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An Albumin-Free Formulation for *Escherichia coli*-Derived Interferon Beta-1b with Decreased Immunogenicity in Immune Tolerant Mice

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Human serum albumin (HSA)-free formulation of *Escherichia coli*-derived human interferon beta (IFNβ-1b) with a high percentage of monomeric protein and low immunogenicity is developed and characterized in the current study. UV spectroscopy, fluorescence spectroscopy, dynamic light scattering, sodium dodecyl sulfate polyacrylamide gel electrophoresis, Western blotting, Micro-Flow Imaging, resonant mass measurement, size exclusion, and reversed-phase high performance liquid chromatographies were applied to assess the effect of excipients on the stability of IFNβ-1b to establish a HSA-free formulation. The antiviral activity of IFNβ-1b was evaluated using human lung carcinoma cell line. Immune tolerant mice to hIFNβ were used to assess the immunogenicity of the HSA-free formulated IFNβ-1b in comparison to Betaferon® drug product and Avonex® drug substance as standards through IgG titering of plasma. HSA-free formulated IFNβ-1b, including 200 mM L-arginine, 200 mM trehalose, and 0.1% n-dodecyl β-D-maltoside in 10 mM sodium acetate buffer, pH 7.4, showed the highest biological activity. The stability of IFNβ-1b in the HSA-free formulation was monitored for 3 weeks at 4°C and 37°C with relative humidity of 10% and 75%, respectively. Protein aggregation and immunogenicity in transgenic mice were decreased in the HSA-free formulated IFNβ-1b compared to Betaferon. The stability, biological activity, and immunogenicity of the HSA-free formulation and Betaferon were evaluated. Incubation of formulations at 4°C and 37°C for 3 weeks showed that the HSA-free formulated IFNβ-1b was more stable and less immunogenic in transgenic FVB/N mice. Low immunogenicity and the absence of HSA, which reduces the potential risk of viral infection (eg, HIV and HCV), are promising for clinical studies.

Introduction

Interferon beta (IFNβ) is a first-line treatment for relapsing remitting multiple sclerosis. IFNβ-1b (Betaferon®) derived from *Escherichia Coli* is nonglycosylated and contains a mutation of Cys to Ser at position 17. It was the first to be approved by the FDA in 1994 followed by IFNβ-1a (Avonex®) in 1996 (Barnard and others 2013). Although, patients treated with these drugs may encounter less relapses and show reduced disability (Schellekens 2002), repeated administration over prolonged periods may result in the formation of neutralizing antibodies that may lead to reduced clinical efficacy (Schellekens 2002; Sorensen 2008; Sorensen and others 2008; Wolbink and others 2009). For Betaferon drug product, the Nab rate is 28%–47% (Paty and Li 1993; Kappos 1998; Giovannoni and others 2002), whereas for Avonex drug product it is 2%–14% (Jacobs and others 2000; Clanet and others 2002; Sominanda and others 2007). The formation of antibodies is thought to be related to protein aggregation and formulation (Barnard and others 2013). To have a successful formulation of therapeutic proteins, understanding of their physicochemical and biological characteristics is needed. The activity of proteins is highly dependent on their conformational structure (Boublik and others 1990). However, the structure of a protein is flexible and sensitive to external conditions such as those used in the expression, purification, formulation, handling, and storage of the drug (Frokjaer and Otzen 2005). Unfavorable conditions

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may lead to denaturation and formation of soluble and insoluble aggregates, and chemical instabilities including imide formation, hydrolysis, oxidation, racemization, and deamidation. All of these may affect safety and efficacy (Shirley and others 2008).

Several excipients (buffers, sugars, surfactants, amino acids, polyethylene glycols, and polymers) have been added to polypeptide pharmaceutical formulations to increase their stability. The stabilization of polypeptides in pharmaceutical compositions, however, remains largely an area of trial and error (Wang and Hanson 1988; Wang 1999) because the stabilizing effects of these chemical additives differ depending on the protein (Chi and others 2003; Kamerzell and others 2011; Ohtake and others 2011). The current IFNβ-1b product (Betaferon) is suboptimal. First, it contains high levels of aggregates that cause an immune response (Hermeling and others 2005a; van Beer and others 2012). Second, the IFNβ-1b molecule, which is nonglycosylated, has poor solubility and therefore is formulated with human serum albumin (HSA) as a solubility-enhancing agent (Shirley and others 2008). HSA is obtained from human blood and can potentially be contaminated with human viruses and other pathogenic entities. Moreover, HSA interferes with the ability to accurately measure the stability of the IFNβ-1b in the formulation (Shirley and others 2008).

To overcome these issues, there is a need for a HSA-free formulation of IFNβ-1b with lower levels of aggregates and lower immunogenicity. Previous studies of IFNβ-1b demonstrated benefits of excipients such as arginine, trehalose, and N-dodecyl-β-D-maltoside (DDM) on the stability and solubility of this protein (Ishibashi and others 2005; Kim and others 2009; Rifkin and others 2011; Fazeli and others 2013; Haji Abdolvahab and others 2014a; Fazeli and others 2014). In the current study, the combination of these excipients was examined during storage at 4 and 37°C for 3 weeks with relative humidity of 10% and 75%, respectively. A battery of analytical techniques was used to characterize the final formulation, and the immunogenicity was compared to that of Betaferon drug product and Avonex drug substance in the FVB/N immune tolerant mouse model (Haji Abdolvahab and others 2014b).

Materials and Methods

IFNβ-1b (0.78 mg/mL) frozen in 50 mM sodium acetate buffer (pH 5.6) was provided by Zistdaru Danesh Co. Ltd. DDM was obtained from Thermo Fisher Scientific (4870 AA Etten-Leur). L-arginine, D-trehalose, bovine serum albumin (BSA), and polysorbate 20 were from Sigma-Aldrich (Chemie B.V.). Sodium acetate was from Merck (64271 Darmstadt). Betaferon was purchased from Bayer (Groningenweg 1A) and was used within its expiry date. The lyophilized Betaferon powder, which consisted of 15 mg mannitol, 15 mg HSA, and 0.3 mg IFNβ-1b, was reconstituted with 1.2 mL of 0.54% NaCl in a prefilled syringe. The endotoxin level of both IFNβ-1b and Betaferon was quantified and below 0.1 ng/μg (1EU/μg).

Sample preparation

IFNβ-1b (0.78 mg/mL) was thawed and formulated with different excipients at different pHs to a concentration of 0.25 mg/mL in 10 mM sodium acetate buffer. The control sample was 0.25 mg/mL IFNβ-1b in 10 mM sodium acetate buffer (pH 5.6). Excipients were chosen based on the effect of each on stability and solubility of IFNβ-1b (Wang and Hanson 1988; van Beer and others 2012). The effects of 3 concentrations of arginine (150, 200, 250 mM), trehalose (150, 200, 250 mM), and DDM (0.01%, 0.1%, 0.5%) were analyzed individually using UV spectroscopy and dynamic light scattering (DLS) at room temperature, and 200 mM arginine, 200 mM trehalose, and 0.1% DDM were selected as the most effective concentrations of these excipients (Supplementary Tables S1 and S2 ; Supplementary Data are available online at www.liebertpub.com/jir).

In the next step, the combination of 2 excipients was tested and then IFNβ-1b was formulated with all 3 excipients. As the buffer concentration for all samples was relatively low (10 mM sodium acetate buffer), the pH was measured every week during the stability studies.

Furthermore, after determining that 200 mM arginine, 200 mM trehalose, and 0.1% DDM were an appropriate formulation, arginine, trehalose, and DDM were replaced with lysine, sucrose, and polysorbate 20, respectively, to confirm the positive effect of these excipients compared to related excipients. Therefore, 3 additional formulations were analyzed and compared to the 200 mM arginine, 200 mM trehalose, and 0.1% DDM formulation using UV spectroscopy and DLS: the 3 additional formulations are 200 mM lysine, 200 mM trehalose, and 0.1% DDM; 200 mM arginine, 200 mM sucrose, and 0.1% DDM; and 200 mM arginine, 200 mM trehalose, and 0.1% polysorbate (Supplementary Figs. S1 and S2).

UV spectroscopy

UV spectra ($\lambda = 250–360$ nm) of 250 μg/mL IFNβ-1b were measured at 25°C using a UV-2450 UV/VIS spectrophotometer (Shimadzu Co. Ltd.) with an 8-well quartz cuvette and a 1-cm path length. The corresponding sample buffer was used as the blank.

Protein concentration was calculated based on UV absorption at 280 nm and the IFNβ-1b molar extinction coefficient (1.575 mL mg^{-1} cm^{-1}). Potential presence of protein aggregates was considered by looking at an increase in OD at 350 nm and a decrease in the ratio of OD280nm/OD260nm (Haji Abdolvahab and others 2014a; van Beers and others 2010a, 2011).

Dynamic light scattering

Samples were analyzed using DLS to measure an average diameter (Z-ave) and polydispersity index (PDI) of species. 250 μg/mL IFNβ-1b was measured with a Malvern ALV CGS-3 goniometer (Malvern Instruments) equipped with a HeNe laser source ($\lambda = 632.8$ nm, 22 mW output power) at a 90° angle at 25°C. ALV Correlator 3.0 software (ALV) was used to analyze the DLS time correlation.

Antiviral activity

Human lung carcinoma (A549) cells infected with EMCV were used to estimate the antiviral activity of IFNβ-1b (Lallemand and others 2008). Antiviral activities of the individual IFNβ-1b protein samples were calculated by comparison of their anticytopathic effect (CPE) with that of the NIBSC IFNβ-1b standard (code: 00/574).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using an XCell SureLock™ Mini-Cell Electrophoresis System. 2.5 μg of samples with a volume of 10 μL was loaded to each well in a precast gel (Ready Gel, Tris–HCl, 4%–12% NuPAGE®, 10-wells× 1.5 mm; Life Technologies). Protein samples were mixed with 4× sample buffer before loading and run under nonreducing condition at room temperature at 70 V for 30 min, followed by a voltage increase to 150 V for 1 h. The electrophoresis buffer was 192 mM glycine, 25 mM Tris (hydroxymethyl) aminomethane, and 0.1% (w/v) SDS. For molecular weight determination, prestained broad range molecular weight markers (Biorad) were run and a Silver Stain Plus kit (Biorad) was used to visualize the protein bands. The gel was scanned with a Bio-Rad GS-800 densitometer using Quantity One software.

Western blotting

SDS-PAGE gels were blotted onto a nitrocellulose membrane with the iBlot® Dry Blotting System (Life Technologies) according to the manufacturer's instructions. The transfer buffer contained 3 mM sodium carbonate, 10 mM sodium hydrogen carbonate, 0.1% SDS (w/v), and 20% (v/v) methanol at pH 10.0. Blots were blocked with 5% (w/v) nonfat milk powder (Elk; Campina Melkunie) in 0.005% (w/v) polysorbate 20 in phosphate-buffered saline (PBS, consisting of 6.4 mM Na_2HPO_4, 3.6 mM KH_2PO_4, and 145 mM NaCl at pH 7.2) for 1 h at room temperature with constant orbital shaking. After washing with PBS containing 0.005% (w/v) polysorbate 20, blots were incubated with 0.2 μg/mL polyclonal rabbit anti-IFNβ antibody (Acris Antibodies) in 5% (w/v) BSA and 0.005% (w/v) polysorbate 20 in PBS overnight at 4°C with constant orbital shaking. A washing step was then carried out using 0.005% (w/v) polysorbate 20 in PBS. Blots were then incubated with peroxidase-labeled goat anti-rabbit immunoglobulin G (IgG) (Sigma Aldrich), diluted 2,000-fold in PBS containing 5% (w/v) BSA and 0.005% (w/v) polysorbate 20, for 1 h at room temperature with constant orbital shaking. Blots were then washed with 0.005% (w/v) polysorbate 20 in PBS. Protein bands were visualized using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) and a Gel Doc Luminescent Imaging system (Bio-Rad Chemdox XRS). Data were analyzed with the Quantity One software.

Size-exclusion ultra performance liquid chromatography

A Waters ACQUITY UPLC system (Waters) equipped with a sample manager and binary solvent manager was used for sample analysis. The mobile phase consisted of 50 mM sodium phosphate buffer, 200 mM arginine monohydrochloride, and 0.1% DDM at pH 7.5. Before measurement, samples were filtered through a 0.2-μm filter (GE Healthcare). Samples (7.5 μL, 250 μg/mL) were loaded onto a Waters AQUITY BEH 450 SEC (150×4.6 mm, 2.5 μm) UPLC column at a flow rate of 0.25 mL/min with a total run time of 14 min. UV absorption was recorded at 210 nm and 280 nm, and fluorescence emission was measured at 340 nm upon excitation at 295 nm. Data were collected and analyzed using the Empower Software 2. The BEH 450 SEC protein standard mix (Waters) was used to acquire a calibration curve.

Reversed-phase high-performance liquid chromatography

The HSA-free formulated IFNβ-1b and Betaferon drug product (250 μg/mL) were analyzed using reversed-phase, high-performance liquid chromatography (RP-HPLC). A Waters Alliance 2695 coupled with a Waters 2487 UV detector (214 nm) and a Waters 2475 Fluorescence detector (excitation 295 nm/emission 340 nm) was used for the detection of protein samples. The column temperature was set at 30°C and the sample temperature at 20°C. A Jupiter 300 (5 μm, 250×4.6 mm) C4 column was used in combination with a Security Guard (4×3 mm) C4 guard column (Phenomenex). The mobile phases were 10% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA) and 100% ACN, 0.1% TFA. After injection of the sample (20 μL, 250 μg/mL) at 1 mL/min, the elution gradient of 10% ACN at 0 min, 47.8% ACN at 22 min, 59.5% ACN at 45 min, 100% ACN at 50 min, followed by a 5 min equilibration was used (Geigert and others 1988).

Fluorescence spectroscopy

The changes in tertiary structure of the HSA-free formulated IFNβ-1b were monitored with a Horiba Fluorolog fluorometer (Horiba Jobin Yvon) and compared with the bulk IFNβ-1b. Excitation was carried out at 295 nm and the fluorescence emission spectra of both IFNβ-1b samples were recorded from 305 to 450 nm in a quartz cuvette with a 1-cm path length at room temperature. The slits were set at 5 nm.

Resonant mass measurement

Resonant mass measurement (RMM) (Affinity Biosensors) equipped with a ''micro'' format resonant mass sensor (channel cross section $8\times8\,\mu m^2$, resonant frequency 400 kHz) was used to analyze particles smaller than 5 μm in diameter (Burg and others 2007). Before every measurement, the sensor was flushed for 60 s with Milli-Q water. Subsequently, possible impurities in the system were removed by 2 ''sneeze'' operations followed by another flushing with Milli-Q water for 60 s. About 150 μL of sample solution was filtered using 0.2-μm filter (GE Healthcare) and was then loaded into the sensor for 45 s. Each measurement consumed about 0.1–0.3 μL of the loaded sample. Each sample was measured 3 times, obtaining values for 500 particles in each run. Particle Lab software version 1.8.510 (Affinity Biosensors) was used to differentiate and quantify protein aggregates from silicon oil.

Microflow imaging

A MFI-5200 (ProteinSimple) equipped with a saline-coated 100-μm flow cell and controlled using the MFI View System Software (MVSS), version 3.1, was used to image micron-sized particles. Milli-Q water was flushed through the system to provide a clean background, and ''optimize illumination'' procedure was performed before each sample measurement. 0.9 mL samples were drawn from a 1-mL pipette tip at a flow rate of 0.17 mL/min (0.2 mL was used as a prerun volume and 0.7 mL of each sample was monitored). Particle size was calculated as the equivalent circular diameter representing the diameter of a sphere occupying the same projection area as the particle (Sharma and others 2010; Brown 2011). MVAS version 1.3 (ProteinSimple) was used for the data analysis.

Immunogenicity

Animals

FVB/N transgenic mice immune tolerant for hIFNβ (Haji Abdolvahab and others 2014b) were bred at the Central Laboratory Animal Institute (Utrecht University, The Netherlands). The offspring were genotyped using Q-PCR showing the presence or absence of the hIFNβ gene in chromosomal DNA isolated from ear tissue. Both transgenic (PCR-positive) and nontransgenic (PCR-negative) litter mates of 12 weeks of age were used. However, the transgenic mice were genotypically positive for the hIFNβ gene, and they were not confirmed to be phenotypically positive, that is, they actually expressed the human IFNβ protein.

Animal experiments

The animal experiments were approved by the National and Institutional Ethical Committee in agreement with the European guidelines on animal experiments. A group size of 4 was calculated for this experiment (www.cs.uiowa.edu/~rlenth/Power) using power analysis. Food (Hope Farms) and water (acidified) were available ad libitum. Both transgenic (Tg) and nontransgenic (non-Tg) mice were divided into 2 experimental groups, which were injected intraperitoneally (IP) with Avonex drug substance, Betaferon drug product, or the optimized HSA-free formulation of IFNβ-1b (5 μg/injection) on days 1–5, 8–12, and 15–19. Blood was collected before injections on days 1, 8, and 15 through cheek puncture. On day 21, the mice were euthanized using decapitation under isoflurane anesthesia and the final blood was collected. Plasma was isolated by centrifugation (3,000 *g* at 4°C for 10 min) and stored at –20°C.

Enzyme-linked immunosorbent assay

Antibodies were measured using a modified direct sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (Hermeling and others 2005b; Kijanka and others 2013). Plates were coated with Avonex drug substance overnight at 4°C and blocked for 2 h at room temperature with buffer containing 4% (w/v) milk powder and 0.1% (w/v) polysorbate 20 in PBS. Peroxidase-labeled anti-mouse IgG (Invitrogen) was used as the secondary antibody and diluted 1:4,000 in blocking buffer. TMB [3,3′,5,5;-tetramethylbenzidine] (Invitrogen) was used as the substrate. The reaction was initiated by the addition of TMB and stopped with 100 μL of 0.18 M sulfuric acid. One hundred-fold diluted plasma samples were screened and defined positive if their mean absorbance values at 450 nm were 10 times higher than the negative control plasma.

The titer of positive plasma samples was determined by plotting the absorbance values of a serial dilution against log dilution. The plots were fitted to a sigmoidal dose–response curve using the GraphPad Prism version 4.00 (GraphPad Software). The reciprocal of the dilution of the EC_{50} value was defined as the titer. Because antibody levels of some plasma samples considered positive were too low or high to calculate titers as described above, OD450 values using a dilution of 1:20 or 1:500 were used for calculations, respectively.

Statistics

As IgG titers were not normally distributed, nonparametric Mann–Whitney or Kruskal–Wallis tests were applied to measure statistical differences between experimental groups, and a P-value of ≤ 0.05 was considered significant.

Results and Discussion

UV spectroscopy

To determine the effect of excipients on the aggregation of IFNβ-1b, the OD at 350 nm and the ratio of OD280nm/OD260nm were analyzed using UV spectroscopy. Samples of IFNβ-1b with different concentrations of selected excipients were monitored during 21 days of incubation at 4°C and 37°C with a relative humidity of 10% and 75%, respectively (Table 1).

As stated in Materials and Methods, 250 μg/mL IFNβ-1b was prepared first with only arginine, trehalose, or DDM. Then, the combination of 2 excipients was tested, and then IFNβ-1b was formulated with all 3 excipients. Either arginine or trehalose alone or the combination of these 2 excipients could not maintain the protein in its monomeric and native form at pH ranging from 5.5 to 8.5, and the protein precipitated after 1 day of storage at 4°C and 37°C with relative humidity of 10% and 75%, respectively. DDM, as a surfactant, was essential for the stability of IFNβ-1b in all formulations. Results indicated that in the presence of DDM or the combination of DDM and arginine, the amount of aggregates diminished (Kim and others 2009; Rifkin and others 2011; Haji Abdolvahab and others 2014a; Fazeli and others 2014); however, these 2 excipients could not prevent the formation of IFNβ-1b aggregates during storage at 37°C, and trehalose with its thermostabilizing properties was needed (Fazeli and others 2013). The formulation containing 0.1% DDM, 200 mM arginine, 200 mM trehalose, and 10 mM sodium acetate buffer (pH 7.5) showed the lowest OD at 350 nm and the highest at OD280 nm/OD260 nm, indicating lower amount of aggregates in this solution (Table 1). This formulation was selected and is referred to hereafter as ''HSA-free formulated IFNβ-1b.''

Light scattering at high wavelengths was more evident in bulk IFNβ-1b and some solutions, which suggested the presence of large aggregates. These solutions displayed the highest OD350 nm and the lowest OD280 nm/OD260 nm, most likely caused by absorption flattening due to extensive aggregation of the sample (van Beers and others 2010a, 2011; Kueltzo and Middaugh 2005). To finalize the HSA-free formulation of IFNβ-1b, each excipient in this formulation was replaced with related ones. Protein precipitations were observed after the substitution of trehalose with sucrose, and DDM with polysorbate after storage for 1 and 2 months at 25°C and 4°C, respectively; however, formulation with 200 mM lysine, 200 mM trehalose, and 0.1% DDM, in which arginine was substituted with lysine, remained stable and few visible particles were detected in this formulation. Therefore, this formulation together with the formulation containing 200 mM arginine, 200 mM trehalose, and 0.1% DDM appeared to yield improved stability, although the latter exhibited slightly lower OD 350 nm and higher OD 280/260 nm after 6 months storage at 4 and 25°C (Supplementary Fig. S1).

Dynamic light scattering

Bulk IFNβ-1b and most excipient-containing samples showed a large Z-ave at 4°C and 37°C, indicating the presence of large aggregates (Table 2). In concordance with our

observations from UV spectroscopy, the lowest Z-ave was defined for the formulation containing arginine, trehalose, and DDM (pH 7.5) at both 4°C and 37°C. However, the Z-ave of this sample (64 nm) with a corresponding large PDI (0.47) after 3 weeks incubation at 4°C indicated that the solution contained aggregates even though these values were lower than with other excipient combinations (Table 2). Although this sample contained mostly monomeric IFNβ-1b (based on further analytical methods), the strong light scattering because of the presence of aggregates overestimated the size average of the protein solution (van Beers and others 2010a, 2011). The light scattering of the aggregates prevents the detection of monomers, which may represent a much larger fraction by weight than the aggregated material (as also indicated by volume and number average distributions) (Philo 2009; van Beers and others 2010a, 2011). The samples formulated with arginine and DDM with or without trehalose (pH 7.5) showed a lower level of particles at 4°C comparable in size to those in other formulations. However, at 37°C trehalose is needed for the stability of IFNβ-1b (Fazeli and others 2013). Protein precipitation was observed in bulk IFNβ-1b solution, samples formulated with DDM, and most of the samples contained trehalose and DDM (Table 2). After replacement of each excipient, the HSA-free formulated IFNβ-1b showed the best stability after 6 months storage at 4°C and 25°C (Supplementary Fig. S2). Z-ave increased very slowly for formulations containing 200 mM arginine, 200 mM trehalose, 0.1% DDM and 200 mM lysine, 200 mM trehalose, and 0.1% DDM. However, formulations containing sucrose and polysorbate precipitated after 2 months at 4°C and 1 month at 25°C (Supplementary Fig. S2A, B). The heterogeneity of particles decreased during storage at 25°C (Supplementary Fig. S2D), with no distinct pattern of size change at 4°C (Supplementary Fig. S2C).

Antiviral activity assay

IFNβ-1b in its pharmaceutical form contains 12.5 mg/mL HSA to inhibit the absorption of hydrophobic products and maintain biological activity (Lin and others 1996; Runkel and others 1998; Hawe and Friess 2007). The antiviral activity of Betaferon drug product or of IFNβ-1b formulated with different combinations of 200 mM arginine, 200 mM trehalose, and 0.1% DDM was evaluated. Activities were determined using the antiviral activity test after the formulated samples of IFNβ-1b had been incubated at 4°C or 25°C for up to 10 weeks. A significant loss in activity was observed for Betaferon drug product, whereas the activity in samples containing arginine and

Table 1. UV Detection at 350 nm and the Ratio of 280/260 nm of Control (Bulk IFNβ-1b) and IFNβ-1b Formulated with Different Excipients

		350 nm								*280/260 nm*							
Time (day)	*pH*	*1*	*7*	*14*	*21*	*1*	*7*	*14*	*21*	*1*	*7*	*14*	*21*	*1*	*7*	*14*	*21*
Temperature		*4°C*				*37°C*				*4°C*				*37°C*			
Control		0.31	—	—	—	—	—	—	—	0.95	—	—	—	—	—	—	—
DDM[a]	5.5	0.01	0.02	0.02	0.05	0.02	0.05	0.08	0.14	2.02	1.82	1.48	1.54	1.63	1.47	1.25	1.17
	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	7.5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	8.5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tre[b] + DDM[a]	5.5	0.14	0.14	0.40	—	—	—	—	—	1.26	1.21	1.13	—	—	—	—	—
	6	0.12	0.16	0.32	—	—	—	—	—	1.27	1.14	1.23	—	—	—	—	—
	7	0.02	0.10	0.05	0.04	0.19	0.47	—	—	1.64	2.23	1.47	1.47	1.16	1.03	—	—
	7.5	0.09	0.12	0.13	0.20	—	—	—	—	1.34	1.12	1.12	1.10	—	—	—	—
	8	0.04	0.10	0.22	0.12	0.54	0.99	—	—	1.60	1.72	1.21	1.18	1.01	0.98	—	—
	8.5	0.06	0.16	0.13	0.18	—	—	—	—	1.52	1.45	1.18	1.11	—	—	—	—
Arg[c] + DDM[a]	5.5	0.04	0.04	0.04	0.04	—	—	—	—	1.50	1.25	1.26	1.23	—	—	—	—
	6	0.02	0.04	0.04	0.09	—	—	—	—	1.44	1.72	1.22	1.21	—	—	—	—
	7	0.01	0.03	0.03	0.08	0.09	0.99	—	—	1.44	1.31	1.30	1.27	1.08	0.89	—	—
	7.5	0.02	0.03	0.03	0.05	0.04	0.08	0.23	0.33	1.50	1.36	1.30	1.29	1.14	1.03	0.93	0.90
	8	0.03	0.03	0.04	0.08	0.05	0.10	0.23	0.32	1.44	1.15	1.00	1.01	1.13	1.02	0.90	0.85
	8.5	0.04	0.03	0.04	0.08	0.07	0.13	0.25	0.32	1.32	1.25	0.88	0.99	1.10	1.00	0.88	0.81
Arg[c] + Tre[b] + DDM[a]	5.5	0.01	0.04	0.04	0.05	0.09	0.31	0.44	0.55	1.75	1.50	1.32	1.27	1.01	1.05	0.78	1.03
	6	0.02	0.04	0.03	0.03	0.09	0.21	0.38	0.44	1.75	1.52	1.34	1.25	0.99	1.13	0.86	1.04
	7	0.01	0.03	0.02	0.03	0.07	0.03	0.28	0.32	1.80	1.76	1.49	1.29	1.32	1.13	0.90	1.05
	7.5	0.01	0.02	0.02	0.03	0.02	0.03	0.13	0.24	2.01	1.97	1.64	1.44	1.45	1.15	1.12	1.04
	8	0.01	0.04	0.04	0.04	0.03	0.09	0.14	0.25	1.87	1.61	1.42	1.29	1.40	1.15	1.13	1.04
	8.5	0.01	0.03	0.03	0.05	0.06	0.06	0.18	0.28	1.77	1.40	1.30	1.30	1.33	1.14	1.08	1.05

—, Indicates visible precipitation in the solution.
[a]0.1% (w/v) DDM was added to the bulk IFNβ-1b.
[b]200 mM trehalose was added to the bulk IFNβ-1b.
[c]200mM arginine was added to the bulk IFNβ-1b.
Formulations with either 200 mM arginine or 200 mM trehalose and combination of these 2 excipients at different pH values precipitated after 1 day of storage at 4°C and 37°C with a relative humidity of 10% and 75%, respectively; therefore, these results are not shown.

Table 2. Z-ave (nm) and PDI of Control (Bulk IFNβ-1b) and IFNβ-1b Formulated with Different Excipients

		Z-Ave (nm)								*PDI*							
Time (day)	*pH*	*1*	*7*	*14*	*21*	*1*	*7*	*14*	*21*	*1*	*7*	*14*	*21*	*1*	*7*	*14*	*21*
Temperature		*4°C*				*37°C*				*4°C*				*37°C*			
Control		175	—	—	—	—	—	—	—	0.60	—	—	—	—	—	—	—
DDM[a]	5.5	37	47	103	134	50	95	148	259	0.51	0.51	0.67	0.85	0.33	0.32	0.33	0.53
	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	7.5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	8.5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tre[b] + DDM[a]	5.5	2504	6432	8980	—	—	—	—	—	0.49	0.78	0.46	—	—	—	—	—
	6	1081	3572	4490	—	—	—	—	—	0.55	0.56	0.63	—	—	—	—	—
	7	226	320	987	1086	4650	—	—	—	0.96	0.90	0.87	0.97	0.46	—	—	—
	7.5	279	336	1012	2024	—	—	—	—	0.26	1.00	0.97	0.89	—	—	—	—
	8	121	183	550	2351	7573	—	—	—	0.77	0.30	0.29	0.25	0.51	—	—	—
	8.5	157	238	425	3312	—	—	—	—	0.65	0.26	0.19	0.36	—	—	—	—
Arg[c] + DDM[a]	5.5	111	121	165	171	1226	—	—	—	0.50	0.60	0.62	0.43	0.55	—	—	—
	6	112	113	141	165	1323	—	—	—	0.54	0.55	0.65	0.54	0.52	—	—	—
	7	93	171	186	282	126	1240	—	—	0.59	0.71	0.73	0.60	0.51	1.00	—	—
	7.5	60	84	134	157	95	920	1192	3247	0.32	0.46	0.78	0.73	0.56	0.89	0.79	1.00
	8	84	123	241	264	107	2692	3254	3357	0.63	0.81	0.97	0.83	0.52	0.53	1.00	0.58
	8.5	115	228	231	374	128	1064	1691	2356	0.75	0.93	1.00	0.86	0.68	0.47	1.00	1.00
Arg[c] + Tre[b] + DDM[a]	5.5	109	115	192	199	446	640	1075	1127	0.57	0.71	0.54	0.67	0.28	0.66	0.98	0.98
	6	110	118	140	234	415	450	1113	1774	0.75	0.71	0.87	0.94	0.31	0.60	0.90	1.00
	7	63	85	78	124	94	387	1180	1036	0.74	0.44	0.64	0.56	0.24	0.41	0.89	0.89
	7.5	59	71	67	64	86	449	580	621	0.41	0.40	0.44	0.47	0.26	0.45	0.77	0.77
	8	86	149	154	160	101	340	635	755	0.56	0.96	0.92	0.99	0.51	0.38	0.58	0.58
	8.5	152	164	182	304	123	542	858	967	0.75	0.93	0.97	1.00	0.54	0.46	0.87	0.87

—, Indicates visible precipitation in the solution.
[a]0.1% (w/v) DDM was added to the bulk IFNβ-1b.
[b]200 mM trehalose was added to the bulk IFNβ-1b.
[c]200 mM arginine was added to the bulk IFNβ-1b.
Formulations with either 200 mM arginine or 200 mM trehalose and combination of these 2 excipients at different pH values precipitated after 1 day of storage at 4 and 37°C with a relative humidity of 10% and 75%, respectively; therefore, these results are not shown.
PDI, polydispersity index.

DDM or arginine, DDM, and trehalose showed a smaller loss of activity (Fig. 1 and Supplementary Table S3).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The HSA-free formulated IFNβ-1b was analyzed using SDS-PAGE and compared to Betaferon drug product. SDS-PAGE under nonreducing conditions was applied to assess the relative molecular masses of the IFNβ-1b monomer and any covalent aggregates (Runkel and others 1998; Walker 2002; van Beers and others 2011). The HSA-free formulation contained monomers without any covalent dimer, trimer, or larger cross-linked aggregates, whereas the Betaferon drug product consisted of monomers, dimer, and covalent cross-linked aggregates of IFNβ-1b and/or HSA (Fig. 2A).

The 18.5-kDa band corresponds to the monomeric form of IFNβ-1b (Runkel and others 1998) detected in both samples (Fig. 2A). HSA with the molecular mass of 67 kDa was detected in the Betaferon drug product. The larger covalent cross-linked aggregates observed in this sample could be IFNβ-1b aggregates and/or HSA aggregates. A recent report indicated that Betaferon drug product contains about 15% aggregates (Barnard and others 2013), whereas others

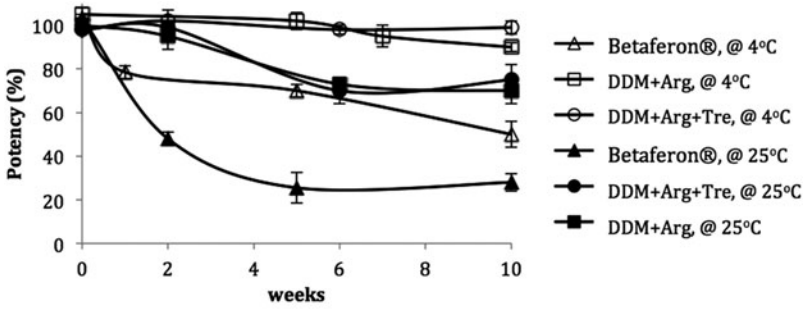

FIG. 1. Antiviral activity (potency) of different formulations of IFNβ-1b.

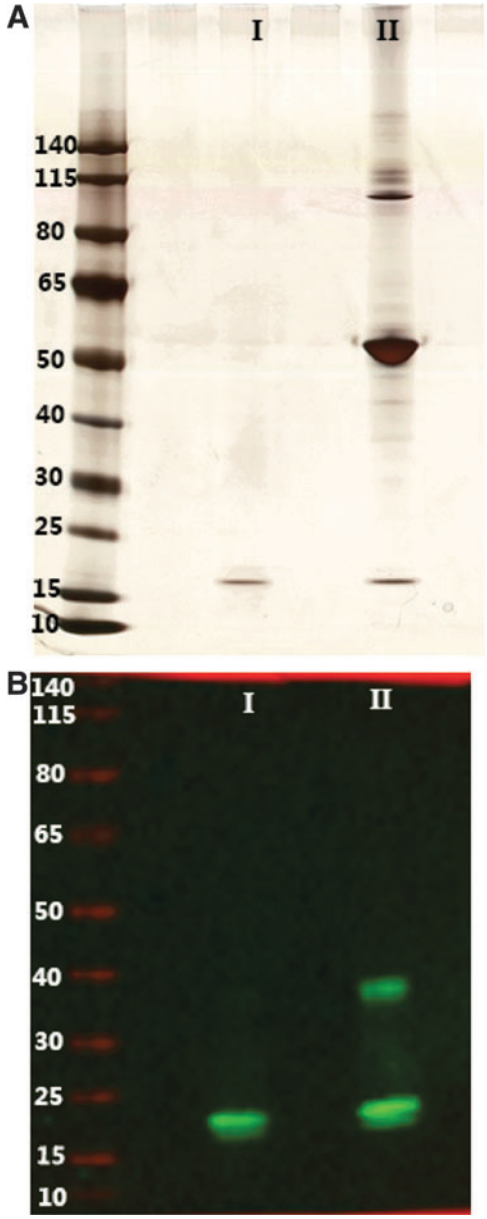

FIG. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis under nonreducing conditions **(A)** and the corresponding Western blot of the gel **(B)** of human serum albumin (HSA)-free formulated IFNβ-1b and Betaferon® drug product. Numbers on the *left* and *right* represent band positions (in kDa) of the molecular weight markers. Lane I, HSA-free formulated IFNβ-1b; Lane II, Betaferon drug product. Color images available online at www.liebertpub.com/jir

indicate levels up to 60% (Runkel and others 1998). The results reported here showed that only monomeric forms were detected using SDS-PAGE when IFNβ-1b was formulated in 200 mM arginine, 200 mM trehalose, and 0.1% DDM, and the larger aggregates that cause an increase in z-average values from DLS may have simply dissolved under conditions used for SDS gel electrophoresis.

Western blotting

The monomeric band of IFNβ-1b in the HSA-free formulation and Betaferon drug product was clearly detected under nonreducing conditions, whereas the dimeric form (37 kDa band), which is present in both samples, was barely detectable in the HSA-free formulation (Fig. 2B). Interestingly, larger aggregates detected using SDS-PAGE were not observed with Western blotting indicating that they were either related to HSA and/or possibly the amount of IFNβ-1b aggregates are too low (less than 0.2% of total protein), to be detected using Western blotting (Gomes 2009) (Fig. 2B). However, the latter is less likely being that Western blotting is generally more sensitive than SDS-PAGE.

In concordance with SDS-PAGE results, no multimeric protein bands were detected in the HSA-free formulation of IFNβ-1b, suggesting that the protein remained in its monomeric form (Fig. 2B).

Size-exclusion ultra performance liquid chromatography

The HSA-free formulated IFNβ-1b was analyzed with a Waters AQUITY BEH 450 SEC UPLC column. The mobile phase contained 0.1% (w/v) DDM to reduce the interaction of the protein with the column matrix, and 200 mM arginine and 50 mM sodium acetate buffer were used to keep the protein stable.

HSA is commonly used in formulations to prevent adsorption (Hawe and Friess 2008). The absence of HSA can cause low protein recovery using this procedure (Hawe and Friess 2008). The use of 0.1% DDM in the mobile phase decreased the adsorption of the protein to the column matrix and also increased the resolution. The HSA-free formulated IFNβ-1b was analyzed using SDS-PAGE before size-exclusion ultra performance liquid chromatography (SE-UPLC) analysis. The results of SE-UPLC showed that the sample contained only monomers and confirmed the results obtained using SDS-PAGE (Figs. 3 and 2A). This observation suggests that the aggregates detected using DLS were likely larger than 0.2 μm and removed during the filtration step before analysis.

Reversed-phase high-performance liquid chromatography

RP-HPLC was used to analyze oxidized or degraded forms of IFNβ-1b. The oxidized form of the protein has been reported to elute before the main peak, followed by other peaks containing oligomerized protein (van Beers and others 2011; Fazeli and others 2014). Oxidized IFNβ-1b was run as a control to qualify the method (data not shown). Neither the HSA-free formulated IFNβ-1b nor the Betaferon drug product showed detectable degradation and oxidation peaks (Fig. 4A, B). Native IFNβ-1b protein peaks eluting at 30–32 min in both samples were as reported previously (Geigert and others 1988; Fazeli and others 2014). Furthermore, Betaferon drug product showed an extra peak eluting at 20–22 min that corresponds to that of HSA (Fig. 4A, B). Because of the gradient elution applied, hydrophilic proteins should elute sooner and hydrophobic molecules should elute later. Therefore, HSA, which is more hydrophilic, is eluted first and then IFNβ-1b, which is known to be hydrophobic in the absence of the glycan chain attached to Asn-80, is eluted later (Fig. 4A, B).

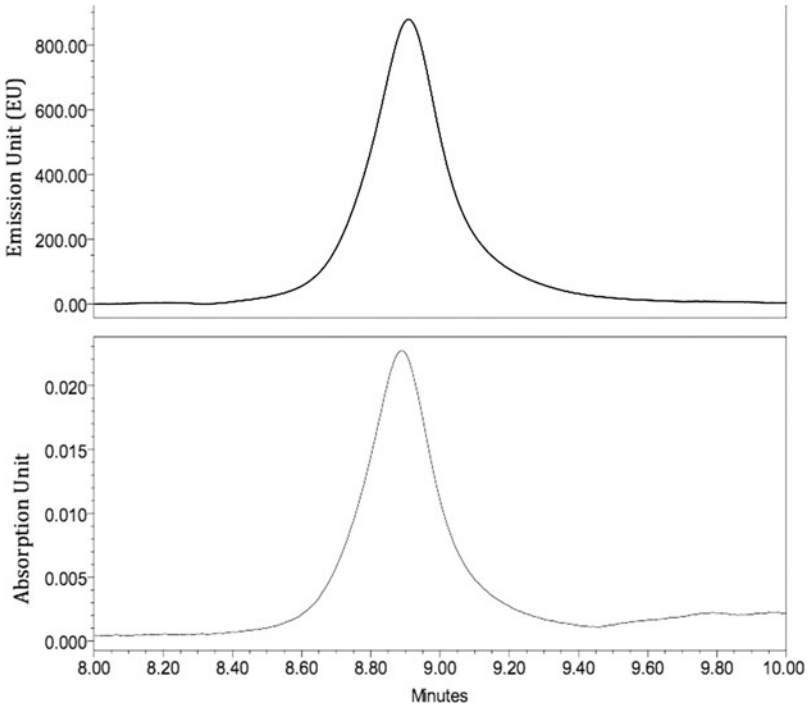

FIG. 3. Size-exclusion chromatograms of the HSA-free formulated IFNβ-1b. The size-exclusion ultra performance liquid chromatography peaks were recorded using a UV detector at 280 nm and the emission florescence peak using a florescence detector at 340 nm with excitation at 295 nm.

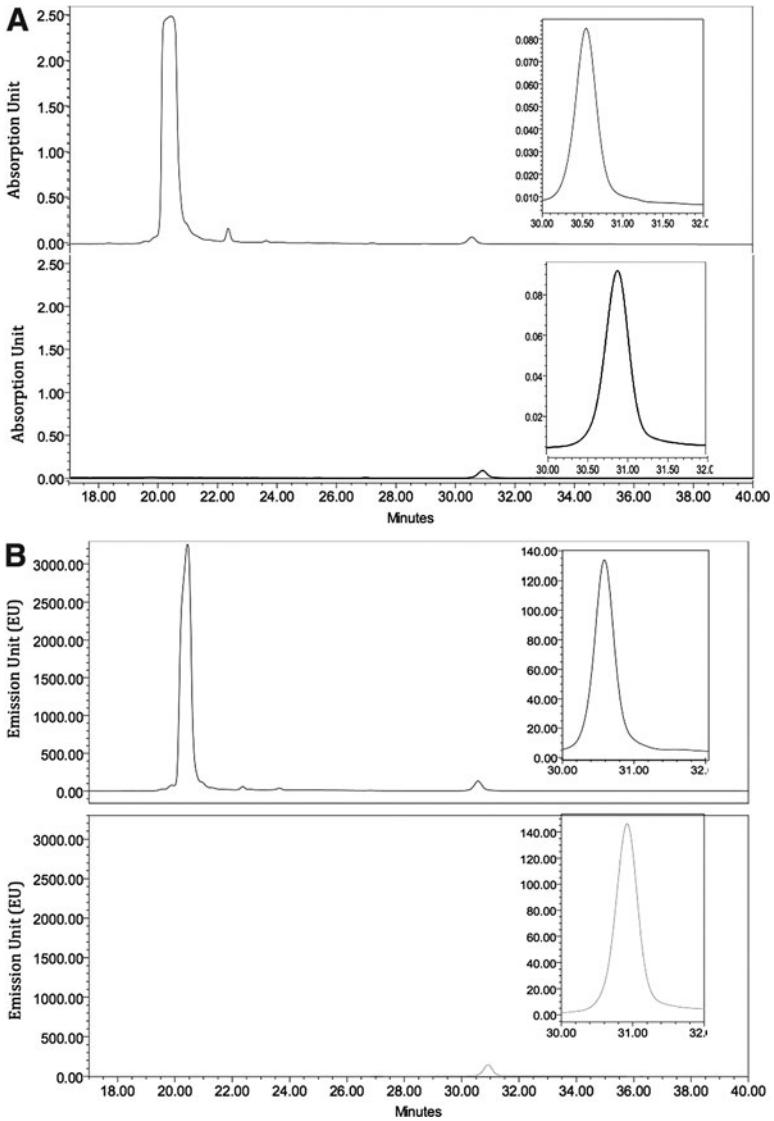

FIG. 4. Reversed-phase high performance liquid chromatography analysis of the HSA-free formulated IFNβ-1b and Betaferon drug product. **(A)** UV absorption chromatogram at 214 nm for Betaferon drug product (*upper panel*) and the HSA-free formulated IFNβ-1b (*lower panel*). **(B)** Fluorescence chromatogram of Betaferon drug product (*upper panel*) and the HSA-free formulated IFNβ-1b (*lower panel*). The peak eluting at 20–22 min is HSA, whereas the peak eluting at 30–32 min is IFNβ-1b. An expanded view of the IFNβ-1b peak is shown as an *inset* in each panel.

Fluorescence spectroscopy

The HSA-free formulated and bulk IFNβ-1b were excited at a wavelength of 295 nm, and emission spectra from 305 to 445 nm were recorded (Fig. 5). The observed intensity provides information on the structure around the tryptophan residues of IFNβ-1b at positions 22, 79, and 143 (Karpusas and others 1997, 1998; Runkel and others 1998, 2000). The maximum fluorescence intensity of the HSA-free formulated IFNβ-1b (at 338 nm) was normalized –, and the fluorescence intensity of another sample was calculated relative to this value. Compared to the bulk IFNβ-1b, HSA-free formulated IFNβ-1b showed a 2-nm blue-shift and a 44% increase in the intensity of fluorescence emission due to the absence of aggregates and/or more retention of tertiary structure. Blue-shift of Trp emission generally means lower exposure of Trp to the aqueous environment—a more ''folded'' structure. Nevertheless, small changes in a fraction of the protein may not be picked up in the intrinsic fluorescence signal (Chen and Barkley 1998; Fan and others 2005; Qiu and others 2008; Fazeli and others 2013).

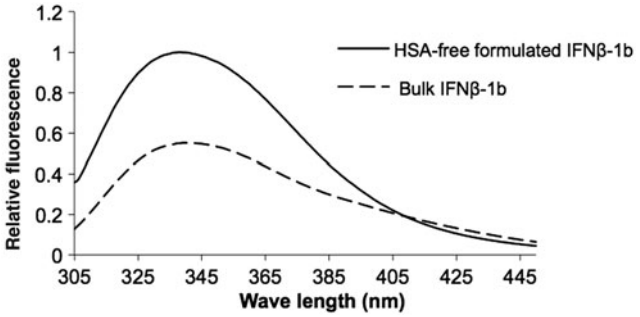

FIG. 5. Fluorescence emission spectra of the HSA-free formulated and bulk IFNβ-1b.

Resonant mass measurement

Submicron and micron particles were measured using RMM. The results showed that the HSA-free formulated IFNβ-1b contained a low particle concentration (7×10^5 particles/mL) with a size range of 0.2–0.4 μm with few larger particles (Fig. 6). By contrast, Betaferon drug product contained heterogeneous particles ranging from 0.2 to larger than 2 μm in size and, more significantly, in the size range of 0.5–0.8 μm. The total particle concentration of Betaferon drug

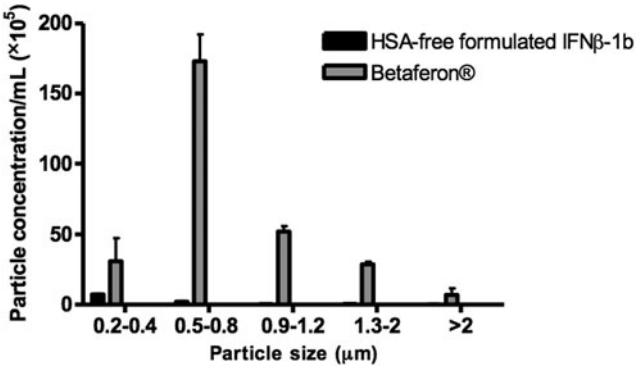

FIG. 6. Particle size distribution of the HSA-free formulated IFNβ-1b and Betaferon drug product determined using resonant mass measurement. Error bars represent standard deviations from triplicate measurements.

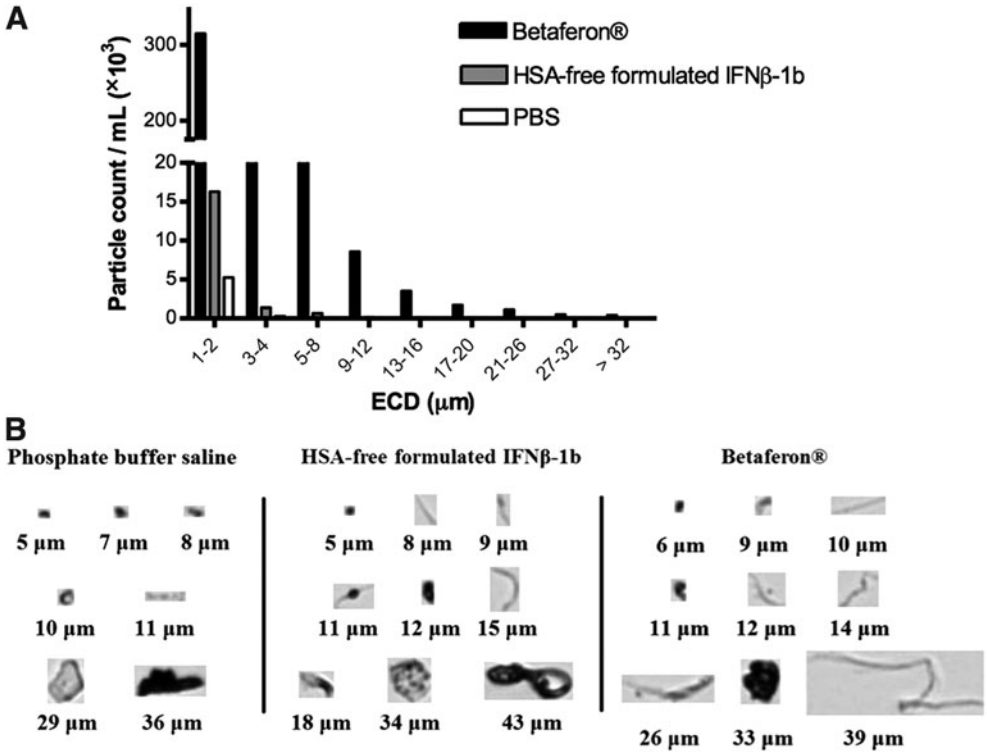

FIG. 7. **(A)** Total number of particles (size range from 1 to >32 μm) detected using microflow imaging in phosphate-buffer saline (PBS), the HSA-free formulated IFNβ-1b, and Betaferon drug product. **(B)** Representative images of protein particles observed in phosphate buffer saline, the HSA-free formulated IFNβ-1b, and Betaferon drug product. The protein particles were observed to be heterogeneous in shape, ranging from small *circles* to large ribbon-like aggregates.

product (3.2×107 particles/mL) is 46 times higher than the number of protein particles in the HSA-free formulated IFNβ-1b (Fig. 6). Increased particle concentration in Betaferon drug product sample might be due to HSA and/or IFN particles.

Microflow imaging

Particles larger than 0.75 μm, which are difficult to measure with other conventional techniques, were detected using microflow imaging (MFI) (Sharma and others 2010). The HSA-free formulated IFNβ-1b showed a lower particle count (1.8×10^4 particles/mL) compared to Betaferon drug product (3.8×10^5 particles/mL). The buffer control contained 5.7×10^3 particles/mL. Results showed that Betaferon drug product has a higher number of particles compared to the HSA-free formulated IFNβ-1b (Fig. 7A). The images of samples are depicted in Fig. 7B. The protein particles in both samples were highly heterogeneous in shape and size, ranging from small circle shape with a size of 5 μm to large ribbon-like aggregates, which were about 40 μm (Fig. 7B).

The presence of other undesired particles like silicone oil, air bubbles, and exogenous contaminants is one of the main complications in protein particle measurement, which could be discriminated and isolated with this technique (Sharma and others 2010).

Immunogenicity

The immunogenicity of therapeutic proteins is influenced greatly by the physicochemical characteristics of the molecule, including the state of aggregation (Chen and Barkley 1998; van Beers and others 2011). To investigate the effect of aggregates on immunogenicity, HSA-free formulated IFNβ-1b and Betaferon drug product were tested in transgenic FVB/N immune tolerant mice.

The level of antibodies in both Tg and non-Tg mice was determined using ELISA and is shown in Fig. 8. In general, all formulations induced high immune responses in the non-Tg animals. IgG titers of Tg mice were significantly lower than non-Tg ones after 14 ($*P = 0.0138$) and 21 days ($**P = 0.0038$) of treatment. Both Tg and non-Tg mice produced antibody after 1 week of treatment with Betaferon, whereas antibody formation was detected after 2 weeks of treatment for both HSA-free formulated IFNβ-1b and Avonex drug substance in non-Tg and Tg FVB/N mice. Tg mice treated with Betaferon drug product showed more than twice the antibody titer

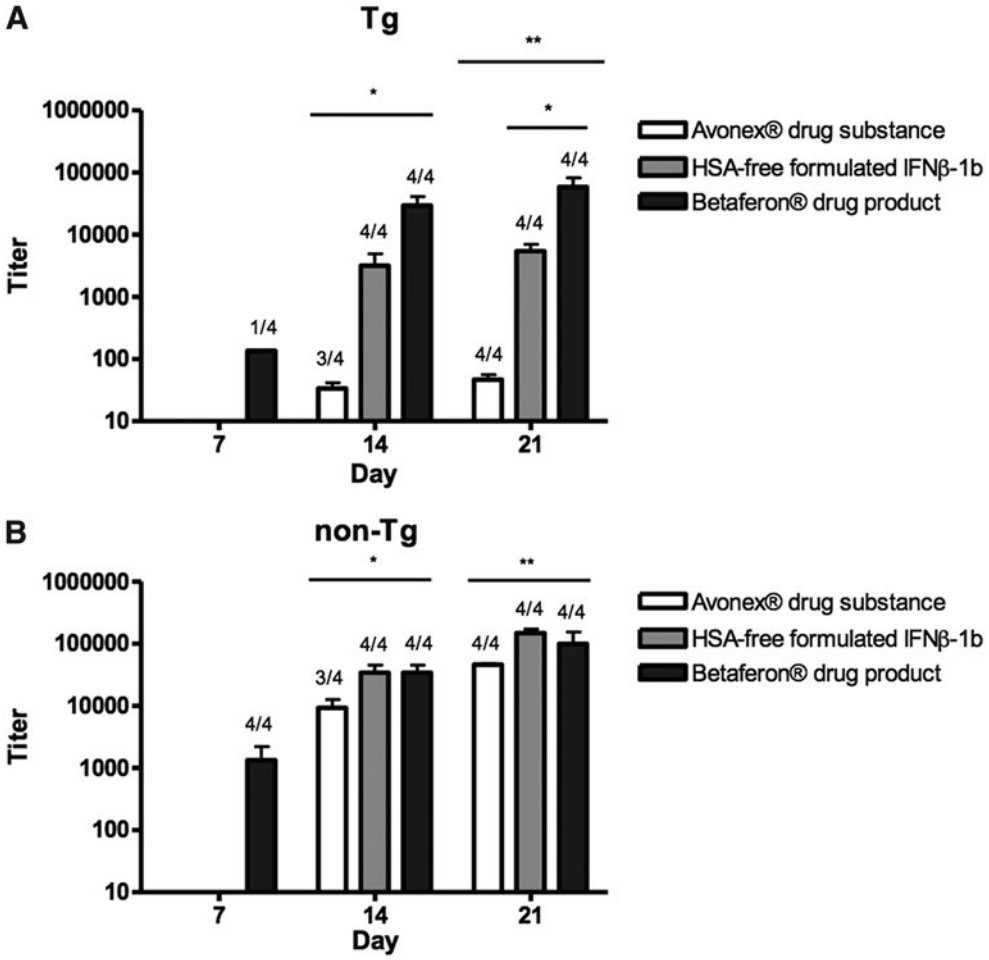

FIG. 8. Formation of anti-IFNβ-1b antibodies in **(A)** Tg and **(B)** non-Tg **(B)** FVB/N mice treated with Avonex® drug substance, HSA-free formulated IFNβ-1b, and Betaferon drug product. Mice received 5 μg of the respective IFNβ 5 times per week for 3 weeks. Bars represent the average titer of IgG-positive mice and the corresponding SEM. IgG levels were significantly higher in non-Tg than Tg mice on day 14 ($*P = 0.0138$) and day 21($**P = 0.0038$). Furthermore, P-value indicates significant lower titers of Tg mice after 21 days treated with the HSA-free formulated IFNβ-1b compared to Betaferon drug product ($*P = 0.0286$). $*P < 0.05$; $**P < 0.01$.

compared to the HSA-free formulated IFNβ-1b (Fig. 8). Although Avonex drug substance (glycosylated IFNβ-1a) in accordance with previous studies (Brown 2011; Hermeling and others 2005b; van Beers and others 2011; Kijanka and others 2013) demonstrated the lowest immunogenic response, of the 2 formulations of IFNβ-1b, the HSA-free formulated IFNβ-1b was the less immunogenic after 21 days of treatment ($*P = 0.0286$). The aggregates in Betaferon drug product likely play a significant role in its immunogenicity (Wang and others 1996; Braun and others 1997; van Beers and others 2010b, 2010c; Hermeling and others 2005c, 2006).

Conclusion

In conclusion, the HSA-free formulated IFNβ-1b contained mostly monomeric native protein with a low level of aggregates, which was confirmed using several analytical methods, including UV and fluorescence spectroscopies, DLS, SDS-PAGE, SE-UPLC, MFI, and RMM. Furthermore, this formulation resulted in better antiviral activity and long-term stability. In addition, the lower immunogenicity of the HSA-free formulated IFNβ-1b in the transgenic FVB/N mice and the absence of HSA, which reduces the potential risk of viral and other pathogenic infection, indicate that the novel formulation may be promising for clinical studies.

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Author Disclosure Statement

No competing financial interests exist.

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