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Electronic Supplementary Information

Materials and Methods

Materials

Herceptin® was obtained from Genentech (San Francisco, US) as freeze-dried powder containing (858.1 mg): 51.4% trastuzumab, 0.74% L-histidine, 1.15% L-histidine hydrochloride monohydrate, 46.7% trehalose dihydrate and 0.01% polysorbate 20. Millex-GV syringe filter units (0.22 μm, PVDF, 33 mm, gamma sterilized, SLGV033RS), Amicon® centrifugal filters (Ultracell® 50,000 NMWL), di-potassium hydrogen phosphate (EMPROVE® Ph Eur, BP, E 340 grade, 105101) and potassium dihydrogen phosphate (EMSURE® ISO grade, 104873) were purchased from Merck Millipore (MA, USA). All other reagents were obtained from Sigma Aldrich and used without further purification.

Synthesis and characterization of choline dihydrogen phosphate (CDHP)

CDHP was made by neutralization reaction involving 1 mole of choline hydroxide with 1 mole of phosphoric acid as given in the literature[1]. Typically, 10 g of CDHP was made by slow addition of an aqueous solution (85%) of phosphoric acid (5.7g, 58 mmoles) to 20% aqueous choline hydroxide solution (30.1g, 248 mmoles) in an ice bath. The contents were stirred for about 1 hour at room temperature and the mixture was roto-evaporated at reduced pressures to obtain crude CDHP. To the crude compound activated charcoal (approx. 1 g) was added, stirred with water and filtered. The filtrate was again evaporated to get pure white solid in 98% yield. The sample was characterized by Electrospray Mass spectrometry to reveal the presence of the cation and anion.

Electrospray Mass spectroscopy analysis, (cone ±35V): CDHP, *m/z* (relative intensity, %): ES^+, 103.9 ($Me_3N^+CH_2CH_2OH$, 100); ES^-, 96.9 ($H_2PO_4^-$, 100). The acid base stoichiometry was confirmed by determining the pH of a 0.1 M aqueous solution of the material. This was found to be in satisfactory agreement with a control aqueous sample (pH= 5.6).

^{13}C NMR (600 MHz, D_2O) δ: 67.3 (CH_2), 55.5 (CH_2), 53.8 (3 x CH_3)

^{1}H NMR (300.13 MHz in D_20, δ, ppm relative to TMS): 3.16(s,9H), 3.49-3.46(m,2H), 4.04-4.06(m, 2H). The choline OH proton and OH protons from dihydrogen phosphate appear to be exchanged with the solvent D_2O peak at 4.67 ppm.

The purity of the synthesised CDHP was determined to be 99.5% (based on 1H NMR spectrum) and observed only tiny impurities at around 2.7ppm (to an extent of ~0.5 %). The peak integral shows 9 protons (for 3 methyl groups of choline cation), 2 protons for methylene groups (attached to Nitrogen atom) and another 2 protons for methylene groups (attached to oxygen atom).

Elemental analysis (%), calculated: C 29.85, N 6.96, H 7.96; **found:** C 28.10, N 6.87, H 8.09.

Formulation preparation

Two sets of Herceptin® formulations were prepared: (1) 20 mg/mL antibody formulations containing no other excipients besides any added CDHP; and (2) 60 mg/mL antibody formulations containing the excipients present in the Herceptin® formulation as provided by the manufacturer in the same antibody:excipient ratio, in addition to any added CDHP. The formulations prepared were designed to test:

1) The effect of CDHP alone on antibody stability, in addition to its effect in combination with excipients commonly used in marketed antibody products. This way, its compatibility and potential synergy with other stabilizing excipients can be tested.

2) The effect of CDHP on antibody stability in formulations containing high antibody concentrations

3) Whether the influence of CDHP on protein stability is related to the protein:ionic liquid ratio or the ionic liquid:solvent ratio, which would determine its practicality in more highly concentrated protein formulations.

Buffer exchange and concentration of Herceptin® solutions was performed using 50 kDa centrifugal filters to achieve the desired formulation. Due to the high viscosity of CDHP stock solutions concentrated Herceptin® solutions were diluted in CDHP to the desired antibody (20 mg/mL) and IL concentration to minimize unwanted aggregation. For 60 mg/mL formulations, the Herceptin® powder provided (containing the

excipients listed in Materials section) was dissolved in water (control), a solution of 53% w/v CDHP or 30% w/v CDHP such that the final concentration of trastuzumab was 60 mg/mL in each formulation and the antibody:excipient ratio was maintained. Dissolution was achieved by keeping each formulation at 4 °C overnight with gentle pipetting to assist dissolution of all the powder. When necessary, sodium hydroxide and phosphoric acid were used to adjust solution pH to 6 for all formulations. Solutions were filtered using 0.22 μm syringe filter units then the UV absorbance at 280 nm was checked to confirm the concentration of trastuzumab in each formulation was 60 mg/mL. All formulations were stored at 4 °C until needed.

Accelerated aggregation studies at elevated temperature

5 μL of each antibody formulation was pipetted into a 0.2 mL PCR vial and incubated at 68 °C in a Thermal Cycler (Applied Biosystems, CA, USA) for various durations to induce aggregation as previously described[2]. A thermal cycler was used to: 1) minimize sample amount; 2) prevent evaporation of the sample and consequent changes in antibody concentration during incubation (lid/cover of thermal cycler is heated above incubation temperature). Following incubation, incubated samples were removed and immediately stored on ice to halt aggregation and then characterized by size exclusion-high performance liquid chromatography (SE-HPLC). To investigate the nature of aggregates formed in the 60 mg/mL formulations, each of the 60 mg/ml formulations that were incubated at 68 °C were also kept for 20 hours at 4 °C after dilution, then monomer content was re-measured using SE-HPLC.

SE-HPLC

Size exclusion-high performance liquid chromatography (SE-HPLC) was used to quantify antibody monomer loss following incubation at 68 °C. Analysis was performed using an Agilent 1200 Liquid Chromatography system (Agilent Technologies, California, USA) with a Zorbax GF-250 column coupled to a guard column at 22 °C, using a 150 mM potassium phosphate mobile phase, at pH 6.5, and a flow rate of 0.5 mL/min. The incubated 5 μL samples of each trastuzumab formulation were diluted to 50 μL with mobile phase buffer prior to injection to prevent blockage of column with high amounts of protein. 10 μL of the diluted samples were then immediately injected and each injection was repeated three times. Stressed 60 mg/mL samples were re-injected after 20 hours at 4 °C. Monomer peaks were detected using an in-line UV

signal detector set at 280 nm. The area under the curve (AUC) of the monomer peak was averaged over the three runs and the mean relative monomer % was calculated for each sample, by setting the monomer AUC of the unincubated samples as 100% and calculating the change in monomer AUC accordingly. The standard deviations (SD) were plotted as error bars in the figures.

Trastuzumab melting temperature (T_m) and onset temperature of aggregation (T_{agg})

Intrinsic tryptophan fluorescence and static light scattering (SLS) techniques were used to calculate the T_m and T_{agg} respectively, of each formulation. A linear temperature ramp from 15 to 95 °C at 1 °C/min scan rate was performed whilst measuring tryptophan fluorescence and SLS simultaneously through laser excitation at 473 nm using UNcle (Unchained Labs, CA, USA). 8.5 µL of each formulation was pipetted undiluted into the UNcle UNI (a sample holding unit containing 16 quartz cells) in triplicates. A holding time was not used to maximise the frequency of measurements. T_m and T_{agg} were determined by the UNcle Analysis software, and in some cases manually, by using the first derivative (for T_m determination). The barycentric mean (BCM) was used to plot the T_m curves, which is defined by the following equation:

$$\lambda_{BCM} = \frac{\sum_{\lambda} \lambda I(\lambda)}{\sum_{\lambda} I(\lambda)}$$

The equation is defined over the range 300-450 nm, whereby each wavelength value (λ) is multiplied by the tryptophan fluorescence intensity (*I*) at that wavelength, and the sum of that value for all wavelengths between 300 to 450 is divided by the sum of the intensities at those wavelengths. This results in an ‘averaged’ peak wavelength (λ_{BCM}) for a given spectrum which eliminates noise and accommodates for changes in the shape of the spectrum. Since antibodies are multi-domain proteins, up to three T_ms can be measured, corresponding to the CH2, Fab and CH3 domains. For trastuzumab only two T_ms are usually distinguishable as the Fab and CH3 domain have approximately the same Tm, leading to overlap.

UV-Vis Spectroscopy

UV-Vis spectroscopy was used to measure the concentration of soluble antibody after dissolution of powder to ensure complete dissolution, and following incubation at elevated temperatures to confirm the lack of insoluble aggregates. Absorbance was measured at 280 nm over a wavelength range of 220-350 nm using a Shimadzu UV-2600 spectrophotometer (Shimadzu, Japan). Samples were corrected by blank subtraction of the respective solvent. Data was not included as the absorbance of all samples remained unchanged following incubation, and therefore no change in soluble antibody concentration was observed.

References

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