The Effects of Dodecyl Maltoside and Sodium Dodecyl Sulfate Surfactants on the Stability and Aggregation of Recombinant Interferon Beta-1b

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Aggregation often occurs during manufacturing and storage of protein drugs. Detergents such as sodium dodecyl sulfate are commonly used to prevent aggregation but need to be eliminated before final formulation for safety reasons. We studied the ability of dodecylmaltoside (DDM), a nontoxic alkyl saccharide surfactant, to reduce aggregation and increase the stability of interferon beta-1b (IFN)-β-1b. An increase of 8°C in the T_m of IFN-β-1b was observed when 0.1% of DDM was present in the protein solution. The absorption of DDM on hydrophobic surfaces of IFN-β-1b enables the surface to become hydrophilic and non-ionic, and increases the stability of the protein. 0.1% DDM also results in a 62% increase in helical and a 25% decrease in β-sheet structures. 0.1% DDM not only suppresses aggregate formation but also improves IFN-β-1b solubilization. Furthermore, we have showed the protective effect of DDM on the anti-viral activity of IFN-β-1b in solution.

Introduction

RECOMBINANT HUMAN INTERFERON BETA (rhIFN-β) is widely used to treat relapsing-remitting multiple sclerosis. There are 2 different IFN-β product types in the market: IFN-β-1b (eg, Betaferon® and Extavia®) and IFN-β-1a (eg, Avonex® and Rebif®). The products differ in various aspects such as formulation and route of administration. In addition, IFN-β-1b is produced in *Escherichia coli* and is non-glycosylated; whereas IFN-β-1a is produced in CHO cells and is glycosylated. IFN-β-1a has the same amino-acid sequence as endogenous human IFN-β, while IFN-β-1b has its Cys-17 mutated to a serine and the N-terminal methionine is deleted. Furthermore, IFN-β-1b (Betaferon) contains ∼60% non-covalent aggregates and IFN-β-1a (Avonex) contains mainly monomeric IFN-β with a low percentage of non-covalent aggregates (Runkel and others 1998).

Many studies have shown that aggregates in rhIFN-β products are a risk factor for immunogenicity (Schellekens 2002; Frokjaer and Otzen 2005; de Vries and others 2009; Wolbink and others 2009). Anti-rhIFN-β antibodies have been shown to lower treatment efficacy.

Surfactants are widely used in pharmaceutical protein formulations to compete with protein for interfaces that might induce unfolding, aggregation, and precipitation of the protein. They hinder a broad range of physical instability phenomena such as protein–protein association, aggregation, and precipitation. Thus, they are essential components of many protein formulations (Maggio 2012).

Surfactants, or detergents, have been used not only to purify, isolate, or solubilize proteins, but also to maintain biological activity by binding to proteins through electrostatic and hydrophobic interactions (Subramanian and others 1986).

A detergent extensively used during protein purification is sodium dodecyl sulfate (SDS). SDS inhibits aggregate formation; however, it is a toxic surfactant and has been shown to irritate skin and eyes (Marrakchi and Maibach 2006), and it needs to be eliminated during formulation. However, its removal may lead to irreversible aggregation, protein precipitation, and greater production costs (Seddon and others 2004).

Non-ionic-ether-based surfactants show substantial improvements in the stability of proteins (Steinhardt and Reynolds 1969; Stubbs and others 1976; Maggio 2010; Rifkin and others 2011). A broad range of alkylsaccharides (non-ionic surfactants) has been studied as transmucosal absorption enhancers (Pillion and others 1994, 2002; Ahsan and others 2001). More recently, it was discovered that some of these molecules such as dodecylmaltoside (DDM) are highly effective in preventing aggregation (Rifkin and others 2011).

DDM, composed of a hydrophilic maltose and a hydrophobic alkyl chain, has been demonstrated to increase solubilization of diverse membrane proteins (Lopez and others

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2002), to stabilize proteins during lyophilization and subsequent reconstitution (Maggio 2008), and, therefore, to lower aggregation. DDM is non-toxic, non-mutagenic, and non-irritating (Rossi 2005; Rifkin and others 2011) and it metabolizes into fatty acids and sugar after administration (Weber and Benning 1984; Rifkin and others 2011). DDM has been used in the formulation of peptide drugs such as eye-drops of insulin. The biodegradation of alkylglycosides to common products of body metabolism is a significant property for an absorption enhancer, which itself may be absorbed, and minimizes the systemic toxicity of this class of compounds (Pillion and others 1995). Moreover, DDM has been shown to improve the transport of insulin across epithelial monolayers without causing cell damage (Tirumalasetty and Eley 2005).

In this study, the ability of DDM to increase the stability of IFN-β-1b and reduce its aggregation was analyzed and compared with SDS using different physical and mathematical methods.

Materials and Methods

Materials

IFN-β-1b at a concentration of 330 and 150 μg mL^{-1} in 50 mM sodium acetate buffer at pH 5.5 was a kind gift from Zistdaru Danesh Co. Ltd. DDM was purchased from Thermo Fisher Scientific, and SDS was purchased from Sigma-Aldrich. Protein concentration was determined by UV absorbance measurements at a wavelength of 280 nm with an extinction coefficient of 1.58.

Visual inspection

Samples were inspected visually against a dark background before further analysis with water as a control.

UV spectroscopy

UV spectra (λ = 240–370 nm) were recorded automatically during every 5°C increase from 25°C to 70°C using an UV-2450 UV/VIS spectrophotometer in a 8-well quartz cuvette with a 1-cm path length. Samples (150 μg mL^{-1} IFN-β-1b) containing 0.1% SDS or 0.01%, 0.1%, or 0.5% DDM were analyzed. The corresponding sample buffer—50 mM sodium acetate buffer at pH 5.5—was used as a blank.

Dynamic light scattering

The average diameter (Z-ave) and polydispersity index (PDI) of the particles in each sample were obtained using dynamic light scattering (DLS). Samples (330 μg mL^{-1} IFN-β-1b) containing 0.1% SDS or 0.01%, 0.1%, or 0.5% DDM were measured at a 90° angle in a Malvern ALV CGS-3 goniometer (Malvern Instruments) that was equipped with a HeNe laser source (λ) 632.8 nm, 22 mW output power. The DLS time correlation was analyzed with ALV Correlator 3.0 software (ALV).

Fluorescence spectroscopy

The tertiary structure of IFN-β-1b was analyzed with a Horiba Fluorolog fluorometer (Horiba Jobin Yvon). Samples were excited at 295 nm, and the fluorescence emission spectra were measured between 25°C and 70°C from 307 to 450 nm in quartz cuvettes with a 1 cm path length. The slits were set at 5 nm, and the average of 3 scans was used. The corresponding buffer spectra were deducted, and the emission maxima were determined using Siano software provided by Dr. A.O. Doroshenko (Kharkov, Ukraine). The program is based on an iterative nonlinear least-squares method based on the Fletcher–Powell algorithm. The individual emission bands were estimated using a lognormal function (Siano and Metzler 1969).

Circular dichroism spectroscopy

Circular dichroism (CD) was measured in a double beam DSM 1000 CD spectrometer (Online Instrument Systems) using a quartz cuvette with a 0.5-mm path length (Hellma) at 25°C, 40°C, and 70°C. The secondary structure was expressed as molar differential extinction coefficient, ellipticity. Each spectrum was defined as the average of 3 repeated scans, and the background spectrum of the buffer was subtracted. Changes of elipticity at 222 nm were selected to analyze the opening up of helical regions in the protein structure (Kaushik and Bhat 2003). The method of Bohm and others employing CDNN CD Spectra Deconvolution Software (available 163 http://bioinformatik.biochemtech.uni-halle.de/cdnn) was applied to quantify the structural changes.

Thermodynamic calculations

By assuming a 2-state refolding/unfolding mechanism under equilibrium conditions, the Gibb's free energy change, $\Delta G°$, is referred to as the conformational stability of a protein (Fazeli and others 2013). If y is any observable parameter to follow unfolding (eg, fluorescence intensity or CD ellipticity), then

$$y = \frac{y_f + y_u \cdot exp\{-\Delta G°/RT\}}{1 + y_u \cdot exp\{-\Delta G°/RT\}} \tag{1},$$

where y_f and y_u represent the values of y for folded and unfolded protein, respectively, R is the gas constant (1.987 cal mol^{-1} K^{-1}), and T is the absolute temperature (K). A practical form of the Gibbs–Helmholtz equation for data fitting of proteins is given next:

$$\Delta G°(T) = \Delta H_m(1 - T/T_m) - \Delta C_p[T_m - T + T \cdot \ln(T/T_m)] \tag{2},$$

where T_m is the melting temperature (at the midpoint of transition) as the reference temperature, T refers to any temperature where $\Delta G°$ (T) is calculated, and ΔH_m is the enthalpy of denaturation evaluated at the T_m. Therefore, equation 1 becomes

$$y = \frac{y_f + y_u \cdot exp\{-(\Delta H_m(1 - T/T_m) - \Delta C_p[T_m - T + T \cdot \ln(T/T_m)])/RT\}}{1 + y_u \cdot exp\{-(\Delta H_m(1 - T/T_m) - \Delta C_p[T_m - T + T \cdot \ln(T/T_m)])/RT\}} \tag{3}$$

Marquardt–Levenberg routine as offered in Origin Pro v 8.5.1 software (Microcal, Inc.) was carried out for non-linear least-squares analysis to calculate thermodynamic parameters of IFN-β-1b under different solution conditions. For this purpose, equation 3 was used to fit experimental data until a fit coverage chi-square tolerance value of 1×10^{-9} was reached.

Antiviral activity

The human lung carcinoma cell line (A549) and encephalomyocarditis virus were used to evaluate antiviral the activity of IFN-β-1b. Cytopathic effects were assessed by colorimetric changes using an ELISA reader (Bio-TEK Instruments, Inc.), and the bioactivity of IFN-β-1b was subsequently analyzed. Antiviral activities of IFN-β-1b samples were determined by comparing their anti-cytopathic effect with that of the NIBSC IFN-β Ser17 mutein standard (code: 00/574) in 3 replicates.

Results

UV spectroscopy

UV spectroscopy was used to determine the presence of aggregates. The optical density at 350 nm increases, and the

ratio of 280/260 nm decreases in the presence of aggregates (Kueltzo and Middaugh 2005). Compared with IFN-β-1b in the absence of surfactant (which showed the highest OD350 nm values and the lowest OD280/260 nm ratios as the temperature increased from 25°C to 70°C (Fig. 1), most likely caused by extensive aggregation), samples in the presence of 0.1% DDM or 0.1% SDS showed low OD350 nm values and high OD280/260 nm ratios (Fig. 1). These data indicate an inhibition of aggregate formation in the presence of these surfactants.

Dynamic light scattering

Glycosylated IFN-β-1a is a cylindrical-shaped protein of roughly $2 \times 3 \times 4$ nm (Karpusas and others 1997). IFN-β-1b in the absence of surfactant showed a large Z-ave at 25°C, 40°C, and 70°C (4,010, 1,592, and 4,508 nm, respectively), indicating the presence of large aggregates independent of temperature (Table 1). The PDI of all samples decreased with increasing temperature, demonstrating a lower heterogeneity at higher temperatures.

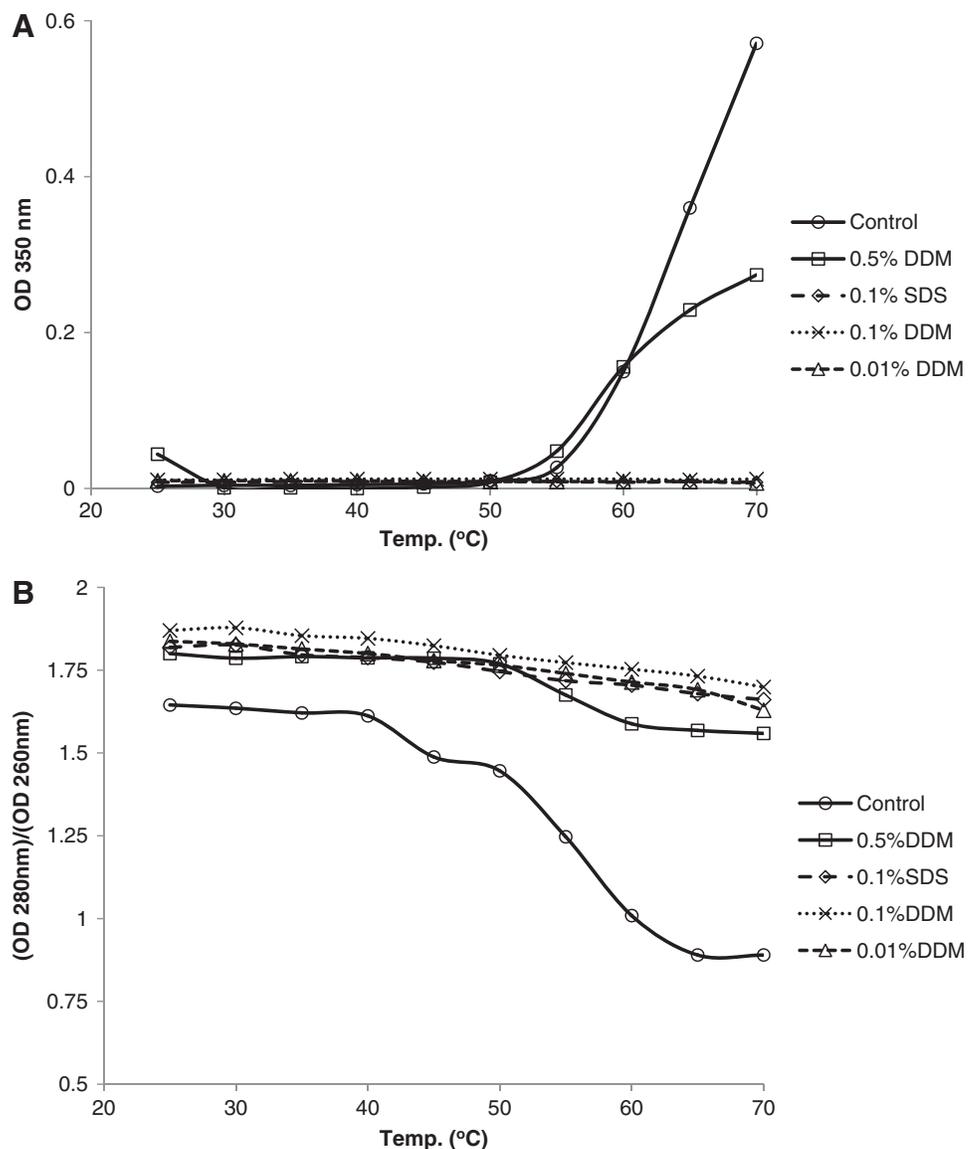The lowest Z-ave and PDI at 25°C was observed for IFN-β-1b monomer samples in the presence of 0.1% DDM,

FIG. 1. UV absorption of interferon beta-1b (IFN-β-1b). **(A)** OD350 nm and **(B)** ratio between OD280/OD260 nm in the presence of dodecylmaltoside (DDM) or sodium dodecyl sulfate (SDS) when increasing the temperature from 25°C to 70°C.

TABLE 1. DYNAMIC LIGHT SCATTERING OF INTERFERON BETA-1B IN THE PRESENCE OF DIFFERENT
CONCENTRATIONS OF DODECYLMALTOSIDE OR SODIUM DODECYL SULFATE AT 25°C, 40°C, AND 70°C

	25°C		40°C		70°C	
Temperature	Z-ave (nm)	PDI	Z-ave (nm)	PDI*	Z-ave (nm)	PDI
IFN without surfactant	4,010	0.389	1,592	0.291	4,508	0.256
IFN + 0.01% DDM	907	0.833	370	0.605	4,621	0.205
IFN + 0.1% DDM	108	0.384	82	0.367	113	0.282
IFN + 0.5% DDM	204	0.88	1,196	0.273	5,405	0.221
IFN + 0.1% SDS	200	0.517	271	0.482	2,132	0.459

DDM, dodecylmaltoside; IFN, interferon; PDI, polydispersity index; SDS, sodium dodecyl sulfate; Z-ave, average diameter.

indicating a smaller size and less heterogeneous particles (Demeester and others 2005; Philo 2009). The intense light scattering of the aggregates hinders the detection of the IFN-β-1b monomer (Van Beers and others 2010) and elevated the Z-ave to 108, 82, and 113 nm at 25°C, 40°C, and 70°C, respectively (Table 1) compared with the size of the soluble IFN-β-1a monomer at $2 \times 3 \times 4$ nm (Karpusas and others 1997).

The addition of 0.1% SDS reduces protein aggregates, but it was not as effective as 0.1% DDM with IFN-β-1b (Table 1). At 25°C, the Z-ave decreased from 4,010 nm in the absence of surfactant to 200 nm with 0.1% SDS, and to 108 nm with 0.1% DDM. However, 0.1% SDS did not suppress aggregation at higher temperatures; thus, the size of the particles increased from 200 nm at 25°C to 2,132 nm at 70°C. To study micelle formation, 0.01%, 0.1%, and 0.5% DDM solutions without protein were analyzed with DLS. The results showed that the Z-ave of particles increased considerably in the 0.5% DDM solution when increasing the temperature (Table 2); while at lower concentrations of DDM, the Z-ave did not change significantly. It seems that DDM starts forming micelles at concentrations higher than 0.5%. The formation of micelles could negatively affect the anti-aggregation potential of DDM.

Thermal unfolding

Thermal unfolding of IFN-β-1b was analyzed by both fluorescence and CD spectroscopy. The tryptophan residues of IFN-β-1b were excited at a wavelength of 295 nm, and a typical maximum emission was detected around 340 nm (Fig. 2) (Karpusas and others 1998; Runkel and others 2000). A comparison of the fluorescence emission peaks of IFN-β-1b in the absence (Fig. 2A) or presence (Fig. 2B) of 0.1% DDM at 25°C–70°C showed a 6–14 nm red shift. The fluorescence spectrum intensity also increased 42%–78% in

comparison with IFN-β-1b in the absence of surfactant over the temperature range tested. The melting temperatures (T_m) of protein in the presence of different concentrations of DDM were computed by fitting fluorescence spectroscopy data using equation 3. A value of 42°C was obtained for the T_m of IFN-β-1b in the absence of surfactant. While 0.01% and 0.5% DDM had no significant effect on the T_m, 0.1% DDM increased it by 8°C (Fig. 3). The enhanced intensity of the fluorescence spectrum suggests that in the presence of 0.1% DDM, the tryptophan residues are more accessible and less quenched due to the lower degree of aggregation (Chen and Barkley 1998; Fan and others 2005; Qiu and others 2008; Van Beers and others 2010). At higher temperatures, DDM protected the protein structure against denaturation.

Far-UV CD spectra of IFN-β-1b in the absence or presence of different concentrations of DDM or SDS were obtained at 25°C, 40°C, and 70°C (Fig. 4). A negative shift in ellipticity at 222 nm reflects more α-helix in the secondary structure of IFN-β-1b (Fazeli and others 2013). The intensity of the spectrum increased by adding DDM or SDS to the IFN-β-1b solution. 0.1% DDM had the most effect on preserving the helical structure of the protein (Fig. 4). The results showed that the presence of 0.1% DDM leads to a 62% increase and a 25% decrease in α-helical and β-sheet structures, respectively, at 25°C.

Antiviral activity

As described earlier, IFN-β-1b tends to form aggregates with low *in vitro* biological activity when no stabilizing additive is present in solution. IFN-β-1b in its pharmaceutical form (Betaferon and Extavia) is formulated (after reconstitution of the lyophilized drug product) with 12.5 mg/mL human albumin to protect the protein. If DDM is to be used as a potential anti-aggregation agent for IFN-β-1b, its effect on the biological activity of the protein should be determined. Therefore, desalted IFN-β-1b samples were formulated with 12.5 mg/mL human albumin or 0.1% DDM, and their activities were determined using an *in vitro* antiviral assay. Compared with the NIBSC IFN-β-1b standard, potency values of 103% ± 5% and 105% ± 5% were obtained for samples formulated with albumin and DDM, respectively, while desalted samples in the absence of additives had a potency of 23% ± 3%.

Discussion

Key in the downstream processing of rhIFN-β-1b is the stabilization of the extracted protein. The addition of 0.1% DDM to aggregated IFN-β-1b led to the dissolution of the aggregates and a 62% increase in α-helical structure at 25°C.
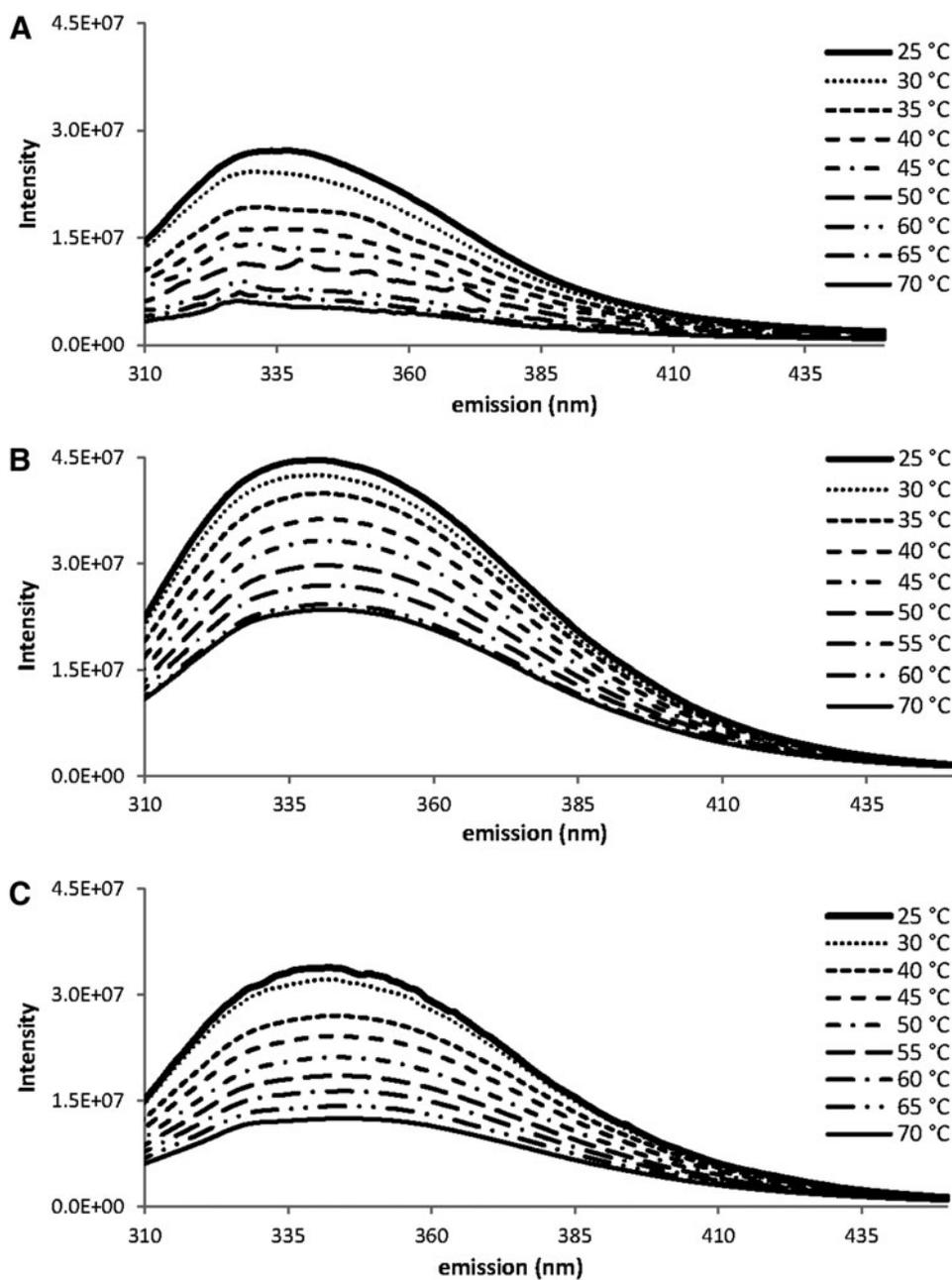
TABLE 2. DYNAMIC LIGHT SCATTERING
OF DIFFERENT CONCENTRATIONS OF DODECYLMALTOSIDE
AND SODIUM DODECYL SULFATE
WITHOUT INTERFERON BETA-1B AT 25°C AND 70°C

	25°C		75°C	
Temperature	Z-ave (nm)	PDI	Z-ave (nm)	PDI
0.5% DDM	11	0.26	194	0.19
0.1% DDM	10	0.13	11	0.29
0.01% DDM	9	0.14	11	0.19
0.1% SDS	10	0.24	19	0.27

FIG. 2. Fluorescence emission spectra of IFN-β-1b when increasing the temperature from 25°C to 70°C in the presence of **(A)** no surfactant (control); **(B)** 0.1% DDM; and **(C)** 0.1% SDS.
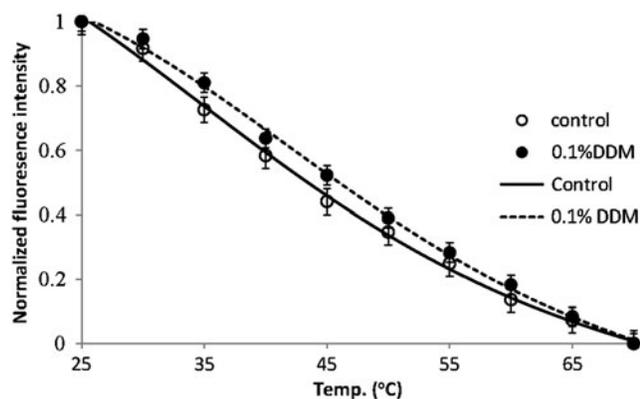

FIG. 3. Effect of 0.1% DDM on melting temperature (T_m) of IFN-β-1b. Lines are the best fit of experimental data using equation 3. $R^2 > 0.99$, $\chi^2 < 0.001$.

Thermodynamic analysis showed that DDM increased stability and did not interfere with the *in vitro* biological activity of the protein. In the presence of 0.1% DDM, IFN-β-1b showed the lowest mean size by DLS and a thermo-stability that protects the protein from aggregation at an increasing temperature. In addition, UV spectroscopy confirmed the reduction in aggregate formation. The T_m of IFN-β-1b is elevated in the presence of 0.1% of DDM, demonstrating an improvement in its stability (Pace and Shaw 2000; Kaushik and Bhat 2003).

Surfactants can bind to proteins by a combination of electrostatic and hydrophobic interactions (Subramanian and others 1986). The addition of SDS (anionic surfactant) to a protein solution results in the induction of protein denaturation by changing the protein conformation. SDS is a long-chain hydrocarbon that is highly negatively charged. There are 2 parts of the molecule, a hydrophilic, negatively
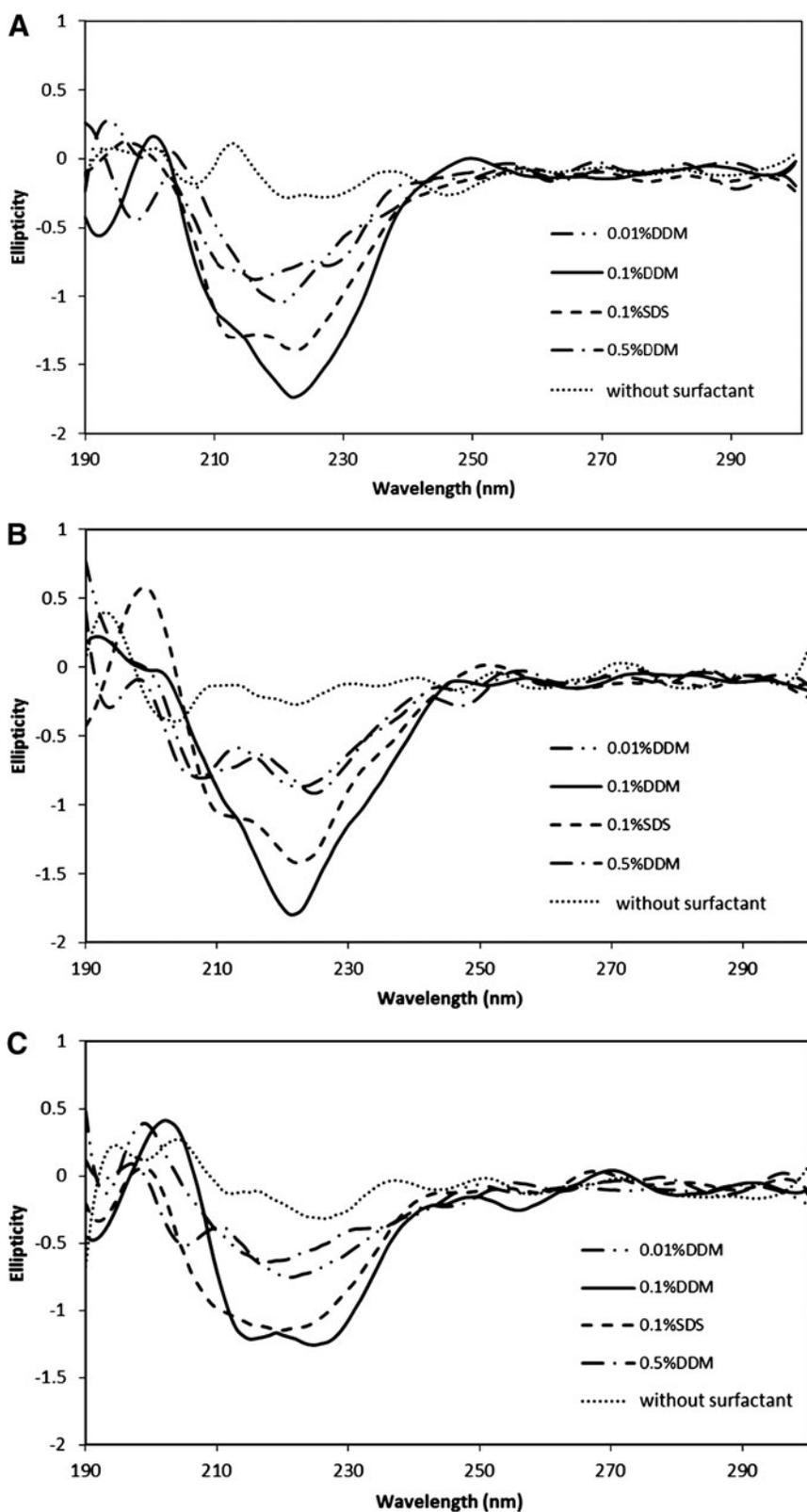
FIG. 4. Far-UV circular dichroism spectrum of IFN-β-1b formulated in the absence or presence of different concentrations of DDM and SDS. Spectra were obtained at **(A)** 25°C; **(B)** 40°C; and **(C)** 70°C.

charged head group, and a long hydrophobic chain (tail). At concentrations above 0.1%, SDS forms micelles in which the charged "head" is exposed to solvent and the hydrophobic tail is buried and the protein becomes denatured. At concentrations below 0.1%, there is generally a minimal effect on proteins (Jacqueline and Tanfordt 1970; Bhuyan 2009). In contrast, the absorption of DDM (non-ionic surfactant that has no charge on the head group) on hydrophobic surfaces of IFN-β-1b covers the protein surface. The surface then becomes hydrophilic and non-ionic due to the enhancement of solubility while preserving protein structures (Zhang and others 1997; Attwood and Florence 2008).

The results show that increasing DDM concentration beyond 0.1% is not necessarily beneficial. While the conformational stability of IFN-β-1b increased considerably by adding 0.1% DDM to the solution, 0.5% DDM did not improve the protein's stability further, possibly due to the DDM becoming less soluble with increasing temperature (Attwood and Florence 2008). The solubilization capacity of a non-ionic surfactant usually decreases with increasing temperature (Attwood and Florence 2008). In the current study, this phenomenon was observed at a concentration of 0.5% DDM using DLS (Table 2).

Indeed, DDM forms larger micelles at a concentration of 0.5% in a temperature-sensitive manner (Table 2) with no detectable large size micelles at lower concentrations (Knudsen and Hubbell 1978; De Grip and Bovee-Geurts 1979).

Furthermore, SDS is a toxic surfactant that results in irreversible aggregation, and it needs to be eliminated during pharmaceutical manufacturing, possibly resulting in higher production costs. By contrast, DDM is a nontoxic, nonmutagenic, and non-irritating (Environmental Protection Agency 2005) surfactant (Weber and Benning 1984), and it has been used in the formulation of peptides (Tirumalasetty and Eley 2005).

Finally, the anti-aggregation effect of DDM prevented the loss of biological activity in the formulated IFN-β-1b solution. We believe that DDM could be an alternative for human albumin in the formulation of IFN-β-1b. While human albumin has some drawbacks that have resulted in the search for albumin-free formulations (Shirley and others 2008), DDM seems to be a safer pharmaceutical adjuvant. Additional studies are planned to investigate this possibility.

Acknowledgments

Author Disclosure Statement

No competing financial interests exist.

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