Optimizing A Therapeutic Humanized Follicle-Stimulating Hormone–Blocking Antibody Formulation By Protein Thermal Shift Assay

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ABSTRACT

Biopharmaceutical products are formulated using several Food and Drug Administration (FDA) approved excipients within the inactive ingredient limit to maintain their storage stability and shelf life. Here, we have screened and optimized different sets of excipient combinations to yield a thermally stable formulation for the humanized follicle-stimulating hormone (FSH)–blocking antibody, MS-Hu6. We used a protein thermal shift assay in which rising temperatures resulted in the maximal unfolding of the protein at the melting temperature (T_m). To determine the buffer and pH for a stable solution, four different buffers with a pH range from 3 to 8 were screened. This resulted in maximal T_ms at pH 5.62 for Fab in phosphate buffer and at pH 6.85 for Fc in histidine buffer. Upon testing a range of salt concentrations, MS-Hu6 was found to be more stable at lower concentrations, likely due to reduced hydrophobic effects. Molecular dynamics simulations revealed a higher root-mean-square deviation with 1 mM than with 100 mM salt, indicating enhanced stability, as noted experimentally. Among the stabilizers tested, Tween 20 was found to yield the highest T_m and reversed the salt effect. Among several polyols/sugars, trehalose and sucrose were found to produce higher thermal stabilities. Finally, binding of recombinant human FSH to MS-Hu6 in a final formulation (20 mM phosphate buffer, 1 mM NaCl, 0.001% w/v Tween 20, and 260 mM trehalose) resulted in a thermal shift (increase in T_m) for the Fab, but expectedly not in the Fc domain. Given that we used a low dose of MS-Hu6 (1 μM), the next challenge would be to determine whether 100-fold higher, industry-standard concentrations are equally stable.

INTRODUCTION

With the approval of the first monoclonal antibody in 1986, the landscape of immunoglobulins as biotherapeutics has undergone transformation [1]. However, the development for therapeutic antibodies is complex, due mainly to protein–protein interactions that lead to antibody aggregation, degradation, deamidation, reduction, oxidation, or interactions between different domains, such as Fab–Fc or Fab–Fab interactions [2]. Aggregated or degraded antibodies can also become immunogenic due to the exposure of 'foreign' epitopes. Given that most therapeutic antibodies are injected intravenously or subcutaneously, and require concentrations >100 mg/mL, human use requires antibodies to be formulated in a way that they remain stable over time [3].

A typical therapeutic antibody is formulated at an acidic pH with salts, sugars, and stabilizers—each of which serve distinct and overlapping functions in ensuring stability [4]. Most antibodies display reduced solubility and increased precipitation at the isoelectric pH (pI) [2, 5], and must therefore be formulated at a pH away from their pI. With highly concentrated antibodies, the use of inorganic salts improves colloidal stability by shielding free residues from self–interaction and precipitation. Salts also maintain isotonicity, which is of particular importance in preventing pain at the injection site. However, higher salt concentrations can lead to precipitation or "salting" out [6] . Sugars likewise contribute to isotonicity [7], and by being preferentially excluded from antibody surfaces, improve stability. However, sugars can also cause antibody glycation, which, in turn, affects half–life. Most therapeutic antibodies thus use non–reducing sugars. Finally, while detergents and stabilizers can cause antibody degradation, very low concentrations protect against degradation caused by agitation and sheer stress that often results from antibody adsorption onto air–solution interfaces [2] .

Based on our studies that have established a causal role for the pituitary hormone, follicle–stimulating hormone (FSH), in regulating bone mass, body composition and neuronal function, we have created a first–in–class humanized antibody that, we find, reduces body fat, induces thermogenic adipose tissue and increases bone density [8-14]. The antibody, *hitherto* termed MS-Hu6*,* was humanized from a parent monoclonal antibody, Hf2, that was raised against a short, 13–amino–acid–long receptor–binding sequence of FSHβ [9]. By binding to FSHβ, MS-Hu6 blocks its interaction with the FSH receptor (FSHR) with high affinity (K_D ~7.5 nM) that approaches that of trastuzumab (K_D ~5 nM, Herceptin®) [8]. Using a protein thermal shift assay (also termed differential scanning fluorimetry), we have previously confirmed the stability of MS-Hu6 and its binding to FSH.

Here, we describe detailed steps to develop a formulation with maximal stability and ligand binding. To do so, we used the protein thermal shift assay to test 217 formulations combining salts, sugars, and stabilizers at different pH values to derive a near–final formulation ensuring maximal stability. The formulation was developed and optimized using Food and Drug Administration (FDA)–approved excipients at concentrations that fall within the inactive ingredient guide limits for excipients. The protocols, methodologies and analytical evaluations were developed, recorded, and archived within MediaLab using our Good Laboratory Practices–complaint platform.

MATERIALS AND METHODS

Protein Thermal Shift Assay

The protein thermal shift assay, a tool for testing protein unfolding in real-time, is utilized routinely to screen conditions for maximum protein stability, determine protein–ligand interactions, and understand protein structure. The fluorescent dye SYPRO–orange is added to the protein

solution, which is exposed to incremental increases in temperature ranging from 25 to 99°C. Protein unfolding exposes hydrophobic surfaces that interact with SYPRO–orange to yield a fluorescence signal at 570 nm. The second–degree derivative is used to determine the melting temperature T_m—a higher T_m indicates greater protein stability. When proteins, such as MS-Hu6, bind ligand, a higher T_m is required for protein unfolding, resulting in a 'thermal shift' derived as a ΔT_m.

Experiments were thus performed using optically clear 0.1 mL PCR tubes (TempAssure, # 1402-2300). MS-Hu6 was diluted into 20 µL reactions containing buffer and/or excipient of interest, and 10X SYPRO–orange and sterile water or formulation buffer (respective buffers). MS-Hu6 in water or phosphate buffer at pH 6.2 were used as controls for baseline thermal stability (N=6 or 8, respectively). The run conditions in the thermocycler (StepOne Plus, Applied Biosystems) were: 25 ºC for 2 minutes, then to 95 ºC, with temperature increments of 0.3 ºC every 15 s. Fluorescence intensity was measured at each temperature. Each condition was run in duplicate (n=2). The ΔT_m was calculated based on the inflection point of the protein thermal shift melting curve, and the thermal shift was calculated using the following equation (1),

$$\Delta T_m = T_m\text{A} - T_m\text{B} \quad \text{.....(1)}$$

<u>Optimizing Antibody Concentration</u>

MS-Hu6 (IgG1 isotype) was obtained from Genscript (Lot# DD1912741) as liquid formulation in phosphate–buffered saline (PBS) at a concentration of either 5.17 or 19.06 mg/mL. ~2 mg was buffer exchanged with sterile cell culture grade water (Corning, Cat # 25-055-CV) using ultra–centrifugal filtration units (Amicon, # UFC903024; MWCO, 30,000 kDa) equilibrated with water. MS-Hu6 was concentrated to 500 µL and re–diluted with water—this process was repeated 5 times. The final antibody concentration in water was 4.34 mg/mL (~400 µL) as measured on Nanodrop (OD 280 nm). To optimize the concentration for use in the protein thermal

shift assay, MS-Hu6 was diluted into a 10 µM working stock solution, which was diluted serially (1:3) into 20 µL reaction tubes containing buffer and dye to yield the following concentrations: 10, 3.3, 1.08, 0.359, 0.119, 0.039, 0.013 and 0.004 µM. Assay in triplicates yielded a final optimal concentration of 1 µM for our studies, although concentrations as low as 4 nM could be detected.

Buffer and Excipient Preparation

For phosphate, citrate, acetate and histidine buffers, 1 M weak acid/base for each was prepared and mixed in different proportions to give 0.2 M (for phosphate), 0.1 M (for acetate and citrate) or 0.05 M (for histidine) working concentrations at various pH levels (Supplementary Table 1). For detergents, a 10% (v/v) stock was prepared for each buffer. For sugars, 1 M stocks were prepared, sterile–filtered, and diluted into 20 µL final reaction volumes.

Data Analysis

At each temperature T, the first derivative ($\Delta RFU/\Delta T$) was calculated to obtain the rate of change of fluorescence. To generate smooth melting curves, a rolling average of 10 such values were plotted against temperature. Peaks, annotated as T_m and calculated from the slope of the melting curve (i.e., second derivative), were plotted against pH for each condition.

Molecular Dynamics Simulations

The models of MS-Hu6 were generated based on human IgG template (PDB id: 1GFV and 3AUV) using a protocol described in detail by Gera et al [8]. For the molecular dynamics (MD) simulations, two systems were prepared at 1 mM and 100 mM NaCl, each following the same protocol. The side chains of the protein were protonated using the protein–prepare implemented in the high–throughput molecular dynamics (HTMD) suite [15]. This was followed by the parameterization of the protein using the Amber ff14SB force field [16]. The systems were

then solvated using a TIP3P water box, with edges extending to 10 Å from the solute. Each system was then equilibrated for 5 ns in an NPT ensemble, where the protein backbone was frozen and the solvent with counter ions were allowed to move. This was followed by an unrestrained production run of 1000 ns in NVE ensemble at 300 K using the ACEMD MD engine [17]. The time step was kept at 4fs; periodic boundary condition was utilized and the accuracy of the particle mesh Ewald was increased, while direct sum tolerance was reduced by an order of magnitude (0.000001). The root mean–square deviation (RMSD), radial distribution function analysis was carried out using gromacs analysis tools. The electrostatic surface charge on the protein was calculated on the final snapshot of the simulation using Adaptive Poisson Boltzmann Solver [18] implemented in PyMol [19].

Size Exclusion Chromatography (SEC)

Size exclusion chromatography (SEC) was performed to detect monomer loss using an AKTA Pure Fast Performance Liquid Chromatography system (Cytiva, Marlborough, MA, USA). Prepacked SEC columns (Superdex 200 10/300 GL 1×30 cm, particle diameter 13 µm) with TSKgel guard column SwXL (6 mm× 40 mm) were used for the analysis. SEC was used to separate the native monomeric proteins from any soluble aggregates in the antibody formulations. Immediately before the analysis, all samples were diluted to 1 mg/ml using the formulation buffer, loaded onto the SEC column (500 µl), and eluted isocratically at a flow rate of 0.4 ml/min. The mobile phase consists of 20 mM phosphate buffer at pH 6.2, 260 mM sucrose, 0.001% w/v Tween 20, and 1 mM NaCl at 25°C. The protein concentration was measured by absorbance at 280 nm. The area under curve of absorption peaks in the chromatogram was used to determine the % monomer loss in the antibody formulations. Representative chromatographs were reported.

Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) was performed to determine the size (hydrodynamic radius, rh) and homogeneity (polydispersity index, PDI) of the antibody; this provides a readout for the colloidal stability of the antibody in various formulations. Briefly, 20 µl of the MS-Hu6 formulation was diluted into 1 ml using the formulation buffer in a 1 ml disposable micro cuvette (Malvern Cat. ZEN0040). Using the Zetasizer Nano-ZS 90 system (Malvern Instruments Ltd., Malvern, PA, USA), the diluted sample was analyzed on a fixed scattering angle (90°) at 4°C for 60 s. The refractive index of the medium was set at 1.33 and the dynamic viscosities of the samples were measured for each sample. The data were collected and reported as the Z-average of the hydrodynamic radius and representative DLS graphs were reported.

RESULTS

To determine the buffer and pH for stable solution of MS-Hu6, four different buffers with a pH range from 3 to 8 were screened using protein thermal shift assay. These buffers included 20 mM citrate (pH 2.9 to 6.1), 20 mM acetate (pH 3.6 to 5.6), 20 mM phosphate (pH 5.6 to 8.0) and 25 mM histidine (pH 5.0 to 7.0) as shown in (Supplementary Table 1). IgG unfolds with increasing temperature with two peaks corresponding to unfolding of Fc and Fab regions. Figure 1A shows representative thermal traces of the Fc and Fab peaks in the four buffers, with each at two pHs within the aforementioned ranges. Figure 1B displays cumulative data on change in T_m with increasing pH in different buffers. Both Fab and Fc regions were relatively stable between pH 5.5 and 6.5, with T_m dropping precipitously at lower pH values (Figure 1B). Maximum stability was attained at pH 5.62 for Fab in phosphate buffer, and at pH 6.85 for Fc in histidine buffer (Figure 1C). We therefore focused on further developing phosphate and histidine buffers.

We screened for concentration effects to find no difference between 10, 20 and 40 mM phosphate for the Fab fragment (Figure 1D). However, for the Fc fragment, 10 mM phosphate

showed better stability, although at pH 6.2 all three concentrations had similar T_ms. Given that our main aim was to optimize the stability of Fab domain, the next set of experiments used 20 mM phosphate buffer at pH 6.2. This was therefore tested in multiple runs within the 5.6 to 7.0 pH range. Average T_ms for Fab and Fc for Formulations 51–58 are shown in Table S2. Maximum stability was obtained at pH 5.76 (T_m = 79.96 ± 0.13) for Fab and pH 7.01 (T_m = 71.12 ± 0.36) for Fc.

Salts reduce viscosity, allow stable ionic interactions, increase solubility, and provide isotonicity. Whether salts stabilize or aggregate a protein depends on its structure and type of interactions [20, 21]. We used isotonic NaCl (150 mM) without (Formulations 67–73) or with 5 mM KCl (Formulations 74–81) (Table S3). For the Fab domain, T_m was 79.3°C (pH 6.09), whereas for the Fc domain, it was 70.9°C (pH 6.78) (Figure 1E and Table S3). Addition of KCl led to no change or a reduction in T_ms for Fab (79.03°C at pH 5.76) and Fc (70.58°C at pH 6.8), respectively. Fab was most stable with phosphate buffer without salt, at a pH of 5.76 for Fab and 7.01 for Fc (Figure 1E). Thus, the presence of KCl had a negative effect on antibody stability, with a reduction in T_m by ~1°C. This is because, it could increase the hydrophobic interactions, thus destabilizing and denaturing antibodies [20, 22, 23].

Stabilizers are required to maintain colloidal stability of monoclonal antibodies; their effect depends upon the type and concentration of the stabilizer. During stabilizer optimization, two critical parameters are considered—antibody aggregation and detrimental structure perturbation. We tested the effect of three stabilizers, namely Tween 20 and Tween 80, and Poloxamer 188, a copolymer of polyoxypropylene and polyoxyethylene. The two former molecules have been used traditionally to improve antibody solubility [24-27]. All stabilizers were first screened at 0.001% (w/v) in the presence of 150 mM NaCl and 5 mM KCl (Figure 2A and 2B). For Tween 20, the Fab region displayed a T_m of 79.21 ^oC (pH 6.2), whereas Fc showed a T_m of 70.32 ^oC (pH 7.01) (Formulations 82–89, Table S4). For Tween 80, the T_m for the Fab region was lower at

78.93 °C (pH 5.99) (Formulations 90–97, Table S5), while the Fc region melted at 70.58 °C (pH 7.01). For poloxamer 188 (Formulations 98–105, Table S4), T_ms for Fab and Fc were 78.8°C (pH 5.99) and 70.43 (pH 6.62), respectively. This showed that, while the addition of KCl reduced T_m by 0.5 °C (Figure 1E), this reduction was reversed by the addition of Tween 20. Moreover, high stabilizer concentration, which leads to strong binding, could destroy and destabilize antibody structure [27].

MS-Hu6 stability was also tested in combinatorically with the following—150, 100, 50 or 1 mM NaCl plus 0.001% w/v Tween 20 in 20 mM phosphate buffer (Figure 3A and 3B). At 150 mM NaCl (Formulations 114–121, Table S5), the highest T_ms for Fab and Fc were at pH 5.99 (78.78°C) and 6.8 (70.51°C), respectively. At 100 mM NaCl (Formulations 122–129, Table S5) T_ms for Fab and Fc increased to 79.25°C (pH 6.42) and 70.88°C (pH 7.01), respectively. At a still lower NaCl concentration of 50 mM NaCl, the T_m further increased to 79.57°C (pH 5.99) for Fab and 71.04°C (pH 6.81) for Fc. At a 50-fold lower concentration of NaCl (1 mM), T_m for Fab was 80.2°C (pH 5.99) and that for Fc was 71.15°C (pH 6.81). Overall, therefore, as the salt concentration decreased the T_m (stability) increased by ~0.3 to 0.5°C as shown in Figure 3B.

Monoclonal antibodies are generally more soluble in dilute salt solutions, as salts in their ionic forms are attracted towards opposite charges within the antibody moiety, which enhances surface hydration. However, very high salt concentrations increase water surface tension, and in doing so, trigger competition between the antibody and salt ions for hydration. Salts strip the vital layers of water molecules from the protein surface, resulting in a decrease in the intermolecular hydrogen bonds, antibody solubility and ligand antibody binding, as well as an increase in hydrophobic interactions; the latter destabilizes and denatures the antibody. This could be the reason for the noted decrease in T_ms for antibodies at higher salt concentrations [20, 22, 23].

To rationalize the effect of salt concentration on the stability of the MS-Hu6, MD simulations were performed under two ionic conditions of 1 mM and 100 mM NaCl. MD simulations have been used to investigate the conformational changes under the influence of ionic salt concentration [28, 29]. The Cα-RMSD was used to check the stability of MS-Hu6. The Cα-RMSDs were comparable and minor differences between the two systems at both salt concentrations. The Cα-RMSD of the two chains are illustrated in Figure 3C. Overall, MS-Hu6 in 1 mM displayed higher stability than in 100 mM NaCl. Next, the localization of the ions relative to the protein surface was assessed using the radial distribution function (RDF). The minimal distance between the ions and the protein were calculated as a variation of time. The distance varies from a contact distance of ~2 Å and was cutoff at 5 Å. In the simulations with 1 mM NaCl, the minimal distances between the ions and protein was greater than that observed under 100 mM (Figure 3D). The RDF analysis indicates that the ions are located closer to the protein surface within the cutoff distance, in accordance with the higher concentration of ions in the solution. The charged ions localized around the protein surface have the ability to influence side chain interactions and alter the electrostatic surface charge pattern of the complex (Figure 3E). This will, in turn, influence protein–protein interactions and possible binding affinities.

Polysaccharides and their derivatives minimize the aggregation of antibody during stress [30]. To determine whether the addition of a sugar enhanced the stability of MS-Hu6, we tested the above formulation (1 mM NaCl, 0.001% w/v Tween 20 and 20 mM phosphate) with sucrose, dextran 40, trehalose, sorbitol, or mannitol (260 mM) (Figure 4). With the addition of 260 mM sucrose (Formulations 146–153, Table S6), the highest T_ms for Fab and Fc were 80.84°C (pH 5.76) and 72.39°C (pH 6.6), respectively. Of note is that we achieved the highest T_m for Fab with the addition of 260 mM trehalose (Formulations 170–177, Table S6) at 81.67°C (pH 6.2), while for Fc, the T_m also improved to 73.29°C (pH 6.8). Overall, when compared against 20 mM phosphate buffer alone, the presence of nonreducing disaccharides led to stabilization of both

Fab and Fc region by ~2 °C. It is known that polyols and sugars can stabilize an antibody's native structure by increasing the free energy of unfolding, which thermodynamically favors the natively folded states of antibodies [30-32].

The alcoholic sugars, namely, sorbitol and mannitol (260 mM each), displayed slightly different characteristics. In the presence of 260 mM sorbitol (Formulations 202–209, Table S6) the maximum stability of Fab was 80.15°C (pH 5.76). For Fc, the highest T_m was 72.35°C (pH 6.81). For mannitol (Formulations 210–217, Table S6), the T_m for Fab and Fc were 80.77°C (pH 5.6) and 71.72°C (pH 6.62), respectively. These alcoholic sugars thus gave very similar T_ms to those observed with just 20 mM phosphate buffer, and better T_ms than those noted with salts. Formulations containing 260 mM dextran 40 were also screened for thermal stability (Formulations 194–201, Table S7). At pH 5.76, it showed maximum stability of Fab at 80.17°C and for Fc at 71.03°C (pH 6.8). In all, a comparison of T_ms at pH 6.2 (Figures 4A and 4B) showed that maximum stability was with trehalose, followed by sucrose, sorbitol, mannitol and finally dextran 40. From these experiments it could be surmised that trehalose improved the stability of both Fab and Fc domains to the highest T_m.

We next added sucrose and NaCl in different concentrations such that total osmolality remained constant (Figure 4E and F). The addition of 200 mM sucrose and 50 mM NaCl with 20 mM phosphate and 0.001% w/v Tween 20 (Formulations 154–161, Table S7) resulted in a highest T_m of 80.18°C (pH 5.8) for Fab, with a lower T_m of 71.65°C (pH 7.01) for Fc. Furthermore, changing the sucrose and NaCl concentrations to 100 mM for each led to shifts in thermal stability. For Fab, the highest T_m noted was 79.54°C (pH 5.99), and for Fc was 71.57°C (pH 6.81). This indicated that increasing salt and decreasing sugar concentration reduced thermal stability by ~0.7°C for Fab, with minimal effects on Fc (Figure 4E and 4F). However, the T_ms of antibody determined with 260 mM sucrose were still higher at 80.8 (~0.5°C higher) for Fab and 72.44°C (~0.8°C) for Fc.

A similar pattern was seen in trehalose (Figure 4C and 4D). The addition of 200 mM trehalose and 50 mM NaCl (Formulations 178–185, Table S7) resulted in the highest T_m of 80.42°C (pH 5.99) for Fab and 71.96°C (pH 6.81) for Fc. On decreasing trehalose concentration to 100 mM and increasing NaCl to 100 mM (Formulations 186–193, Table S7), the highest T_m for Fab and Fc both decreased slightly to 79.85°C (pH 5.76) and 70.93 °C (pH 6.62), respectively. Thus, while decreasing trehalose led to slight decline in T_ms for Fab and Fc (Figure 4C and 4D), 260 mM trehalose gave maximum stability at 81.67°C for Fab and 72.88°C for Fc. Thus, once again, the presence of increasing amounts of salts led to a decrease in thermal stability.

This trend was again reflected at pH 6.2. Highest T_ms for Fab were noted for Formulation 181 that contained 200 mM trehalose followed by Formulation 157 that contained 200 mM sucrose. The next most stable condition was Formulation 189 containing 100 mM trehalose followed by Formulation 125 containing 100 mM NaCl. This was followed by Formulation 133 containing 50 mM NaCl, and Formulation 165 that contained 100 mM sucrose and 100 mM NaCl. Finally, while screening across different pHs, salts, sugars, and detergents, as well as optimizing their concentrations, we found that the best condition configured was a pH 6.2 formulation in 20 mM phosphate, 260 mM trehalose, 1 mM NaCl and 0.001% w/v Tween20 (Formulation 173, Table S6) as shown in Figure 4.

After confirming the conditions for optimal stability of MS-Hu6, we tested its binding to FSH. For this set of experiments, 1 µM MS-Hu6 was incubated with 10 µM of FSH and its thermal stability was measured. This was performed in the following conditions: 20 mM phosphate at four different pH levels (5.76, 5.99, 6.20 and 6.42), plus, for each condition, 0.001% w/v Tween20 and 1 mM NaCl. Binding was tested in 260 mM trehalose and 260 mM sucrose in the presence or absence of FSH. ΔT_m was calculated from the changes measured within each condition (Figure 5A). At pH 6.2, in case of phosphate buffer, ΔT_m for Fab was 3.07°C. On addition of excipients with 260 mM trehalose (Figure 5B), the ΔT_m for Fab was 3.01°C. In the presence of 260 mM

sucrose, the ΔT_m was 2.68°C (Figure 5C). Thus, the presence of FSH led to a shift in T_m to the right for Fab by ~2.5 to 3°C (Figure 5A–C). Notably, and as a control, Fc remained relatively unchanged (~0.5°C to the left). In the presence of trehalose at pH 6.2, ΔT_m on addition of FSH was ~3°C higher irrespective of whether it was with phosphate or histidine (Figure 5B). Likewise, in the presence of sucrose, ΔT_m was ~2.7°C (Figure 5C).

Using SEC, MS-Hu6 in this optimized formulation and in PBS were evaluated for aggregation (formation of high molecular weight species, HMS) and fragmentation (formation of low molecular weight species, LMS). MS-Hu6 in the optimized formulation retained a slightly higher fraction of monomers (99.41%, Peak 2) compared with MS-Hu6 in PBS (99.27%) (Figure 6A). The % monomer loss in either buffer was <1%, indicating that there was minimal fragmentation or aggregation, and is within the acceptable limit (5–10%).

Furthermore, formulation (1 mg/ml) and MS-Hu6 standard (1 mg/ml) were tested to confirm the heterogeneity and aggregation at the nano level using DLS. This study recorded size in terms of hydrodynamic radius and PDI as indicators of aggregation and heterogeneity. The DLS data of the MS-Hu6 (in PBS) were found to be polydisperse in nature, with a PDI value of 0.74 (Figure 6B). And the hydrodynamic radius of the major volume (99.07%) was found to be 4.50 nm (Figure 6B). The MS-Hu6 sample could contain 0.3 % soluble subvisible aggregated particles with a hydrodynamic radius of 96.04 nm (0.1% volume) and 2789 nm (0.20 % volume), respectively. However, in the case of formulated MS-Hu6, PDI was reduced and found to be 0.41 (Figure 6B). The hydrodynamic radius of the major volume (99.90 %) was found to be 4.05 nm (Figure 6B), suggesting the reduction of size and PDI compared to unformulated antibodies. The soluble subvisible aggregated particles in formulated antibodies were found to be decreased to 0.1% volume as compared to unformulated samples. That was not even detectable due to the minimal volume. In the formulation, these aggregated particles could be dissolved or solubilized in the presence of a stabilizer and other excipients and could maintain colloidal stability.

DISCUSSION

In this study we determined the thermal stability of an FSH–blocking antibody MS-Hu6 using thermal shift assay to estimate T_ms in 217 different formulations consisting of combinations of pH, salts, sugars and detergents/stabilizer using our GLP–compliant platform. Beginning by determining the pH range within which the Fab and Fc showed stability, we noted that these two regions behaved distinctly [7]. Phosphate buffer provided higher stability to the Fab region, whereas histidine buffer improved Fc stability. The Fab region seemed to be stable at a pH of 5.6, whereas Fc had improved stability at a pH of 6.9. In contrast, concentration of buffer ions impacted the behavior of Fc region, but did not appreciably alter the Fab region.

The Fab region of an IgG molecule contains the antigen binding domain, and the structural conformation and stability of this region is paramount when targeting a ligand for inhibition. The Fc region is also important in that it ensures a longer half–life and optimal pharmacokinetics for a therapeutic antibody. Therefore, in our studies, while we selectively focused on conditions that would give maximum stability to the Fab region, we also ensured the Fc region was within an acceptable range of stability. In addition, we ensured that the pH of the formulation would be as further away as possible from the isoelectric point, where the solubility would be minimal and the tendency for aggregation would increase. With this in mind, we screened for the stability of the Fab and Fc regions in the range of pHs between 5.6 to 7.0, with excipients added, while also concentrating on pH 6.2 (determined to be optimal pH for Fab in some conditions) [33].

We tested excipients that are most prevalent in marketed therapeutic antibody formulations [34, 35]. Around 50% of commercial formulations tend to have NaCl [35, 36]. Thus, we first screened NaCl and KCl, at concentrations that closely matched physiology. Interestingly, we saw a decline in the stability of both Fab and Fc regions, especially in the lower pH range (for the Fc region). Particularly at pH 6.2, we noted both Fab and Fc stability decline by 0.8 ^{o}C upon

the addition of salt, suggesting that salts reduce stability. Next, we screened detergents (stabilizers) as vital for preventing antibody degradation with agitation stress. To determine if the presence of detergents/stabilizers would improve thermal stability after the decline with salts, we tested three stabilizers in the presence of salts. We noted that Fab was more stable upon the addition of 0.001 % w/v Tween 20, whereas Tween 80 performed better for Fc stability. However, 20 mM phosphate buffer still gave better stability in comparison, and the stabilizers could not rescue the salt–induced decline in thermal stability.

Concluding that the presence of salts seemed to have a destabilizing effect on the antibody, in the next set of experiments we moved to reduce salts to 1 mM and added 260 mM sugars with 0.001% w/v stabilizer. Here, we saw a remarkable improvement in melting temperatures for both Fab and Fc. In the presence of each sugar, we saw a rise in T_ms by at least 1–2 ^{o}C at best, and T_ms comparable with 20 mM phosphate at worst. Thus, the presence of sugars benefited the stability of MS-Hu6, potentially through interactions of glycosylated amino acids with the sugars. We also confirmed the stability of MS-Hu6 in sugar and salt titration experiments, where increasing amounts of salts and decreasing amounts of sugars were tested, either combinatorically or separately. Here, we again noted that decreasing salt concentrations had a marginally positive effect on the T_ms of Fab and Fc regions. Surprisingly, adding sugars to salts did not strongly mitigate the negative effect of salts on the Fab region. The best noted T_m was with 20 mM phosphate buffer at pH 6.2 in the presence of 260 mM trehalose or sucrose, 1 mM NaCl and 0.001% w/v Tween 20—this was deemed as our final low–dose formulation. This experimental observation was further corroborated using MD simulations, which highlighted improved stability of MS-Hu6 at low salt concentration, consistent with the reduced localization of ions around the protein and their ability to influence side chain interactions.

Finally, we tested the binding of MS-Hu6 to FSH in the presence of this final formulation within a narrow pH range. We compared this to alternative formulations consisting of 10 mM

histidine (instead of 20 mM phosphate) and sucrose (instead of trehalose). We saw a 3 ^{o}C shift in the Fab region, which is comparable to previous experiments. Importantly, the final formulation did not seem to impact the ability of MS-Hu6 to interact with its ligand. We did not see major differences in binding capability between histidine and phosphate buffers, as in both instances, a shift in stability of Fab through interaction with FSH was evident.

Monomer loss in the formulated antibody and MS-Hu6 in PBS, determined in our SEC study, was less than 1% and within the permissible range of 5-10% observed in commercially available biopharmaceutical products. Typically, a maximum of 5% monomer loss is the acceptable threshold [37]. Dimers, which are reversible structures formed from soluble subvisible particles, contribute to minimum aggregation and are not critical in biopharmaceutical product development [4]. These particles may diminish in the presence of an efficient stabilizer. However, antibody fragmentation or multimerization forming insoluble irreversible clumps are critical in the development of biopharmaceuticals. For this, excipients are used to stabilize the monomeric form of antibodies at higher concentrations [32]. Any protein formulation must achieve high long-term stability, which requires maximizing the monomeric fraction and minimizing multimer formation or fragmentation [37].

In the DLS study, no significant aggregation was observed in either sample, which is less than 1% of the total volume. In both cases, samples maintained the monomeric form with minimal or no aggregated particles. Because of their reversible nature, these aggregates are impractical to eliminate below certain levels, so that these subvisible soluble particles are well-accepted by the US pharmacopeia (USP) and US FDA (5-10%) in the final optimized formulation [38]. Furthermore, they could not affect the colloidal stability and structural integrity of antibody in the formulation [39]. Most notably, bigger and irreversible particles need to be controlled because they could precipitate out of solution when protein exceeds the solubility limit. According to FDA guidelines, these particles are of different sizes; while particles of 150 µm are visible [40], most

particle below this sizes could elicit immune reactions [41, 42]. The particles with sizes of ≤50-100 µm are considered to be subvisible particles. However, a size ≥10 µm can obstruct or interfere with blood flow [43]. Chapter 788 of the USP is on the subvisible particle counting method, which set acceptable limits for particulate matter in a container (≤100 ml to be 6000 particles ≥10 µm and 600 particles ≥25 µm) [43]. However, because the majority of biological products are currently administered intravenously or subcutaneously, the FDA has strict regulatory guidelines in place that take the 10 µm threshold limit of aggregates into account in order to prevent blood vessel occlusion. Additionally, this might help to maintain long-term colloidal stability of the biopharmaceutical product [44]. In all, while we have successfully formulated MS-Hu6 at a low concentration of 1 µM, the next is to determine whether the formulation will provide equal stability to a 100–fold higher, industry–standard, concentration. Furthermore, it would be critical to compare if each of the excipients offers protection against antibody degradation pathways, particularly in response to heating, freeze–thaw cycles, and agitation.

AUTHOR CONTRIBUTIONS

D.S., S.R., S.G., and S.H.: Conceptualization, data curation, formal analysis, validation, investigation, visualization, methodology, writing—original draft, writing—review and editing. A.R.P. and T.-C.K.: Data curation, formal analysis, investigation, and visualization. A.P., F.K., L.C., J. Chatterjee, E.S., S.M., F.S., and J.G.-R.: Data curation, formal analysis, investigation, and visualization. O.B. and A.M.: Data management and provenance. C.R.: Conceptualization. M.M. and J. Caminis: GLP management and methodology. C.J.R.: Conceptualization and methodology. D.L., V.R., and S.-M.K.: Data curation, formal analysis, investigation, and methodology. T.Y. and M.Z.: Conceptualization, supervision, writing—original draft, writing—review and editing.

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COMPETING INTERESTS

M.Z. is an inventor on issued patents on inhibiting FSH for the prevention and treatment of osteoporosis and obesity (U.S. Patent 8,435,948 and 11,034,761). M.Z. is also an inventor on a pending patent application on the composition and use of humanized monoclonal anti–FSH antibodies and is a co-inventor of a pending patent on the use of FSH as a target for preventing

Alzheimer's disease. These patents are owned by Icahn School of Medicine at Mount Sinai (ISMMS), and M.Z. would be recipient of royalties, *per* institutional policy. M.Z. also consults for several financial platforms, including Gerson Lehman Group and Guidepoint, on drugs for osteoporosis and genetic bone diseases.

LEGENDS TO FIGURES

Figure 1: Screening of different buffer conditions and pHs to obtain a stable MS-Hu6 solution. (**A**) 20 mM citrate buffer (pH 2.9–6.1), 20 mM acetate buffer (pH 3.6–5.6), 20 mM phosphate buffer (pH 5.6–8.0), and 25 mM histidine buffer (pH 5.0–7.0). (**B**) Representation of the cumulative data on change in T_m with increasing pH in different buffer conditions. (**C**) Maximum stability of the Fab and Fc domains; at pH 5.62 for Fab in phosphate buffer, and at pH 6.85 for Fc in histidine buffer. (**D**) Effect of phosphate buffer concentrations (10, 20, and 40 mM) on stability of the Fab and Fc domains of MS-Hu6. (**E**) Effect of 20 mM phosphate buffer containing 150 mM NaCl and 5 mM KCl on the stability of the Fab and Fc domains of MS-Hu6.

Figure 2: (**A**) Effect of stabilizers (Tween 20, Tween 80, and poloxamer 188) on the stability of the Fab and Fc domains of the MS-Hu6 in a buffer containing 20 mM phosphate, 150 mM NaCl, and 5 mM KCl at pH 5.5–7.0. In this formulation, Tween 20 at 0.001% yielded the highest stability at pH 6.2 (**B**).

Figure 3: (**A**) Effect of NaCl concentrations (150, 100, 50, and 1 mM) on the stability of the Fab and Fc domains of the MS-Hu6 in a buffer containing 20 mM phosphate and 0.001% Tween20 at pH 5.5–7.0. The most stable formulation at pH 6.2 was with 1 mM NaCl (**B**). (**C**) Molecular dynamics simulation of MS-Hu6 at 1 and 100 mM NaCl concentrations. The Cα-RMSDs were comparable and minor differences were observed between the two systems at both ionic concentrations. The Cα-RMSDs of the two chains of the MS-Hu6 are colored red and black. (**D**) Ion distribution around the protein surface was assessed using the radial distribution function (RDF). The minimal distance between the ions and the protein was calculated as a variation of time. RDF analysis for 100 mM salt simulation indicates that the ions are located closer to the complex surface within the cutoff distance, in accordance with the higher concentration of ions in

the solution. (**E**) The charged ions localized around the protein surface can influence side chain interactions and alter electrostatic surface charge pattern.

Figure 4: (**A**) Effect of sugar (260 mM each, sucrose, trehalose, mannitol, dextran 40, and sorbitol) on the stability of the Fab and Fc domains of MS-Hu6 in a buffer containing 20 mM phosphate, 1 mM NaCl, and 0.001% w/v Tween 20 at pH 5.5–7.0. The addition of trehalose yielded the highest stability at pH 6.2 (**B**). (**C**) Effect of trehalose and NaCl concentrations on the stability of the Fab and Fc domains of the MS-Hu6 at pH 5.5–7.0. Trehalose at 260 mM combined with NaCl at 1 mM yielded a formuation that is most stable at pH 6.2 (**D**). (**E**) Effect of sucrose and NaCl concentrations on the stability of the Fab and Fc domains of the MS-Hu6 at pH 5.5–7.0. Similar to trehalose, sucrose at 260 mM combined with NaCl at 1 mM yielded the most stable formulation at pH 6.2 (**F**).

Figure 5: Effect of FSH binding to MS-Hu6 on the stability of Fab and Fc domains of MS-Hu6 in (**A**) 20 mM phosphate buffer at pH 5.76–6.42, (**B**) 20 mM phosphate buffer, 1 mM NaCl, 0.001% w/v Tween 20, and 260 mM trehalose at pH 5.76–6.42, and (**C**) 20 mM phosphate buffer, 1 mM NaCl, 0.001% w/v Tween 20, and 260 mM sucrose with at pH 5.76–6.42.

Figure 6: (**A**) Representative size exclusion chromatography (SEC) chromatographs of MS-Hu6 in PBS and in formulation (1 mg/ml). (**B**) Representative particle size volume distribution graphs obtained from dynamic light scattering (DLS).

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