Synthetic glycopolymers as modulators of protein aggregation: influence of chemical composition, topology and concentration

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Abstract. Novel drug excipients are required to achieve stable formulations of protein drug candidates. Synthetic glycopolymers have been shown in some cases to improve protein formulation stability, although their structure-function relationship remains unknown. Here we report the synthesis of linear or 4-arm star glycopolymers with different molecular topology and chemical composition, with mannose, galactose, arabinose, N-acetyl glucosamine, lactose and trehalose pendant units - and investigate their modulation of conformational stability and aggregation propensity of a model monoclonal antibody (mAb1). Mono-and di-saccharides with free reducing ends are not frequently utilised as protein stabilisers, due to potential reactivity with a protein's amine groups. In this study this was circumvented through the use of a stable acetal linker connecting the polymer backbone to the sugar pendant residues, which made the latter virtually non-reactive with amines. The general destabilisation the antibody was determined as anunfolding transition temperature (Tm) of CH2 and Fab structural domains, and aggregation temperature (Tagg). The most prominent effect of the glycopolymers on a temperature induced stress in a low concentration solutions was a decrease in Tm and Tagg, regardless of sugar composition or glycopolymer topology - in contrast to the stabilising effect of the corresponding mono- and di-saccharide constituents. The exceptions of linear-lactose and star-trehalose glycopolymers, which increased Tm of the mAb Fab region and Tagg, however, highlights a more complex structure-function relationship. Accelerated stability studies of the high concentrated mAb solutions (50 mg mL-1) revealed that the increased glycopolymer concentrations generally decreased the mAb stability, as judged by the amount of mAb1 'monomer' molecules in solution, with star- and linear-trehalose glycopolymers further generating visible aggregates. Interestingly the latter effect could not have been predicted from the Tm or Tagg experiments conducted at a low concentration regime. Taken together, the data demonstrate a complex interplay of sugar chemistry and molecular topology of the synthetic glycopolymers on their modulation of protein conformational stability and aggregation propensity. Solution concentration was also an important parameter contributing to the stability modulation, and suggests that the stabilising properties of a sugar as the mono- or di-saccharide cannot be extrapolated to the corresponding glycopolymers.

Introduction

Biopharmaceuticals are a large segment of the pharmaceutical industry, representing over 40% of the products currently in the industrial R&D pipeline, with an estimated annual revenue of about $75 billion worldwide.[1] Development of protein pharmaceuticals is challenging, with a number of hurdles that need to be overcome.[2, 3] Most biotherapeutics can degrade at various stages of their preparation/utilisation - e.g. bioprocessing, storage and transport - hence

requiring special precautions, such as controlled temperature and pH, and the addition of stabilising excipients.[4-7] Amongst the physical instability pathways, protein aggregation is arguably the most common and often problematic, and can result from physical and/or chemical insults at all phases of the development process.[2] The underlying challenge in protein formulation is to retain the native conformation of the biotherapeutic, which relies on a complex and often fragile network of intramolecular non-covalent interactions. These include ion pairing, H-bonding and other dipolar forces, π-effects, and van der Waals interactions, all of which can be affected by the physico-chemical conditions of the surrounding medium. Upon unfolding and subsequent irreversible aggregation, protein therapeutics may present reduced or even suppressed activity, decreased solubility, and altered immunogenicity.[8] A number of strategies have been developed to address this issue, with variable success.[9]

An important approach for stabilising protein formulations involves the use of excipients. These can be diverse in chemistry and function, and include salts, carbohydrates, proteins and polymers.[10-13] Low molecular weight sugars - e.g. mono- or disaccharides - and polyols are important classes of protein stabilisers which can minimize both physical and chemical degradations pathways.[14, 15] Although their mechanisms of stabilisation are not fully understood, the preferential exclusion/hydration hypothesis describes the exclusion of a sugar is excluded from the protein surface. In turn, this can thermodynamically favour the protein conformation with the smallest surface area (generally corresponding to its native state).[16,17, 18]

A recent study by Maynard and co-workers has shown that appropriately designed trehalose glycopolymers are superior to their isolated low molecular weight trehalose disaccharide components in their ability to help a model protein, lysozyme, to retain its enzymatic activity after stress stimuli, when.[19] This pioneering work suggested that both the 'local' concentration and spatial arrangement of sugar within glycopolymers played a role in preventing protein aggregation and retaining enzymatic activity. Similar to low molecular weight sugars, the mechanisms by which glycopolymers influence protein structural stability and aggregation are still largely unknown, especially in regards to the role of macromolecular features – size, macromolecular architecture and dispersity - and the chemical nature of the carbohydrate repeating units.

The present study addresses this important point, with a specific focus on how synthetic glycopolymers can affect the conformational and colloidal stability of formulations of monoclonal antibodies (mAbs). Here, two different homopolymer libraries were synthesised: linear glycopolymers containing a range of carbohydrates - mannose, galactose, arabinose, *N*-

acetyl glucosamine, lactose or trehalose- in their repeating units, and their corresponding 4-arm star shaped glycopolymer analogues. More specifically, this work was aimed at investigating structure/function relationships for this class of synthetic macromolecules by systematically varying polymer structural parameters. The nature of the sugar repeating units and the macromolecular architecture were assessed for their influence on protein structural stability and aggregation behaviour.

Experimental

MATERIALS AND METHODS

Monoclonal antibody. The monoclonal antibody (mAb1) used in this study is a bi-specific antibody (IgG1), with a molecular weight of 204 kDa and was provided by MedImmune (Cambridge, UK). The mAb has three distinct unfolding transition temperatures ($T_{m,CH2}$, $T_{m,Fab}$, $T_{m,CH3}$), with an experimentally determined isoelectric point (pI) of 9.

Equipment. All polymerisation reactions were carried out using standard Schlenk techniques under an inert atmosphere of oxygen-free nitrogen, unless otherwise stated. TLC was performed using pre-coated silica gel 60 F254 and developed in the solvent systems indicated. Compounds were visualized by $KMnO_4$ staining. Merck 60 (230-400 mesh) silica gel was used for column chromatography. Molar mass distributions were measured using size exclusion chromatography (SEC).

Size exclusion chromatography - Multi-angle static light scattering (SEC-MALS). SEC-MALS was carried out with a Wyatt dawn 8+ 1200 Infinity series in a system equipped with a SEC Analytical Column for membrane proteins, TSKgel® Size Exclusion (SW-Type) HPLC Column, G3000SWxl, L, 5 µM, 300 Å. Standard Dulbecco's Phosphate Buffered Saline (DPBS) was used as the mobile phase, with a flow rate of 1 mL min^{-1}. Number average molecular weight (M_n) and dispersity (Đ) were calculated by a standard calibration method using poly(ethylene glycol) narrow standards (Polymer Laboratories, AqGPC). The resulting chromatograms were analysed using ASTRA® software, V.6.1.2.84 (Wyatt Tech Corp).

Nuclear Magnetic Resonance. ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) analysis was carried out using a Bruker DPX400 UltraShield™ Spectrometer. The spectra were processed with MestReNova 6.0.2© 2009 Mestrelab Research S.L. All chemical shifts are reported in ppm (δ) relative to tetramethylsilane, referenced to the chemical shifts of residual solvents resonances.

Mass Spectrometry. Mass Spectrometry was carried out using a Micromass LCT KC453 spectrometer. Data were processed with OpenLynx software.

Differential Scanning Calorimetry (DSC). Measurements were performed on a Nano DSC (TA Instruments, USA), scanning once from 25 to 110 °C at 2 °C/min following an equilibration time of 10 min and using a data interval of 5 s; reproducibility was confirmed using water-water baseline tests. Protein samples were prepared at a concentration of 1.0 mg mL^{-1} and filtered through of 0.2 µm filter. Lysozyme standards of 3.0 mg mL^{-1} were measured at start and end of the protein samples and verified to yield a single T_m of 60.1 ± 2.0 °C and ΔH of 415 ± 20 kJ/mol. 900 µL of each protein sample, standard, and blank buffer, were loaded into a 96 well plate, the wells sealed with a silicon mat and the plate loaded into the autosampler. Thermograms were analysed using TA Instruments software, NanoAnalyze, with the protein contribution to the change in molar heat capacity (Cp) determined by subtracting a scan for the blank buffer from the protein sample. A baseline correction for each scan before and after the endothermic peak was made and the data then fitted to a two-state (scaled) model of protein unfolding to calculate the melting temperature (T_m).

Static light scattering (SLS) and intrinsic fluorescence. SLS and intrinsic fluorescence measurements were conducted simultaneously, using an Optim 2 system (Avacta Group plc, Thorp Arch Estate, Wetherby, United Kingdom). Data were processed using the standard Optim analysis software provided, as per manufacturer's recommendations. Protein conformational stability was assessed by measuring the temperature of the onset of melting, that is, the mid-point temperature of the three unfolding transitions ($T_{m,CH2}$, $T_{m,Fab}$, $T_{m,CH3}$). This was monitored by following the change in intrinsic fluorescence intensity ratio (350/330 nm), which occurs as the protein unfolds, due to different tryptophan solvent exposure. SLS at λ = 266 nm was used as an indicator for colloidal stability, and was used to measure the aggregation onset temperature (T_{agg}), defined as the temperature where a 10% of the total change in signal was observed.[20] For SLS and intrinsic fluorescence studies, a mAb1 stock solution (50 mg mL^{-1}) was diluted to a 2.0 mg mL^{-1} concentration, in 25 mM histidine buffer, pH 6.4.[21] The excipients to screen were dissolved in the same buffer at double the concentration required in the measurement sample. These solutions were then mixed in a 1:1 vol:vol ratio, yielding a final protein concentration of 1.0 mg mL^{-1}, at the desired mAb1:excipient ratio. Control mAb1 samples without additional excipients were prepared by diluting the 2.0 mg mL^{-1} solution of mAb1 with equal volumes of 25 mM histidine buffer, pH 6.4. T_{agg} was automatically determined using the software supplied by the manufacturer. The molar ratios were calculated considering the molecular weights of mAb1, mono- and di-

saccharides, and polymer saccharide repeating units. Ratios were therefore calculated based on mAb1 *vs.* concentration of sugar units, either as isolated low molecular weight mono- and di-saccharides, or as carbohydrate moieties included within polymer chains.

All polymers were analysed in an extensive formulation screening, with 9 repeats per polymer formulation resulting from three different runs with 3 repeats *per* run. For every glycopolymer or low molecular weight sugars, all excipient: mAb1: ratios were analysed in the same run, along with three repeats for mAb1 alone, in the absence of any excipients (controls), to enable easier and more accurate comparison of data. Due to mAb1 controls being run for each excipient tested, non-stabilised mAb1 was thus analysed for a total of 108 repeats.

In the SLS and intrinsic fluorescence studies, the samples were heated from 25 to 90 °C using 1 °C increments, with an equilibration time of 30 s before each point measurement. The sample measurements were run in triplicate (*vide infra*).

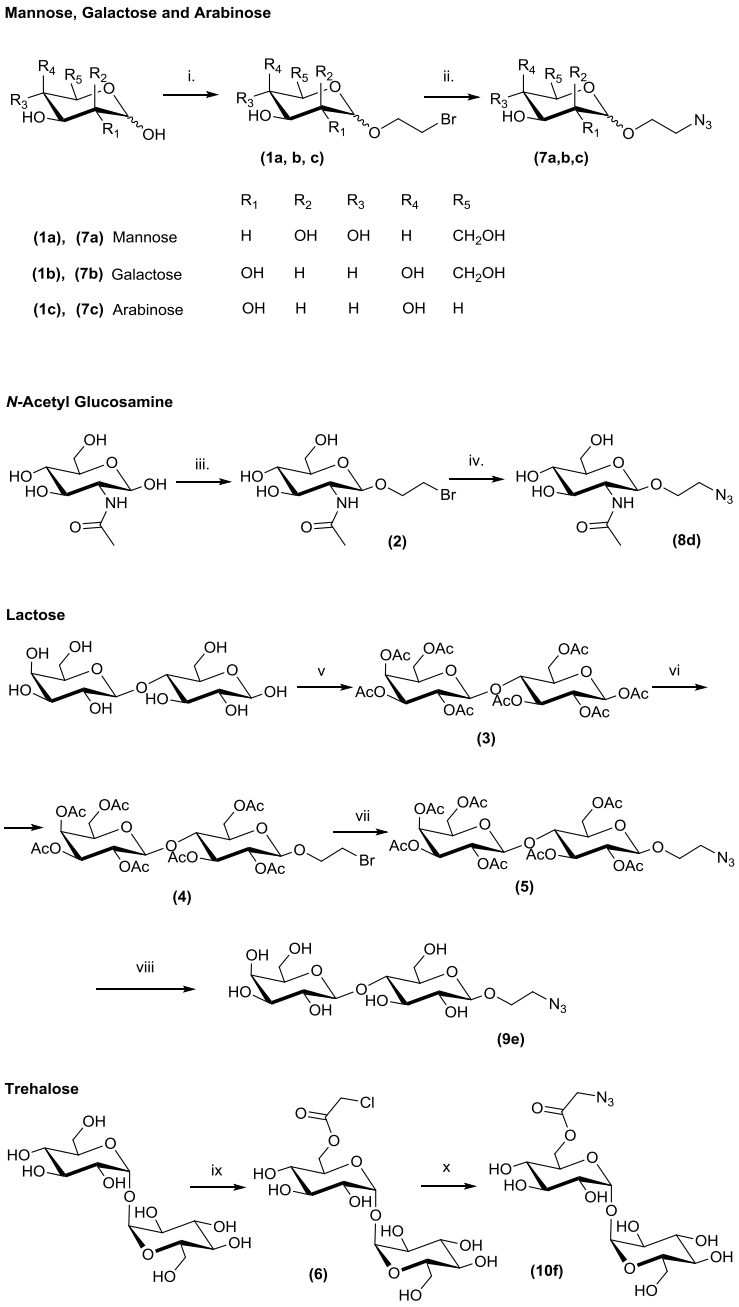
Accelerated stability studies. Accelerated stability studies were carried out at a 50 mg mL^{-1} mAb1 concentration, formulated in 25 mM histidine buffer, pH 6.4. The glycopolymers were added as solids to 50 mg mL^{-1} mAb1 solutions to achieve the [mAb1]:[polymer sugar repeating units] ratios required. The resulting solutions were transferred to glass vials and stored for the required number of weeks at 25 °C (accelerated conditions) and 40 °C (stress conditions), with three individual replicate samples per [mAb1]:[polymer sugar repeating units] ratio. At the required time point, samples were analysed individually and the mean with standard deviation of these readings was reported for each [mAb1]:[polymer sugar repeating units]. Protein degradation was assessed at regular time points by Size Exclusion-High Performance Liquid Chromatography (SE-HPLC). At time zero samples were run and the area under the curve (AUC) of the peak for native mAb1 (here referred to as *monomer* as a reference to oligomeric or aggregated and/or degradation products) was calculated. The degradation of mAb1 was assessed via integration of the AUC of the monomer peak in comparison to the integration of the AUC of all the peaks appearing in subsequent weeks, as described by Kheddo, *et al*.[22] The samples were tested weekly by SE-HPLC, using a Shimadzu HPLC system (LC-20 AP pump), equipped with a SPD-M20A UV detector and SIL-20A autosampler. A Tosoh TSKgel column was used, with 5 μL samples being injected each time, with DPBS as the mobile phase, with UV detection at λ = 280 nm.

Statistics. Statistical analyses were carried out using GraphPad Prism v6. The statistical significance of differences between groups was determined using one-way ANOVA, comparing the values of the mAb alone with mAb runs in the presence of excipients. Comparisons were considered non-significant, * $p < 0.05$, or ** $p < 0.01$.

Results and discussion

Synthesis of glycopolymers. In this work, sugar-based polymers were synthesised by post-polymerisation modification of functional polymers with a range of complementarily reactive carbohydrates, as described by Ladmiral *et al*,[23] to create two libraries of glycopolymers with different compositions and topology. Linear- or star-shaped four-arm architectures were selected with a view to investigate the effect of the polymer architecture on protein stability in solution. By generating all materials from common macromolecular precursors, all polymers within each library have identical degree of polymerisation (DP, that is, the number of polymer repeating units) and molecular weight dispersity, and only vary for the nature of the pendant sugar units. This allowed us to isolate and deconvolute the effect of a single structural variable - the nature of the carbohydrate repeating units – on protein stabilisation. To prepare the required glycopolymers, the low molecular weight sugar (mono- or disaccharide) employed in this study were modified to introduce alkylazido chemical 'handles' in their molecular structure, to chemically graft these modified saccharides to preformed poly(propargyl methacrylate) polymer precursors, by 'click' CuAAC reaction[23] (Scheme 1).

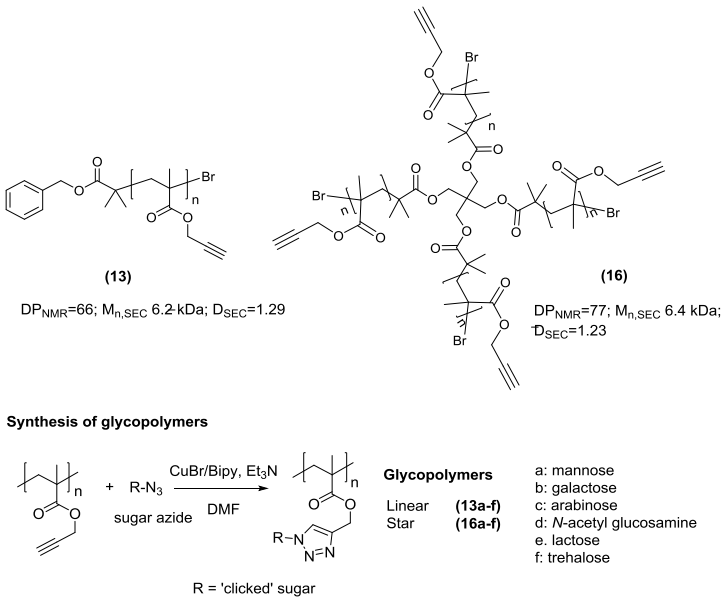
For mannose, galactose and arabinose, the monosaccharide starting materials were suspended in an excess of 2-bromoethanol and heated at 90 °C in the presence of Amberlite 120H acid catalyst.[24] The resulting 2'-bromoethyl glycoside intermediates were then treated with NaN_3 in DMF to give the sugar azides required for polymer conjugation. A similar protocol was utilised for the synthesis of 2'-azidoethyl *N*-acetyl glucosamine, with the difference that HBr generated *in situ* with acetyl bromide was utilised as the catalyst to generate the required 2'-bromoethyl glycoside intermediate **(8d)**. This procedure – direct conversion of sugars into their corresponding alkyl bromide glycosides followed by treatment with NaN_3 – was found to be particularly suited for the functionalization of monosaccharides, which was achieved in only two steps from commercially available carbohydrates, and did not require protection/deprotection of the sugar hydroxyl groups.



Scheme 1. Synthesis of the mannose, arabinose, galactose, *N*-acetyl glucosamine, lactose, and trehalose azides employed in this study as precursors to the required linear and star glycopolymers. *Reagents and conditions:* i. 2-bromoethanol, Amberlite H120, 90 °C, 2.5 h; ii. NaN_3, DMF, 80 °C, 24 h; iii. 2-bromoethanol, acetyl bromide; iv. NaN_3, DMF, 50 °C, 20 h; v. I_2, Ac_2O; vi. $BF_3{\cdot}OEt_2$, 2-bromoethanol; 2-bromoethanol; CH_2Cl_2; vii. NaN_3, DMF, 50 °C, 20 h, viii. CH_3ONa, CH_3OH; ix. chloroacetyl chloride, 2,4,6-collidine, DMF; NaN_3, DMF. Letter labels (a: mannose, b: galactose, c: arabinose, d: *N*-acetyl glucosamine, e. lactose, f: trehalose) are included in the code name for final sugar azides to facilitate the identification of their corresponding final glycopolymers (see also Table 1).

Application of this general protocol to the synthesis of lactose azide resulted in undesired transacetalyzation side-process, where 2-bromoethyl gluco- and galactopyranosides were formed due to cleavage of the β-1→4 glycosidic linkage between glucose and galactose in the lactose molecule. An alternative route was then identified, where lactose was first peracetylated with acetic anhydride and I_2 as the catalyst,[25] followed by treatment with 2-bromoethanol and $BF_3{\cdot}OEt_2$, to introduce the required alkylhalide functionality. Subsequent reaction with NaN_3 followed by deprotection with CH_3ONa/CH_3OH, afforded the desired lactose azide **(9e)**.

Trehalose consists of two glucose units connected via an α,α-1,1-glycosidic bond, hence the general approach utilised to synthesize the other sugar azides - introducing an azide-containing *O*-alkyl glycoside group - was not applicable, as it would have resulted in the cleavage of the linkage holding together the two glucopyranoside residues. Thus, an alternative strategy was utilised that involved reaction of D-(+)-trehalose dehydrate with chloroacetyl chloride in DMF using 2,4,6-collidine as a base. This was carried out at -50 °C to favour reaction of the sugar primary hydroxyl groups over the more hindered secondary ones. Treatment of the reaction crude product – consisting of a statistical distribution of trehalose starting material, and mono and bis sugar alpha-chloroacetates - with sodium azide in DMF followed by flash chromatography on SiO_2 afforded the desired trehalose monoazide **(10f)**.



Scheme 2. Synthesis of linear **(13a-f)** and star **(16a-f)** glycopolymers. Top: clickable linear (**13**) and star (**16**) poly(propargyl methacrylate) precursors; and 'click' synthesis of the required glycopolymers (linear **13a-f**, and star **16a-f**). a: mannose, b: galactose, c: arabinose, d: *N*-acetyl glucosamine, e: lactose, f: trehalose.

The synthesis of the required functionalizable polymer precursors and all required intermediates was carried out following a general strategy previously reported by Haddleton and ourselves and subsequently utilised in a number of studies[24, 26-31] for quantitative polymer functionalization with sugar azides.[23] Linear and 4-arm star poly(propargyl methacrylate)s were synthesized via ATRP, using trimethylsilyl-propargyl methacrylate monomer and mono- and tetravalent initiators prepared from benzyl alcohol and pentaerithrol, respectively (Scheme 2).[23]

Polymerisations were carried out using the required initiator and *N*-(ethyl)-2-pyridinmethanimide / Cu(I)Br catalytic system, in toluene at 70 °C. The resulting polymers were deprotected by removing the TMS group with tetrabutylammonium fluoride (TBAF) and acetic acid, thus affording the desired clickable linear (DP 66, $Đ_{SEC}$ 1.29) and star (DP 77, $Đ_{SEC}$ 1.23) polymers.

Finally, the polymers were functionalised *via* Copper(I)-catalysed azide-alkyne cycloaddition (CuAAC) in DMF in the presence of the required sugar azides, using Cu(I)Br/Bipy as the catalyst. Quantitative polymer functionalisation was confirmed by the complete disappearance of the propargyl methacrylate repeating unit signals in the ^{1}H NMR spectra of the final, linear **(13a-f)** and 4-arm star **(16a-f)** glycopolymers (Table 1).

Table 1. Glycopolymers synthesized and utilised in this work and their macromolecular features.

Polymer code[a]	Architecture	Sugar	DP[b]	M_n[c] (kDa)	Đ[c]
13a	Linear	Mannose	66	23.9	1.22
13b	Linear	Galactose	66	22.5	1.23
13c	Linear	Arabinose	66	23.5	1.26
13d	Linear	NAc Glucosamine	66	27.0	1.29
13e	Linear	Lactose	66	25.5	1.25
13f	Linear	Trehalose	66	37.6	1.18
16a	Star	Mannose	77	27.2	1.25
16b	Star	Galactose	77	24.5	1.28
16c	Star	Arabinose	77	20.5	1.23
16d	Star	NAc Glucosamine	77	29.5	1.29
16e	Star	Lactose	77	42.6	1.29
16f	Star	Trehalose	77	55.9	1.17

[a] Polymer code as *per* numbering in Schemes 1 and 2. [b] Degree of polymerisation (DP) calculated by ^{1}H-NMR of the glycopolymers by comparing the area of the signals of the pendant propargyl ester repeating groups to those from the polymerisation initiators. [c] M_n and Đ determined by SEC in DPBS (see Methods).

Design of stability studies of mAb1. Amongst sugars investigated in this study, trehalose is a commonly used formulation excipient, due to its demonstrated ability to stabilise conformational and solution colloidal properties of antibodies.[32,33, 34] In contrast, monosaccharides mannose, galactose, arabinose and *N*-acetyl glucosamine, or di-saccharide lactose, are not frequently utilised.[35] at least in part due to the hemiacetal ends of these sugars

being reactive towards protein amino groups, through a series of side-processes that include formation of imines and Maillard reactions.[36,37,38,39] In the present study, this issue was circumvented through the use of a stable acetal linker connecting the polymer backbone to the sugar pendant residues, which made the glycopolymers virtually non-reactive against amines. We tested the effects of polymer architecture (linear *vs.* 4-arms star) and nature of the repeating sugar units on model antibody conformational and colloidal stability. In addition, we also investigated the effects of concentration and polymer:mAb1 ratios (by varying the [polymer sugar repeating units]:[mAb1molar ratio) on these parameters, as the concentration of polyhydroxylated excipient is known to affect the stability of monoclonal antibodies in solution. [40,41,42]

The effect of sugars and glycopolymers on mAb1 conformational stability. Initial experiments focused on mAb1 in the absence of excipients, which showed distinct transitions for the unfolding of its structural domains by DSC: T_m = 69.46 °C for CH2, T_m = 78.70 °C for Fab and T_m = 85.05 °C for CH3 (Figure S46), where CH2 and CH3 are constant regions of the heavy chain of the Fc (fragment crystallisation), and Fab is the antigen-binding fragment. In this study intrinsic fluorescence and light scattering measurements were carried out simultaneously, to investigate protein conformational stability and solution aggregation, respectively, on the same samples and experimental conditions. Conformational stability of mAb1 was assessed from its intrinsic fluorescence,.[20] as upon protein unfolding tryptophan (Trp) emission (λ_{max}) undergoes a bathochromic shift, typically from ~335 to 355 nm, which occurs when, upon heat-induced denaturation, Trp residues buried within hydrophobic protein domains become exposed to a more polar aqueous environment,.[20,43,44] It should be noted that the intrinsic fluorescence measurements reported in Figure 1 (values for mAb1 alone) are in agreement with DSC data for the mAb1 unfolding transitions (Figure S46 and Table S1). T_m indicates the mid-point temperature of the transition from native to a fully unfolded conformation for mAb1 in solution, and an increase in T_m in the presence of an excipient is considered to be indicative of enhanced conformational stability.[20,40,41]

Changes in mAb1 unfolding transition temperatures in the presence of low molecular weight sugars and glycopolymers ranged from 0.1 °C to 3.22 °C (Figure 1). In previous studies differences of ± 0.2 °C have been reported, and utilised to discriminate between different formulations.[45]

Amongst low molecular weight mono-and di-saccharide excipients, trehalose produced an overall stabilising effect on mAb unfolding, as judged from an increase of T_m for all three mAb domains (Figure 1); which is in agreement with previous studies.[33, 34,46,47] Similarly, the stabilising profile of lactose was in agreement with reports for other disaccharides, such as sucrose[41, 48]. For other tested sugars, increases in $T_{m,CH2}$ and $T_{m,Fab}$ were observed; the latter being particularly prominent for galactose and arabinose. The effect on T_m of CH3 domain was found to be more dependent on the nature of the sugar utilised, with, in general, d estabilising effects of monosaccharides, particularly galactose, and stabilisingstabilisation provided by disaccharides, particularly trehalose. Since reducing monosaccharides are not commonly used to stabilise protein formulations, comparative data are not easily available.

For the linear and star glycopolymers **(13a-f)** and **(16a-f)**, respectively, and at low mAb1 concentration (1.0 mg mL^{-1}), the most prominent and consistent effect on temperature-induced stress was a general decrease in T_m of CH2 domain, regardless of molecular topology, chemistry of the sugar repeating units, or sugar:protein molar ratio. This general effect was in clear contrast to the predominantly stabilising effect seen for corresponding mono- and di-saccharides. Interestingly, decreased $T_{m,CH2}$ values are pronounced for monosaccharide-based linear- and star-glycopolymers (e.g. mannose and galactose), very low for disaccharide lactose, but pronounced for disaccharide trehalose.

The effect of glycopolymers on $T_{m,Fab}$ and $T_{m,CH3}$ of mAb1 was less consistent, with unclear patterns. It is however noticeable that glycopolymers did not show the general stabilisation of the Fab domain seen for the corresponding mono- and di-saccharides. Comparisons between individual sugars and the corresponding glycopolymers revealed that, for instance, arabinose monosaccharide caused a prominent increase in T_{mFab} and small effect on $T_{m,CH3}$, whilst linear arabinose glycopolymer (**13c**) produced a prominent effect on lowering $T_{m,CH3}$ and a small effect on lowering $T_{m,Fab}$.

Arabinose-based polymers behaved differently than monosaccharide mannose- and galactose-glycopolymers, whilst *N*-acetyl glucosamine-star polymer (**16d**) had a pronounced destabilising effect on the Fab domain.

This might suggest that, amongst mono-saccharide-based glycopolymers, the size and nature of the sugar repeating units may be an influencing factor in affecting the unfolding temperatures of mAb1 domains. For di-saccharide based glycopolymers,an increase in $T_{m,Fab}$ induced by lactose linear and 4-arms (**13e** and **16e**), and a decrease elicited by trehalose linear and 4-arms glycopolymers (**13f** and higher ratios of **16f**) was observed; a result that at this stage is difficult to rationalise.

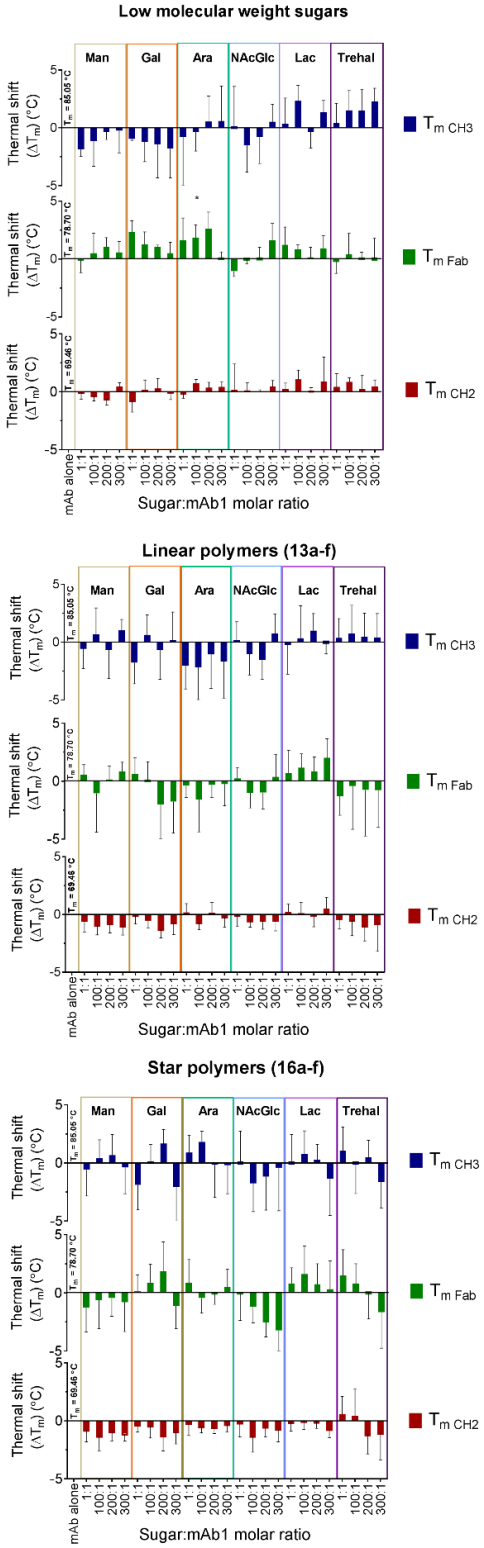


Figure 1. Change in Tm values obtained from intrinsic fluorescence readings of CH2, Fab, and CH3 mAb1 domains in the presence of low molecular weight mono/di-saccharides, and linear **(13a-f)** and star **(16a-f)** glycopolymers, relative to mAb1 in histidine buffer only ($T_{m,CH2}$ = 69.46 °C, $T_{m,Fab}$ = 78.70 °C, $T_{m,CH3}$ = 85.05 °C), as measured by Optim 2 instrument. In all samples [mAb1] = 1.0 mg mL-1, in 25 mM histidine buffer, pH 6.4. Statistical significance, * = $p < 0.05$.

Variations in sugar:protein molar ratio did not result in clear trends on modulation of T_m of CH2, Fab, and CH3 domains, with the exception of the trehalose-based star polymer (**16f**), which produced a stabilising effect at lower molar ratios (1:1 and 100:1) and a destabilising effect at higher ratios (200:1 and 300:1) (Figure 1).

Published work on structural stability of IgGs in the presence of different excipients suggests that the CH2 domain, the most unstable in response to thermal and chemical insults, may determine the overall physical stability of mAbs.[41,49] Applied to the present study, the overall decrease of the $T_{m,CH2}$ induced by all the tested glycopolymers regardless of composition or molecular architecture might therefore suggest that under the exerimental conditions investigated, these glycopolymers mostly exert an overall destabilising effect on the mAb.

The effect of sugars and glycopolymers on mAb1 thermally induced aggregation. SLS analysis provided an insight into the colloidal stability of mAb1 samples towards aggregation (Figure 2). It has been proposed that, upon unfolding, non-native protein conformations expose hydrophobic 'patches' which can initiate self-association through non-reversible and non-specific assembly pathways, leading to the formation of soluble and insoluble aggregates.[50] T_{agg} reflects the temperature at which a noticeable change in light scattering of the protein solution is measured, as a consequence of the presence of insoluble aggregates.[20] Values of T_{agg} shown here represent the temperature where a 10% of the total change in signal is observed.

Simultaneous monitoring of both unfolding (Figure 1) and protein aggregation (Figure 2) enabled us to assess the effects of the glycopolymers on unfolding (T_m) and aggregation (T_{agg}) recorded for the same mAb1 samples. It should be noted that T_m and T_{agg} values are not expected to overlap, as they are related to distinct structural and kinetic phenomena.[51]

Aggregation temperatures (T_{agg}) lower than T_m have been described for a number of proteins,[52] and could be attributed to thermally induced self-association prior to denaturation, which in turn may lead to subsequent denaturation. In addition, T_m is defined as the mid-point of unfolding transition, thus it does not accurately define the onset of the process. In systems where a relatively low proportion of unfolded proteins can initiate the formation of aggregates, $T_{agg} < T_m$ may be observed.

Under the stress conditions employed in this work, T_{agg} of mAb1 alone (60.6 °C) was found to be lower than T_m values of CH2, Fab and CH3 domains (Figure 1), indicating the antibody propensity to initiate aggregation at low levels of molecular unfolding.

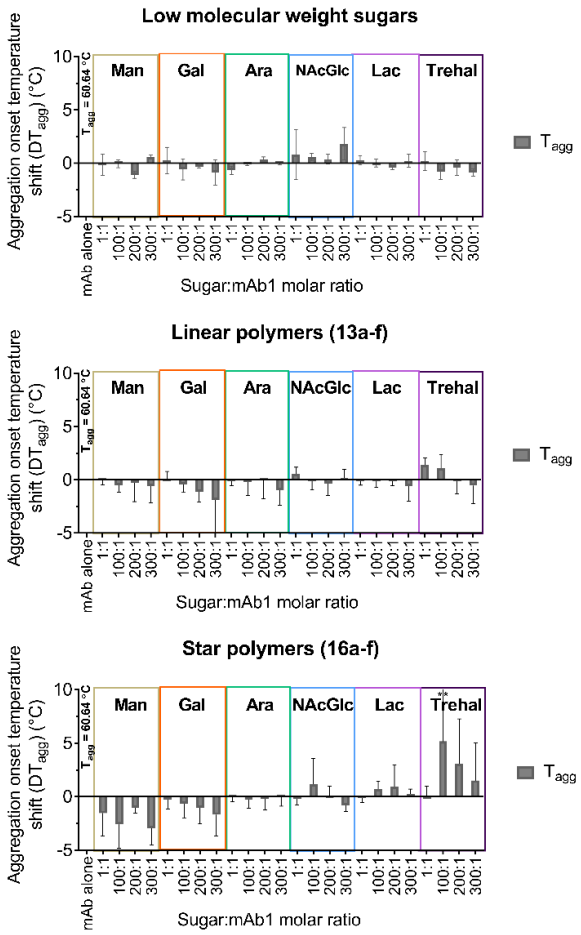


Figure 2. Change in mAb1 T_{agg} in the presence of low molecular weight mono- and di-saccharides, and linear **(13a-f)** and star **(16a-f)** glycopolymers, relative to mAb1 in histidine buffer alone (T_{agg} = 60.64 °C), as measured by SLS. In all samples [mAb1] = 1.0 mg mL^{-1}, in 25 mM histidine buffer, pH 6.4. Statistical significance, ** = $p < 0.01$.

A more detailed inspection of SLS profiles for the mAb1 alone, and its sugar and glycopolymer formulations (representative examples shown in Figure S47) revealed a two-step aggregation process with a small increase just above 60 °C and an incremental, larger increase at temperatures above ~ 70 °C. It should be noted that trehalose glycopolymers showed a less pronounced initial step compared to the other glycopolymers(Figure S47), resulting in higher estimated T_{agg} values. In general, the addition of mono- or di-saccharides to mAb1 solutions over all concentrations ratios tested did not result in a significant modulation of the antibody's T_{agg} over all concentrations tested (Figure 2). In contrast, the linear- and star-glycopolymers based on monosaccharides mannose and galactose appeared to promote thermally induced mAb1 aggregation, resulting in a decrease in T_{agg} of up to 3 °C. The opposite was observed for di-saccharide-based glycopolymers,

where 4-arm lactose and trehalose glycopolymers, as well as the linear trehalose glycopolymer at specific concentrations, generally raised the T_{agg} of mAb1 (although the T_{agg} values estimated for trehalose polymers need to be viewed with some caution, as discussed above).

In terms of sugar:mAb ratios, the linear glycopolymers , induced a more pronounced decrease in the T_{agg} at the higher ratios with the exception of the *N*-acetyl glucosamine (**13d**) polymer at the highest ratio. In formulations containing trehalose linear glycopolymer (**13f**), a stabilising effect was observed at lower concentrations (1:1 and 100:1 molar ratios) and an increasing destabilising effect at higher concentrations (200:1 and 300:1), similar to the other linear polymer excipients.. Such concentration-dependent effect was found to be less pronounced for the 4-arms star library, with exception of the galactose polymer (**16b**).

From the protein conformation stability and aggregation studies undertaken in this work, some initial structure-function correlations can be established for the glycopolymer-containing formulations. In general, glycopolymers based on monosaccharide repeating units in linear or 4-arm star configurations in general induced a decrease in the T_m of CH2 and Fab domains of mAb1 ($T_{m,CH2}$ and $T_{m,Fab}$) which occurred along with a decrease in T_{agg}. For disaccharides, lactose-based glycopolymers did not show a significant destabilising effect on the CH2 domain ($T_{m,CH2}$), while increasing stabilisation of the Fab domain ($T_{m,Fab}$). This destabilising effect may have resulted in the increase of T_{agg} observed for the star glycopolymer (**16e**) but not for the linear (**13e**). Interestingly, trehalose based polymers **(13e)** and **(13f)** appeared to show concentration-dependent effect in influencing both T_m and T_{agg} of mAb1, with stabilising effects at lower concentrations, and an increasingly destabilising effect at higher concentrations.

In the context of modulation of antibody structural stability, properties such as the ability to form hydrogen bonding networks between individual sugar units and sugar-water have been shown to be specific to the chemistry of the sugar utilised, with consequent distinct effects on protein stability.[53] This bonding landscape is likely to vary for the different sugars incorporated in our glycopolymers, and consequently one could expect distinctive interactions with protein surface that might induce divergent modulation of aggregation behaviour.

Accelerated stability studies. To assess the effect of glycopolymers on long term storage stability of mAb1, accelerated stability studies of highly concentrated solutions (50 mg mL^{-1}) were undertaken. In these experiments, glycopolymers with both linear and 4-arm star architectures, mannose, **(13a)** and **(16a)**, lactose, **(13e)** and **(16e)**, and trehalose, **(13f)** and **(16f)** were selected. Mannose-glycopolymers were utilised as examples of polymers with monosaccharide repeating unit which showed clear destabilising effect on mAb1 - i.e. consistently decreased $T_{m,CH2}$ and T_{agg} (Figures 1 and 2).

Both lactose- and trehalose-based disaccharide-glycopolymers were investigated due to their rather different behaviour in previous conformational and colloidal stability experiments (Figures 1 and 2). Trehalose as low molecular weight disaccharide displayed a clear positive effect on mAb1 conformational stability (increasing T_m values), which was lost as trehalose linear or star glycopolymers. In addition, colloidal stability SLS profiles for trehalose based were somewhat altered, compared to other glycopolymers(Figure S47). Lactose glycopolymers were selected as they induced less well-defined trends in the protein conformation and colloidal stability studies.

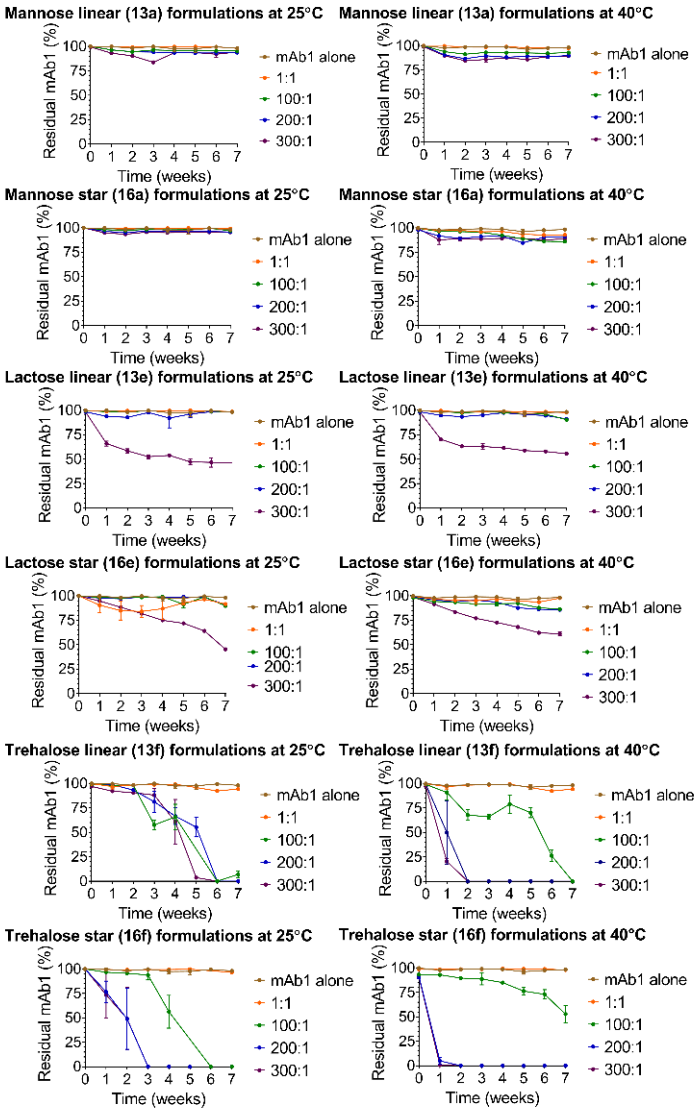


Figure 3. Physical stability of mAb1 (50 mg mL^{-1} in 25 mM histidine buffer, pH 6.4) vs time, in solutions formulated with linear and star glycopolymers of mannose (**13a** and **16a**), lactose (**13e** and **16e**), and trehalose (**13f** and **16f**), at different [sugar repeating units]:[mAb1] molar ratios, at 25 °C (left, accelerated conditions) and 40 °C (right, stress conditions). Residual native mAb1 (monomer) % in solution was determined via SE-HPLC using DPBS as the mobile phase, and calculated via integration of the AUC of the mAb1 and peaks of degradation products.

In these experiments, the decrease in concentration of mAb native protein in solution - referred to as mAb1 *monomer* to differentiate it from its oligomeric aggregation products formed over time - was monitored over seven weeks. mAb1 solution at 50 mg mL^{-1} was used in all formulations tested in this part of the study to mimic the high concentrations of antibody solutions often required for pharmaceutical products. Additionally, the use of a high concentration solution of mAb1 also allowed for better assessment of the loss of mAb1 monomer.[54] As in the previous experiments, the glycopolymers were added in four different sugar:mAb1 molar ratios.

The resulting stability profiles indicate that, in general, increasing sugar:protein molar ratio resulted in a decrease in native mAb1 (*monomer*) content over time, for all formulations tested, at both 25 and 40 °C (Figure 3). Interestingly, mannose glycopolymers (**13a)** and **(16a)**, which showed clear destabilising effect in protein conformation and stability tests, appeared to produce relatively modest destabilising effect on long term stability of the mAb1 solutions, even at 300:1 sugar:mAb1 ratios, i.e. concentrated glycopolymer solutions. This is in contrast to mAb1 solutions containing trehalose glycopolymers (**13a)** and **(16a)**. Stabilization of antibody unfolding, i.e increased T_m and T_{agg} was seen at lower sugar:mAb1 ratio (100:1), whilst at the higher mAb1 concentration utilised in these accelerated stability studies (50 mg mL^{-1}) both linear and star trehalose glycopolymers induced visible precipitation from week two, as confirmed by a decrease of the mAb1 *monomer* content over time observed by SE-HPLC analysis, already at 100:1 molar ratio. Formulations containing mannose and lactose linear or star glycopolymers, were visually free of precipitated material throughout the seven-week experiment.

It is commonly accepted that hydrophilic polymers can increase the conformational stability of mAbs by the excluded volume effect and/or preferential exclusion mechanisms.[41] The glycopolymers should, therefore, exclude themselves from the protein surface, forcing the protein to stay in a preferential hydrated state, adopting more compact conformations, which often correspond (or are closely related) to the protein native conformation.[24,55] However, such an exclusion of excipients from the vicinity of protein macromolecules can sometimes result in microenvironments with higher protein local concentration, which favours protein-protein interactions, and may eventually lead to protein aggregation. This effect is often more pronounced at high protein concentrations,[56,57] such as 50 mg mL^{-1} utilised for this study. Accordingly, particular glycopolymer compositions or molecule architectures lead to different macromolecular crowding effects, hence resulting in mAb1 aggregation to a different extents. Interestingly, SEC analysis of trehalose glycopolymers (**13f**) and (**16f**) in aqueous conditions

showed that, whilst possessing a narrow dispersity Đ (1.18 for linear **(13f)** and 1.17 for star **(16f)** which are slightly narrower than that of the other glycopolymers), these polymers had hydrodynamic volumes higher than expected when compared to poly(ethylene glycol) narrow standards used as SEC calibrants, a phenomenon that is in line with a previous report by Maynard and co-workers for styrene-based trehalose polymers.[19] In our study we found that trehalose glycopolymers (**13f**) and (**16f**) had a molecular weight slightly higher than that of disaccharide lactose (**13e**) and (**16e**) (37.6 *vs.* 25.5 kDa for linear, 55.9 *vs.* 42.6 kDa for star glycopolymers, for trehalose and lactose, respectively; Table 1). Although studies on the detailed mechanism of these protein aggregation phenomena are currently on-going, the higher hydrodynamic volume of trehalose glycopolymers might explain the distinctive behaviour of solutions containing these glycopolymers in the accelerated stability studies. These results are in agreement with previous work by Miura and co-workers, who showed that the fibrillation of Aβ(1-40) was favoured for polyacrylamide-based linear glycopolymers with trehalose moieties present at each polymer repeating unit - thus structurally analogous to (**13f**)..[58] Interestingly, these authors also found that when their trehalose monomer was copolymerised with an excess of acrylamide, a significantly smaller, hydrophilic monomer, the resulting materials could reduce fibrillation of Aβ(1-40). This trend could be ascribed, at least in part, to the different macromolecular crowding provided by trehalose homo- and co-polymers investigated in their work.

Simultaneous study of the conformational and colloidal stability of protein formulations are of great importance to understand the phenomena underpinning the lack of long-term stability of a number of potential mAb biotherapeutics. The stability of the CH2 domain appeared to be governing the overall behaviour of mAb1; the glycopolymers solutions that decreased $T_{m,CH2}$ resulted in decreased mAb1 *monomer* content, *i.e.* presence of antibody aggregation in the accelerated stability studies. For the latter, trehalose polymers overall produced the most marked effect on the colloidal stability of mAb1, with visible precipitation observed from as early as week two. Although the distinctiveness of the colloidal stability profile of the trehalose polymers (T_{agg}, Figure S47) was noticed during initial characterisation stage, it was only during the accelerated stability studies that the extent of mAb1 aggregation was appreciated, due to the visible precipitates.

Overall, the trend of decreased conformational and colloidal stability of mAb1, assessed from intrinsic fluorescence and static light scattering measurements ($T_{m,CH2}$ and T_{agg}) of thermally stressed formulations, appears to be generally in line the trends from accelerated stability

studies, despite high antibody concentrations in the latter, therefore suggesting that screening of T_m and T_{agg} could be employed to predict the stability of selected mAbs.

Conclusions

Two libraries of well-defined glycopolymers with linear and four-arm star architectures were successfully synthesised via ATRP and post polymerisation modification via 'click chemistry'. Applied as solution excipients to a model antibody solution, these glycopolymers modulated both the conformational and colloidal stability of a model monoclonal antibody, mAb1. Initial structure-function relationships for these macromolecular excipients were identified, with effects dependent on the nature of sugar monomer, concentration in solution and polymer architecture. The glycopolymers mostly decreased the stability of formulations of the model antibody at lower concentrations, opposite to what was observed to the corresponding low molecular weight sugars. Moreover, the polymers often destabilised the protein in solution to a greater extent at higher concentrations. Interestingly, this induction of mAb1 aggregation was particularly pronounced for trehalose-based glycopolymers, especially at their higher concentrations, and was only appreciated in long term stability studies. It is notable that effects of low molecular weight sugar excipients cannot be extrapolated to effects of corresponding glycopolymers and that often at higher concentrations of glycopolymers in solution induction of antibody unfolding and/or aggregation, rather than stabilisation to aggregation, prevails. More studies of the mechanisms involved are still required to understand this phenomenon and are currently being undertaken. This study also suggests that the use of glycopolymers as excipients of protein liquid formulations is very much dependent on the physico-chemical characteristics of these macromolecular materials (and possibly on the nature of the protein therapeutic of choice), hence their suitability as stabilisers for protein liquid formulations needs to be evaluated on a case by case basis.

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21. This histidine-containing buffer was utilized as mAB1 is routinely stored into this medium to ensure mAb1 conformational integrity during storage. mAb1 was tested in this medium in the presence and in the absence of polymers, thus the effect of the polymers on the overall stability of mAb1 could be assessed.

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