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Assessing the impact of viscosity lowering excipient on liquid-liquid phase separation for high concentration monoclonal antibody solutions

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ABSTRACT

With continued interest in high concentration monoclonal antibody drug products to meet subcutaneous administration requirements, there is heightened attention on balancing protein-protein interactions, solution properties and overcoming instabilities such as increased in viscosity, particle formation, loss in potency, and aggregation of drug products. L-arginine hydrochloride is a commonly used viscosity reducing excipient used to influence protein-protein interactions of high concentration of mAbs. Contrary to literature, we observed that slight modifications to L-arginine hydrochloride concentrations in model drug product formulations can result in liquid-liquid phase separation if excipient and pH conditions are not well tightly controlled. We utilized a biophysical toolkit to assess the potentials of liquid-liquid phase separation (LLPS) that informs the limits of excipient and pH levels using structural- and molecular interaction-based assessments. While liquid-liquid phase separation observed in this study is reversible and does not impact inherent protein folding and structure, we demonstrated that increased ionic content in the formulations can significantly alter the balance of osmolarity toward the occurrence of LLPS. The aim of this work is to demonstrate the diversity of the toolbox used to evaluate the observed LLPS and the decision-making for optimization of formulation development.

Introduction

Delivering monoclonal antibodies (mAbs) via the subcutaneous route of administration is increasingly desired during drug product development as it yields to better patient-centric, making new therapies more accessible and easing the burden on the healthcare system.[1,2] As mAbs are highly selective therapeutics, larger subcutaneous doses are typically employed to achieve maximum therapeutic levels for optimal pharmacokinetic exposure compared to an IV based regimen therapy.[3] This consideration is under the assumption that doses between IV and SC are different based on clinical pharmacology attributes, blood pharmacokinetic parameters such as Cmax, Cmin, Tmax, AUC, and concentration within the therapeutic window. For subcutaneous delivery, the dose can be altered depending on the injection volume or the drug product (DP) concentration, while the delivery can be managed with a host of device options.[4,5] Historically, for subcutaneous injections, volumes within the ranges of 0.5–2.25 mL are used without causing substantial injection site related pain, however up to 5 mL may be tolerated in the abdomen due to the limiting size of the subcutaneous space.[5] More recently, work has focused on trying to increase subcutaneous injection volume to greater than 3 mL (i.e. up to 25 mL has been tried in the clinic.[6]) and this area of drug product delivery is growing.[1] Another approach for high dose mAb product development is to push the drug product concentrations beyond the 100 mg/mL paradigm and evaluate its potential in aiding for a robust subcutaneous delivery. The aim is to achieve the high concentration while understanding key structural and analytical focal areas enabling stable higher concentration DPs. Alternative technologies have looked at atypical approaches to managing high to ultra-high concentrations of drug products that can be delivered either in small volumes or moderately large volumes managed via viable device optionality.

With increasing mAb concentrations, the propensity of protein-protein interactions increases substantially and the need to control these interactions by varying different excipient levels takes priority in drug substance/drug product formulation and process development. The excipient levels in the formulation require modulation for

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favorable solution properties that enables ease of manufacturing, aiding stable products over its shelf life, and patient acceptability and useability.[7] Often there is a fine balance between achieving optimal solution properties and colloidal stability in a high concentration mAb formulation that is indicative of a stable DP. A big watch-out for high concentration mAb formulations is the propensity to form highly viscous solutions primarily due to intermolecular interactions and changes to associated rheology, changes to intramolecular-excipient interactions, and sheer sensitiveness.[8]

A typical formulation strategy (primarily driven to reduce viscosity and improve manufacturability) is by modulating protein-protein interactions and behavior of physiochemical properties in different buffer systems. Increased ionic strength is typically known to decrease viscosity through shielding protein charges on either hydrophobically or solvent exposed surfaces and weakening the long-range attractive interactions between protein molecules.[9] While increased ionic strength decreases repulsive interactions, it may also increase protein-protein attractive forces and hydrophobic interaction between the heterogenous distribution of charge and hydrophobic residue of mAbs.[10] This can in turn result in an alteration of balance of osmolarity and self-association interaction causing either greater reversible or non-reversible protein self-associations such as opalescence, and liquid-liquid phase separation (LLPS).[11] While opalescence is more of an aesthetic problem, LLPS can inhibit dose administration and have a detrimental effect on colloidal stability impacting overall drug product stability, ability to meet batch release criteria's, and use-case under different administration settings. It can also lead to rejection of manufactured clinical or commercial DP batches, and is shown to be associated with long-term stability impacts.[12] LLPS is usually a thermodynamically reversible phenomenon in which mixtures of different components separate into two liquid phases.[13] During LLPS there is nucleation of droplets or protein domains (in mAb formulations), which is dependent on temperature and composition, explaining an interplay between thermodynamics and kinetics.[14] As lower temperatures reduce the activation energy of molecules, movement is restricted leading to the nucleation that may otherwise be prevented at higher temperatures with higher energy reserves.[15] The temperature reversibility can be limiting for the DPs stability, storage and use at various temperature conditions. While it is favorable to increase number of excipients or the excipient concentrations to improve solution properties such as viscosity and modulate protein-protein interactions, care and optimization must be taken to ensure excipients do not shift the balance of attractive-repulsive forces between proteins, leading to potential LLPS. For the production of highly concentrated monoclonal antibodies (mAbs) during drug substance downstream processing, understanding the Donnan effect via DLVO theory on electrostatic interactions, protein charge density, and excipient levels is vital for ultra-diafiltration of buffer systems. Chemistry manufacturing controls (CMC) for drug substances and drug products considers DLVO theory (Derjaguin−Landau−Verwey−Overbeek theory) to optimize high concentration mAb formulations for yields, purity, and process efficiencies. Strategies in formulation and processing must balance both protein repulsion and self-association like phenomenon's.

Modifying excipients, whether by adding new ones or adjusting quantities, can impact mAb interactions, solution properties, and stability. These changes depend on alterations to surface charge, hydrophobic/hydrophilic exposure, water molecule exclusion, and dynamic behavior of self-association, protein structure dynamics, folding-unfolding kinetics of the solution. Previously, it has been demonstrated that L-arginine hydrochloride (HCl) is an effective excipient at reducing viscosity and LLPS for majority of the IgG formulations at either 50 or 100 mg/mL and marketed products in the market.[16,17] It was summarized that the LLPS phenomena was reduced likely due to decreases in attractive forces between proteins by shielding protein charges and reducing key intermolecular interactions between either Fab-Fc or Fc-Fc. Due to the ability to shield protein charges, L-arginine HCl has also been used as a viscosity reducing agent. However, as explained earlier, it must be noted that while increasing excipients can shield charge, it may have other downstream effects, leading to other intermolecular interactions with the protein and excipients and may further contribute to LLPS.

The regulation of excipient incorporation, along with the maintenance of rheologically pertinent solution properties, balanced protein-protein interactions, and optimal colloidal stability, presents a significant challenge.[18] During the formulation development of an IgG1 monoclonal antibody, L-arginine HCl was used primarily to reduce viscosity. Although the formulation showed favorable results in molecular and biophysical assessments, an increase of 10 % in excipient concentration and a 0.8-unit increase in pH resulted in reversible liquid-liquid phase separation (LLPS). The occurrence of LLPS has prompted additional investigation into its effects on the IgG isotype formulation and the contributing factors involved. Biophysical characterization techniques such as diffusion interaction parameter and thermal melting temperature are commonly used to predict the stability of mAb formulations.[19] However, there are limitations to these tools for high concentration mAb formulations as they are typically conducted at low concentration and predict stability at accelerated conditions.[20,21]

To investigate the impact of varying formulation conditions on LLPS formation, we utilized a biophysical toolbox to evaluate the potential occurrence of LLPS across different formulations with variations in excipients and pH levels. Our assessment involves implementing molecular assessments of protein species via size-exclusion chromatography, isoelectric focusing capillary electropheris (iCE), and microfluidic modulation FTIR infrared (MM-IR) spectroscopy, in addition to biophysical characterization by dynamic light scattering, and differential scanning fluorimetry. We used a plate-based method with poly-ethylene glycol (PEG) to study forced precipitation and assess the apparent solubility of the mAb formulation. Lower solubility in PEG-6000 indicated liquid-liquid phase separation (LLPS). An inverse linear relationship was found between osmolality and solubility, suggesting that adjusting ionic strength/osmolality could reduce LLPS risk during processing or storage. While changes in excipients may not affect viscosity, thermal stability, or protein interactions, they can alter protein solubility and increase LLPS risk, potentially leading to failure to meeting drug product specifications.

Materials and methods

All materials used in the study were purchased from Fisher Scientific. The model IgG monoclonal antibody was generated in-house at Pfizer.

Monoclonal antibody formulation

Monoclonal antibody formulations of IgG1 were formed through ultrafiltration/diafiltration process using tangential flow filtration. Four different formulations were produced with the excipient and pH listed in Table 1.

Molecular assessments

Size exclusion chromatography (SEC)

As previously described in Jogdeo et al.[22], size variants of mAb formulations were assessed by size exclusion chromatography (SEC) on a Waters e-2695 HPLC system at 30°C using a YMC-Pack Diol-300 column (300 × 4.6 mm, 2 μm, Catalog # DL30S02−3046PTH). Samples (25 μg) were loaded onto the column at a flow rate of 0.2 mL/min and eluted for 30 mins using 20 mM sodium phosphate, 400 mM sodium chloride, pH 7.2 as mobile phase, with absorbance monitored at 280 nm. The Empower 3® software (Waters) was used for data

Table 1
Monoclonal antibody formulations examined.

Variable	Formulation 1	Formulation 2	Formulation 3	Formulation 4
Monoclonal antibody (mg/mL)	160	160	160	100
L-Histidine/L-Histidine monohydrochloride monohydrate (mM)	20	20	10	20
Sucrose (mg/mL)	45	55	30	85
L-Arginine hydrochloride (HCl) (mM)	50	55	20	0
Disodium EDTA (mg/mL)	0.05	0.0625	0.0375	0.05
PS80 (mg/mL)	0.2	0.3	0.1	0.2
pH	5.8	6.6	5.3	5.8

analysis to detect the percentage area of intact IgG1 compared to high molecular mass species (%HMMS) and low molecular mass species (%LMMS).

Capillary isoelectric focusing (iCE)

As previous described by Nichols et al.[23], the pI values for the mAb and variants were determined *via* capillary isoelectric focusing (cIEF) using a ProteinSimple iCE3 instrument and the iCE Chemical Test kit (ProteinSimple, part no. 101801). Anolyte and catholyte solutions were prepared as per the manufacturer instructions. The samples for injection contained 0.3 mg/ml protein, 4 % Pharmalyte pH 3−10 (GE part no. 17-0456-01), 2.0 M urea, 0.25 % methyl cellulose (ProteinSimple, part no. 101876), 0.01 mg/ml pI Marker 6.14 (ProteinSimple, part no. 102220), and 0.01 mg/ml pI Marker 9.50 (ProteinSimple, part no. 101996). The capillary was cIEF Cartridge Column (Fluorocarbon Coated, 100 mm ID × 50 mm, ProteinSimple part no. 101701). The samples were prefocused at 1500 V for 1 min followed by a 3000 V focusing period of 6 min. UV detection wavelength was at 280 nm and the iCE CFR software was used to calibrate the UV image with the pI markers to determine the sample pI.

Microfluidic modulation infrared (MM-IR) spectroscopy

To determine the secondary structure of the IgG Formulations after LLPS, multi-molecular infrared (MM-IR) spectroscopy was employed.[24,25] MM-IR was performed on an automated Apollo system (Redshift Bio, Burlington, MA, US). All samples and corresponding buffers were loaded at 5 mg/mL in triplicates in a 96-well plate for real-time referencing. The MM-IR alternated injections of the sample and its buffer into the flow cell using compressed dry air (back pressure: 5 psi, modulation rate: 1 Hz). The detector simultaneously measured sample and buffer absorbance, allowing real-time subtraction of buffer absorbance from the sample. Differential absorbance spectra were recorded across the Amide I band. For data analysis, the absorbance values were collected at various wavelengths and the AQS3® Delta software (Redshift Bio) was used for analysis. The higher-order structural elements were calculated following the procedure by Ivancic et al. and Liu et al.[26,27]

Biophysical characterization

DSF and DLS by UNcle

The UNcle instrument by Unchained Labs was used to determine the thermal melting temperature (Tm) and diffusion interaction parameter (kD) of the formulations by differential scanning fluorimetry (DSF) and dynamic light scattering (DLS), respectively.[28] The equipment was used according to manufacturer recommendations. DLS data was collected alongside fluorescence data under the "Tm & Tagg with optional DLS". Concentration ranges of 1 mg/mL to 15 mg/mL of mAb formulations were plated in UNcle specialized cartridges, called “UNI” in triplicates (9 uL per channel). Samples were diluted in its respective formulation buffers. Samples were heated from 15 °C to 95 °C, at 0.5 °C/min Linear rate. DLS data was collected before and after heating. The DLS data at 15 °C (pre-heating) was used to determine the Diffusion Coefficient of each sample. The corresponding Tm data (determined by fluorescence at 266 nm and 473 nm) was collected over the linear heating intervals. The Tm (thermal melting temperature) is calculated at the temperature where the fluorescence begins to decrease due to protein unfolding.

kD by Dynapro and Stunner instruments

The Wyatt DynaPro III DLS instrument was used to determine the diffusion coefficient according to concentration of the IgG1 formulations. DLS was run according to manufacturing instructions on 20 μL samples in 384-microwell plates. Samples were measured in triplicates at a concentration range from 1 mg/mL to 30 mg/mL for the mAb formulations.

The DLS/kD function was used in Stunner (by UNchained Labs) under Protein assay, the B_{22} & k_D attributes setting was selected. System standards were applied by using IgG as the model protein. A generic Histidine with excipients buffer was used for background. Concentration ranges of 1 mg/mL to 12 mg/mL of the mAb formulations were plated in the Stunner plate (2 μL per well). MilliQ water was used as the control. Samples were run in triplicates. From the data generated, the diffusion coefficient was plotted against the concentration of the mAb formulations, and a linear regression function was fitted to calculate the slope and y-intercept. kD was calculated as: kD = slope/y-intercept.

Forced liquid-liquid phase separation with PEG-6000

In a standard buffer (20 mM histidine, 85 g/L sucrose, 0.05 g/L EDTA and 0.2 g/L polysorbate 80, pH 5.8), a 40 % w/v stock of PEG-6000 was made. For the plate-based assay, as described by Li, Kantor and Warne.[29] different concentrations of PEG-6000 were made from the 40 % w/v stock from dilution in standard buffer. In order to account for 1:8 dilution with the mAb formulations, 25 μL of PEG-6000 at the following concentrations were plated: 40 % w/v (equivalent to 5 % w/v when diluted), 32 % w/v (equivalent to 4 % w/v when diluted), 24 % w/v (equivalent to 3 % w/v when diluted), 16 % w/v (equivalent to 2 % w/v when diluted), 8 % w/v (equivalent to 1 % w/v when diluted), 4 % w/v (equivalent to 0.5 % w/v when diluted), 2 % w/v (equivalent to 0.25 % w/v when diluted), and 0 % PEG-6000.

To the different concentrations of PEG-6000, 175 μL of the 160 mg/mL mAb formulations were added and mixed via up and down pipetting movements. To note, for Formulation 4, the maximum concentration sample available was 100 mg/mL. Plates were sealed with a parafilm sheet and incubated at 2–8 °C for 15 minutes. The plates were assessed for cloudiness against a black background and then measured for absorbance at 500 nm on a plate reader (SoftProMax Plate reader).

For the centrifugation assay in Eppendorf tubes, a 25 % w/v stock of PEG-6000 was made in platform buffer. In Eppendorf tubes, 900 μL of mAb formulations were mixed with 100 μL of the 25 % w/v PEG-6000 stock. A control of 900 μL of mAb formulations were mixed with 100 μL of standard buffer. All tubes were incubated at 2-8 °C for 30 minutes before being centrifuged at 4000 rpm for 60 minutes at 4 °C. The supernatant of the samples was collected, and concentrations were measured via UV spectroscopy (SoloVPE) using an extinction coefficient of 1.56.

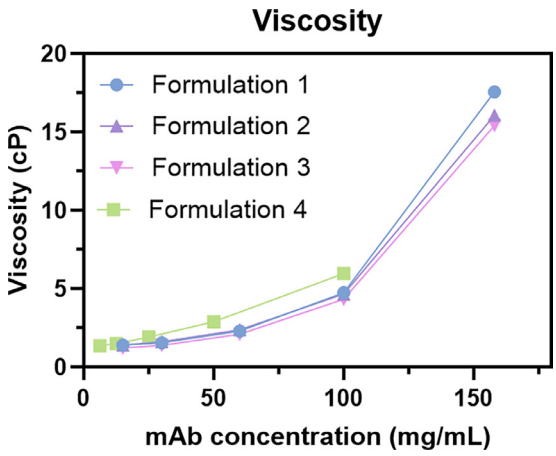

Fig. 1. Viscosity as a function of concentration at 20 °C.

Results and discussion

For the development of a robust drug product, the selection and optimization of excipients in monoclonal antibody (mAb) formulations are crucial. These excipients are tailored to achieve desirable solution properties that align with the quality target drug product profile (QTPP) and adhere to stringent manufacturing quality requirements.[30] Excipients can have different functions, including acting as a tonicifier, chelator, buffer, or surfactant to name a few.[31] In the context of high concentration mAb products, the addition of viscosity-decreasing excipients is a common approach to ensure that the viscosity remains within an optimal range for patient administration and manufacturing ease.[9] Elevated viscosity often results from increased protein-protein interactions, which can be mitigated by enhancing the ionic strength with suitable excipients. L-arginine HCl is frequently used to lower viscosity by shielding these interactions, though salts such as sodium chloride and amino acids with both hydrophilic and hydrophobic residues, including lysine, histidine, and proline, are also effective.[16,32] In this study, L-arginine HCl was integrated into the IgG1 mAb formulations to evaluate its impact on viscosity reduction. As illustrated in Fig. 1, the use of L-arginine HCl at relatively low concentrations resulted in a minimal impact on viscosity for Formulations 1-3 compared to Formulation 4, which did not contain L-arginine HCl. Notably, Formulation 2, which included the highest concentration of L-arginine HCl (55 mM), demonstrated a reversible liquid-liquid phase separation (LLPS) that was not observed in the other formulations (see Fig. 2). This phase separation was reversible upon warming the formulation to room temperature (~25 °C), unlike the stability observed at 2–8 °C after two weeks of storage. Fig. 3.

The phenomenon of LLPS is thermodynamic in nature, where the nucleation of protein domains is influenced by temperature and the composition of the formulation (Brown et al., 2015). For Formulation 2, extended storage at 2-8 °C periodically induced LLPS, forming a gel-like material, which was reversible by returning the formulation to room temperature (~25 °C) similar to the observations in (Smith et al., 2016). Further investigation into the precise mechanisms and optimal concentrations of such excipients is essential to enhance formulation design, implementation of process controls during formulation, and understanding potential impacts to therapeutic efficacy.

Moreover, it is important to consider the molecular and biophysical characterization of mAb formulations to understand the impact of excipients on drug product quality and stability. Techniques such as size exclusion chromatography (SEC) and iCE are employed to assess parameters like monomer content, high molecular mass species (HMMS), low molecular mass species (LMMS), and charge variants (Chumsae et al., 2013). Despite differences in excipients across the formulations, and the LLPS observed in Formulation 2, studies showed no significant change in the percentage of monomer across the formulations, with values consistently above 99.4 %. The amount of HMMS and LMMS remained low, below 0.5 % and 0.08 % respectively. Additionally, charge variant analysis revealed that the percent main species were similar for Formulations 1–3, while Formulation 4 exhibited a ~20 % increase in acidic species, likely due to process changes in the monoclonal antibody drug substance manufacturing (Chumsae et al., 2015). These findings underscore the importance of a detailed understanding of formulation excipients and their impact on mAb properties to optimize drug product development and ensure effective therapeutic outcomes.

After two weeks of storage at 2–8 °C (40 % humidity), Liquid-liquid phase separation (LLPS) was observed in Formulation 2, resulting in the formation of a gel-like material. Notably, this phenomenon was absent in Formulations 1, 3, and 4. Upon warming to approximately 25 °C, the separation was reversed; however, recurring storage at 2–8 °C led to periodic LLPS, as illustrated in Fig. 2. The occurrence of LLPS is influenced by both temperature and the composition of the formulation.[14]

To elucidate the underlying mechanisms, we conducted a comprehensive investigation of LLPS in four different formulations using advanced molecular and biophysical techniques. This analysis aims to provide a deeper understanding of the conditions that promote LLPS and the specific factors within the formulation compositions that contribute to this reversible transition.

Formulation and liquid-liquid phase separation does not impact key molecular properties

Size exclusion chromatography (SEC) remains an indispensable technique for discerning potential fragments and high molecular

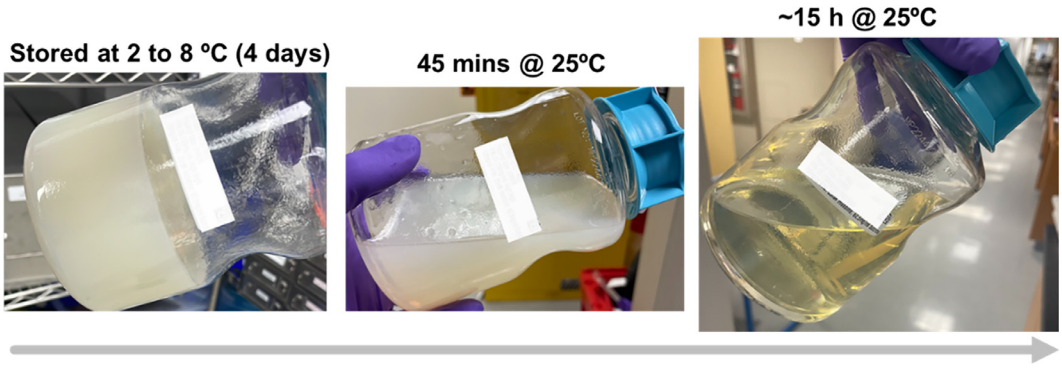

Fig. 2. Representative images of IgG1 Formulation 2 following LLPS and the reversibility at different temperature incubations.

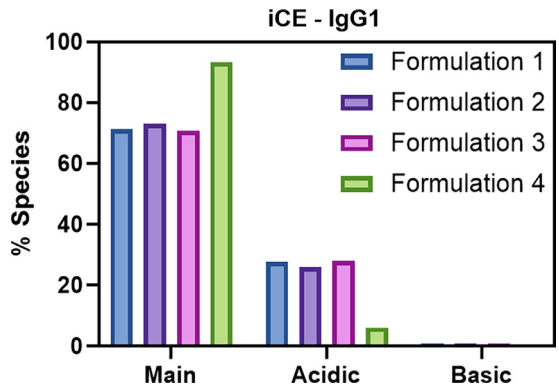

Fig. 3. Ion Exchange Chromatography (iCE) determines the percent main, acidic and basic species for all formulations.

mass species (HMMS) in monoclonal antibody (mAb) formulations, facilitating size-based separation.[33] The presence of HMMS can provoke liquid-liquid phase separation (LLPS) and consequently affect the drug product's quality, biological activity, and immunogenic profile.[34] To ascertain the impact of LLPS on mAb fragmentation, four formulations were subjected to SEC-HPLC analysis post-reversion to a homogenous liquid state following the LLPS event in Formulation 2. Despite varying excipient compositions among the formulations, including the LLPS observed in Formulation 2, the percentage of monomer mAb remained consistently high (>99.4 %) across all samples, as delineated in Table 1. Additionally, the quantities of HMMS and low molecular weight species (LMMS) were minimal, registering below 0.5 % and 0.08 %, respectively.

To further investigate potential discrepancies in charge species among the formulations, imaged capillary electrophoresis (iCE) was utilized.[35] Table 2 delineates that the percentage of main species was comparable across Formulations 1-3, with Formulation 4 exhibiting a ~20 % increase. This variation correlates with a heightened percentage of acidic species in Formulation 4 compared to Formulations 1-3, which is likely attributable to procedural alterations in the monoclonal antibody drug substance manufacturing process. Enhanced sugar content in the cell culture media can induce glycation, thereby increasing acidic species, as documented by Chumsae et al.[36] The observed glycation and acidic species variation are ostensibly influenced more by manufacturing process variations than formulation excipients.

Multi-modulation infrared (MMIR) spectroscopy was employed to evaluate the secondary structure of the four mAb formulations, as described by Smith et al.[37,38] The analysis was conducted to determine any structural alterations post-LLPS in Formulation 2, which had returned to a liquid state.

The MMIR spectroscopy results, depicted in Fig. 4, revealed identical spectra across all four formulations, indicating no observable changes in the secondary structure. This finding is critical as it suggests that the phase separation event did not perturb the protein folding or conformation. Furthermore, the analysis showed that the ratio of beta-sheets to alpha-helical structures remained consistent across the formulations, corroborating the hypothesis presented by Johnson et al.[24]

Table 2
Size exclusion chromatography determined the %monomer and %high molecular mass species (HMMS) and %low molecular mass species (LMMS).

	SEC		
Sample	%HMMS	%Monomer	%LMMS
Formulation 1	0.385	99.6	NMT 0.2
Formulation 2	0.503	99.4	NMT 0.2
Formulation 3	0.409	99.5	NMT 0.2
Formulation 4	0.400	99.6	NMT 0.2

These observations imply that the LLPS is a reversible thermodynamic process that does not adversely affect the IgG structure or its functional integrity. This conclusion aligns with previous studies, such as those by Williams et al.[39], which demonstrated that LLPS could serve as a concentrating technique for mAb formulations without compromising the protein's bioactivity. Hence, while the thermal melting temperature (Tm) assesses the temperature at which protein unfolding occurs, it does not predict protein aggregation or interaction under cold conditions, as noted by Lee et al.[21]

In summary, the MMIR spectroscopy data and thermal melting temperature measurements collectively underscore the stability of mAb formulations under varying conditions and the non-impacting nature of LLPS on protein structure and function. This reinforces the viability of these formulations for therapeutic applications, ensuring prolonged stability and efficacy in clinical settings

Formulation did not impact critical biophysical properties

Establishing the thermal melting temperature (Tm) values of formulations is a critical approach widely employed to understand the stability of monoclonal antibodies (mAbs) by determining the temperature at which protein denaturation occurs, as previously described in the literature.[40] However, similar to the limitations observed with diffusion interaction parameter (kD) measurements, Tm assessments were not effective in predicting the potential for liquid-liquid phase separation (LLPS). Differential scanning fluorimetry (DSF) is typically utilized to assess thermostability and broadly eliminate less stable formulations from screening assessments.[41] In this study, the thermal melting temperature was evaluated for four different formulations using the DSF method via the UNcle instrument. Samples were subjected to a controlled heating protocol from 15 °C to 95 °C at 0.5 °C/min increments, with fluorescence spectra measured continuously. The Tm, defined as the temperature at the onset of unfolding, is reported in Fig. 5.

Formulation 1 exhibited a Tm of 65.8 °C. In contrast, Formulation 4 demonstrated a higher Tm of 68.9 °C, which was comparable to Formulation 2 (Tm of 68.7 °C). The increased Tm of Formulation 4 can likely be attributed to the higher concentration of stabilizing excipients, particularly sucrose (85 g/L). Similarly, Formulation 2, which contains 55 g/L sucrose and 55 mM L-arginine HCl, showed a higher Tm compared to Formulation 1, which has 45 g/L sucrose and 50 mM L-arginine HCl. Formulation 3, with reduced excipient content (30 g/L sucrose and 30 mM L-arginine HCl), exhibited a lower Tm of 62.4 °C, further confirming the correlation between excipient concentration and thermal stability.

These findings underscore the importance of excipient composition in influencing the thermal stability of mAb formulations. The data presented aligns with previous studies, demonstrating that higher excipient concentrations contribute to enhanced protein stability by delaying the onset of thermal denaturation. This understanding is crucial for the development of robust therapeutic mAb formulations, ensuring their stability and efficacy under varying storage and handling conditions.

While the thermal melting temperature (Tm) values of the four formulations exhibited consistency correlating with excipient content, it is hypothesized that higher salt concentrations may improve protein shielding, thus delaying the onset of thermal denaturation. This phenomenon might result in false positive values regarding Tm. However, the Tm parameter is unlikely to predict protein aggregation or interactions under cold conditions where LLPS occurs, as supported by previous studies. Despite the occurrence of LLPS, the structural integrity and folding of the IgG remained unaffected, indicating that LLPS does not compromise the bioactivity of the monoclonal

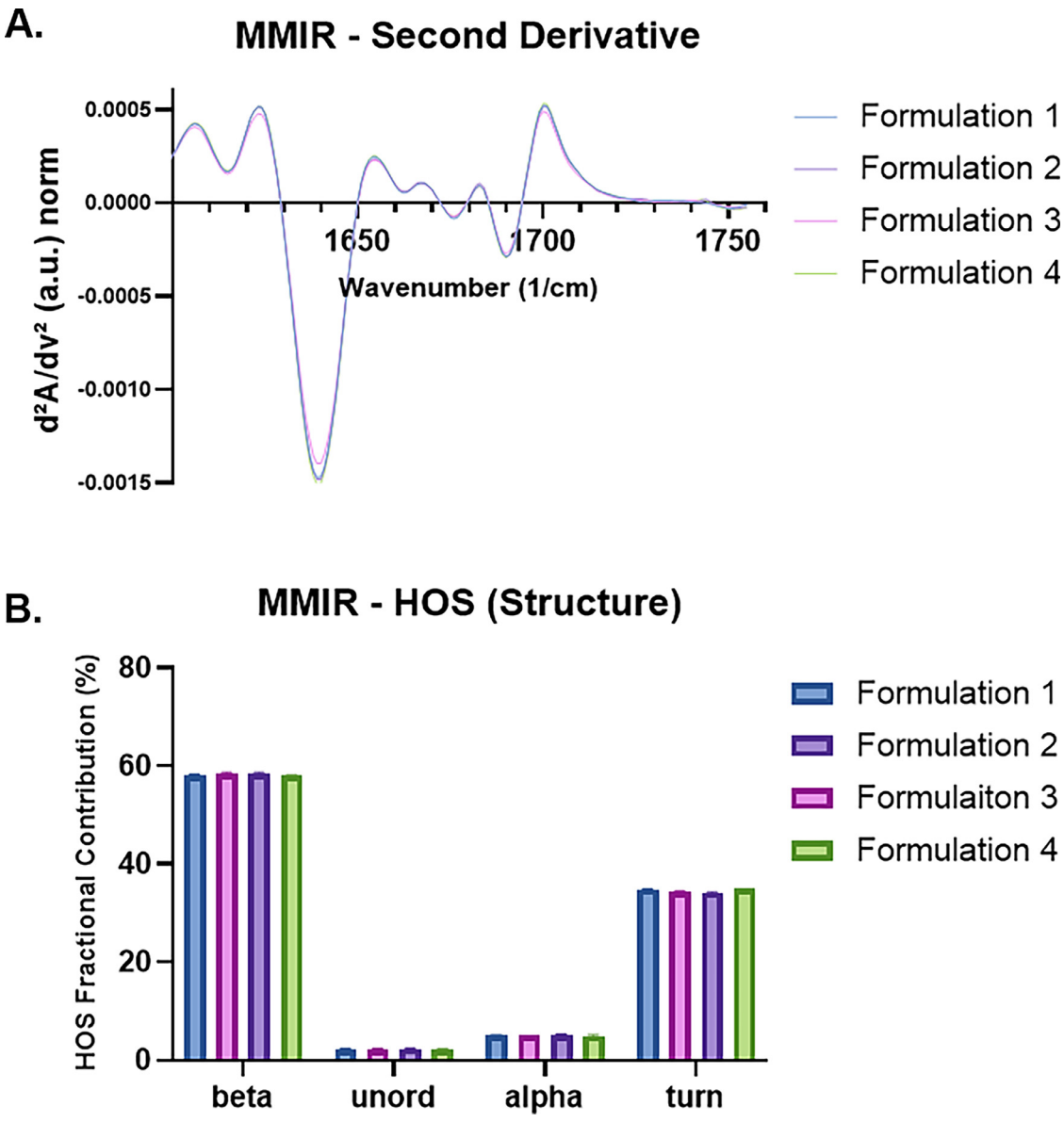

Fig. 4. MM-IR data: (A) second derivative spectrum of the mAb formulations and (B) HOS (structure) comparison.

antibody (mAb). This observation is in line with prior research, which demonstrates that LLPS can be utilized as a method to concentrate mAb formulations, leading to a phase enriched with protein compared to a phase devoid of protein.

Orthogonal approaches to assess protein formulation interactions are widely recognized as essential.[19] The diffusion interaction parameter (kD) is frequently reported as the most important predictor of colloidal self-interaction.[44] However, a major limitation of kD assessment is that it is measured over a range of low protein concentrations, where protein molecules are inherently more separated from each other, which differs from a highly concentrated mixture.[45] Using dynamic light scattering (DLS), kD was quantified by plotting the diffusion coefficient over concentration and fitting a linear regression. All plots had a linear regression coefficient greater than 0.85. The kD is calculated as the slope divided by the y-intercept and converted into units of mL/g. Since DLS is a variable experimental technique, three different instruments were used to assess the diffusion coefficient versus monoclonal antibody concentration (1 mg/mL to 10 mg/mL). These included the Stunner, UNcle, and DynaPro plate-based reader. Fig. S1 shows the kD for the four formulations, which are similarly negative, indicating a positive attraction between molecules in the formulation. Subtle differences are observed across the different instruments, but overall, the values are similar, and in terms of kD, they demonstrate similarity in the attractive interaction. A noticeable trend is that Formulation 3 tends to have a less negative kD value, which correlates with the lower concentration of excipients in the formulation. These findings confirm that the IgG monoclonal antibody is inherently attractive in nature.

Previous reports have demonstrated that kD evaluations only capture pairwise interactions between the dilute protein solution and do not expose close-range interactions that have potential issues related to protein precipitation.[19,45] Forced precipitation with PEG to assess protein solubility has been suggested as a better alternative to kD measurements. While kD measurements have been shown to correlate well with protein solubility predictions through forced precipitation with PEG, exceptions arise when ionic excipients are used within the formulation.[45] Further explanation of the differences observed between the two stability-indicating assays is warranted.

Additionally, it is important to consider the impact of formulation pH on protein-protein interactions. Variations in pH can significantly alter the electrostatic environment of the protein, influencing both kD

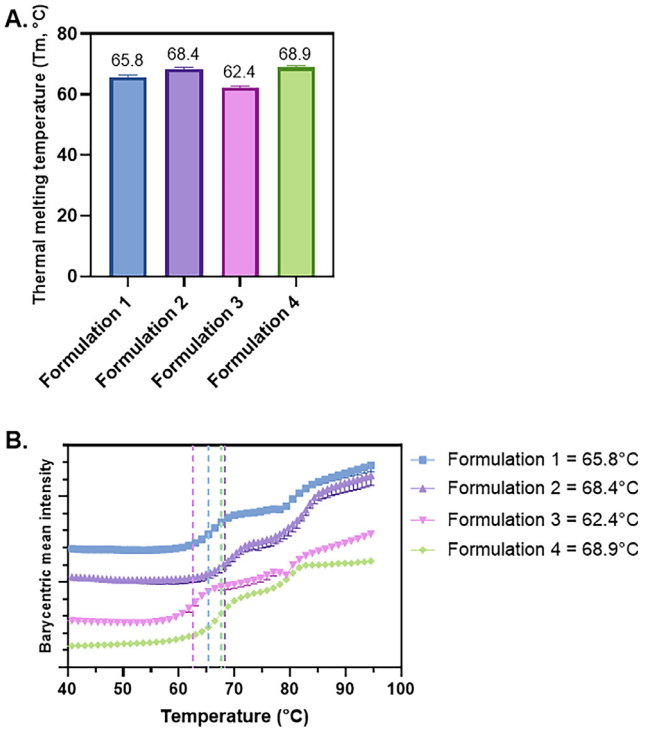

Fig. 5. Thermal melting temperatures across formulations. (A). Thermal melting temperature for each formulation (data represented as mean ± standard deviation, n = 3). (B). Barycentric mean intensity verus temperature profiles for each formulation which determined the thermal events, data represented as mean ± standard deviation, n = 3.

values and solubility assessments.[42,43,48] For instance, formulations with higher histidine base to histidine HCl ratios demonstrated different behaviors in precipitation studies, indicating a need to balance pH alongside excipient concentration for optimal formulation stability.

Furthermore, the role of excipient concentration in modulating protein stability cannot be understated. As observed, formulations with higher sucrose and L-arginine HCl concentrations exhibited increased thermal stability but with lower solubility. This suggests that excipients not only act as stabilizers but also play a crucial role in maintaining the bioactivity of the monoclonal antibody, ensuring appropriate solution properties, and structural performance under varying conditions.

Finally, the interplay between excipients and protein concentration highlights the complexity of formulation development. While higher excipient concentrations generally enhance stability, the specific combinations and ratios of excipients, as well as protein concentration, must be carefully optimized to prevent adverse interactions such as aggregation or precipitation.

Forced liquid-liquid phase separation using PEG-6000 as a crowding agent

The analysis confirmed that despite the similarities in solution properties and biophysical characteristics indicating stability, different monoclonal antibody (mAb) formulations with varying ionic components exhibited distinct propensities for liquid-liquid phase separation (LLPS). To systematically evaluate the LLPS potential, formulations were subjected to forced precipitation using polyethylene glycol (PEG-6000) as a crowding agent. PEG-6000 facilitates protein-protein interactions at lower concentrations, thereby inducing precipitation.[46] To examine liquid-liquid phase separation, eight variations of the formulations were exposed to different PEG-6000 concentrations, ranging from 5 % w/v to 0 %, to force precipitation.

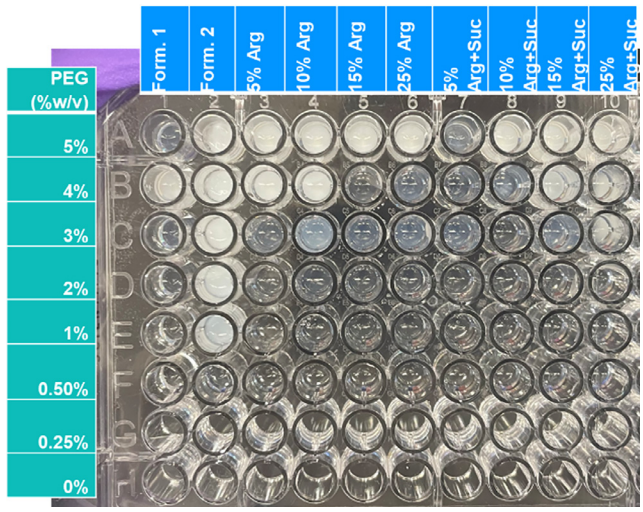

Fig. 6. Representative image of forced liquid-liquid phase separation with PEG-6000 at various concentrations in plate-based assay with a range of formulations.

PEG-6000 acts as a crowding agent that can force protein-protein interactions at lower concentrations, stimulating precipitation.[47,49] The concentration was consistent after an 8:1 dilution with PEG-6000, where the final monoclonal antibody concentration was 138 mg/mL, except for Formulation 4, which had a lower starting concentration, thus the final concentration after dilution was 87.6 mg/mL. The mixtures were examined in 0.2 mL volume in a microwell plate, according to a method previously described by Li, Kantor and Warne.[29] By design, to assess the LLPS at cold temperatures, the microwell plate was kept at 2 to 8 °C and precipitation was observed by cloudy wells as well as absorbance at 500 nm.

After incubation for 15 minutes at 2 to 8 °C, the forced LLPS can be observed by an increase in absorbance at 600 nm in Fig. 7. For the standard formulations tested (Fig. 6), Formulation 2 precipitated with the lowest amount of PEG-6000, specifically at 1 % w/v and above. The lowest amount of PEG-6000 that does not cause precipitation can be surmised as the apparent solubility score for the formulation,[3,49] for which it was 0.5 % w/v PEG-6000 for Formulation 2. The apparent solubility score for Formulation 1 was higher at 2 % w/v PEG-6000. For Formulation 3, where all of the excipients and pH were lowered compared to Formulation 1, the apparent solubility increased to 3 % w/v PEG-6000. While for Formulation 4, which contained no L-arginine HCl and was at a lower protein concentration, the apparent solubility score further increased to 4 % w/v PEG-6000.

When Formulation 1 that had L-arginine HCl removed and then added in at 5 % (i.e. 52.5 mM), 10 % (i.e. 55 mM), 15 % (i.e. 57.5 mM) and 25 % (i.e. 62.5 mM) higher than the base case of 50 mM (Fig. 7B), all variations had a similar reaction to formulation 1, where the apparent solubility score for all four formulations was 2 % w/v PEG-6000, indicating the variations of limiting or increasing L-arginine HCl had limited effect in causing LLPS.

When these formulation variations were taken and additional sucrose was then added to the same extent as the increased L-arginine HCl (i.e. 5 %, 10 %, 15 % and 25 % increase in L-arginine HCl and sucrose), the absorbance after exposure to different concentrations of PEG-6000 is shown in Fig. 7C. The additional 5 % and 10 % of L-arginine HCl and sucrose in the formulation did not change the apparent solubility compared to formulation 1 (i.e. 2 % w/v PEG-6000). Interestingly, the apparent solubility for the formulation with a 10 % rise in L-arginine HCl and sucrose, was lower than formulation 2, which had similar excipient levels. The only difference between these formulations was the pH, where formulation 2 had a pH of 6.6, resulting from a higher ratio of histidine base to histidine HCl. However,

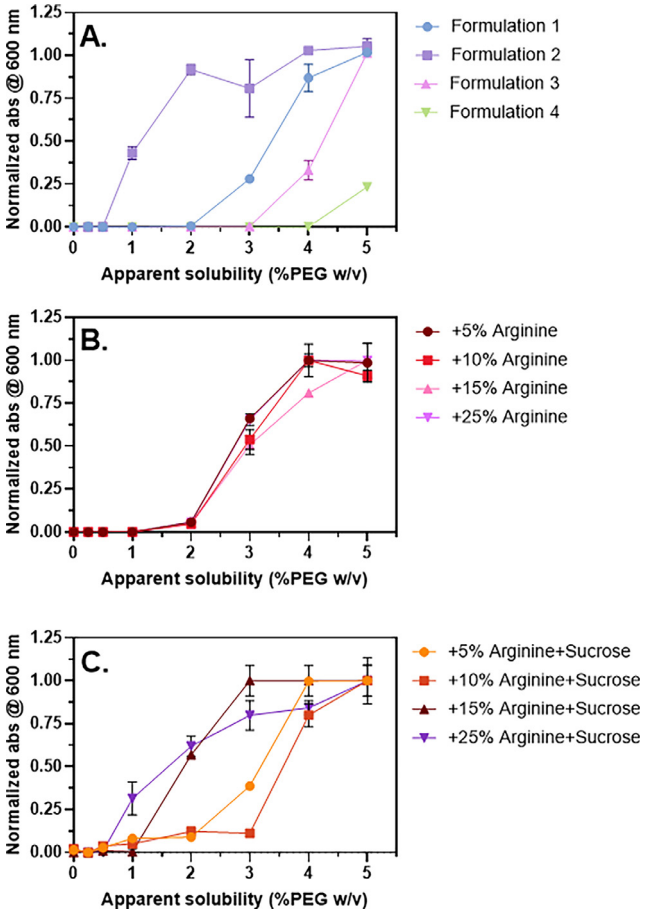

Fig. 7. Forced liquid-liquid phase separation (LLPS) with PEG-6000 quantified by absorbance and normalized to the highest value = 1. Data represented as mean ± standard deviation, n = 3.

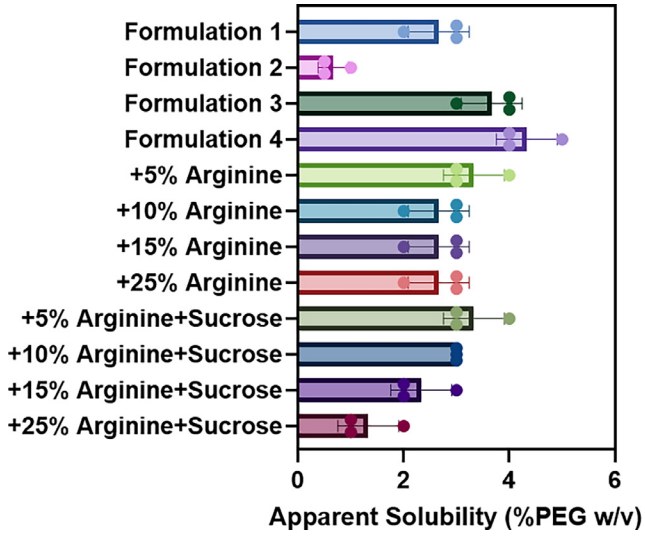

Fig. 8. Forced liquid-liquid phase separation with PEG-6000 with different formulation variations. Apparent solubility based on concentration of PEG-6000 that did not precipitate mAb. Data represented as mean ± standard deviation, n = 3.

maintaining an approximate pH of 5.8, in coordination with formulation 1 and further increasing the L-arginine HCl and sucrose by 15 % and 25 % resulted in increased apparent solubility scores of 1 % w/v PEG-6000 and 0.5 % w/v PEG-6000, respectively. These findings suggest that the interplay between pH, L-arginine HCl, and sucrose is critical to the formulation's propensity for LLPS. The apparent solubility scores for all formulations are comprehensively depicted in Fig. 8. emphasizing the importance of optimizing excipient levels and having process controls during formulation development to balance stability and mitigate LLPS risk.

Through the identification of the lowest concentration of PEG-6000 that did not cause precipitation of the mAb formulation, the apparent solubility was determined as a surrogate for the potential of LLPS.[50] Previously, L-arginine HCl at 200 mM has been utilized as an excipient to reduce the potential LLPS.[17] Contrary, our findings reveal that in a formulation with 55 mM L-arginine and 55 g/L sucrose with pH 6.6, LLPS occurs where the apparent solubility was 0.5 % w/v PEG-6000. When the L-arginine HCl and sucrose concentration were reduced by 10 % and the pH reduced by 0.6 units, the potential of LLPS was considerably less with the formulation having an apparent solubility of 2 % w/v PEG-6000. Moreover, maintaining a pH of 5.8 and increasing the L-arginine HCL and sucrose content by +25 %, further resulted in a reduced apparent solubility of 1 % w/v PEG-6000, suggesting propensity of LLPS. The increased potential for LLPS is predicted to be related to the increased ionic components from increased excipient levels and pH.

To further validate the forced LLPS experiment with PEG-6000, the study was replicated with larger sample volumes to meticulously assess the monoclonal antibody post-LLPS induction. A standardized concentration of 2.5 % w/v PEG-6000 (slightly exceeding the apparent solubility for most formulations) was incubated with the various formulations and kept at 2–8 °C prior to centrifugation at the same temperature 2–8 °C. Subsequently, the supernatant was collected and analyzed for monoclonal antibody concentrations. As depicted in Fig. 9, a notable change in the supernatant concentration was observed exclusively with Formulation 2, indicative of LLPS post-PEG-6000 addition. In contrast, the monoclonal antibody concentration in the supernatant remained consistent with and without PEG-6000 across all other formulations, suggesting the absence phase separation or settling of the monoclonal antibody in solution at cold temperature of 2–8 °C.

PEG-600 (samples represented as mean ± standard deviation, n = 3)

Prior studies have employed solution-state Nuclear Magnetic Resonance (NMR) spectroscopy to predict the propensity of mAb to undergo phase separation.[51] This advanced, specialized technique necessitates expensive equipment and specialized expertise to conduct experiments and analyze heightened characterization data analysis, making it impractical for high-throughput applications. Conversely, the forced precipitation of proteins via incubation with PEG and subsequent determination of apparent solubility score have been traditionally utilized to assess protein stability and formulation suitability. Herein, we present the use of forced precipitation with PEG-6000 as a predictor for LLPS occurring in mAb formulations. This method offers a practical alternative to NMR, providing a robust high throughput plate based, cost-effective means to monitor excipient levels to prevent LLPS during formulation development.

Liquid-liquid phase separation as a function of osmolality

The observed correlation between increased concentrations of L-arginine HCl and sucrose in the formulation is a likely a contributing factor to the observed LLPS. To establish a quantitative relationship between the ionic strength and LLPS occurrance, the osmolality of the different formulations was theoretically calculated and plotted against the apparent solubility of the formulation in Fig. 10. A linear regression analysis revealed a significant negative correlation was found between the osmolality of the formulations compared to their apparent solubility calculated from the PEG-6000 assay, with an R^2

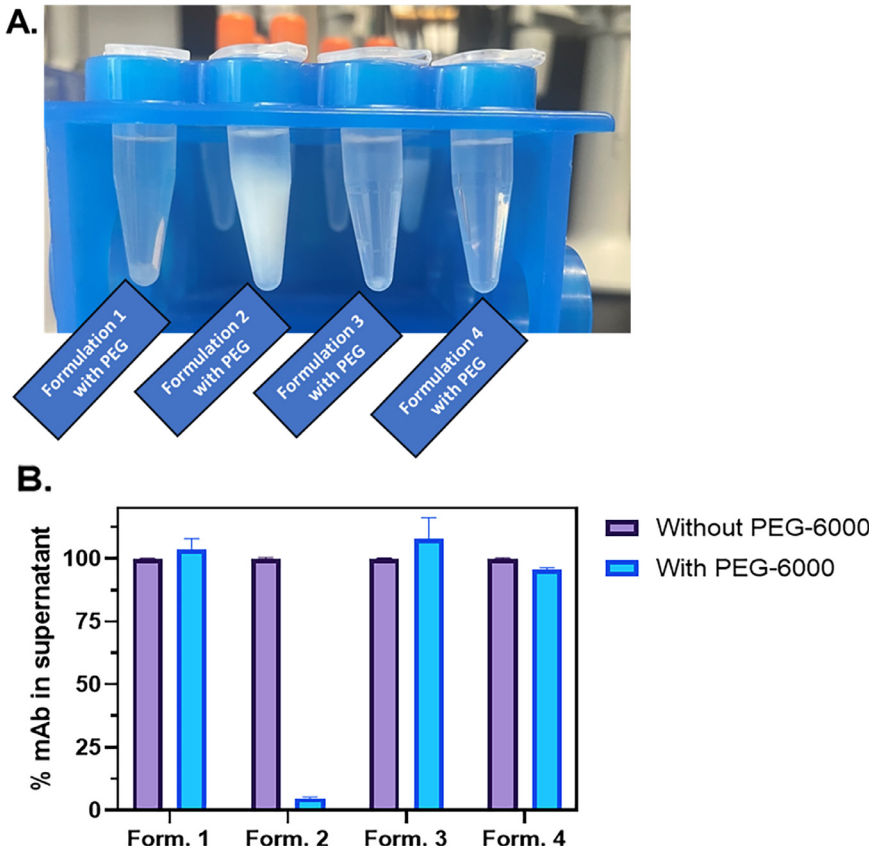

Fig. 9. Monoclonal antibody concentrations in the supernatant with and without PEG-6000 incubation, (A) representative image of formulations incubated with PEG-6000 post centrifugation and (B). %mAb in supernatant, post centrifugation, with and without PEG-600 (samples represented as mean ± standard deviation, n = 3).

value of 0.7365. This correlation indicates the higher osmolality is associated with reduced solubility, thereby enhancing the propensity of LLPS occurring for this IgG1 mAb. Elevated osmolality presents a critical challenge in the administration of high concentration mAb products due to risk of hemolysis and injection site pain and discomfort.[52] The US Pharmacopeia recommends an osmolality range of 285 ~ 310 mOsmol/kg for parentral formulations to mitigate risks.[53] Although only theoretical osmolality values were considered, formulations exhibiting higher LLPS potential (e.g., those containing 55 mM L-arginine HCl and 55 g/L sucrose) slightly exceeded this recommended osmolality range, raising additional concerns during drug product development.

Therefore, while isotonicity is conventionally prioritized for patient safety, our findings underscore the importance of maintaining isotonic conditions not only for patient comfort but also for ensuring the stability and quality of the protein drug product. This dual consideration is particularly pertinent for formulations susceptible to LLPS.

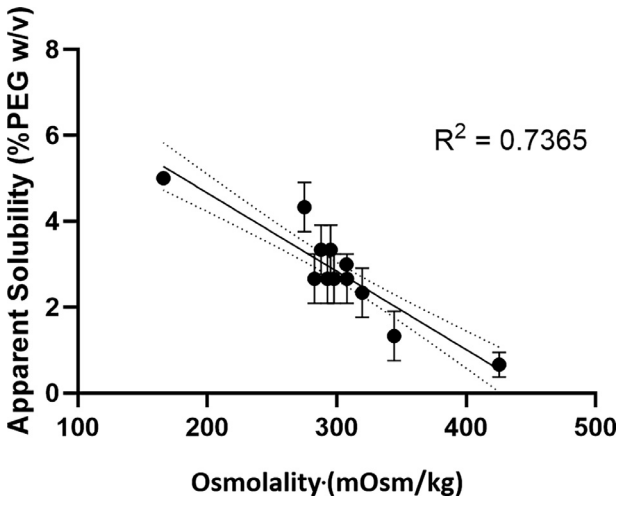

Fig. 10. The theoretical osmolality of formulations plotted against apparent solubility.

Conclusion

In summary, a comprehensive evaluation using molecular and biophysical analyses was conducted to investigate liquid-liquid phase separation (LLPS) in a series of IgG1 formulations. While limited differences were observed between molecular and most biophysical assessments, a plate-based assay incorporating PEG-6000 was employed to induce and predict LLPS in the monoclonal antibody formulations. Notably, Formulation 2, which contained 55 mM L-arginine HCl and 55 g/L sucrose at a pH of 6.6, exhibited LLPS and had the lowest apparent solubility score with PEG-6000, demonstrating a significant difference in monoclonal antibody concentration between the two phases; minimal mAb was detected in the supernatant. This observation indicates that LLPS results in two distinct phases, one being protein-rich and the other not.

The study also revealed that Formulation 1, when maintained at a constant pH of 5.8 and subjected to increased levels of L-arginine HCl and sucrose (+25 % excipient levels), exhibited LLPS due to a similar apparent solubility score as Formulation 2. Formulation development of high concentration mAbs necessitates optimizing ionic content and excipient concentrations during drug product development for robust, stable, and quality drug products. These insights are pivotal,

particularly when considering prior knowledge regarding IgG structure, glycosylation patterns, and drug product concentrations, to design robust drug product formulations.

Conflict of interest

No conflict of interest to be reported.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.xphs.2025.103804.

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