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Liquid-liquid phase separation of a monoclonal antibody at low ionic strength: Influence of anion charge and concentration

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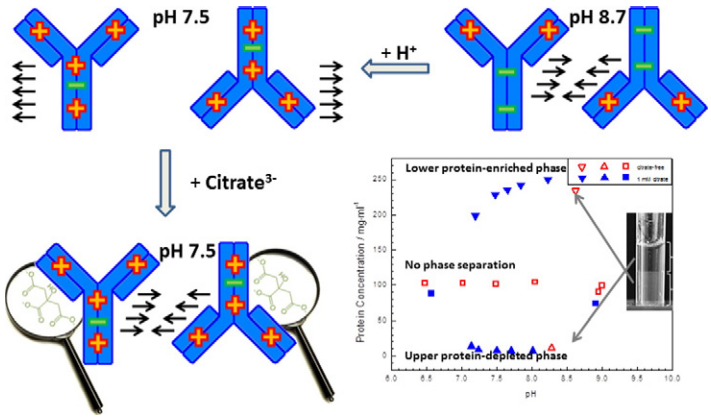
HIGHLIGHTS

- Antibody solution undergoes liquid-liquid phase separation (LLPS) at the isoelectric point.
- Citrate and oligovalent anions induce LLPS at pH below the isoelectric point.
- Citrate is assumed to bind to the antibody resulting in charge neutralization.
- Citrate-antibody binding was complementarily proven by using buffer equilibration.

GRAPHICAL ABSTRACT



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ABSTRACT

Liquid-liquid phase separation (LLPS) of a monoclonal antibody solution was investigated at low ionic strength in the presence of oligovalent anions, such as citrate, trimellitate, pyromellitate and mellitate. Phase separation was observed at the isoelectric point of the antibody at pH 8.7 as well as in more acidic pH regions in the presence of the tested oligovalent ions. This can be attributed to charge neutralization via binding of the oligovalent anions to the positively charged antibody. The influence of the anion concentration on liquid-liquid phase separation with respect to the net charge of the antibody was examined. Similarities to the formation of a complex coacervate were shown to apply. These findings enable us to understand the usage of excipients to rationally induce or avoid liquid-liquid phase separation at low ionic strength. Furthermore we present a method to directly examine the competition of different ions for the solvation shell, called buffer equilibration.

1. Introduction

1.1. Relevance of liquid-liquid phase separation (LLPS)

Liquid-liquid phase separation (LLPS) due to reversible self-association of antibody molecules is a complex phenomenon already

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observed by several groups [1–8]. In this work, we will focus on solution conditions that induce LLPS at low ionic strength (<150 mM) in the absence of polyethylene glycol (PEG) or other molecular crowding substances. Phase separation observed in the presence of high salt concentrations in the molar range or PEG should be regarded separately as these additives induce phase separation via a different mechanism, namely the competition for water of hydration, or salting-out [7,9,10].

The importance of LLPS in biotechnology and production ranges from unwanted phase separation during antibody production and storage to a systematic application as membrane-free concentration technique or column-free approaches for protein purification [11,12]. In detail, the purification process ends after the last process step, namely the ultra-/diafiltration step with the aim of conditioning the protein into a specific formulation and/or increase protein concentration. This solution is called bulk drug substance and is often stored in large stainless steel tanks. These tanks are then stored up to few days at 2–8 °C prior to the fill and finish process. During this storage time the potential formation of an LLPS may induce a protein concentration gradient in the tank. It is avoided to mix the protein solution in the tank in order to reduce potential protein aggregation. As a consequence of the protein gradient in the tank, fill and finish into e.g. pharmaceutical vials, induce a batch inhomogeneity with vials having different protein concentrations. This has strictly to be avoided in order to meet batch consistency.

Our work deals with factors associated with the phenomenon of LLPS (lower temperature, pH close to the isoelectric point (pI), higher valency of counterions and lower salt concentration) and with solution properties such as high turbidity, which precedes LLPS. The induction of LLPS is related to specific medical causes. For example, certain immunoproliferative and autoimmune diseases such as cryoglobulinemia are associated to reversible precipitation of immunoglobulins upon cooling below 37 °C [13,14]. Some types of cataract are associated to opacity of crystallins, a protein class which is performing liquid-liquid phase separation [15–18]. In certain cases, LLPS is metastable with respect to crystal formation [19–28]. For such cases the exact position of the phase boundary in the phase diagram (excipient concentration vs. protein concentration) can be used to predict and optimize conditions for protein crystallization at specific environmental conditions [28].

1.2. Solution conditions that favor LLPS

LLPS occurs spontaneously upon changing the solution conditions such as temperature, pressure [29], pH, the presence of cosolutes or their concentration. Thereby a homogeneous protein solution separates into a lower phase with high protein concentration and high density and an upper phase with low protein concentration and lower density. Points on the phase boundary, called coexistence curve, describe the concentrations of the two coexisting protein solutions. At temperatures above the maximum of the coexistence curve, termed upper critical point or upper critical solution temperature (UCST), no phase separation takes place and a single phase is formed. The protein concentration at the critical temperature is termed critical concentration. In currently published studies, the critical concentration is similar for most investigated antibodies (approximately 90 mg/ml) [2,3,8] whereas the critical temperature strongly depends on the unique antibody and the solution composition. Published data of the critical temperature of monoclonal antibodies vary from −3 °C to ≥25 °C [2,3,8].

Even though not yet systematically summarized, it has been shown in several studies that LLPS often occurs at conditions close to the pI [1–6,30]. To our knowledge, up to now just two studies have been published in which LLPS in antibody solutions was also observed more than one pH unit distant from pI at low ionic strength and in the absence of polyethylene glycol [3,31]. In general, LLPS has been shown to be more pronounced at lower temperature (upper critical solution temperature: UCST phase behavior) and lower ionic strength [2–5,7,8,14]. A strong correlation between LLPS and opalescence of antibody solutions has been found, probably due to attractive protein-protein interactions which form the basis for both phenomena [2–5].

1.3. Definition and properties of complex coacervates

A well-studied sub-type of LLPS is complex coacervation. Complex coacervation was first mentioned by Tiebackx in 1911, naming a liquid-liquid phase separation as a result of electrical interaction between opposite charges on polyelectrolytes. Later, in 1938 Bungenberg de Jong studied the phase behavior of protein solutions [32]. Complex coacervation was observed for isolabile globular proteins at the isoelectric point and for biocolloids that interact with oppositely charged ions, such as ferrycyanide or picrate [32]. These days, complex coacervation is defined as a spontaneous liquid-liquid phase separation that occurs in solution of oppositely charged macromolecules, including both polyelectrolytes and charged colloidal particles [12,33].

By current definition, the formation of LLPS by an antibody (charged colloidal particle) interacting with oligovalent anions as shown in Fig. 1 is not explicitly a complex coacervate. However, in the following work we want to point out the large number of similarities between complex coacervates and LLPS observed here.

Complex coacervates have some properties in common: An optimum mixing ratio between the oppositely charged molecules exists, at which the formed complex is electrically neutral and phase separation is most pronounced [12,32,34–38]. The presence of salt has a dissociating effect on the complex of oppositely charged molecules and coacervation is weakened or even suppressed [32,35,38,39]. Finally, the charge density of the interacting partners has an influence on the physical appearance of the complex. Weak neutralizing interactions between the involved macromolecules result in liquid-liquid phase separation. When the interacting partners are more densely charged, liquid-solid phase separation (LSPS) i.e. precipitation might occur [35,40].

Another concept that describes the effect of concentration dependent citrate-induced interactions with antibodies is the concept of LLPS of effective particles [41,42]. Effective protein-protein interactions depend strongly on the presence of other additives in solution, such as salts, buffers, sugars etc. If an additive interacts with the protein, the formed particle is called “effective particle” [42]. By changing the solvent composition, effective protein-protein interactions are modified and the phase behavior can thereby be adjusted [42].

1.4. Mapping the ion atmosphere

Binding between anions and monoclonal antibodies (positively charged below pI) has an impact on intermolecular antibody-antibody interactions [43–46]. In these studies, anion binding was assumed due to differences in the electrophoretic mobility of the antibody in different ion environments. In the following work, we present a direct quantitative approach to measure the competition of different ions for the composition of the ion atmosphere. The ion atmosphere, i.e. the mobile sheath of ions surrounding the protein, has no discrete structure and interactions between ions and proteins follow complex thermodynamic behavior, which cannot be described by simple binding isotherms [47,48]. Therefore Bai and co-workers [47] suggest the determination of a competition constant of competing ions rather than a dissociation constant. We adopted the method of Bai et al. [47], originally developed for DNA, to antibodies by measuring the anion content via ion chromatography subsequent to buffer equilibration (BE IC). Our results confirm that citrate ions preferentially bind to the protein compared to chloride ions.

2. Materials and methods

2.1. Analytics

The antibody concentration was determined by UV absorbance at 280 nm (Perkin Elmer UV/Vis Spectrometer, Lambda35) in plastic