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Research paper

Freezing and glass transitions upon cooling and warming and ice/freeze-concentration-solution morphology of emulsified aqueous citric acid

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ABSTRACT

Although freeze-induced phase separation and the ice/FCS (freeze-concentration solution) morphology of aqueous solutions play an important role in fields ranging from life sciences and biotechnology to geophysics and high-altitude ice clouds, their understanding is far from complete. Herein, using differential scanning calorimetry (DSC) and optical cryo-microscope (OC-M), we have studied the freezing and glass transition behavior and the ice/FCS morphology of emulsified 10–60 wt% CA (citric acid) solutions in the temperature region of ~308 and 153 K. We have obtained a lot of new result which are understandable and unclear. The most essential understandable results are as follows: (i) similar to bulk CA/H_2O, emulsified CA/H_2O also freezes upon cooling and warming and (ii) the ice/FCS morphology of frozen drops smaller than ~3–4 μm is less ramified than that of frozen bulk solutions. Unclear results, among others, are as follows: (i) in contrast to bulk solutions, which produce one freezing event, emulsified CA/H_2O produces two freezing events and (ii) in emulsions, drop concentration is not uniform. Our results demonstrate that DSC thermograms and OC-M images/movies are mutually supplementary and allow us to extract important information which cannot be gained when DSC and OC-M techniques are used alone.

1. Introduction

In aqueous solution, citric acid (CA) forms strong hydrogen bonds between CA molecules themselves and with H_2O [1–3]. The hydrogen bonds between CA molecules are responsible for the formation of CA-nanoclusters [4–9] whose size and population increase with concentration. As temperature decreases, the CA-nanoclusters greatly contribute to increasing viscosity because their translational and rotational mobility is much smaller than that of unbound CA and H_2O molecules. CA is widely used in food industry [10] and as a buffer for the lyophilization (freeze-drying) of biopharmaceutical formulations [11,12], because of a broad buffering range, minimal pH changes with temperature, and its resistance to crystallization under typical freeze-drying conditions [13]. It is also used for the preparation of multicomponent excipients with increased T_g in order to reduce protein denaturation in lyophilized products [14,15]. Emulsified formulations containing CA are used in biotechnology, biopharmaceutics, tissue engineering, etc. For example, emulsions are used for the preparation of oil-based formulations as drug delivery systems [16] and porous scaffolds needed in biopharmaceutics and tissue engineering [17–19]. Three-dimensional (3D) porous scaffolds for tissue engineering are prepared from lyophilized double water/oil/water (W/O/W) emulsions [20]. Rapidly frozen and lyophilized W/O emulsions are also used as transdermal delivery carrier as a solid-in-oil nano-suspension [21].

Lyophilization consists of three main steps: the freezing step, which takes place upon cooling of formulations, and the primary and secondary drying steps which are performed upon subsequent warming [22–27]. During freezing, formulations/solutions can separate into pure ice and FCS which freezes/crystalizes or transforms to glass upon further cooling. This freeze-induced phase separation (FIPS) produces a specific for each formulation ice/FCS morphology which controls (i) resistance to the vapor flow of sublimated ice during the primary drying, (ii) formation of the porous medium of a resulting cake, and (iii) desorption of residual moisture from the porous cake during the secondary drying. Thus ice/FCS morphology formed during freezing controls the duration of lyophilization and quality attributes of final lyophilized products [22–31].

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Formulations are usually multicomponent. The knowledge of the freezing and glass transition behavior and the ice/FCS morphology of aqueous components is useful for the preparation of multicomponent formulations with more or less predicted low-temperature behavior and ice/FCS morphology. We recently reported about the low-temperature behavior and ice/FCS morphology of bulk CA/H_2O and sucrose/H_2O [32,33]. The main factors that influence FIPS and, consequently, ice/FCS morphology of bulk *binary* formulations are the concentration and molecular structure of solutes. Whether the ice/FCS morphology of frozen emulsified formulations differs from that of bulk formulations was not investigated before. What now is known is that the heterogeneous freezing temperature of *diluted* bulk solutions is much warmer than the homogeneous freezing temperature of emulsified micrometer-scaled drops. In contrast, bulk and emulsified *concentrated* solutions transform to glass at approximately similar T_g. Physically, the much colder freezing temperature of small drops may impact freezing rate and, consequently, ice/FCS morphology in comparison with that of bulk solutions. Another factor that may influence the ice/FCS morphology of emulsified drops is a surfactant/oil matrix which envelops drops in emulsions. To our best knowledge, there are few works which reported that a surfactant/oil matrix may influence the low-temperature behavior of aqueous drops [34]. Besides biotechnology, biopharmaceutics, etc., the knowledge of the low-temperature behavior and ice/FCS morphology of small CA/H_2O drops may be important for the atmosphere where aqueous drops containing CA have been observed [35].

In this paper, we present the results of the measurements of freezing and glass transition behavior and ice/FCS morphology of emulsified 10–60 wt% CA solutions. The measurements were performed using differential scanning calorimetry (DSC) and optical cryo-microscope (OC-M). The paper is structured as follows. In experimental Section 2, we describe DSC and OC-M measurements and in Section 3, we recall the main features of the ice/FCS morphology of bulk CA/H_2O. In Section 4, we rationalize the ice/FCS morphology of emulsified drops from the comparison of bulk and emulsion DSC thermograms and analyze OC-M images obtained *in situ* during the cooling/warming of emulsions. Conclusions are collected in Section 5.

2. Experimental

We prepared 10–60 wt% CA solutions by mixing >99% anhydrous citric acid (Merck) with the corresponding amount of ultrapure water. Solution-in-oil emulsions were produced according to a widely used emulsification technique described elsewhere [36–38]. As an oil-surfactant matrix we used a mixture of 80 wt% Halocarbon 0.8 oil (Halocarbon Products Corp.) and 20 wt% Lanolin (Sigma Aldrich) (thereafter the HL-matrix). Emulsions were prepared by magnetic stirring of the solution/HL-matrix mixtures of 1/10 by volume.

We investigated the freezing, melting and glass transition behavior of emulsified solutions with a Mettler Toledo DSC 822 calorimeter at the scanning cooling/warming rate of 3 K/min in the temperature range between ~308 and 153 K. The calibration of calorimeter and details about measurements are described elsewhere [37–39]. Shortly, the emulsion samples of 20–40 mg were loaded and then hermetically cold sealed in a DSC aluminum (Al) crucible of 40 μl by volume. Weighing of emulsion samples before and after measurements showed no weight change due to H_2O evaporation. The HL-matrix vitrifies at ~123 K [36] and, consequently, does not perturb the glass transitions of CA/H_2O which are observed between ~176 and 220 K [32,33]. We also used an optical cryo-microscope (OC-M) Olympus BX51 equipped with a Linkam cold stage, Linksys32 temperature control and video capture software for the *in situ* observation and recording of the freezing process and ice/FCS morphology of emulsified drops. To this end we placed emulsions in between a standard 75 × 25 mm microscope slide and a cover glass. OC-M measurements were performed at the cooling/warming rate of 3 and 5 K/min between 300 and 193 K [32,33].

The main body of DSC and OC-M measurements was performed on emulsions in which the diameter of drops varied between the smallest observable size of ~1 μm and the largest size of ~5 μm. The freezing process and ice/FCS morphology of such drops are obscured by the HL-matrix and resolution limit imposed by light wavelength. In order to better understand how ice/FCS morphology change with decreasing drop size, we also employed emulsions in which the diameter of largest drops was ~200 μm and larger. Such emulsions were prepared by varying the rate and time of magnetic stirring.

3. ICE/FCS morphology of frozen bulk CA/H_2O

In this section, we shortly recall the low-temperature behavior and ice/FCS morphology of bulk CA/H_2O because this information is necessary for better understanding the low-temperature behavior and ice/FCS morphology of emulsified CA/H_2O. Concentrated solutions (more than ~65 wt% CA) do not freeze upon cooling and warming [33]. Instead they undergo liquid-glass (CA/H_2O-glass) and reverse glass-liquid transitions upon cooling and warming, respectively. Upon cooling less concentrated solutions (~62–64 wt% CA), increasing viscosity does not prevent the appearance of numerous ice nucleation events [33], but completely suppresses the growth of tiny ice crystals i.e., incipient freezing process is terminated by a CA/H_2O-glass transition. This terminated freezing process resumes upon subsequent warming above a reverse glass-CA/H_2O transition. The solutions of concentration between ~58 and 62 wt% CA freeze upon both cooling and warming [33]. The solutions of concentration less than ~56 wt% CA freeze only upon cooling. FIPS, which occurs during the freezing of CA/H_2O, produces a mixed-phased system: an ice framework (IF) and two freeze-concentrated solutions, FCS_1 and FCS_2 [32,33]. Because of its unique molecular/CA-nanocluster structure, CA/H_2O is very convenient for the *in situ* observation of freezing process and ice/FCS morphology using OC-M. Fig. 1 displays the OC-M images of frozen bulk CA/H_2O solutions of different concentrations. It is seen that IF is entangled with FCS_1 which is *maximally* freeze-concentrated, ~81 wt% CA. Image 1d shows that the IF/FCS_1 as a whole is enveloped by FCS_2 which is a less concentrated, ~75 wt% CA [32,33]. Images 1a–1d show how IF/(FCS_1 + FCS_2) morphology changes with increasing solution concentration.

4. Results and discussions

4.1. The comparison of bulk and emulsion thermograms

In Fig. 2a, we present typical cooling and warming DSC thermograms of bulk CA/H_2O [32,33]. The cooling and warming thermograms of emulsified 10–60 wt% CA are displayed in Fig. 2b. As will be seen below, the comparison of bulk and emulsion thermograms is useful for better understanding the ice/FCS morphology of frozen emulsified drops. Fig. 2a shows that the bulk cooling thermogram contains one exothermic event T_f which is due to the freezing out of pure ice [32,33]. Upon subsequent warming, the melting of ice produces a prolonged endothermic peak T_m with a long low-temperature tail. In DSC method, the number of freezing events usually corresponds to the number of melting events. However, in Fig. 2b, emulsion 10–50 wt% CA cooling thermograms contain two freezing events T_{f1} and T_{f2}, whereas the corresponding