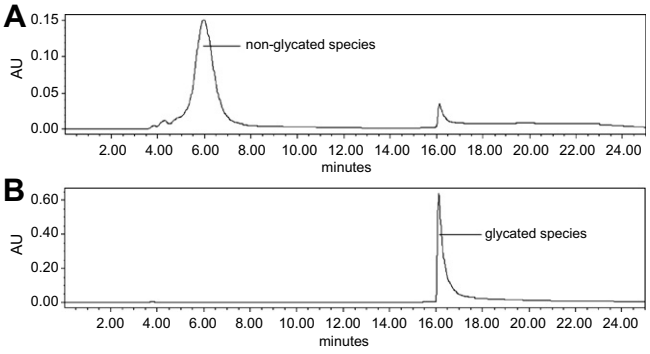

Fig. 4. Typical boronate affinity chromatograms of non-glycated and glycated MAbs. The upper panel (A) shows the negative control of MAb 1; the lower panel (Panel B) shows the artificially glycated MAb 1 upon incubation with 500 mM glucose at 40 °C for one week.

antibodies (MAb 1, 2, 3, 4, 5 and 6) were examined. To address the question on both protein-to-protein variation of glycation products and on lot-to-lot variability, three different lots of MAb 3 have additionally been analyzed using boronate affinity chromatography. Results of the quantification of glycation of those samples are summarized in Table 2. The antibodies differed significantly in their degree of glycation, which was found to be in the range from about 2% to 14% (Table 2). MAb 3 did not exhibit significant lot-to-lot variants; however, it should be noted that the signals for the glycated species were in the range of the limit of quantitation of this method (ca 2%).

Table 2
Degrees of glycation [%] of different unstressed MAb formulations as assessed by boronate affinity chromatography

Antibody	pH/presence of sugar in the formulation	Degree of glycation [%] ± SD[a]
MAb 1	pH 5.5 (240 mM sucrose)	6.9 ± 0.3
MAb 2	pH 6.0 (no sugar)	14.2 ± 0.2
MAb 3 (Lot A)	pH 6.0 (no sugar)	2.0 ± 0.1
MAb 3 (Lot B)	pH 6.0 (no sugar)	1.9 ± 0.1
MAb 3 (Lot C)	pH 6.0 (no sugar)	2.1 ± 0.1
MAb 4	pH 6.0 (no sugar)	5.6 ± 0.4
MAb 5	pH 5.5 (no sugar)	14.3 ± 0.1
MAb 6	pH 6.0 (no sugar)	2.3 ± 0.1

[a] As assessed from triplicate injections.

To analyze whether sucrose-containing antibody formulations are critical in terms of glycation, we analyzed different samples of MAb 1 and MAb 2. More precisely, both lyophilized and liquid formulations containing 240 mM sucrose at pH 5.5 stored for 52 weeks at −80 °C (liquid only), −20 °C (liquid only), 2–8, 25 and 40 °C (MAb 2 only) were analyzed. Boronate affinity chromatography of stability samples of MAb 1 revealed that no major differences in the degree of glycation could be detected under the conditions tested (Fig. 5A). The degree of glycation for the differently stored MAb 1 samples were in the range from 6.8% to 7.9%. This also included the sample stored frozen at −80 °C, where an increase of glycation due to hydrolysis of sucrose appears highly unlikely. Although being statistically significant for the 25 °C liquid sample over the corresponding −80 °C sample, the differences observed are considered to be in the range of method variability. Overall, the MAb 1 samples did not differ prominently irrespective of storage conditions (−80, −20, 2–8 or 25 °C) and irrespective of the dosage form (freeze-dried versus liquid formulation). For MAb 2, up to 52 weeks at −80, −20, 2–8 and 25 °C, no significant increase in the level of glycation occurred; however, a significant increase of glycation was found for the 40 °C/52-week liquid sample (Fig. 5B). For the liquid formulations of MAb 1, those findings were verified using LC–ESI-MS. Selected samples of MAb 1 formulations were analyzed, i.e. samples stored for 52 weeks at −80, 2–8 and 25 °C. For all three conditions, no changes in mass in the heavy chain (theoretical mass of 49,747 Da) were measurable, suggesting that no further glycation occurred during storage (Fig. 6). In the light chain spectra, we found for all stress conditions a single species with a mass change of +162 Da (indicating glycation as compared to the light chain peak (theoretical mass of 23,859 Da) (Fig. 7). Though exact quantification of MS peaks is not considered to be adequate here, the signal intensities for respective peak were compared for the differently stored samples including the sample stored frozen at −80 °C and found not to differ significantly. These results are in accordance with the results from boronate affinity chromatography suggesting no significant increase in glycation over storage time.

Finally, to assess whether glycation can occur under (simulated) administration conditions in dilutions containing antibody product and glucose (Dextrose) as a function of storage time, two different MAb were diluted into 5% Dextrose infusion bags to a concentration of 2 mg/ml, stored at ambient temperature and analyzed after different storage time via boronate chromatography on their level of glycation. For MAb 7, the degree of glycation increased proportionally to the time of incubation (Fig. 8A). After 3 h of incubation, no significant change in the degree of glycation was observed. After 24 h of incubation of the antibody in 5% Dextrose, a slight increase in the degree of glycation was observed. After 3 days of incubation, the degree of glycation had approximately doubled from initially ca 7% to ca 14% glycation products.

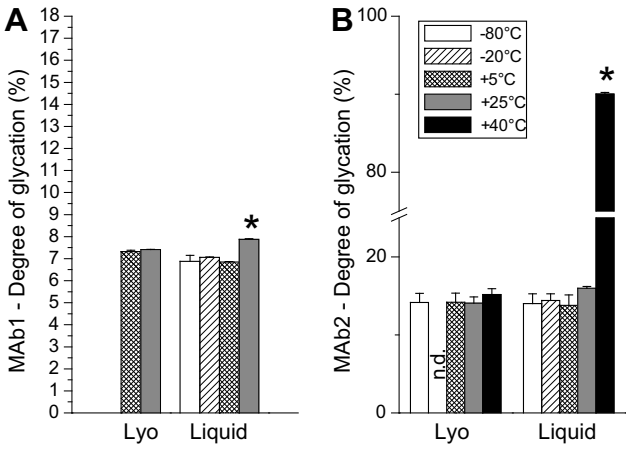

Fig. 5. Degrees of glycation [%] of sucrose-containing formulations (of two different huMAb as assessed by boronate affinity chromatography); all samples were analyzed after 52 weeks storage at indicated conditions (A: MAb 1, B: MAb 2).

The degree of glycation as a function of incubation time of the diluted MAb 2 formulation in Dextrose 5% is given in Fig. 8B. Here, the non-glycated flow through peak (MAb 2) was overlapping with the glucose peak. Therefore, we could not derive the area ratio and modified the data analysis of the boronate affinity chromatography and analyzed the AUC of the glycated peak over time as a marker for glycation (Fig. 8B) instead of using the ratio – avoiding a major change in the performance of the analytical method itself. However, also the AUC of the glycated peak showed a linear increase with storage time. This suggests that glycation occurred in both MAb 2 and MAb 7 formulations after dilution in 5% Dextrose with incubation time. Samples diluted in 0.9% NaCl did not show any increase in the level of glycation with storage time (data not shown).

4. Discussion

Without doubt, not only the physical but also the chemical stability of proteins is of crucial importance [37]. Possible chemical modifications include the covalent addition of moieties and are of interest for various research disciplines including pharmaceutical sciences. A typical addition reaction is the glycation reaction [22]. It describes the reaction of glucose with primary amines in proteins, e.g. the N-terminus or lysine residues [22,37].

During their lifetime, therapeutic proteins can encounter some potential sources of glucose. Obviously, glycation of monoclonal antibodies can occur during the fermentation process, where glucose is routinely fed to the MAb-producing cells, This was recently supported by the findings of Quan et al., where the degree of glycation was strongly affected by the total sugar feed [42]. We also found glucose being highly reactive towards

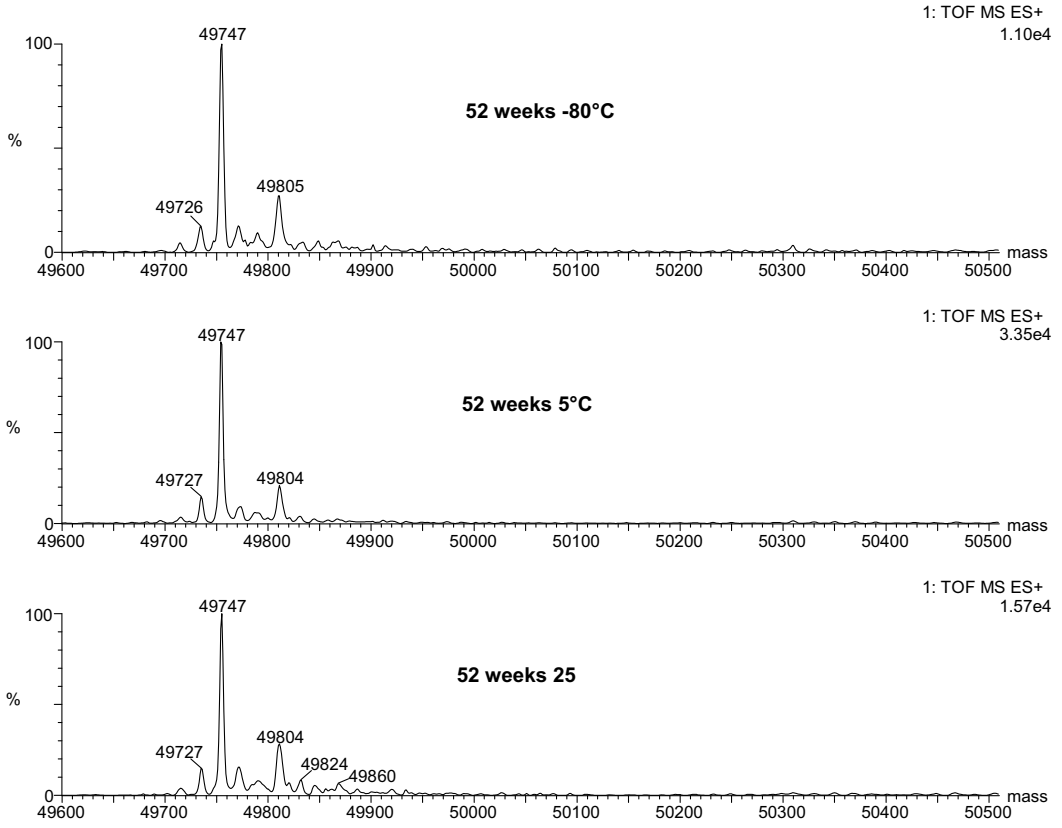

Fig. 6. Deconvoluted heavy chain spectra of three different sucrose-containing liquid MAb 1 formulations after 52 weeks; storage temperatures are given in the figure.

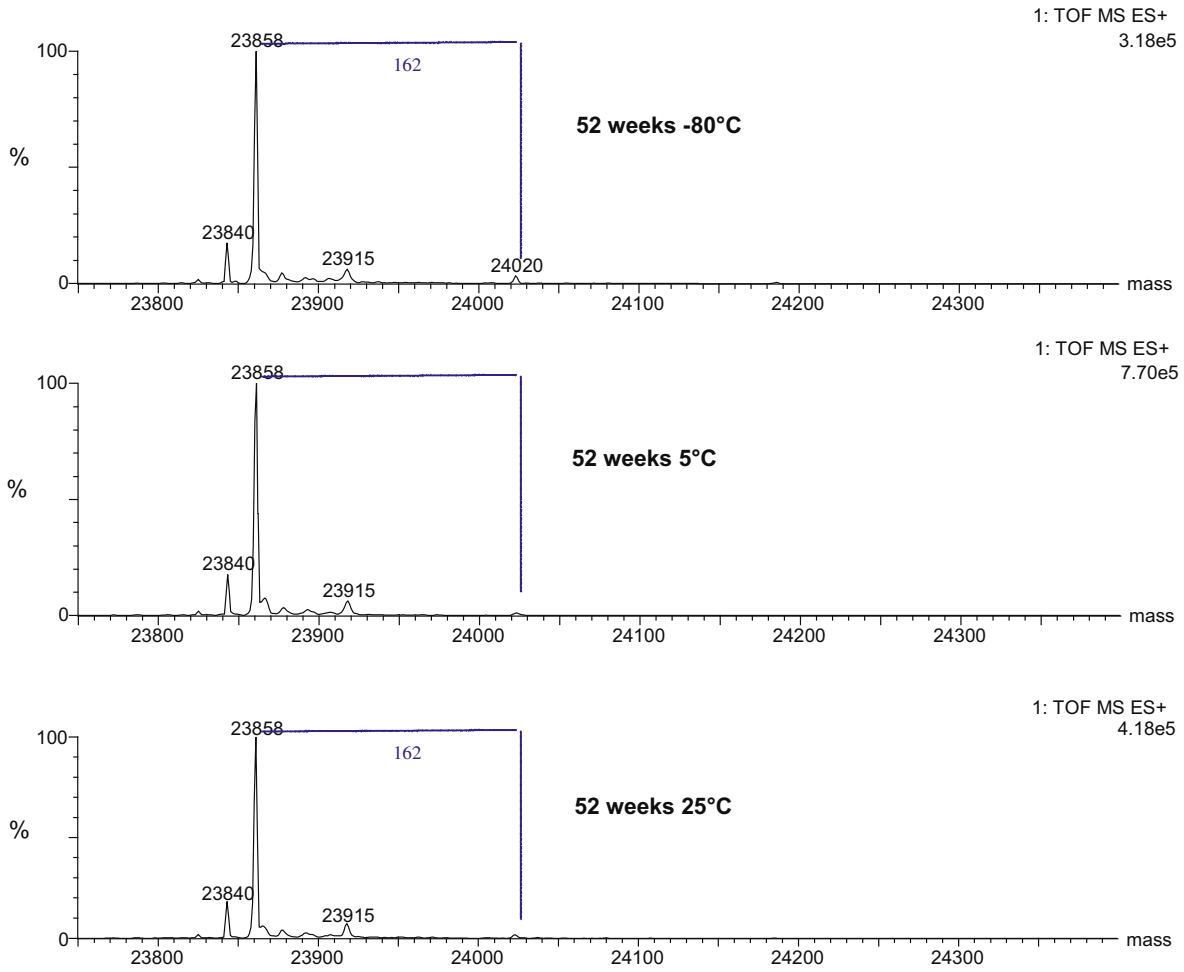

Fig. 7. Deconvoluted light chain spectra of three different sucrose-containing liquid MAb 1 formulations after 52 weeks; temperatures are given in the figure; mass changes induced by glycation are indicated by 162 (which corresponds to the mass change of +162 Da).

our antibodies tested, as seen during the preparation of our working standard in the course of incubation with 500 mM glucose at 40 °C for one week (glycated MAb 1) (Figs. 2 and 4). Under these conditions, ca 100% glycation occurred. However, the glycation reaction did not induce major clipping products or aggregates as tested by SEC and SDS–PAGE (Table 1 and Fig. 3). Moreover, we found all antibodies examined to be glycated whereas their glycation levels differed prominently (Table 2). For MAb 3, no lot-to-lot variabilities could be detected; we can just speculate about the reasons for the observed differences in glycation levels, as the glucose feed during fermentation does not allow for high variation. Very likely, proteins differ in susceptibility to glycation due to differences in lysine content and – probably even more importantly – their surface accessibility. In this context, Gadgil et al. suggested a more structure-related estimation of glycation probability [40]. In this context, Zhang et al. demonstrated that the likelihood of lysine glycation in therapeutic MAb's may depend on the spatial proximity of certain amino acids inducing glycation hot spots [43]. Obviously, this is beyond the scope of this study, but needs to be addressed in future studies. Further work also needs to address the potential impact of glycation on the charge heterogeneity of the obtained material.

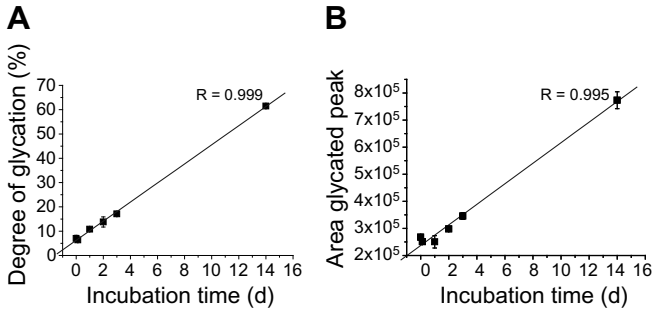

Fig. 8. Change of glycation degree during simulated administration in 5% Dextrose infusion bags (A: MAb 7, B: MAb 2) as assessed by boronate affinity chromatography; Panel B: due to overlapping of non-glycated MAb 2 and glucose peaks in the affinity chromatograms (data not shown), the glycated peak area increase served as means to indicate glycation over time; for both Panels, data is expressed as means from three injections ±SD.

Another cause of Maillard-like covalent modifications are excipients; Quan et al. reported on the reactions of proteins with reducing sugars like lactose even in the dry-powder state [38]. Therefore, reducing sugars are usually not any longer used for protein formulation development. However, reducing sugars such as glucose may also occur as degradation products of higher order non-reducing carbohydrates like sucrose [39,40], which is commonly used in dry-powder and liquid protein formulations. Therefore, we examined the level of MAb glycation in sucrose-containing formulations in both liquid and freeze-dried state. Samples of MAb 1 formulations containing 240 mM sucrose at pH 5.5, stored for 52 weeks at different conditions (−80 °C (liquid only), −20 °C (liquid only), 2–8 °C and 25 °C), were analyzed via boronate affinity chromatography (Fig. 5A). The degree of glycation was comparable irrespective of storage temperature and state (freeze-dried or liquid). Even though the 25 °C liquid formulation differed significantly from other formulations, we consider this difference to be within the standard error of the method. For MAb 2, similar results were obtained in boronate affinity chromatography, as no significant differences were found. Only liquid formulations of MAb 2 stored at 40 °C for 52 weeks did actually show a significant increase in glycation (Fig. 5B). From this data we conclude that sucrose-containing formulations did not show an increase in glycation within one year of storage of the two MAb examined. For liquid MAb 1 formulations, this finding was confirmed via ESI-MS, where no differences were found as compared to the frozen control (−80 °C) (Figs. 6 and 7). Perhaps under very accelerated conditions (40 °C for 52 weeks), it is conceivable that non-reducing disaccharide sugars can hydrolyze to form reducing monosaccharides such as glucose to react with primary amino groups of the protein to glycation products. In a recent study by Gadgil et al., a liquid sucrose-containing formulation of a MAb stored at 4 °C up to 18 months showed no evidence of glycation [40]; in contrast, glycation could already be observed after one month of storage at 37 °C. It should be noted that pH differed from our formulation samples; precisely, formulations were of pH 5 in respective study [40], which probably could increase the hydrolysis rate of sucrose, as it is catalyzed by protons. Interestingly, preliminary data from Liu et al. [44] indicates that upon initial glycation of MAb – depending on the pH – the formation of advanced glycation end products (AGEs) is possible. However, under conditions leading to glycation, other, more significant protein damage would also be occurring, for example protein aggregation, deamidation and other pathways of protein degradation. Therefore, the importance of long-term 40 °C data remains elusive. Long-term stability data at the recommended storage temperature of 2–8 °C did not show any significant increase in the MAb glycation, both in liquid or freeze-dried dosage forms, thus not suggesting to avoid using sucrose in those formulations. Obviously, reducing sugars such as lactose are not recommended to be used in formulations due to high likelihood of covalent reaction.

Finally, we studied the level of glycation during simulated administration of two MAb formulations after dilution and storage up to 14 days at ambient temperature in 5% Dextrose infusion bags. 5% Dextrose, apart from 0.9% NaCl is used commonly as solution for infusion to dilute various drug products in the market for administration via the i.v. route. To test compatibility of the drugs in the diluted solution, compatibility studies in infusion bags are routinely performed; based on this data, recommendations for handling and dilution are included by pharmaceutical companies in product information leaflets or the summary of product characteristics of marketed products. For both antibodies examined, the degree of glycation increased almost linearly as a function of incubation time (Fig. 8). Interestingly, the glycation kinetics are described to strongly depend on the protein itself and the buffer species used for incubation [45]. However, it should be noted that in regular practice in clinical pharmacies, the storage times of diluted products are – due to microbial concerns – usually limited to much shorter storage times than 14 days. In summary, the authors suggest to specifically test the glycated products when considering diluting protein drug products in infusion solutions containing reducing sugars such as glucose (5% Dextrose) during simulated administration testing.

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