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Droplets-based millifluidic for the rapid determination of biopolymers phase diagrams

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ABSTRACT

Liquid-liquid phase separation is a phenomenon occurring spontaneously upon changing the physico-chemical conditions of (bio-)polymers mixtures such as pH, ionic strength, total concentration, mixing ratio or temperature. The establishment of a phase diagram is a useful approach to highlight interactions and phase separation conditions of a mixture. Such approach generally performed in bulk is highly time and raw material consuming. In the present work, we developed a droplets-based millifluidic device for rapid phase diagram determination of liquid-liquid phase separated system. The proof of concept was first performed using a colloidal suspension of titanium dioxide (TiO_2). The developed millifluidic device was then applied to a model protein-polysaccharide mixture made of β-lactoglobulin (BLG) and Gum Arabic (GA) in water and at pH 4.2. In this model, a specific type of liquid-liquid phase separation occurs: complex coacervation. Biopolymers droplets of about 2.5 μL of various compositions were generated to investigate the impact of total biopolymers concentration (0.1–5 wt %) and protein-polysaccharide mixing ratio (1:8 to 8:1). Initiation and suppression of phase separation were then evidenced through turbidity measurements within the droplets using image analysis. High similarities between cloud points probed using the millifluidic device and the binodal curve determined in bulk were found in the total biopolymers concentration range explored. Droplets-based millifluidic represents an efficient and highly versatile tool to probe liquid-liquid phase separation of various biopolymers mixtures.

1. Introduction

Liquid-liquid phase separation is a universal phenomenon occurring spontaneously in nature. For example, mussels (*Mytilus edulis*) and sandcastle worms (*Phragmatopoma California*), two sessile marine organisms, are able to secrete glue displaying strong underwater adhesives properties. The key step in this adhesion mechanism is a liquid-liquid phase separation between several oppositely charged proteins (Waite, 1987; Waite, Andersen, Jewhurst, & Sun, 2005; Zhao, Sun, Stewart, & Waite, 2005). Also, growing evidence suggests that liquid-liquid phase separation underlies cellular compartmentalization (Hyman & Brangwynne, 2011; Hyman & Simons, 2012; Hyman, Weber, & Jülicher, 2014; Keating, 2012). From a technological point of view, liquid-liquid phase separation is commonly used as a process for protein separation (Wang, Gao, & Dubin, 1996) or for microencapsulation of oil or active ingredients (Burgess & Ponsart, 1998; Weinbreck, Minor, & De Kruif, 2004) with potential applications in cosmetics, pharmaceutics or food. For these applications, one sub-type of liquid-liquid phase separation, known as complex coacervation (Bungenberg de Jong & Kruyt, 1929), have received intense research effort to uncover the associated theoretical aspects and mechanisms (Kizilay, Kayitmazer, & Dubin, 2011; Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998; Turgeon, Beaulieu, Schmitt, & Sanchez, 2003). Few years ago, a new type of coacervation has been evidenced on protein-protein mixtures (Nigen, Croguennec, Renard, & Bouhallab, 2007). Recent advances in this new field were reviewed by Croguennec, Tavares, and Bouhallab (2017). Complex coacervation is mainly driven by electrostatic interactions between two oppositely charged molecules. Generally, it results in a two phase system: one biopolymers-rich phase named coacervate phase and one very dilute phase, named the diluted or equilibrium phase.

Liquid-liquid phase separation of (bio-)polymers is driven by the variation of the Gibbs free energy. This free energy that governs interactions is known to be strongly impacted by several physico-chemical parameters such as pH, ionic strength, concentration

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and temperature (Harnsilawat, Pongsawatmanit, & McClements, 2006; Mekhloufi, Sanchez, Renard, Guillemin, & Hardy, 2005; Nigen et al., 2007; Weinbreck, De Vries, Schrooyen, & De Kruif, 2003). Under specific conditions, a phase separated systems minimize the free energy and is therefore more thermodynamically favourable compare to a one phase system. Understanding and highlighting the specific interactions and/or phase separation conditions is therefore still a challenge that usually goes through the establishment of a phase diagram. Phase diagram possesses two main regions: the monophasic region for which (bio-)polymers coexist in one phase and the biphasic region, where macroscopic phase separation occurs. These two regions are separated by an equilibrium line, also known as binodal curve, which is determined by the biopolymers composition of the two phases at equilibrium. Cloud point determination, based on turbidity measurements in a non-equilibrium state, is also classically used to evidence the biphasic area (Thomson, Schurtenberger, Thurston, & Benedek, 1987). Such studies, usually performed in bulk, are highly time consuming and require large quantities of raw materials, not always available especially when working with purified or recombinant proteins of biomedical interest. To overcome these major drawbacks, alternative strategies recently emerged taking advantage of system miniaturization.

Microfluidics has become over the past few years a tool of interest, which is suitable for liquid-liquid phase behaviour investigation (Leng, Joanicot, & Ajdari, 2007; Selimović, Gobeaux, & Fraden, 2010; Shim et al., 2007; Silva et al., 2014). It is an attractive tool that can be coupled to many analytical techniques but such devices are based on expensive microfabrication technologies which require specific equipment and expertise. For phase diagram determination, another approach, as relevant as microfluidics, is known as millifuidics. Millifluidics consists of an assembly of low price and commercially available tubings of about millimetre-sized and chromatography connectors. It is easy to implement and does not require specific equipment except syringe pumps. The emergence of millifluidic is relatively new. To the best of our knowledge the first millifluidic device which was referred as “simplified microfluidic device” was developed by Quevedo, Steinbacher, and McQuade (2005). It offers a great versatility and the fast creation of modular set-up. It is an efficient tool to investigate polymerization reactions (Lorber, Pavageau, & Mignard, 2010a, 2010b) or to create microparticles of controlled and tuneable size and shape (Engl, Tachibana, Panizza, & Backov, 2007; Lukyanova et al., 2013; Martins, Poncelet, Marquis, Davy, & Renard, 2017; Schmit, Courbin, Marquis, Renard, & Panizza, 2014; Tadmouri et al., 2012).

In this study, we developed a low material consuming droplets-based millifluidic device for rapid phase diagram establishment of biopolymers mixtures. The proof of concept was first performed using colloidal suspensions of titanium dioxide (TiO_2). β-lactoglobulin (BLG)/Gum Arabic (GA) mixture was chosen to validate the approach as it is a case-study of complex coacervation (Mekhloufi et al., 2005; Sanchez, Mekhloufi, & Renard, 2006; Sanchez et al., 2002; Schmitt et al., 2000; Schmitt, Sanchez, Thomas, & Hardy, 1999).

2. Material and methods

2.1. Material

2.1.1. β-Lactoglobulin (BLG) powder

Bovine β-Lactoglobulin (BLG) powder (Batch number JE 001-8-415) was obtained from Davisco Food International (USA). The protein content was of 97% on a dry basis, using the Dumas Method (Bremner & Mulvaney, 1982, pp. 595–624) with a corrective factor of 6.25. Ash content, obtained by thermogravimetric analysis under nitrogen atmosphere, was equal to 1.5%. The molecular mass was determined by ESI mass spectroscopy (SYNAPT, Waters, France). Two major species corresponding to the variant A of $M_w = 18\,277$ Da and the variant B of $M_w = 18\,363$ Da were detected. BLG powder was stored under vacuum at 4 °C until use. The presence of BLG aggregates in the initial stock dispersions was reported to significantly affect coacervation phenomenon (Schmitt et al., 2000). Prior use, the non-native protein was removed from the powder as follows (Jung, Savin, Pouzot, Schmitt, & Mezzenga, 2008): the protein was dispersed overnight at 10 wt % in Milli-Q water under magnetic stirring, and adjusted to pH 4.6 using 1 M HCl solution to precipitate non-native forms of BLG. The resulted dispersion was centrifuged at 15 000 g (KR25i, Jouan, France) at 20 °C for 15 min. The supernatant was then adjusted to pH 4.2 using 1 M HCl solution before freeze-drying. The resulting BLG powder was stored under vacuum at 4 °C until use, and placed at 20 °C in a desiccator containing $MgCl_2 \cdot 6H_20$ saturated salt during whole time of experiments.

2.1.2. Gum Arabic (GA) powder

Gum Arabic (GA) powder from Acacia Senegal trees (batch number 97J716) was provided by Nexira (France). High performance size exclusion chromatography combined to multi angle laser light scattering detector (HPSEC-MALLS, WYATT, France) gave an average molecular weight (M_w) of 648 900 Da. The polydispersity index value was 2.27. The sugar composition, obtained by the alditol acetates method as described by Lopez-Torrez, Nigen, Williams, Doco, and Sanchez (2015), was as follows: 38.8% Galactose, 29.2% Arabinose, 18.9% Glucuronic acid, 9.9% Rhamnose, 1.6% 4-O-Me glucuronic acid, and 0.3% Mannose.

2.1.3. Chemicals

Titanium dioxide (TiO_2) (Sepic, France) was used for the implementation of the millifluidic device. Hydrochloric acid (HCl), sodium hydroxide (NaOH) and sunflower oil were supplied from Sigma. Milli-Q water was used in all sample preparations.

2.2. Biopolymers stocks dispersions

2.2.1. BLG dispersions

Dispersions of BLG at 6.2 wt % were prepared at room temperature. Freeze-dried BLG powder was first solubilized overnight in Milli-Q water under magnetic stirring. The resulting dispersion was adjusted to pH 4.2 with 1 M HCl before being filtered on a 0.2 μm cellulose acetate membrane (Sartorius, France). The protein concentration was determined by UV absorption spectrophotometry at a wavelength of 278 nm using $\varepsilon_{BLG} = 0.96$ L g^{-1} cm^{-1} as extinction coefficient (Townend, Winterbottom, & Timasheff, 1960). By dilution in Milli-Q water, a range of BLG stocks dispersions from 0.1 wt % to 5 wt % was obtained. pH was readjusted to 4.2 if required.

2.2.2. GA dispersions

GA dispersions at 6.5 wt % were prepared overnight at room temperature in Milli-Q water, using magnetic stirrer. pH was adjusted to 4.2 using 1 M NaOH and residual insoluble part was removed by centrifugation (15 min, 15 000 g, 20 °C). The supernatant was filtered on a 0.2 μm acetate cellulose membrane and polysaccharide concentration was determined by dry matter analysis. A range of GA concentrations from 0.1 wt % to 5 wt % was prepared by dilution in Milli-Q water. pH was readjusted to 4.2 if required.