



Ovalbumin-stabilized concentrated emulsion gels

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Abstract. In this paper, ovalbumin is used as the sole emulsifier to produce stable concentrated oil (oil phase = 70 wt%) in water emulsion near ovalbumin isoelectric point. The emulsions display excellent stability against flocculation and coalescence at ovalbumin concentration ranging from 0.6 to 1 wt%. The oil–water interfacial tension decreases in the presence of ovalbumin, indicating the adsorption of ovalbumin at the interface. Although the increase in ovalbumin from 0.1 to 1 wt% does not significantly influence the equilibrium interfacial tension, the surface mean diameter of the emulsion droplets decreases as the concentration increases from 0.1 to 1 wt%. These emulsions exhibit solid gel-like behaviour. At low ovalbumin concentrations (0.1–0.4 wt%), emulsions undergo a phase separation after 24 h. We hypothesize that emulsions with higher ovalbumin content contain oil droplets with multilayer adsorbed films. The steric stabilization due to adsorbed multilayers and the ability of rigid interfacial films to withstand external droplet pressures upon contact contributes to emulsion stability.

Keywords. Emulsion gels; ovalbumin; isoelectric point; protein concentration; bulk rheology; interfacial tension.

1. Introduction

Soft materials are composed of entities that are much larger than an atom but still smaller than the overall size of the material [1]. These large building blocks give rise to elastic behaviour and influence the ability of the material to withstand deformation [2]. Such materials can be disordered, metastable and can exhibit non-linear mechanical response [3,4]. Some common examples of soft materials include colloids [5], liquid crystals [6], emulsions [7,8], membranes [9], polymers [10,11], granular systems [12] and foams [13,14]. Soft polymer thin films under confinement undergo instability leading to the formation of different patterns and morphologies, which can be controlled to prepare functional coatings to form super-hydrophobic or super-oleophobic surfaces [15,16].

An oil in water emulsion is composed of oil droplets dispersed in a continuous water phase. Such emulsions find applications in the food and beverage industry, pharmaceuticals, cosmetics, paints and polymers [17–20]. Concentrated emulsions (oil fraction ≥ 50 wt%) are often commonly used by food manufacturers to achieve desired properties in products such as creams, salad dressings and butter [21]. Different types of colloidal and nano-sized particles, of synthetic or natural origin, such as nanocrystals [22,23], colloidal silica and zirconia particles [24,25], polysaccharides [26,27], protein-polysaccharide complexes [28–30] and microgels [20,31–33] have been used to stabilize concentrated oil in water emulsions. A recent demand

towards natural ingredients has shifted interest towards the use of particles of biological origin as stabilizing agents in oil–water emulsions especially for food and biomedical applications [34–36]. Amphiphilic protein molecules adsorb at the oil–water interface created by homogenizing oil and water phases. These molecules reduce the interfacial tension, facilitating the formation of small oil droplets and stabilize them by forming an adsorbed monolayer around the oil droplets [37,38].

Even though the emulsifying properties of proteins have been widely studied, limited literature is available on the use of protein as the sole stabilizer of highly concentrated emulsions [39–46].

The stability of an emulsion depends on the inter-droplet interactions, the thickness and the strength of the adsorbed interfacial films [47]. The net interaction potential between two emulsion droplets is the sum of attractive Van der Waals, electrostatic potentials and some short-range interaction [4]. The Van der Waals forces are long-range forces, while electrostatic forces between similarly charged emulsion droplets can be repulsive over a short range or long-range depending on the screening of charges in the medium. Short-range interactions such as depletion, bridging interaction and hydrophobic forces influence the stability of emulsions [48,49]. It has been reported that protein stabilized emulsions undergo considerable droplet flocculation near the isoelectric point of protein [43,50,51]. This is because the net charge on the protein is zero, and therefore electrostatic repulsion between two emulsion droplets is

negligible which would result in droplet aggregation. This reduces the stability of the emulsion against creaming.

This paper investigates the effect of protein (ovalbumin) concentration on the stability of concentrated soya bean oil in water emulsions near the isoelectric point. The oil content is fixed at 70 wt%, and pH of the system is maintained at 4.2 (isoelectric point of ovalbumin). The commercial oil is not subjected to purification to ensure closeness to real-life conditions. Different analytical techniques including optical and confocal microscopy, interfacial tension, bulk rheology, zeta potential (ζ -potential) and dynamic light scattering are used to study the microstructure, surface charge, stability and rheology of the emulsions. To the best of the author's knowledge, this study reports for the first time the excellent stabilizing properties of ovalbumin in vegetable oil in water emulsions at concentrations as low as 0.6 wt% near the isoelectric pH.

It is observed that the emulsions containing 0.6–1 wt% ovalbumin are stable against creaming and coalescence. The emulsions form solid-like gels and display remarkable long-term stability up to at least 2 weeks. The microstructure of the emulsion gels show tightly packed oil droplets with adsorption of ovalbumin at the soya bean oil–water interface. It is hypothesized that the emulsion stability may be due to steric stabilization between the multilayer adsorbed films of droplets and the rigidity of thick interfacial films that can withstand external droplet pressures upon contact.

2. Materials

Ovalbumin from chicken egg white (62–88%) was purchased from Sigma Aldrich (M.W. 42.7 kDa, CAS no. 9006-59-1). Citric acid monohydrate (M.W. 210.14 g mol⁻¹, CAS no. 5949-29-1) and disodium hydrogen orthophosphate (177.99 g mol⁻¹, CAS no. 13472-35-0) were bought from Sisco Research Laboratories and Qualikems Fine Chem, respectively. Soya bean oil was purchased from a local store and used without further purification. Fluorescent dye Ponceau S was obtained from HiMedia Laboratories (M.W. 760.57 g mol⁻¹, CAS no. 6226-79-5). Aqueous solutions were prepared using de-ionized (DI) water obtained from milli-Q water purification system (resistivity 18.3 M Ω cm at 25°C). All other reagents were of analytical grade.

3. Methods

3.1 Ovalbumin characterization

3.1a Dynamic light scattering: The hydrodynamic diameter of ovalbumin is measured by dynamic light scattering using Nanobrook Omni (Brookhaven Instruments Corp.) at 25°C. A volume of 300 μ l of stock solution is diluted with 9.7 ml

of buffer solution to avoid multiple particle effects. A 3 ml diluted ovalbumin solution (0.1 wt%) is added to disposable polystyrene cuvettes and placed for analysis. The buffer solution is passed through 0.22 μ m Nylon filter to separate the impurities. The reported results are averaged over five readings (mean $\pm$ standard deviation (S.D.)).

3.1b Zeta potential: The ζ -potential of ovalbumin is measured at pH 2.6, 4.2 and 6 using Zetasizer Nanobrook Omni (Brookhaven Instruments Corp.) at 25°C. A 4 ml diluted ovalbumin solution (0.1 wt%) is added to disposable polystyrene cuvettes and placed for analysis. The reported results are averaged over five readings (mean $\pm$ S.D.).

3.2 Emulsion preparation

Before emulsification, a stock solution of ovalbumin (33.33 mg ml⁻¹) is prepared by mixing 6.67 g of ovalbumin in 200 ml of DI water using a magnetic stirrer at 1000 rpm for 10 h at 25°C. The stock solution is centrifuged at 4000 rpm for 15 min to separate the impurities. This solution is then diluted to obtain ovalbumin concentration (0.1, 0.2, 0.4, 0.6, 0.8, 1 wt%) and pH is adjusted to 4.2 by using citrate-phosphate buffer. The diluted solutions are stored at 4°C overnight to ensure complete hydration of ovalbumin. The emulsions are produced by homogenizing soya bean oil (70 wt%, dispersed phase) and aqueous ovalbumin dispersion (0.1–1 wt%, continuous phase) at 8000 rpm for 4 min using D 130 Wigger Hauser homogenizer. The emulsions are either directly used for analysis or are stored at ambient temperature.

3.3 Emulsion stability—visual observation

The stability of emulsion is characterized based on the visual inspection of the emulsions left quiescently at room temperature over a period of 24 h. Similarly, long-term stability is determined by visual inspection of the emulsions after 2 weeks. Each sample is prepared twice to ensure reproducibility.

3.4 Emulsion characterization

3.4a Zeta potential of oil droplets: The ζ -potential of oil droplets with no adsorbed ovalbumin is measured using Zetasizer Nanobrook Omni (Brookhaven Instruments Corp.) at pH 2.6, 4.2 and 6 at 25°C. Emulsions are prepared by method described in section 3.2 without addition of ovalbumin to the aqueous buffer solution. Each sample is diluted 500 times with the buffer solution to maintain a constant pH. The reported results are averaged over five readings (mean $\pm$ S.D.).

3.5 Dynamic interfacial tension

The evolution of interfacial tension with time is obtained using optical tensiometer (Attention Theta, Biolin Scientific) in pendant drop mode. A 5 μl droplet of ovalbumin solution is formed using a glass syringe (needle diameter 0.71 mm). The needle is lowered into a disposable polystyrene cuvette containing soya bean oil and thereafter the droplet is formed. The drop profile is monitored for 3600 s at room temperature. Each measurement is repeated 3 times to ensure repeatability.

3.6 Microstructure analysis

3.6a Optical microscopy: The droplet size distribution is evaluated based on the images obtained from optical microscopy (Zeiss Axio Lab.A1 laboratory microscope). A 40 μl emulsion droplet is placed into an observation chamber, which consists of parallel strips of parafilm sandwiched between a glass slide (22 $\times$ 50 mm, No. 1) and a second coverslip (18 mm circular). Prior to the assembly, both the coverslips and glass slides are cleaned using acetone. The slides are transferred to the microscope and observed under brightfield mode. Images are taken at 3–4 random locations (parallel to the plane of the slide).

3.6b Confocal microscopy: The microstructure of freshly prepared emulsions is studied using confocal microscopy (TCS SP8 Leica Microsystems). A volume of 3 ml of freshly prepared emulsion and 0.3 ml of Ponceau S (0.1%w/v) solution are added into glass vials. The dye is mixed into the emulsion by gently stirring the vial. The dyed emulsion is kept for at least 30 min to ensure complete ovalbumin staining. A 30 μl emulsion droplet is placed onto a glass slide (22 $\times$ 50 mm, No. 1) and covered using a second coverslip (18 mm circular).

3.6c Droplet size distribution: The surface average diameter ($d_{3,2}$) of the emulsion droplets is measured by analysing the images of emulsions obtained using optical microscopy. The diameter of the droplets is measured using ImageJ. The number of droplets used for analysis varied between 200 and 800. Equation (1) is used to calculate the surface average diameter:

$$d_{3,2} = \frac{\sum_i n_i d_i^3}{\sum_i n_i d_i^2} \quad (1)$$

where d_i is the diameter of droplet i and n_i is the number of droplets with diameter d_i .

3.7 Bulk rheology

The storage (G') and loss modulus (G'') of the emulsion gels are measured at 25°C using Modular Compact Rheometer

(Anton Paar, MCR 302). The geometry used for the experiments is a micro-roughened parallel plate geometry (diameter = 25 mm). The plates are separated by 0.5 mm. The emulsion samples are stored for 24 h prior to the analysis. Stable emulsions are directly used for analysis but samples exhibiting phase separation are first centrifuged at 10,000 rpm for 15 min to separate the cream from the emulsion and then the cream is used to measure bulk rheological properties. G' and G'' are measured as angular frequency (ω) is varied from 100 to 0.1 rad s^{-1} at a constant oscillatory strain of 0.1% in the linear viscoelastic region. Large-amplitude oscillatory strain (LAOS) sweep is conducted at 6.28 rad s^{-1} with strain% varying from 0.1 to 500%.

4. Results and discussion

4.1 Ovalbumin characterization

The volume average diameter of ovalbumin at pH 4.2 is 9.7 ± 5 nm (mean $\pm$ S.D.). Table 1 shows the ζ -potential of ovalbumin measured at pH 2.6, 4.2 and 6. It is observed that the net charge on ovalbumin is nearly 0 at pH 4.2 (isoelectric point). This value is near the values reported in the literature which range between 4.4 and 4.6 [52]. As expected, ovalbumin carries a positive charge below the isoelectric point while it takes a negative charge above the isoelectric point.

Protein adsorption and interfacial rheological properties are affected by the electrostatic interactions between the protein and the adsorbent [53–55]. Hence, ζ -potential of oil droplets dispersed in aqueous buffer at different pH is measured. Table 2 shows that the ζ -potential of soya bean oil droplets with no adsorbed ovalbumin (a bare interface). The oil droplets carry a similar charge as the ovalbumin above and below its isoelectric point indicating unfavourable electrostatic interactions for ovalbumin adsorption while near the isoelectric point, the oil droplets carry a mildly negative charge. Also, the ζ -potential values are very small, indicating the rapid flocculation or aggregation of the oil droplets without ovalbumin.

Table 1. Variation in ζ potential of ovalbumin with pH.

Emulsion pH	Zeta potential (mV)
2.6	18 ± 1.2
4.2	0.3 ± 1.3
6	-9.7 ± 1.3

Table 2. Variation in ζ potential of oil droplets with pH.

pH	Zeta potential (mV)
2.6	7.5 ± 0.8
4.2	-2.2 ± 0.6
6	-5.3 ± 0.8

4.2 Formation of gels

As demonstrated in figure 1, we observe that at low ovalbumin concentrations (0.1–0.4 wt%), the emulsions undergo coalescence and creaming after 24 h. However, at higher ovalbumin concentration (0.6–1 wt%) emulsions exhibit stability against flocculation and coalescence up to 2 weeks. This is surprising since the pH of the emulsion is near the isoelectric point of ovalbumin. Furthermore, these emulsions do not flow when the vials are inverted, exhibiting solid gel-like behaviour.

4.3 Dynamic interfacial tension

The dynamic interfacial tension of oil–water interface is measured for a period of 1 h without addition of ovalbumin using DI water and buffer solution. The oil–water interfacial tension should remain constant at the theoretically predicted value $\sim 29 \text{ mN m}^{-1}$ in the absence of ovalbumin. But it is observed that the interfacial tension reduces to 12.5 mN m^{-1} at pH 4.2 (table 3). The reduction in interfacial tension at DI water/oil interface indicates that the commercial soya bean oil contains impurities which act as surface-active agents. Gaonkar *et al* [56] reports that a mixture of monoglycerides in commercial vegetable oils contributes to a reduction in the interfacial tension. Furthermore, the reduction in interfacial tension at aqueous buffer (pH 4.2)–oil interface shows that the ions in the buffer solution also influence the interfacial tension.

Figure 2 illustrates the systematic decrease in interfacial tension upon the addition of ovalbumin at pH 4.2 from

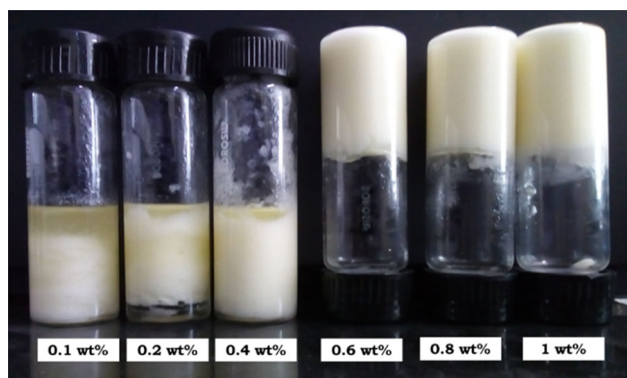


Figure 1. Visual observation of emulsion stability for 24 h (gel-like behaviour observed for 0.6–1 wt% ovalbumin).

$\sim 18 \text{ mN m}^{-1}$ to $\sim 4 \text{ mN m}^{-1}$. This effect can be attributed to the ovalbumin adsorption at the oil–water interface [57].

Increasing the ovalbumin concentration from 0.1 to 1 wt% does not have a significant effect on the interfacial tension of the system after 1 h. The interfacial tension of the droplet containing 0.1 wt% ovalbumin is 3.8 mN m^{-1} whereas the interfacial tension of the droplet containing 1 wt% ovalbumin is 2.7 mN m^{-1} after 1 h. Each measurement is repeated 3 times and the maximum uncertainty is $\pm 0.5 \text{ mN m}^{-1}$.

4.4 Microstructure analysis

Figure 3 shows the images of the emulsion microstructure using optical microscopy. It is visible that the emulsion droplets are aggregated at 0.1 wt% ovalbumin concentration while a jammed packing is observed at 1 wt% ovalbumin concentration with droplets compressing against each other. The emulsion droplets in the compressed network form flattened inter-droplet layers (denoted by rectangle in figure 4) upon contact. Plateau borders are formed where three inter-droplet films meet (denoted by circle in figure 4).

Figure 4 shows the confocal microscopy images of emulsions. The images indicate that the oil droplets are

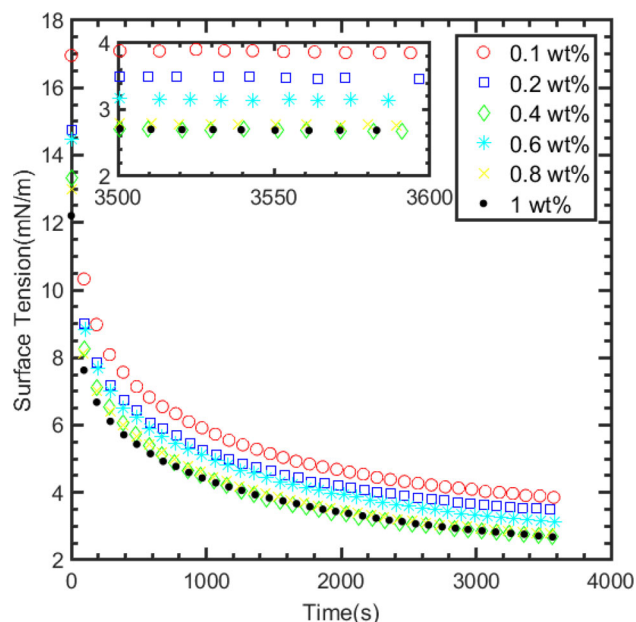


Figure 2. Dynamic interfacial tension at soya bean oil and water interface with increasing ovalbumin concentration at pH 4.2.

Table 3. Interfacial tension for soya bean oil and water interface at time $t = 0$ and $t = 3600 \text{ s}$.

	Interfacial tension (mN m^{-1}) at $t = 0 \text{ s}$	Interfacial tension (mN m^{-1}) at $t = 3600 \text{ s}$
Soya bean oil—DI water	28.1 ± 0.8	19.7 ± 0.6
Soya bean oil—pH 4.2 buffer	25.1 ± 0.5	12.5 ± 0.4

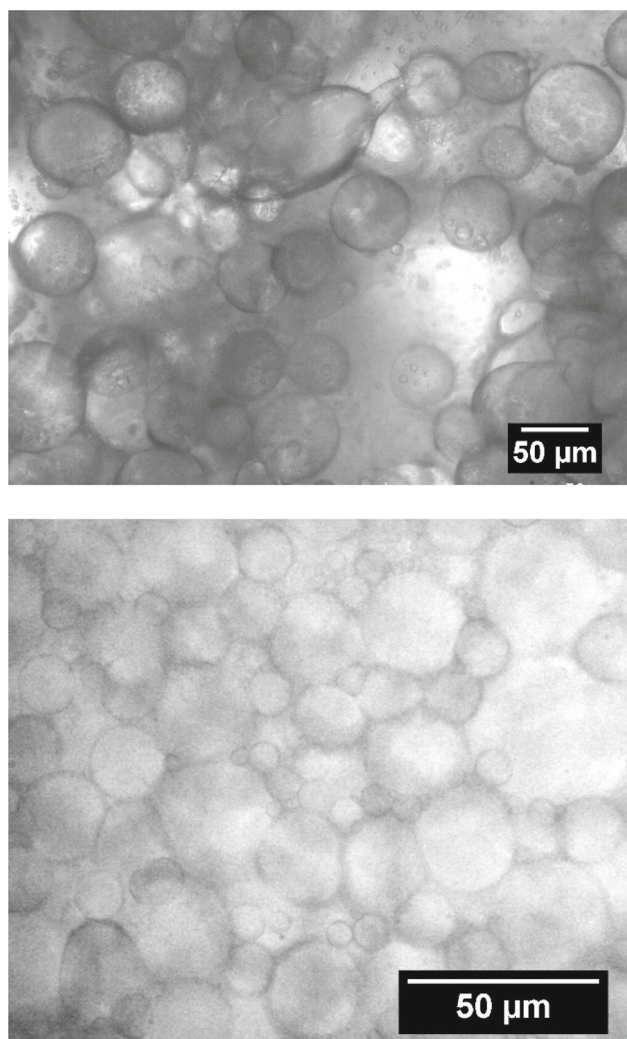


Figure 3. Optical microscopy images of fresh emulsions with 0.1 wt% ovalbumin (top) and 1 wt% ovalbumin (bottom) at pH 4.2.

partially covered at low ovalbumin concentration. Furthermore, the droplets coalesce together to form larger droplets. At high ovalbumin concentration, the droplets are smaller with a qualitatively thicker interface. They are compressed against each other thereby deforming their shape but resist coalescence. This agrees well with the observed phase separation in emulsions with low ovalbumin concentration and stable solid-like gels formed at high ovalbumin concentration.

Figure 5 shows that the average surface diameter ($d_{3,2}$) of the emulsion droplets decreases as the concentration of ovalbumin increases (except for 0.2 wt%).

A significant reduction in the droplet size is observed from $52 \pm 0.9 \mu\text{m}$ (0.1 wt% ovalbumin) to $20 \pm 0.5 \mu\text{m}$ (1 wt% ovalbumin). This is consistent with the observation that at high protein concentration, more protein is available to stabilize the smaller oil droplets with higher interfacial area [35,58].

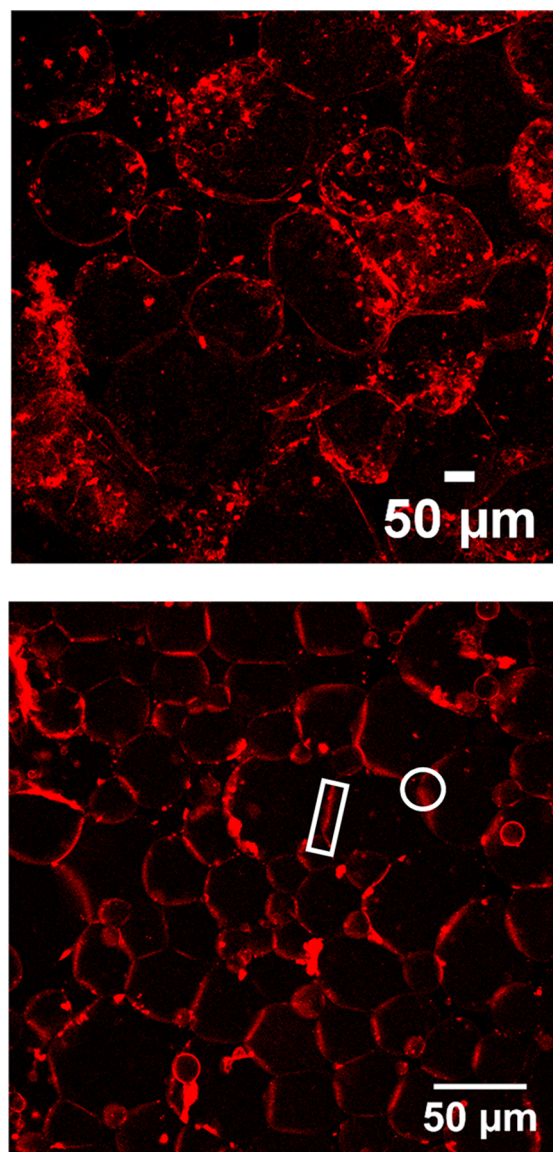


Figure 4. Confocal microscopy images of fresh emulsions with 0.1 wt% ovalbumin (top) and 1 wt% ovalbumin (bottom) at pH 4.2. The rectangle represents the flat inter-droplet layer, and the circle represents the plateau border.

4.5 Bulk rheology

Figure 6 represents the frequency and amplitude sweep spectra of emulsions (ovalbumin concentration 0.1 and 1 wt%) at pH 4.2. The frequency sweep profiles show that throughout the frequency range, the emulsion containing 1 wt% ovalbumin and the cream phase of emulsion containing 0.1 wt% ovalbumin exhibit a prominently solid-like response with storage modulus (G') greater than the loss modulus (G''). The storage and loss modulus show a mild increase with frequency.

The amplitude sweep profiles show that G' of the emulsion with 1 wt% ovalbumin is nearly constant till the

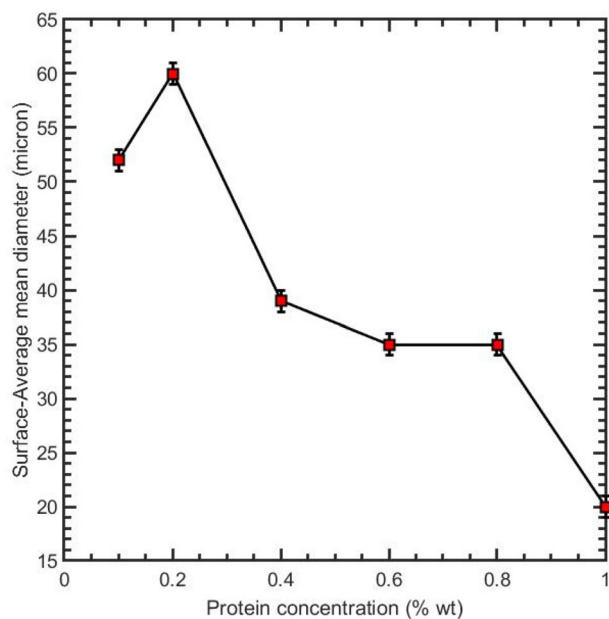


Figure 5. Surface mean diameter of emulsion droplets with increasing ovalbumin concentration at pH 4.2.

oscillatory strain (γ) of 3% (linear viscoelastic regime or LVR) beyond which it decreases sharply with an increasing oscillatory γ . Interestingly, G'' remains constant until $\gamma = 7\%$ beyond which it begins to decrease with the oscillatory γ . At low strain, the $G' \gg G''$ indicating an elastic behaviour, however, at higher strain values, the interdroplet structure might undergo some rearrangement and therefore elastic modulus decreases. Qualitatively similar trends are observed on decreasing the ovalbumin concentration from 1 to 0.1 wt%, except that the LVR shifts to a higher oscillatory strain of 10%.

The plateau value of storage modulus of emulsions within the LVE regime containing 1 wt% ovalbumin (~ 220 Pa) is an order of magnitude higher than those of emulsions with 0.1 wt% (~ 10 Pa) ovalbumin, which indicates that emulsion with higher ovalbumin concentration is more solid-like than emulsions with lower ovalbumin concentration. This is consistent with our hypothesis that at higher ovalbumin concentration, thick multilayer interfacial films are formed, which enhances the viscosity of the emulsion.

5. Hypothesis

Initially, ovalbumin molecules adsorb on the oil droplets to form a monolayer. We hypothesize that at high ovalbumin concentrations, the excess ovalbumin can reversibly adsorb on the monolayer to generate multiple adsorbed layers [59,60]. Upon close contact between two droplets, the thick multilayers can undergo steric stabilization, and the rigid interfacial multilayers can withstand pressure upon contact, thereby preventing flocculation [47,60]. The rigidity of the

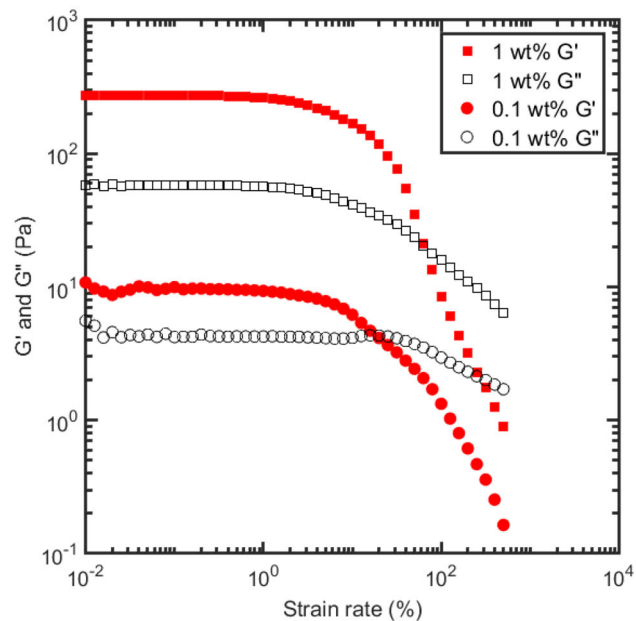
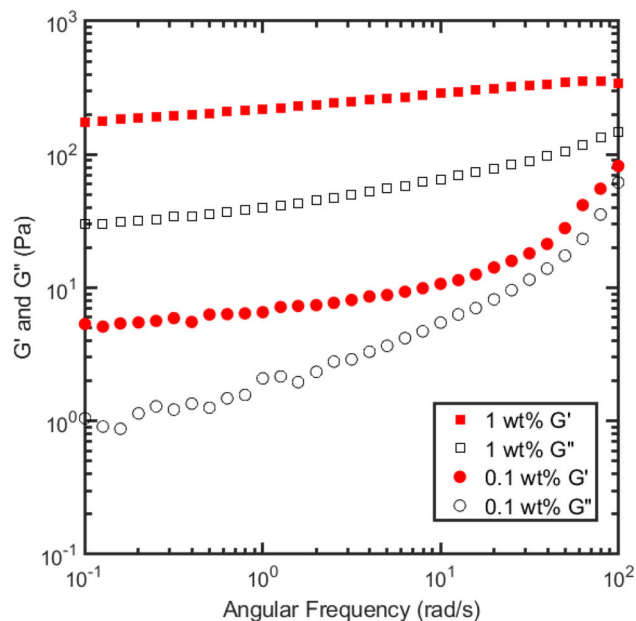


Figure 6. Frequency sweep (top) and amplitude sweep (bottom) for oil water emulsion with ovalbumin concentration 0.1 and 1 wt% at pH 4.2.

film is higher near the isoelectric point since the neighbouring ovalbumin molecules adsorbed on the interface pack closely and possibly form intermolecular bonds [54]. At low ovalbumin concentrations, the ovalbumin molecules do not entirely cover the oil droplets. Incomplete monolayer coverage and lack of electrostatic repulsion does not prevent the emulsion droplets from aggregating together through bridging flocculation and ultimately undergoing coalescence [61].

Figure 7A provides a schematic representation of steric stabilization between two emulsion droplets completely

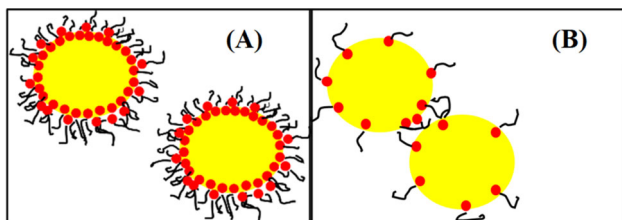


Figure 7. Schematic representation of steric stabilization between two emulsion droplets at 1 wt% ovalbumin concentration (A) and bridging flocculation between two emulsion droplets at 0.1 wt% ovalbumin concentration (B) at pH 4.2. Oil droplets represented by yellow spheres, ovalbumin hydrophobic head (red) and hydrophilic tail (black).

covered by ovalbumin molecules in a tight monolayer and reversibly bound multilayers at 1 wt% ovalbumin concentration. Figure 7B demonstrates bridging flocculation between two emulsion droplets at 0.1 wt% ovalbumin.

6. Conclusion and future work

In this paper we report for the first time the excellent stabilizing properties of ovalbumin in vegetable oil–water emulsion at concentrations as low as 0.6 wt% near the isoelectric pH. The existing literature on the use of proteins alone for stabilizing concentrated emulsions is sparse [29,39–41,43,44]. The resulting emulsions display gel-like properties ($G' > G''$) and demonstrate excellent stability against creaming and coalescence. This emulsion stability might be due to steric stabilization between multilayer adsorbed films and the rigidity of thick interfacial films that can withstand external droplet pressures upon contact. Emulsions containing 0.1–0.4 wt% ovalbumin exhibit coalescence within 24 h. This might be due to bridging flocculation between incompletely covered oil droplets. The oil–water interfacial tension after 3600 s is relatively independent of ovalbumin concentration but the droplet size of emulsions decreases significantly with an increase in ovalbumin concentration.

In the future, we plan to conduct a parametric study to understand the influence of protein concentration (>1 wt%), dispersed phase oil fraction (up to 90 wt%) and pH (above and below isoelectric point) on the stability, microstructure, surface coverage, bulk and interfacial rheology of concentrated oil in water emulsions and determine variables that can be used to design food emulsions rationally.

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