Pharm Res (2012) 29:683–694
DOI 10.1007/s11095-011-0593-4

RESEARCH PAPER

Opposite Effects of Polyols on Antibody Aggregation: Thermal *Versus* Mechanical Stresses

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Received: 12 July 2011 / Accepted: 14 September 2011 / Published online: 27 September 2011

ABSTRACT

Purpose To investigate the physical stability of antibody-polyol formulations under thermal and mechanical stresses.

Methods mAb-U was analyzed in buffer, trehalose, sucrose, glycerol and ethylene glycol solutions at pH 7.0. T_{m1} of mAb-U was determined using DSC. Thermal stress studies were performed by incubating mAb-U-polyol solutions at 40°C (2 months), 50°C (3 weeks) and 65°C (5 days). Mechanical stress studies were conducted by shaking mAb-U-polyol solutions at 200 rpm for 5 days at 25°C.

Results Trehalose and glycerol increased the T_{m1} of mAb-U, whereas ethylene glycol decreased it. The trend observed in the order of increasing aggregation of mAb-U after thermal stress (40°C and 50°C) was buffer = trehalose = sucrose< glycerol<ethylene glycol. A similar trend in aggregation was observed for 65°C studies. An inverse relationship was observed in aggregation trends upon exposure to mechanical and thermal stresses.

Conclusions Preferentially excluded polyols increase the conformational stability of proteins but also increase their chemical potential in the solution phase. This increase in free energy can promote precipitation and interfacial adsorption of a protein as these reactions result in a decrease in its free energy. Therefore, addition of polyols can be destabilizing for the physical stability of aqueous protein formulations

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KEY WORDS antibody aggregation · preferential exclusion · protein adsorption · protein conformational stability · protein solubility

ABBREVIATIONS

DSC	differential scanning calorimeter
mAb-U	monoclonal antibody-U
PHI	polyol hydrophobicity index
SE-HPLC	size exclusion high performance liquid chromatography
T_m	thermal transition temperature
Trp	tryptophan
UV	ultraviolet

INTRODUCTION

Aggregation of proteins poses a major challenge in formulation development as it can render a product unsuitable for release as a marketed product and it can also lead to an immunogenic response in a patient (1). Since a protein formulation needs to be stable over the shelf life of 18–24 months, it is important to predict and minimize aggregation in a formulation. Proteins can undergo aggregation through both chemical and physical reaction pathways. Physical destabilization can occur upon denaturation of proteins, adsorption of proteins onto surfaces and precipitation.

Denaturation or unfolding of proteins involves the loss of their secondary and tertiary structures. Upon perturbation of the tertiary structure of proteins, the nonpolar groups are exposed which are otherwise, buried inside a protein. Exposure of such groups in solution leads to a decrease in the entropy of water (2) resulting in an increase in the free energy of the system. To offset this thermodynamically

unfavorable condition, unfolded molecules can interact and form aggregates. Phase separation occurs when the aggregates exceed the solubility limit and fall out of the solution. The tendency of a protein to unfold in a solution is given by the difference in the free energy between its unfolded and native states (ΔG^{o}_{unf}) (3). A positive value of ΔG^{o}_{unf} means that the native state is more stable and a negative value means that the unfolded state is more stable in the solution. One of the ways of estimating the ΔG^{o}_{unf} or the conformational stability of proteins is from their thermal transition temperatures (T_m) using differential scanning calorimeter (DSC). T_m of proteins is defined as the temperature at which 50% of the protein molecules are unfolded. An increase in T_m is indicative of a protein being conformationally stabilized and vice versa. Since ΔG^{o}_{unf} is temperature dependent and it decreases with an increase in temperature, denaturation of proteins can be induced upon thermal stress. Thus, proteins are subjected to high temperatures during preformulation studies to investigate the effect of different solution conditions and excipients on their stability.

Proteins, being amphiphilic molecules, can also undergo physical destabilization through interfacial adsorption (1,4). Upon adsorption, hydrophobic groups are exposed towards air, which can result in unfolding. Interaction between unfolded molecules can lead to aggregation. Since proteins encounter a number of surfaces during purification, filling, storage and shipping, interfacial adsorption of proteins can be a major challenge in the development of protein therapeutics (5). Hence, mechanical stress is an important tool to monitor the physical stability of protein formulations. During pharmaceutical development, both temperature and mechanical (shaking, stirring, etc.) stresses are used to accelerate the aggregation process to predict long-term stability of protein formulations.

Polyols such as sucrose and trehalose are widely used as excipients in protein formulations as they have been shown to increase the T_m of proteins (6,7). The effect of such polyols on the conformational stability of proteins has been attributed to them being preferentially excluded from the vicinity of proteins (8,9). An increase in the chemical potential of a protein is observed upon preferential exclusion of polyols because the interactions between the two are unfavorable. To offset the unfavorable increase in free energy, proteins tend to reduce the contact area with the surrounding solvent. As the contact area is greater for the unfolded state than the native state, the equilibrium is shifted towards the native state, resulting in conformational stabilization of proteins in the bulk.

Polyols can also affect the solution phase stability of proteins, which can be defined as the tendency of proteins to stay as a single phase (solution phase in the case of aqueous formulations). For a protein to remain soluble, its chemical potential in the solution phase should be lower than its chemical potential in the solid state (10). Since preferentially excluded polyols increase the chemical potential of proteins, the solubility of proteins should consequently decrease (relative to water). The increase in the free energy of a protein in the solution phase can also result in its adsorption onto air/water interface because adsorption leads to a decrease in the unfavorable solvent exposed area of a protein, which in turn reduces its free energy. Hence, preferentially excluded polyols are anticipated to promote aggregation through interfacial adsorption of proteins, under mechanical stress.

However, this effect can be not generalized because the stabilizing or the destabilizing effect of polyols on the physical stability of a protein will also depend on its inherent ability to self-associate. Self-association is referred to as reversible formation of higher order species by non-covalent interactions between monomers resulting in a decrease in the solvent exposed area of a protein and thus its free energy. Hence, self-association and adsorption of proteins will be competing mechanisms upon addition of preferentially excluded polyols. If a protein is inherently more prone to self-association, then the addition of such polyols will result in stabilization under interfacial stress. However, for proteins less prone to self-association, the effect of polyols will be destabilizing because of an increase in their tendency to adsorb onto the air/water interface.

Attempts have been made to correlate T_m of proteins in different solution conditions with the trend in aggregation observed upon high temperature incubation of proteins (11,12). Since both, DSC and incubation, involve subjecting proteins to thermal stress, it is likely that the observed correlation arises from unfolding in the bulk solution being the governing mechanism of aggregation in these studies. However, besides unfolding in the bulk, there are various other pathways such as interfacial adsorption and precipitation, through which proteins can aggregate. Addition of polyols can promote these reactions as they decrease the solubility of proteins i.e. increase the free energy of proteins in solution. Since the storage temperatures of protein formulations are much lower than their T_m, it is predicted that the use of polyols can be destabilizing for some proteins. The purpose of this manuscript is to investigate the effect of polyols on the physical stability of a monoclonal antibody under both thermal and mechanical stresses.

MATERIALS AND METHODS

Materials

All buffer reagents were of highest purity grade available from commercial sources and were used without further

purification. Sucrose, trehalose dihydrate, glycerol, ethylene glycol were purchased from Sigma-Aldrich (St.Louis, MO). The monoclonal antibody, U, (mAb-U) (Genentech, Inc.) was supplied as a 20 mg/ml solution in a 20 mM histidine acetate buffer (pH 5.5) containing 4% sucrose. Deionized water equivalent to Milli-Q™ grade was used to prepare all the solutions.

Sample Preparation

All the antibody-polyol solutions were analyzed at pH 7.0 and an ionic strength of 20 mM. For this purpose, phosphate buffer at a buffer strength of 10 mM was prepared and the ionic strength was adjusted to 20 mM with NaCl. 10% w/v polyol solutions were then prepared using this buffer. All buffers and polyol solutions were filtered through Millipore's (Billerica, MA) Durapore® 0.22 μm membrane filters. Prior to analysis, mAb-U was buffer exchanged to prepare stock solutions in the phosphate buffer (pH 7.0 and ionic strength 20 mM) using the Amicon Ultra centrifugation tubes (Millipore) with a molecular weight cut-off of 10 kDa obtained from Fisher Scientific (Fair Lawn, NJ). The final concentration of the stock solutions was determined using Varian's (Palo Alto, CA) Cary 50-Bio UV–vis spectrophotometer. An absorptivity of 1.73 $(mg/ml)^{-1}cm^{-1}$ was used to measure the concentrations. The prepared stock solutions were subsequently used to prepare 5 mg/ml samples for the studies.

Differential Scanning Calorimetry

A nano-DSC (TA Instruments, New Castle, DE) was used to determine the T_m of mAb-U in buffer, 10% w/v trehalose, glycerol and ethylene glycol solutions. The instrument was allowed to run through the night with multiple scans of the blank solutions in both the sample and reference cell before the measurement of the actual samples. 1 mg/ml of mAb-U solutions (pH 7.0 and ionic strength 20 mM) were analyzed at a scan rate of 1°C/min from 25°C–90°C with a pre-scan thermostat of 10 min. From the scans, only T_{m1} could be determined accurately because of aggregation of the protein before the completion of its second unfolding transition (T_{m2}). All buffers and samples were degassed for 5 min in the degassing accessory attached with the instrument before their introduction into the DSC cells. The thermal scans were baseline subtracted and analyzed using the NanoAnalyze software (TA Instruments).

Incubation of mAb-U at 40°C, 50°C and 65°C

5 mg/ml of mAb-U solutions (pH 7.0 and ionic strength 20 mM) were prepared in buffer, 10% w/v trehalose, sucrose, glycerol and ethylene glycol solutions. 200 μl (40°C and 50°C) and 250 μl (65°C) were filtered through Millex® (Millipore) syringe filters and filled in sterile Fisherbrand® (Fisher Scientific) 0.5 ml microcentrifuge tubes with loop and O-ring. Three tubes per solution condition were incubated at 40±0.1°C for 2 months, 50±0.1°C for 3 weeks and 65±0.1°C for 5 days in an isotemp oven (Fisher Scientific). After the incubation period, the samples were centrifuged at 10,000 rpm for 5 min using an Eppendorf minispin (Hamburg, Germany).

Second Derivative Fluorescence Spectroscopy

Fluorescence measurements were carried out using Photon Technology International's (PTI) spectrophotometer (Birmingham, NJ). mAb-U samples were prepared at 0.1 mg/ml in pH 7.0 phosphate buffer at an ionic strength of 20 mM. The excitation wavelength was fixed at 295 nm (to record emission from tryptophan only) and the emission spectra were collected from 305 to 450 nm. The excitation and emission slit widths, set at 2 nm, were used to collect all the emission scans at a scan speed of 2 nm/s. Eight spectra were collected for each solution condition and averaged to obtain the final spectrum. Raman peak was subtracted from the spectrum by collecting the emission scan of buffer blank. All the emission scans were normalized to 1.0 (FeliX32™ software, PTI) before derivatization. This helped to compare the intensity of the bands in the second derivatives. FeliX32™ software uses a five point Savitzky-Golay algorithm for derivatization. For these studies, second order derivatization was used.

Surface Pressure of mAb-U Solutions at 25°C

Surface pressure (π, dyne/cm) was determined by measuring the surface tension difference between the polyol solution (γ_{m_3}) and the protein-polyol solution (γ_{2,m_3}):

$$\pi = \gamma_{m_3} - \gamma_{2,m_3} \tag{1}$$

All measurements were made using a semiautomatic Surface Tensiomat 21 (Fisher Scientific). A petri dish (60 mm×15 mm) was used for the measurements utilizing a sample volume of 15 ml. The instrument uses a platinum-iridium Du Nöuy ring based on the principle, that the force, required to detach the ring from the surface of the solution, is proportional to the surface tension. The instrument measures the apparent surface tension (S). Using the following relationship, S is converted to give the true surface tension, P.

$$S = P \times F \tag{2}$$

F, in the above equation, is a correction factor, which is dependent on the size of the ring, wire used in the ring, apparent surface tension and the densities of the two phases.

mAb-U solutions were prepared at a concentration of 0.1 mg/ml in buffer, 10% w/v sucrose and ethylene glycol solutions. All solutions were filtered using 0.22 μm filters and were stored for 24 h prior to making measurements.

Shaking of mAb-U Solutions at 25°C

5 mg/ml of mAb-U solutions (pH 7.0 and ionic strength 20 mM) were shaken using an analog multipurpose rotator (Model 2309) from Barnstead International (Dubuque, IA). The shaker provides a 9″×9″ inch platform and rotation through 0.75″ (1.9 cm) orbit. The solutions were shaken at 200 rpm for 5 days at 25.0±0.1°C. 1 ml of the solutions were filtered using 0.22 μm filters and filled in Fisherbrand® (Fisher Scientific) 1.8 ml glass vials with screw thread caps (03-339-21A). After the shaking period, the solutions were transferred to 2 ml Eppendorf tubes and centrifuged at 10,000 rpm for 15 min using the Eppendorf minispin.

Size-Exclusion High Performance Liquid Chromatography (SE-HPLC)

Before stressing, all the samples were analyzed for monomer and soluble aggregate content. For this purpose, SE-HPLC was used with an inline UV detector set at a wavelength of 280 nm. 10 mM phosphate buffer (pH 7.0) at a total ionic strength of 300 mM (ionic strength adjusted with NaCl) was used as the mobile phase. A 300×8.0 mm inner diameter YMC-Pack Diol-200 column, DL20S05-3008WT (YMC America, Inc., Allentown, PA) was used at an isocratic flow rate of 1 ml/min with an injection volume of 30 μl. All the solutions were diluted 1:5 times in phosphate buffer (pH 7.0 and ionic strength 20 mM) prior to injection. The chromatograms recorded for the samples before stress were labeled as $t=0$.

After centrifugation of the stressed samples, the supernatant was analyzed by SE-HPLC for monomer and soluble aggregate content. All the conditions and parameters were similar to those used for the analysis of the $t=0$ samples. The software, Peak Simple 3.88 (SRI Instruments, Torrance, CA), was used to analyze the chromatograms. Percent loss in monomer is reported as the difference in the area under the peak before ($t=0$) and after the stress period. The content of soluble aggregates in the supernatant of the samples after centrifugation was calculated as the difference in the ratio of the area under the soluble aggregate peak to the total area of the chromatogram (all peaks) before ($t=0$) and after the stress period. Percent fragmentation was calculated as the ratio of the area under the fragment peak of the stressed samples to the total area of the chromatogram (all peaks).

RESULTS AND DISCUSSION

Conformational Stability

Effect of Polyols on the T_{m1} of mAb-U

To investigate the effect of polyols on the conformational stability of mAb-U, its T_{m1} was determined using DSC. Figure 1 shows the thermal scans obtained for mAb-U in buffer, 10% w/v, trehalose, glycerol, and ethylene glycol solutions. The results show that trehalose and glycerol increase the T_{m1}, with a higher increase seen for trehalose. On the other hand, ethylene glycol decreases the T_{m1} of mAb-U. The results are consistent with the published reports on the use of trehalose and glycerol as thermal stabilizers whereas, ethylene glycol has been shown to thermally destabilize various proteins (13–15).

Aggregation of proteins through unfolding is generally shown to follow the Lumry-Eyring model (16):

$$N \leftrightarrow D \rightarrow A$$

The first step of this mechanism is the unfolding/denaturation (D) of the native protein (N). At this stage, the two species are in reversible equilibrium with each other. However, the formation of aggregates (A) from the denatured protein is an irreversible process. Polyols can affect the conformational stability of proteins by affecting the free energy of unfolding of the first step of the reaction ($N \leftrightarrow D$). The increase in conformational stability imparted by polyols such as trehalose and sucrose has been explained on the basis of preferential exclusion of these polyols from the vicinity of proteins (8,9). Preferential exclusion implies

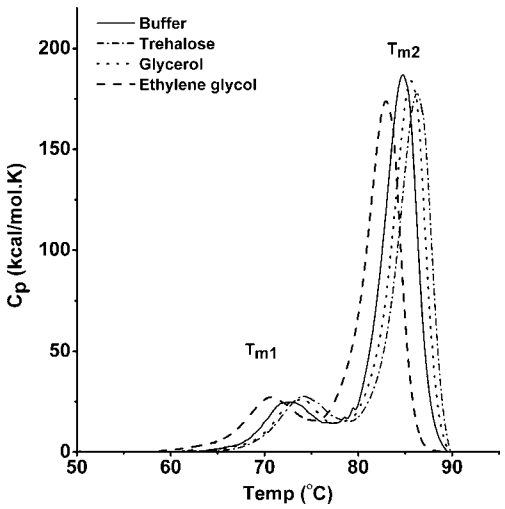

Fig. 1 DSC scans of mAb-U. 1 mg/ml solutions of mAb-U (pH 7.0 and ionic strength 20 mM) in buffer, 10% w/v trehalose, glycerol and ethylene glycol solutions were analyzed at a scan rate of 1°C/min.

unfavorable interactions between polyols and proteins, which leads to an increase in the chemical potential of proteins. However, upon expansion of the native state during unfolding, unfavorable interactions between proteins and polyols increase further as the zone of exclusion around proteins increases. Therefore, the protein conformation with the least contact area with the surrounding solvent is preferred, which is generally the native conformation of the protein. This effect on the conformational stability of proteins by polyols is described by the following equation (9):

$$\delta\Delta G^{o} = \Delta G_{m_3}^{o(D-N)} - \Delta G_{w}^{o(D-N)} \quad (4)$$

$\delta\Delta G^{o}$ is given by the difference in the standard free energy of the unfolding reaction in a polyol solution $\left(\Delta G_{m_3}^{o(D-N)}\right)$ and water $\left(\Delta G_{w}^{o(D-N)}\right)$. Since $\Delta G_{m_3}^{o(D-N)} > \Delta G_{w}^{o(D-N)}$ in preferentially excluded polyols, it results in a positive value of $\delta\Delta G^{o}$. A positive value indicates stabilization of the native state of proteins in the presence of polyols.

The shift in the equilibrium reaction towards the native state of the protein results in a corresponding increase in its T_m. It has been reported that trehalose is more preferentially excluded than glycerol (8) and thus a greater increase in T_{m1} is observed for trehalose. On the other hand, it has been shown that it is favorable to transfer the amino acid side chains from water to ethylene glycol solutions (17,18), which implies favorable interactions between a protein's amino acids and ethylene glycol. The free energy of unfolding will consequently be reduced in ethylene glycol, as it will be favorable for the protein to unfold and expose its hydrophobic groups. This in turn decreases the T_{m1} of mAb-U in 10% ethylene glycol (Fig. 1).

Effect of Polyols on the Physical Stability of mAb-U under Thermal Stress: Unfolding in the Bulk

One of the widely used accelerated methods for testing stability of proteins is storing the formulations at elevated temperatures and investigating the aggregation pathways of proteins (19). High temperatures accelerate the reaction by decreasing the energy barrier between the native and the unfolded state and increasing the collision frequency between the unfolded molecules (20,21). To study the protective effect of polyols under thermal stress, samples of mAb-U in buffer and different polyol solutions were incubated at 40°C, 50°C and 65°C. Figure 2 shows the percent soluble aggregates and fragmentation observed for mAb-U after 2 months of incubation at 40°C. The amount of soluble aggregates generated upon storage was found to be similar amongst buffer, trehalose and sucrose solutions. However, an increase in soluble aggregates is observed in the solution of glycerol and ethylene glycol, with a higher increase seen in ethylene glycol. On the other hand, fragmentation is only observed in buffer, trehalose and sucrose solutions. Figure 3a shows the chromatograms for the mAb-U before ($t=0$) and after incubation at 50°C for 3 weeks in buffer, trehalose, sucrose, glycerol and ethylene glycol. Figure 3b shows the percent soluble aggregates and fragmentation. At 50°C, fragmentation is also observed in glycerol along with buffer, trehalose and sucrose solutions. For studies conducted at 40°C and 50°C, solutions were clear after storage and only soluble aggregates and fragments were observed. Figure 4a shows the chromatograms for mAb-U before and after storage at 65°C for 5 days in buffer, 10% w/v trehalose and glycerol solutions. The chromatograms show a significant decrease in the monomer peak and the shape of the monomer peak changes significantly, which indicates major perturbation of the monomer conformation upon thermal stress. After storage at 65°C, the solutions were turbid indicative of formation of insoluble aggregates. Figure 4b shows the percent loss in monomer observed after storage. The solution without any polyol (buffer) shows highest aggregation followed by glycerol, whereas presence of trehalose results in the lowest amount of aggregation of mAb-U.

To investigate the aggregation trend observed for mAb-U in different polyols under thermal stress, its tertiary structure was analyzed as a function of temperature. It has been shown previously (22) that second derivative fluores-

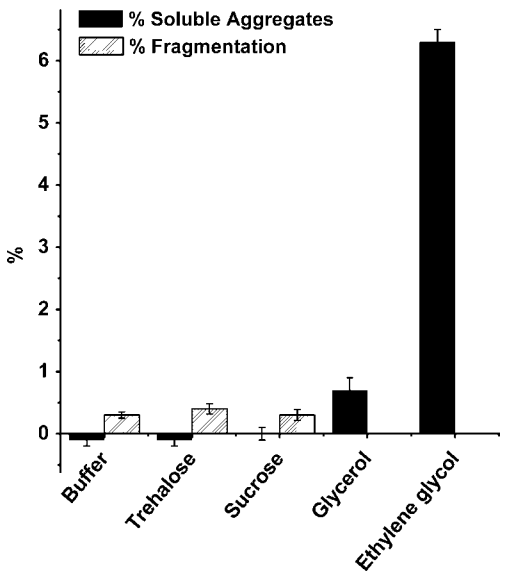

Fig. 2 Percent soluble aggregates and fragmentation observed for mAb-U at 40°C after incubation for 2 months in buffer, 10% w/v trehalose, sucrose, glycerol and ethylene glycol solutions. Samples were analyzed at a concentration of 5 mg/ml in phosphate buffer (pH 7.0) at 20 mM ionic strength. Percent soluble aggregates and fragmentation were calculated using SE-HPLC (Mobile phase: Phosphate buffer pH 7.0 and ionic strength 300 mM). Error bars are standard deviations ($n=3$).

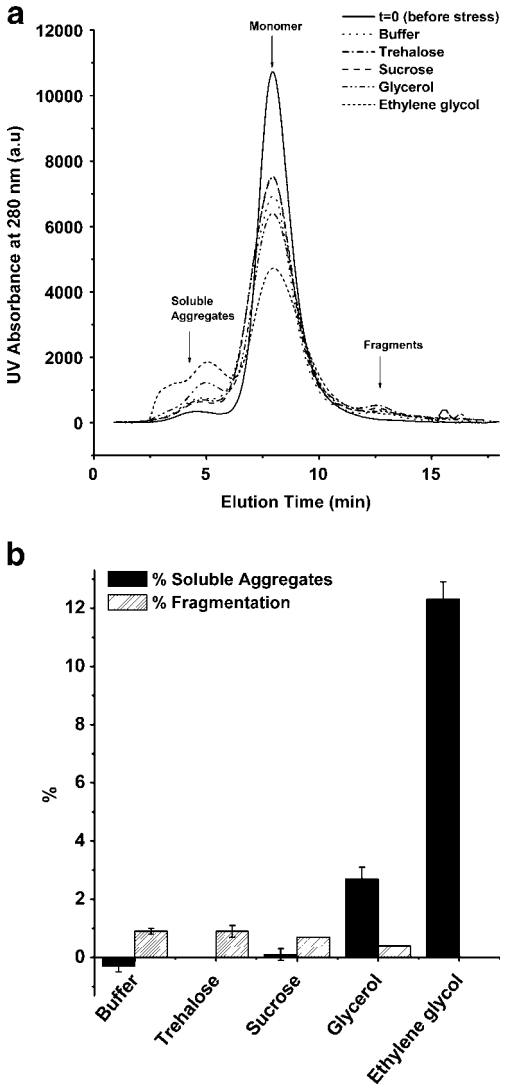

Fig. 3 Effect of polyols on the physical stability of mAb-U solutions upon storage at 50°C for 3 weeks. Samples were analyzed at a concentration of 5 mg/ml in phosphate buffer (pH 7.0) at 20 mM ionic strength. (**a**) UV chromatograms (280 nm) for mAb-U solutions before ($t = 0$) and after storage in buffer, 10% w/v trehalose, sucrose, glycerol and ethylene glycol solutions. 10 mM phosphate buffer at pH 7.0 with a total ionic strength of 300 mM was used as the mobile phase. (**b**) Percent soluble aggregates and fragmentation observed for mAb-U solutions after storage at 50°C for 3 weeks in buffer, 10% w/v trehalose, sucrose, glycerol and ethylene glycol solutions. Percent soluble aggregates were calculated using SE-HPLC (Mobile phase: Phosphate buffer pH 7.0 and ionic strength 300 mM). Error bars are standard deviations ($n = 3$).

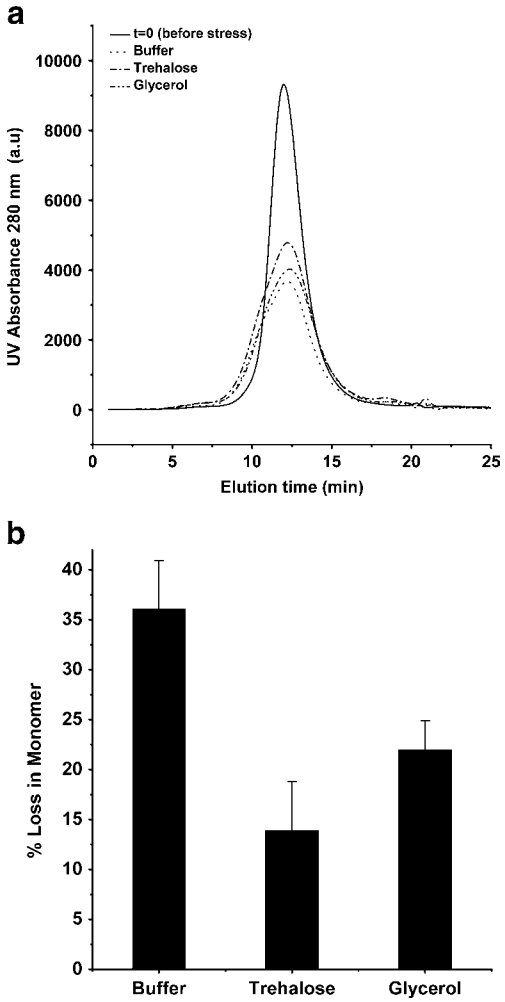

Fig. 4 Effect of polyols on the physical stability of mAb-U solutions upon storage at 65°C for 5 days. Samples were analyzed at a concentration of 5 mg/ml in phosphate buffer (pH 7.0) at 20 mM ionic strength. (**a**) UV chromatograms (280 nm) for mAb-U solutions before and after storage in buffer, 10% w/v trehalose and glycerol solutions. 10 mM phosphate buffer at pH 7.0 with a total ionic strength of 300 mM was used as the mobile phase. (**b**) Percent loss in monomer observed for mAb-U after storage at 65°C for 5 days in buffer, 10% w/v trehalose and glycerol solutions. Percent loss in monomer was calculated using SE-HPLC (Mobile phase: Phosphate buffer pH 7.0 and ionic strength 300 mM). Error bars are standard deviations ($n = 3$).

cence spectroscopy is a useful technique in studying the tertiary structure of proteins and therefore, was used in this study. A steady state emission scan reflects only the average emission behavior of all the tryptophans (Trps) present in a protein. To study the subtle changes in the microenvironment of the Trps, second derivative is performed on the steady state scans. The changes in the tertiary structure of a protein are reflected as shifts and/or increase or decrease in the intensity of the different bands in the derivatized spectrum. The main bands for a protein with two or more Trps are around 325 nm, 340 nm and 350 nm corresponding to the three classes of Trps (23,24). These classes of Trps have also been observed for a mAb (25). The first band (325 nm) in the second derivative spectrum corresponds to a Trp residue lying in a relatively hydrophobic environment, whereas the bands around 340 nm and 350 nm correspond to the surface exposed Trps.

Figure 5a shows the normalized steady state emission scans of mAb-U at different temperatures in phosphate buffer (pH 7.0) at 20 mM ionic strength. No shift in the emission maximum is observed with an increase in

temperature. Figure 5b shows the corresponding second derivatives of the normalized emission scans. Bands at 327 nm, 339 nm and 351 nm are observed in the second derivative of the native state (25°C) consistent with the three classes of Trps. In this study, the change in the tertiary structure of mAb-U is investigated by comparing the second derivative of the spectra recorded at elevated temperatures with that recorded at 25°C. From the spectra (Fig. 5b), it is seen that the tertiary structure of mAb-U is native-like at 40°C and 50°C (the bands overlap with those observed for 25°C) whereas a significant change is observed at 65°C as the shoulder band around 339 nm is lost and a decrease in the intensity of the band around 327 nm is observed. The DSC scans also show 65°C as the onset of the unfolding of the CH_2 domain (T_{m1}) of mAb-U (Fig. 1) and hence a change in the tertiary structure of mAb-U is expected. Since the structure of mAb-U is expanded at 65°C, trehalose, which is excluded more than glycerol (shows a greater increase in T_{m1}), subsequently results in lower aggregation upon incubation. Ethylene glycol, on the other hand, is a thermal destabilizer (Fig. 1) and hence shows higher aggregation at 40°C and 50°C than the thermal stabilizers, sucrose, trehalose and glycerol.

Glycerol showed lower aggregation than buffer at 65°C, whereas, it showed higher aggregation than buffer at both 40°C and 50°C (Figs. 2 and 3b) even though it increases the T_{m1} of mAb-U. The difference in the stabilization imparted by glycerol can arise from the difference in the mechanism of protein aggregation at the two temperatures. At 65°C the tertiary structure of mAb-U is perturbed and the addition of glycerol results in its stabilization upon storage because it increases the ΔG^{o}_{unf} of mAb-U (increases T_m). Thus, aggregation is dominated by the preferential exclusion mechanism near the unfolding temperature of the protein. However, glycerol has been shown to interact favorably with the hydrophobic amino acid side chains (18,26) and has been reported to increase the solubility of some proteins (27,28). Such binding of glycerol to a protein can cause conformational changes that can lead to formation of higher order aggregates as observed in this study at 40°C and 50°C for mAb-U (Figs. 2 and 3b). Therefore, at temperatures, at which a protein is still in its native conformation, aggregation is dominated by binding induced unfolding.

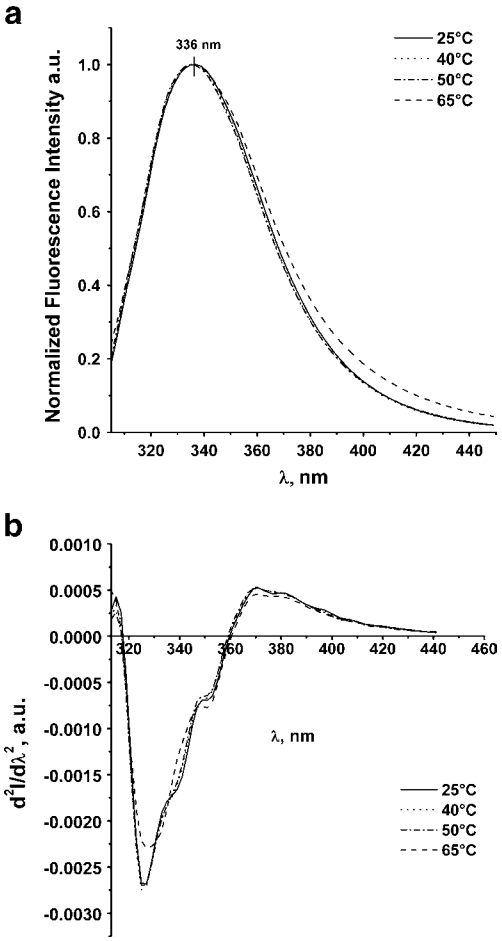

Fig. 5 Effect of thermal stress on the fluorescence emission of mAb-U (pH 7.0 and ionic strength of 20 mM) in solution. (**a**) Trp fluorescence emission scans normalized to the fluorescence intensity of 1.0 at the emission maximum. The emission scans were recorded from 305 to 450 nm using an excitation wavelength of 295 nm. (**b**) Second derivatives of the normalized Trp emission scans of mAb-U.

Solution Phase Stability

Polyols, besides imparting conformational stability to proteins, also modulate other reactions in solution such as solubility, precipitation and interfacial adsorption of proteins (29–31). Such processes affect the solution phase stability of proteins. The enhancement or inhibition of such reactions by polyols can be explained by the effect they have on the chemical potential of proteins (32). For a protein to remain in the solution phase, its chemical potential in the solution phase should be lower than its chemical potential in the precipitated state (10,33). Since preferentially excluded polyols increase the chemical potential of a protein, its tendency to precipitate increases. Upon precipitation, there is a reduction in the unfavorable contact area between the protein and the surrounding solvent, which results in a decrease in the chemical potential of the protein (30,34,35). This effect of polyols on the solubility of proteins is given by the following equation (33):

$$\Delta\mu_{tr} = \mu_2^{m_3} - \mu_2^{w} = RT \ln \frac{S_{2,w}}{S_{2,m_3}} \tag{5}$$

In the above equation, $\Delta\mu_{tr}$ is the transfer free energy of the protein molecule from water $\left(\mu_2^{w}\right)$ to the polyol solution

$\left(\mu_2^{m_3}\right)$ and $S_{2,\ w}$ and S_{2,m_3} are the solubilities of the protein in water and polyol, respectively. Since $\mu_2^{m_3} > \mu_2^{w}$, the solubility of the protein should decrease in polyols relative to water.

Effect of Polyols on the Surface Pressure of mAb-U

It has been shown that the driving force of protein adsorption is the chemical potential gradient between the bulk and the interface (36). In light of the above discussion that polyols increase the chemical potential of proteins in solution by being preferentially excluded, an increase in the adsorption gradient can lead to an increase in adsorption of a protein onto air/water interface. The effect of polyols on the propensity of proteins to adsorb onto air/water interface can be investigated by studying the surface pressure of polyol solutions upon addition of the protein. In the present study, surface pressure of mAb-U at pH 7.0 in different polyol solutions was determined using a Du Nöuy ring surface tensiometer (Fig. 6). Surface pressure of a solution is determined by the difference in the surface tension of the solvent with and without the protein as shown by the following equations:

$$\pi_1 = \gamma_0 - \gamma_{2,0} \tag{6}$$

$$\pi_2 = \gamma_{m_3} - \gamma_{2,m_3} \tag{7}$$

In the above equations, γ_0 is the surface tension of the buffer solution, and $\gamma_{2,\ 0}$ is the surface tension of mAb-U in buffer solution. The difference in the surface tension of the two solutions gives the surface pressure, π_1. π_2 is the surface pressure of mAb-U solution upon addition of a polyol and is given by the difference between the surface tension of the polyol solution, γ_{m_3}, and the surface tension of the mAb-U-polyol solution, γ_{2,m_3}. It is observed from the plot that surface pressure of mAb-U solution is higher in the presence of polyols compared to the surface pressure of the mAb-U solution without any polyol (buffer) ($\pi_1 < \pi_2$). The results are in agreement with the expected increase in surface pressure of mAb-U in the presence of preferentially excluded polyols. Since the protein is more prone to go to the interface in the presence of a preferentially excluded polyol, it will cause a greater decrease in the surface tension compared to a solution without any polyol $\left(\gamma_{2,m_3} < \gamma_{2,0}\right)$ thus resulting in a higher surface pressure. The trend seen in the order of decreasing surface pressure is sucrose > ethylene glycol > buffer. The results are also consistent with the published reports where an increase in surface pressure has been observed for proteins in various polyols (37,38).

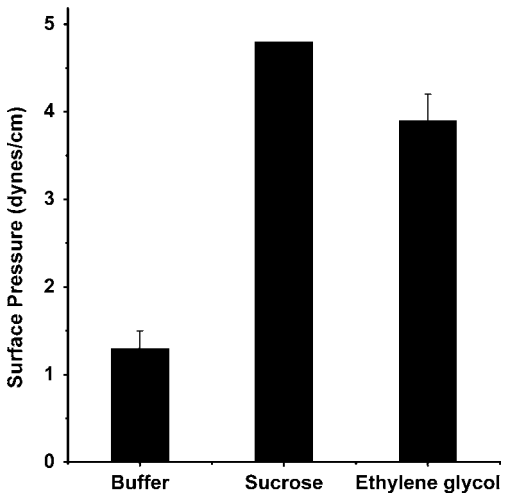

Fig. 6 Surface pressure of mAb-U in polyols. Surface tension measurements were performed on 10% w/v polyol solutions and 0.1 mg/ml mAb-U in 10% w/v polyol solutions at room temperature. The difference between the two measurements is reported as surface pressure. Error bars for buffer and ethylene glycol represent standard deviation ($n = 3$) and for sucrose it represents deviation from the mean ($n = 2$).

Effect of Polyols on the Physical Stability of mAb-U under Mechanical Stress: Air/Water Interface Adsorption

The addition of preferentially excluded polyols can increase the air/water interface adsorption of proteins, which are less prone to self-association. This can be destabilizing for liquid formulations because proteins are exposed to different interfaces during the development process. Furthermore, fill volumes less than the total vial capacity introduce air-water interface which can lead to protein aggregation upon mechanical agitation (39). To study the effect of polyols on the physical stability of proteins under mechanical stress, solutions of mAb-U were shaken at 200 rpm for 5 days at 25±0.1°C in different polyols. The results are shown in Fig. 7 and the trend observed in the order of decreasing percent loss in monomer of mAb-U is trehalose > sucrose > glycerol > ethylene glycol = buffer.

Proteins can adsorb spontaneously onto an air-water interface given that (5)

$$\Delta G_2^{ads} = \left(\Delta H_2^{ads} - T\Delta S_2^{ads}\right) < 0 \tag{8}$$

where ΔG_2^{ads} is the change in the free energy of the protein upon adsorption. For proteins, in the presence of an air-water interface, ΔG_2^{ads} is generally negative (favorable) with the entropy term driving the reaction. One of the dominant forces in protein folding arises from hydrophobic interaction, which is an aversion of the nonpolar groups of proteins for water (2,40). Exposure of nonpolar groups to water results in a decrease in the entropy of water, which is thermodynamically unfavorable. Hence, the nonpolar groups are buried inside, which gives rise to the tertiary structure of proteins. However, it is known that not all

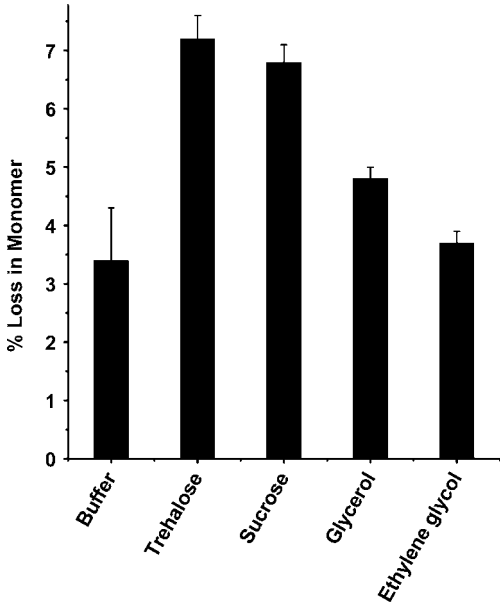

Fig. 7 Effect of polyols on the physical stability of mAb-U under mechanical stress. 5 mg/ml solutions of mAb-U (pH 7.0 and ionic strength of 20 mM) were shaken at 200 rpm for 5 days at 25.0 ± 0.1 °C in buffer, 10% w/v trehalose and glycerol solutions. Percent aggregation was calculated using SE-HPLC (Mobile phase: Phosphate buffer pH 7.0 and ionic strength 300 mM). Error bars are deviations from average of triplicate studies.

nonpolar groups are buried inside the proteins and some lie on the surface, which can lead to formation of water clathrates around them causing the entropy of water to decrease. Adsorption of proteins leads to the removal of these nonpolar groups away from water and towards air, resulting in a gain in entropy by water. Depending on the time of exposure and the conformational stability of a protein, the exposure of nonpolar groups towards air can cause a protein to unfold which leads to an increase in its conformational entropy (41). Upon constant expansion and contraction of the interface, as experienced during shaking and stirring, the unfolded protein molecules are forced back into the bulk and the interface is renewed for more proteins molecules from the bulk to adsorb (42,43). The presence of unfolded molecules in the bulk is unfavorable as the number of hydrophobic groups exposed increases, leading to a greater decrease in the entropy of water. To counteract this increase in free energy, unfolded protein molecules can interact to form aggregates, which can eventually phase separate.

The results obtained upon shaking of mAb-U are consistent with the predicted effect of polyols on the physical stability of a protein under mechanical stress (Fig. 7). Trehalose, as shown by DSC, increases the T_m more than glycerol. This suggests that trehalose is more preferentially excluded than glycerol and will therefore increase the chemical potential of mAb-U to a greater extent. Higher increase in chemical potential can lead to greater adsorption on to the air/water interface and will therefore result in higher aggregation. Hence, addition of trehalose shows higher aggregation than glycerol upon shaking. On the other hand, ethylene glycol decreases the T_m of mAb-U, which indicates preferential binding of ethylene glycol to mAb-U. Preferential binding will decrease the chemical potential of mAb-U in solution and will therefore decrease the tendency of the protein to adsorb onto the air-water interface, resulting in lower aggregation. The results also agree with those obtained by Serno *et.al* (44) and Charman *et.al* (45) where an increase in aggregation upon mechanical stress has been observed for protein-polyol solutions.

Hydrophobicity of Polyols

In an earlier publication, it was shown that polyols have both a hydrophobic and a hydrophilic part. Based on the negative transfer free energies of the hydrophobic amino acids from water to polyol solutions, it was proposed that polyols could interact with proteins via hydrophobic interactions. A Polyol Hydrophobicity Index (PHI,φ) was previously established based on molecular calculations. According to PHI, the trend in the order of increasing hydrophobicity for polyols observed was trehalose < sucrose < glycerol < ethylene glycol (18). A polar polyol is expected to interact less with the protein resulting in higher preferential exclusion and thus greater conformational/thermal stability. The relationship between PHI and percent aggregation of mAb-U under thermal stress is shown in Fig. 8a-c. A parallel relationship is observed between percent aggregation and PHI, as a more polar polyol, trehalose, causes less aggregation than a relatively less polar polyol, ethylene glycol at all the temperatures. On the other hand, an inverse relationship is observed between the percent loss in monomer obtained upon shaking of mAb-U and PHI (Fig. 9). Since hydrophobic polyols are expected to interact favorably with the hydrophobic patches on a protein's surface, they will increase the tendency of the protein to stay in solution. Therefore, ethylene glycol, one of the most hydrophobic polyol studied, shows the least amount of aggregation, whereas trehalose shows the highest aggregation.

Opposite Effects of Polyols on the Physical Stability of Proteins

The use of polyols as stabilizers in lyophilized formulations is common practice where they are hypothesized to act as hydrogen bond substitutes in the absence of water (46). However, an aqueous formulation is a dynamic system where the mobility of proteins increases the number of degradation

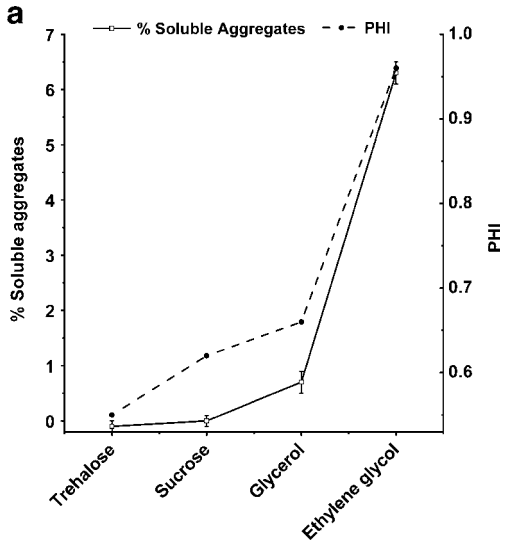

Fig. 8 Relationship between Polyol Hydrophobicity Index (PHI) and percent aggregation for mAb-U obtained upon incubation at (**a**) 40°C (**b**) 50°C and (**c**) 65°C. Numbers in parenthesis are the PHI values.

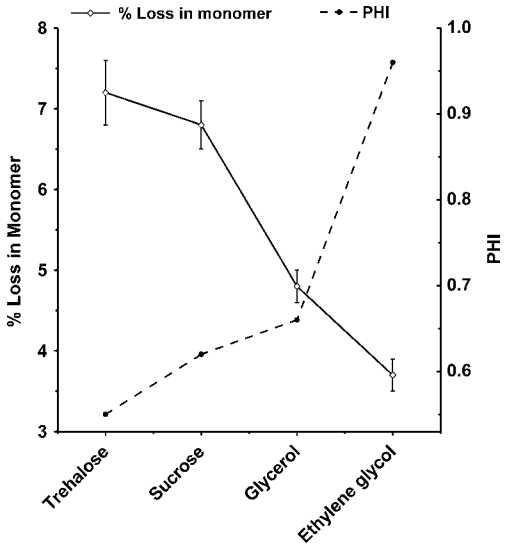

Fig. 9 Relationship between Polyol Hydrophobicity Index (PHI) and percent loss in monomer of mAb-U obtained upon shaking at 25°C.

routes and therefore, addition of polyols can have complex consequences. According to widely accepted belief, polyols in solution formulations are predicted to increase the overall physical stability of proteins because of their ability to increase their thermal stability. However, the results of this study show that thermal stabilizers can increase aggregation upon mechanical stress. At temperatures close to T_m of a protein, its native structure is prone to unfolding because the free energy of unfolding decreases with an increase in temperature. Therefore, polyols, which are thermal stabilizers, will also decrease aggregation upon incubation of a protein at high temperatures. However, the storage temperature of a protein formulation is much lower than its T_m and therefore it is highly probable that the protein native state is unperturbed. This can lead to factors such as the tendency of proteins to adsorb onto interfaces, protein solubility, protein-protein interactions and precipitation to have a greater impact on the physical stability of proteins. Thus, addition of polyols can have a destabilizing effect on the physical stability of aqueous protein formulations near storage conditions because they can induce aggregation by increasing their propensity to adsorb on to air/water interfaces. These effects can be more pronounced in high concentration protein formulations where proteins are already in a crowded environment and the effect of added excipients on their free energy in solution and solubility are more likely to govern their overall physical stability.

CONCLUSIONS

From the results of this study, it is deduced that polyols can have dual and opposite effects on the physical stability of

proteins. Besides increasing the conformational stability of proteins, preferentially excluded polyols can enhance interfacial adsorption of proteins, which can lead to aggregation. Since proteins encounter various interfaces during development, addition of such polyols can be destabilizing for aqueous protein formulations. Thus, screening methods, which are relevant to the storage conditions of proteins, should be employed to accurately investigate the effect of excipients on their physical stability, as high temperature studies may not be representative of the aggregation mechanism of a protein near storage temperatures.

ACKNOWLEDGMENTS & DISCLOSURES

The authors gratefully acknowledge financial and material support from Genentech, Inc. Support from graduate school at University of Connecticut in the form of summer fellowship is also acknowledged.

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