European Journal of Pharmaceutics and Biopharmaceutics 120 (2017) 1–8

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Elucidating the weak protein-protein interaction mechanisms behind the liquid-liquid phase separation of a mAb solution by different types of additives

Guoliang Wu [a], Shujing Wang [a], Zhou Tian [a], Ning Zhang [b], Han Sheng [b], Weiguo Dai [c], Feng Qian [a,*]

[a] School of Pharmaceutical Sciences and Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Tsinghua University, Beijing 100084, China
[b] China R&D and Scientific Affair, Shanghai Discovery Center, Janssen Research & Development, Johnson & Johnson, Shanghai 200030, China
[c] Janssen Research & Development, Johnson & Johnson, Malvern, PA 19355, USA

ARTICLE INFO

Article history:
Received 8 May 2017
Revised 9 July 2017
Accepted in revised form 24 July 2017
Available online 25 July 2017

Keywords:
Monoclonal antibody
Liquid-liquid phase separation
Interaction parameter
Phase diagram
High concentration of protein
Protein stability

ABSTRACT

Liquid-liquid phase separation (LLPS) has long been observed during the physical stability investigation of therapeutic protein formulations. The buffer conditions and the presence of various excipients are thought to play important roles in the formulation development of monoclonal antibodies (mAbs). In this study, the effects of several small-molecule excipients (histidine, alanine, glycine, sodium phosphate, sodium chloride, sorbitol and sucrose) with diverse physical-chemical properties on LLPS of a model IgG1 (JM2) solutions were investigated by multiple techniques, including UV–vis spectroscopy, circular dichroism, differential scanning calorimetry/fluorimetry, size exclusion chromatography and dynamic light scattering. The LLPS of JM2 was confirmed to be a thermodynamic equilibrium process with no structural changes or irreversible aggregation of proteins. Phase diagrams of various JM2 formulations were constructed, suggesting that the phase behavior of JM2 was dependent on the solution pH, ionic strength and the presence of other excipients such as glycine, alanine, sorbitol and sucrose. Furthermore, we demonstrated that for this mAb, the interaction parameter (k_D) determined at low protein concentration appeared to be a good predictor for the occurrence of LLPS at high concentration.

1. Introduction

In recent years, monoclonal antibodies (mAbs) have been widely used to treat various human diseases including cancer and autoimmune disorders because of their excellent target specificity and safety profile [1–3]. As large molecules with complex structure, the administration of mAbs confronts an array of challenges in formulation and delivery [4]. Although the high daily doses (100–200 mg) make intravenous infusion the easiest administration route, subcutaneous injection is more convenient for patients and therefore more desirable [5–7], the high-concentration (>100 mg/mL) mAb solution necessitated by low injection volume (<1.5 mL) presents many challenges in characterization, manufacturing, long-term stability over the shelf-life and ease of injection to patients [8]. At high concentrations, the increased chance of molecular interactions results in various formulation challenges such as high viscosity, protein aggregation, excessive opalescence and increased tendency of solution phase separation [9,10].

Liquid-liquid phase separation (LLPS), a widely observed metastable phenomenon in protein solutions, is one of such formulation challenges for mAb drug development [11–14]. It is usually induced by noncovalent interactions between protein molecules at low temperature, without formation of any irreversible precipitates [11]. During LLPS, two definite phases, a heavy phase with a higher protein concentration and a light phase with a lower protein concentration, are formed. This phase separation is governed by the thermodynamic equilibrium between protein-protein and protein-water interactions in a specific solution condition. The progression and final equilibrium of LLPS are dictated by conditions including temperature, pH, ionic strength, buffering agents, the species and concentrations of other excipients [11]. LLPS could induce several formulation challenges, including reduced aesthetic appeal of protein solution, increased protein aggregation potential in the heavy phase, decreased physical stability due to the shift of pH or ionic strength in the two protein phases [12]. Despite all challenges mentioned above, LLPS could also be exploited as a time-saving and cost-effective alternative in protein purification

* Corresponding author.
E-mail address: qianfeng@biomed.tsinghua.edu.cn (F. Qian).

http://dx.doi.org/10.1016/j.ejpb.2017.07.012
0939-6411/

and concentration, along with other sophisticated and expensive techniques such as ultrafiltration, freeze-drying, and chromatography [11,15].

LLPS is a metastable state of protein solution, where proteins aggregate to form a heavy phase due to intermolecular attractions. These attractions could be strengthened or suppressed by changing the formulation environment, such as adjusting pH or adding various excipients. Many excipients of diverse physical-chemical properties have been adopted in protein solution formulations, such as citrate and phosphate as buffering agents, polyols/saccharides as lyo-protectants, amino acids and polymers as viscosity-lowering agents, and salts or sugars as osmo-regulators [16–18]. All these effects could be realized through multiple mechanisms, such as preferential hydration, electrostatic interactions, dispersive forces and hydrogen bonds formation [16,19].

In this article, we studied the LLPS phenomenon of a mAb, previously designated as JM2 [17], in various solution conditions. In order to understand the interaction mechanisms behind LLPS, a series of excipients were used: (1) histidine-hydrochloride (HisHCl) and phosphate (NaH_2PO_4/Na_2HPO_4) as the buffering agents, to study the influence of pH; (2) sodium chloride (NaCl) together with the buffering agents, to study the effect of ionic strength on the process of LLPS; (3) hydrophobic-hydrophilic amino acids, glycine (Gly) and alanine (Ala), to study the possible hydrophobic interactions; and (4) two commonly used saccharides, sorbitol (Sor) and sucrose (Suc), to study other possible non-ionic interactions. The relationship between the interaction parameter (k_D) measured at low protein concentrations and phase behaviors at high protein concentrations was also investigated.

2. Materials and methods

2.1. Materials

JM2, an IgG1-type mAb, was kindly provided as solution by Janssen Research & Development (Johnson & Johnson, Shanghai, China) [17]. All buffer reagents and chemicals used were of the analytical grade and purchased from vendors as follows: L-histidine, Tris, D-sorbitol and sucrose (AMRESCO, Houston, USA). L-glycine (Solarbio, Beijing, China). L-alanine (Sigma-Aldrich, St. Louis, USA). Sodium dihydrogen phosphate and disodium hydrogen phosphate (Xilong Chemical, Guangdong, China). Sodium chloride, sodium hydroxide and hydrochloric acid (Sinopharm, Shanghai, China).

2.2. Methods

2.2.1. Solution preparation

All buffers were prepared with Milli-Q water and filtered through 0.2 μm nitrocellulose membranes before usage (Millex, Merck Millipore Ltd., Ireland). The pH of solution was measured by a pH meter (Mettler Toledo FiveEasy plus) and adjusted to the target value (within the deviation of ±0.05). Buffer exchange of JM2 solutions was realized with dialysis using the dialysis membrane with molecular weight cutoff of 14 kDa (Union Carbide Co., U.S.A.) or ultracentrifugation using centrifugal filter units with the molecular weight cutoff of 30 kDa (Amicon Ultra, Merck Millipore, Germany).

2.2.2. Protein concentration measurement

The concentration of JM2 in solution was determined with a UV–*vis* spectrophotometer (UV-4802, UNICO, Shanghai, China). The UV absorbance at 280 nm (UV_{280}) of dilute JM2 solutions were measured and subtracted by the buffer blank. The JM2 concentration was calculated with its respective extinction coefficient and the corresponding dilution factor.

2.2.3. Protein conformation characterization by circular dichroism (CD)

CD measurements were conducted using a Chirascan plus CD spectrometer (Applied Photophysics, UK). JM2 samples were prepared in 20 mM phosphate buffer at pH 6.7, and loaded in 0.1 mm path-length quartz cuvettes for CD experiments at 25 °C. Far-UV spectra were recorded from 260 nm to 200 nm for samples at 0.3 mg/mL, and near-UV CD were recorded from 340 nm to 260 nm for samples at 10 mg/mL. The instrument was set with a scan rate of 50 nm/ min, a 0.5 nm bandwidth and 1 sec integration time. All samples were recorded three times. The spectra were averaged and smoothened using the Chirascan software.

2.2.4. Protein thermal-stability evaluation by differential scanning calorimetry (DSC)

DSC measurements were conducted using a Manual Microcal VP-Capillary DSC system (GE Healthcare, U.S.A.). The sample at 1 mg/mL was filled into the tantalum cells with an active volume of 130 μL, using the corresponding buffer as reference. The thermogram was obtained by scanning from 25 °C to 100 °C at the temperature-increasing rate of 180 °C/hr. The data were analyzed with the VP-Viewer software. With buffer scans subtracted, the midpoint of the thermal transition temperature (T_m) was determined by fitting the curve with a non-2-state model [17].

2.2.5. Tm and hydrodynamic diameter characterization with Unchained Labs (DSF and DLS)

The general stabilities of JM2, including unfolding and aggregation, were evaluated with an all-in-one UNcle stability platform (Unchained Labs, Norton, MA). Differential scanning fluorimetry (DSF) and dynamic light scattering (DLS) were used to monitor the evolution of protein structure and size distribution upon temperature increasing at a rate of 2 °C/min from 20 °C to 95 °C. The label-free fluorescence from intrinsic aromatic amino acid residues excited by the inset excitation wavelength of 266 nm was collected for melting temperature (T_m) calculation. In addition, the size distribution of the same set of samples was simultaneously determined by a DLS module at 660 nm before and after the heating program. Totally 9 μL of JM2 at 1 mg/mL in 20 mM HisHCl buffer at pH 6.7 was loaded into the sample well for DLS and intrinsic fluorescence test with duplicates.

2.2.6. Colloidal stability evaluation by high-performance liquid chromatography-size exclusion column (HPLC-SEC)

HPLC-SEC was conducted using a Shimadzu LC-20AT HPLC system (Kyoto, Japan) with a Tosoh TSKgel BioAssist G3000SWxl column (7.8 mm × 300 mm) and a TSKgel guard column SwXL (6.0 mm × 40 mm). Each sample of 20 μg was loaded and isocratically eluted at a flow rate of 0.7 mL/min in the mobile phase of 200 mM phosphate buffer at pH 6.0. The protein concentration was determined with UV_{280}. Area under the curve of peaks in the chromatogram was used to quantify the protein monomer and aggregates.

2.2.7. Construction of JM2-water phase diagrams

The JM2-water phase diagrams under different solution conditions were regarded as functions of the protein concentration. JM2 solutions were incubated at 1, 4, 8, 12, 16, 20, 25 and 30 °C for 48 h to induce LLPS. For phase separations that occurred at temperature above 25 °C, the concentration of the heavy phase could not be accurately determined due to the quick formation of gels. Thus the liquid-liquid coexistence curves were only plotted in the temperature range of 1–25 °C. Buffers conditions tested here