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Lipid Oxidation of Fish Oil-in-Water Pickering Emulsions Stabilized With Protein-Based Janus Particles

Thang Tran¹ | Charalampos Tsekeridis² | Fathinah Islami Hasyati³ | Betül Yesiltas¹ | Ana C. Mendes¹ | Ioannis S. Chronakis¹ | Claire Berton-Carabin^{3,4} | Heloisa Nunes Bordallo² | Karin Schroën³ | Charlotte Jacobsen¹

¹National Food Institute, Technical University of Denmark, Kongens Lyngby, Denmark | ²Niels Bohr Institute, University of Copenhagen, Copenhagen, Denmark | ³Wageningen University, Department of Agrotechnology & Food Sciences, Laboratory of Food Process Engineering, Wageningen, the Netherlands | ⁴INRAE, UR BIA, Nantes, France

Correspondence: Charlotte Jacobsen (chja@food.dtu.dk)

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ABSTRACT

Pickering emulsions stabilized with solid particles have a significant advantage over conventional emulsions due to their high coalescence stability. However, their chemical stability, especially in fish oil-in-water Pickering emulsions, needs improvement. Janus particles, with their anisotropic structure that enhances interface binding and allows for antioxidant inclusion, offer a promising solution for improving both physical and oxidative stability in these emulsions. In this study, fish oil-in-water Pickering emulsions were produced with zein-caseinate Janus particles. The latter were produced by electrohydrodynamic co-jetting technique. Confocal laser scanning microscopy and scanning electron microscopy analysis confirmed anisotropic structures of these particles. Emulsions with 2.5 wt% oil and 1.0 wt% protein emulsifier were assessed for the physical and oxidative stability. Emulsions produced with zein-caseinate Janus particles had unchanged size distribution during the entire storage experiment and low number of coalescence events compared to emulsions with sodium caseinate. These results indicate that emulsions stabilized with zein-caseinate Janus particles enhanced physical stability compared to those stabilized with caseinate only. The formation of hydroperoxides and depletion of tocopherols were at the same level for both emulsions, while formation of volatile compounds was slower in the Pickering emulsions with Janus particles. This implies that Janus particles may hinder the degradation of lipid hydroperoxides to secondary oxidation products.

Practical Applications: Zein-caseinate Janus particles can be produced with electrohydrodynamic co-jetting technique. The significant enhancement of both physical and oxidative stability in Pickering emulsions stabilized by these particles suggests their potential as effective Pickering emulsifiers for food emulsions rich in polyunsaturated fatty acids.

Abbreviations: CLSM, Confocal laser scanning microscopy; DSC, Differential scanning calorimetry; EHD, Electrohydrodynamic; FITC, Fluorescein isothiocyanate; IFT, Interfacial tension; pI, Isoelectric point; PV, Peroxide value; SEM, Scanning electron microscope.

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1 | Introduction

Pickering emulsions that are stabilized with solid particles have a remarkable advantage over conventional emulsions, namely their very high coalescence stability [1, 2]. Pickering particles that are located at the oil-water interface have very high desorption energy and are not easily removed, if at all [3]. Besides solid particles that have classically been used for this purpose [4, 5], biobased particles, for instance, zein, gliadins, and other natural particles have also been used in food applications [6, 7]. Studies on the ability of specific Pickering particles to prevent coalescence are still limited, but a few studies have evaluated the coalescence stability of oil droplets stabilized with Pickering particles by a microfluidic set-up [8]. The set-up includes a T-junction and a coalescence chamber. Oil droplets are produced at the T-junction and followed by a coalescence chamber where merging events may happen. A study with caseinate-coated colloidal lipid particles using this technique showed that coalescence stability of oil droplets can be prevented when the surface of oil droplets is covered with an adequate number of particles [8]. Although Pickering particles can protect emulsion droplets against coalescence, they often require modification to effectively enhance the physical stability of emulsions [1]. Zein, which is a food-grade alcohol-soluble protein derived from corn, exhibited suitable colloidal properties as Pickering particles due to its size, charge, and wettability [2, 7]. Yet, high hydrophobicity of zein nanoparticles may have negative effects on the physical stability of emulsions. Combining other ingredients to tune surface wettability may help to improve the physical stability of emulsions [9]. Caseinate has been combined with zein protein to improve physical stability of Pickering emulsions [10]. The last decades have witnessed a growing trend of high-internal phase Pickering emulsions stabilized with zein and caseinate complexes [10, 11]. Very little is currently known about the physicochemical properties of low-fat Pickering emulsions stabilized with zein and caseinate complexes [12]. Therefore, a study of low-fat Pickering oil-in-water emulsions (2.5% oil) stabilized with zein and caseinate complexes is necessary.

The chemical stability of Pickering emulsions is still a point of improvement, particularly for fish oil-in-water Pickering emulsions. This is because fish oil is rich in omega-3 polyunsaturated fatty acids, which are highly susceptible to lipid oxidation. In emulsions, lipid oxidation is initiated and takes place at the oil-water interface, where the oil phase encounters the aqueous phase containing prooxidants, for instance, trace metal ions [13, 14]. The initiation of lipid oxidation generates free radicals, which are followed by formation of new lipid hydroperoxides in the propagation step. These lipid hydroperoxide compounds are the primary oxidation products, and they continuously decompose to volatile compounds (secondary oxidation compounds). These volatile products not only decrease the quality of fish oil-fortified food products but also pose potential health risks for consumers when fish oil-in-water emulsions are introduced into food systems. Lipid oxidation in emulsions is largely driven by the catalytic breakdown of lipid hydroperoxides due to the presence of trace metal ions. This occurs when lipid hydroperoxides, located close to the droplet surface, interact with transition metals in the continuous phase. Preventing such interactions may help to improve the oxidative stability of oil-in-water emulsions. Factors

controlling the catalytic activity of transition metals include the physical location of prooxidants in the continuous phase and the interfacial properties of droplets (surface charge and thickness) [14]. Several studies have demonstrated the ability of Pickering particles to enhance the oxidative stability by inhibiting the activity of metal ions in the continuous phase. This inhibition occurs primarily through the effective chelating activity of Pickering particles or the formation of a network that restricts the movement of metal ions [15, 16]. A positive surface charge also helps to repel prooxidant metals away from the interface. A thick layer of Pickering particles at the oil-water interface may thus contribute significantly to improving the oxidative stability of Pickering emulsions. It can act as a physical barrier to limit the access of prooxidants present in the aqueous phase to the oil phase in some cases. Physical barriers with antioxidant agents can improve the oxidative stability of Pickering emulsions. In experiments investigating the impact of non-covalent and covalent zein-gallic acid nanoparticles, a significant enhancement of chemical stability when gallic acid with antioxidant activity was located at the oil-water interface via covalent modification with zein has been shown. However, gallic acid was not that effective in experiments with non-covalent zein-gallic acid nanoparticles [17]. By modifying Pickering particles to achieve a certain functionality, that is, antioxidant activity, the chemical stability of Pickering emulsions can be improved because of their presence at the oil-water interface or in the continuous phase. It has been demonstrated that caseinate has antioxidative properties due to its ability to chelate metal ions that can otherwise catalyze lipid oxidation [18–20]. Some studies have confirmed that combining zein particles with antioxidants can enhance the oxidative stability of zein-based Pickering emulsions [21, 22]. We therefore hypothesized that it is possible to enhance the chemical stability of Pickering emulsions by using a combination of zein and caseinate complexes as Pickering particles, particularly in fish oil-in-water Pickering emulsions.

The concept of Janus particles was introduced for the first time in 1991 by a Nobel scholar, Pierre-Gilles de Gennes [23, 24]. Janus particles, also known as “patchy” particles, are formed by modifying surface patterns on colloidal particles, resulting in anisotropic surface features. Theoretical and experimental studies comparing the assembly of isotropic and anisotropic particles on liquid-liquid interfaces showed that Janus particles exhibit a good interfacial tension (IFT) value upon adsorption of Janus particles to the oil-water interfaces compared to isotropic particles. The findings highlight the considerable benefits of Janus particles in enhancing the physical stability of emulsions [25, 26]. Furthermore, recent research proved that it is possible to compartmentalize two different proteins on a single Janus particle. These Janus particles exhibited two distinct functions from their active compartments, as proven in different applications [27]. However, the application of Janus particles in food is still limited. A few examples are applications such as emulsions stabilizers, toxin detectors, antimicrobial agents, and food packaging materials [24, 28]. As mentioned before, the structure of Janus particles can be tailored to achieve antioxidant activity by adding antioxidants into one compartment, further broadening their potential applications, particularly in food emulsions. A search of the literature revealed only a few studies that fabricated food-grade Janus

materials [24]. No previous study has investigated fabricating Janus particles with antioxidant activity from food-grade materials, like zein and caseinate, by the electrohydrodynamic (EHD) co-jetting technique, even though this technique offers a versatile method to fabricate Janus particles from distinct inorganic or polymeric materials [29–31]. By producing protein-based Janus particles from zein and caseinate using the EHD co-jetting technique, we expect that the Janus particles can achieve a dual functionality of zein and sodium caseinate. Thus, it can contribute to improving the physical stability and oxidative stability of Pickering emulsions.

Therefore, this study was firstly aimed to explore the potential for fabricating protein-based Janus particles from food-grade materials, zein and sodium caseinate, by the EHD co-jetting technique. Secondly, the coalescence stability of oil droplets stabilized with these protein particles was evaluated with a microfluidic set-up. Finally, these particles were emulsified with fish oil to produce Pickering emulsions. Physical stability and oxidative stability of these Pickering emulsions were examined and compared to emulsions stabilized with sodium caseinate.

2 | Materials and Methods

2.1 | EHD Co-Jetting Fabrication of Zein and Sodium Caseinate

Zein (6 g, Sigma Aldrich, St. Louis, MO, USA) was dissolved in 60% ethanol solution to produce 150 mL of 4 wt% zein solution. The solution was stirred at room temperature for 3 h.

Sodium caseinate (1.5 g, MIPRODAN 30, Arla Foods Ingredients, Viby, Denmark) was added into 60% ethanol solution to produce 150 mL of 1 wt% sodium caseinate solution. The solution was stirred continuously at 60°C for 3 h.

Particles were produced with EHD co-jetting process (side-by-side electrojetting) [30, 31]. Both solutions were loaded into two 12 mL syringes and, these syringes were attached to a dual-syringe pump (New Era Pump Systems, Farmingdale, NY, USA). The voltage was maintained at 25 kV with a high voltage supply (ES50P-10W, Gamma High Voltage Research, Ormond Beach, FL, USA) and the flow rate was set at 0.1 mL/min. The distance was 15 cm between the tips and the collector. Two tips were set-up in side-by-side configuration and the collector was covered with aluminum foil. Protein particles were deposited on the collector.

To prepare a suspension of protein particles, protein sample was detached from the collector, resuspended with ultrapure water and centrifuged at $4472 \times g$ for 10 min to obtain the pellet at the bottom of a centrifuge tube. The suspension of protein particles (~1.0 wt%) was prepared by resuspending the pellet with 10 mM acetate buffer at pH 3, and stirring overnight before homogenizing with Ultra Turrax at 10 000 rpm for 10 min. Centrifugation at $179 \times g$ for 1 min was applied to remove big aggregates. The suspension of anisotropic protein particles (1.0 wt% protein) was emulsified with fish oil to produce Pickering emulsions.

2.2 | Morphological Analysis of Protein Particles

For confocal laser scanning microscopy (CLSM), two solutions were prepared with fluorescence dyes, Nile Red (Sigma Aldrich, St. Louis, MO, USA) and Fluorescein isothiocyanate (FITC, Sigma Aldrich, St. Louis, MO, USA). Nile Red solution (1 mg/mL) was prepared by dissolving 0.001 g of Nile Red in 1 mL of ethanol. FITC solution (2 mg/mL) was prepared by dissolving 0.002 g FITC in 1 mL of ethanol. Zein solution with Nile Red was prepared by adding 40 μ L solution of Nile Red (1 mg/mL) into 10 mL of 4% zein solution and stirring well for 1 h. The bottle was covered with aluminum foil. Sodium caseinate was stained with FITC by adding 40 μ L of FITC solution to 10 mL of 1% sodium caseinate solution and stirring well for 1 h. The bottle was covered with aluminum foil. These two jetting solutions with fluorescent dyes were electrojetted following the same protocol as mentioned above. To collect protein particles for CLSM (Carl Zeiss, Oberkochen, Germany), a glass slide was put on the collector, and protein particles were deposited onto the glass slide for 1 min.

For scanning electron microscope (SEM), protein particles were deposited on a cover glass for 1 min. Samples were coated with a 10 nm-thick platinum (Leica ACE600, Wetzlar, Germany). SEM analysis was carried out using Quanta FEG 200 ESEM and CRYO (FEI, Oregon, USA) at an accelerating voltage of 15 kV.

2.3 | Thermal Analysis of Protein Particles

Protein particles were analyzed with differential scanning calorimetry (DSC: Netzsch 214 Polyma, Netzsch, Selb, Germany) in connection with Proteus software (Bruker, Germany). Zein-caseinate particles detached from the collector were used for this analysis. Zein powder and caseinate powder were used for this analysis as received. Samples with similar mass (5 mg) were packed into aluminum crucibles and sealed with a punch lid in a closed system. Samples were measured from 25°C to 250°C in a liquid N₂ atmosphere purged at 40 mL/min, and the protective gas was 60 mL/min N₂ with heating and cooling rates of 10 °C/min. A reference crucible and an empty crucible were measured to correct the baseline before experiments.

2.4 | Electrostatic Charges of Protein Particles

Zeta potential of protein particles was assessed at different pH values (from 2 to 10) with Zetasizer Nano ZS with the Malvern MPT-2 Autotitrator (Malvern Instruments Ltd., Worcestershire, UK) at 25°C. Suspensions of protein particles (0.1 wt%) were titrated from pH 2 to 10 with 0.1 M HCl or 0.1 M NaOH solutions. The isoelectric point (pI) was determined by the intercept with the X-axis, corresponding to a net charge equal to 0.

2.5 | Analysis of Interfacial Tension

Pendant drop was used to analyze IFT of protein suspensions (zein-caseinate particles and zein particles at 0.02%) using drop tensiometer (OCA 25, Dataphysics Instruments, Filderstadt,

Germany). MCT oil was filled in a quartz cuvette, and the protein suspension was loaded into a glass syringe. The IFT was recorded for 30 min at 25°C. Sodium caseinate solution (0.02%) and water were also measured with the same procedure. All measurements were duplicated, and representative results are shown.

2.6 | Analysis of Coalescence Stability

Microfluidic coalescence cells (Micronit, Enschede, the Netherlands) that were presented in earlier work were used to evaluate coalescence stability of emulsion droplets [8]. In the set-up (Figure 2a), oil droplets were formed at T-junction when two channels of oil phase and aqueous phase joined together (width = 100 µm). Then, oil droplets flowed into the coalescence chamber (width = 500 µm, length = 26.2 mm) where coalescence events may happen, as observed through a change in droplet size. The cell was fixed in a Fluidic Connect Pro Chip Holder (Micronit, Enschede, the Netherlands) and two phases were connected to the cell with Polyether Ether Ketone tubing. The flow rate of both phases was adjusted by controlling the pressure (OB1 Flow Controller, Elveflow, France) and monitored with flow sensors (mini CORI-FLOW sensors, Bronkhorst, The Netherlands). The dispersed phase was n-hexadecane (Thermo Fisher, Kandel, Germany), that was adjusted at 5 µL/min. The aqueous phase was protein solution (sodium caseinate or zein-caseinate particles) with concentration at 10⁻², 10⁻³, and 10⁻⁴ wt%. Zein-caseinate particles were first washed with ethanol and centrifuged before dilution with ultrapure water. The flow rate of the aqueous phase was adjusted to 150, 200, and 250 µL/min.

Images were recorded at the T-junction, position A at the beginning area of coalescence chamber, and position B at the end of coalescence chamber (Figure 2a) at 10× magnification (Axiovert 200 MAT, Carl Zeiss, Oberkochen, Germany) and high-speed camera (Fastcam NOVA S-6, Photron, Tokyo, Japan). The setting for recording images was 1000 frames at 30 frames/s. The mean number of coalescence events was calculated based on the mean droplet area at interested positions (A and B) and the mean initial droplet surface area when entering the coalescence chamber (Figure 2a).

2.7 | Emulsification and Storage Experiments

Sodium caseinate and zein-caseinate particles were used as protein emulsifiers. Sodium caseinate solution (1.0 wt%) and zein-caseinate particle suspension (1.0 wt%) were prepared with sodium acetate buffer (10 mM) at pH 3, and they were stirred continuously at room temperature overnight before emulsification.

Fish oil-in-water emulsions stabilized with either sodium caseinate protein or zein-caseinate particles protein were produced as follows (2.5 wt% oil and 1.0 wt% protein emulsifier). Coarse emulsions (375 g) were prepared by homogenizing fish oil (9.38 g) with 365.63 g of protein emulsifier solution (1.0 wt%) by using a homogenizer (Ultra Turrax, Ystral) at 16 000 rpm for 3 min. Final emulsions (1 wt% protein and 2.5 wt% oil) were produced by homogenization with a high-pressure homogenizer (Microfluidizer, M110L Microfluidics, Newton, MA, USA) equipped with a ceramic interaction chamber (CIXC,

F20Y, internal dimension 75 µm) at 62 MPa for three passes. After emulsification, prooxidant 50 µM FeSO₄ solution was added to all solutions (187.5 µL). Each emulsion was duplicated.

All emulsions were stored in the dark at room temperature (20°C) for 10 days. Emulsions were sampled on days 0, 1, 2, 6, and 10. Emulsions were measured for droplet size and zeta potential right after sampling. For assessing lipid oxidation, emulsions were purged with N₂ and stored at -40°C before further analysis.

2.8 | Physical Properties and Morphology of Emulsions

Droplet size was measured by static light scattering (Mastersizer 2000, Malvern instruments, Worcestershire, UK). Emulsions were diluted with water in sample dispersion unit at 3000 rpm to pass the low threshold of obscuration value. Each sample was measured twice, and the average of these measurements was used for the results.

Zeta potential was measured with a Zetasizer Nano ZS (Malvern instruments, Worcestershire, UK) with a DTS1070 cell at 20°C. Emulsions (1 mL) were diluted with 9 mL of ultrapure water before each measurement. Each measurement was duplicated for average results.

For optical microscopic observation, a droplet of the emulsion was placed on a glass slide, covered with a coverslip, and then examined with Primo Star microscope (Carl Zeiss, Oberkochen, Germany) at a magnification 100× with oil immersion.

For Cryo-SEM, Pickering emulsions were observed by cryo-SEM (QFEG 200 Cryo ESEM, FEI, Oregon, USA) for their interfacial structure. Emulsions were rapidly frozen in liquid nitrogen at vacuum condition. The frozen samples were then transferred to the chamber for sample fracture and sublimation (-90°C, 10 min). This was followed by gold-plating (10 mA current, 60 s). The cold stage was maintained at -140°C, and the acceleration voltage was 15 kV.

2.9 | Preparation of Lipid Extract

Lipids from emulsions were first extracted by using the Bligh and Dyer method with some slight modifications [32]. A 10 g emulsion was homogenized with a 30 mL mixture of methanol/chloroform, 1:1, v/v using an Ultraturrax at 10 000 rpm for 20 s. Two phases of the mixture were then separated with centrifugation (SIGMA 4-16KS, Sigma Laborzentrifugen, Germany) at 1402 × g for 10 min at 18°C. The aqueous phase, which formed the top layer, was decanted. The bottom layer, consisting of the chloroform phase, contained the lipid extract. This lipid extract was then collected, filtered and used for analysis of oil content, peroxide value (PV) and tocopherol concentration.

The oil content in lipid extract was calculated using the mass of the lipid extract and the mass of oil. The mass of oil was determined by the difference in weight before and after drying the lipid extract at 105°C for 3 h.

2.10 | Analysis of Primary Lipid Oxidation Products

Lipid extracts were analyzed for PV by following the official method from International Dairy Federation [33]. Briefly, peroxide compounds were reacted with ferrous ions to produce ferric ions. These ferric ions reacted with thiocyanate and formed a red complex of ferric-thiocyanate. This red ferric complex can be measured with spectrometer at 500 nm (UV-1280, Shimadzu, Japan), FeCl₃ solution at various concentrations was used for standard curve. PV was calculated as meq peroxide/kg oil (meq O₂/kg oil) and PV of lipid extract was duplicated for average results.

2.11 | Analysis of Tocopherols

Lipid extracts were analyzed for tocopherol content by following AOCS Official Method Ce 8–89 with slight modification [34]. Two grams of lipid extract were evaporated under a flow of nitrogen and then dissolved in 1 mL of n-heptane. The resulting lipid solution in n-heptane (1 mL) was injected into a Spherisorb S5 W column (250×4.6 mm) (Phase Separation, Deeside, UK). A guard column (Waters Spherisorb, 5µm Silica, 4.6 mm I.D. × 10 mm) was used as a pre-elution step. The elution was carried out using a mobile phase consisting of n-heptane/2-propanol, 100:0.4, v/v, at a flow rate of 1 mL/min. Tocopherols were identified using a fluorescence detector, with the excitation wavelength set at 290 nm and the emission wavelength set at 330 nm. Tocopherol homologues (alpha, beta, delta, and gamma) were analyzed using a mixture of standard tocopherols with known concentrations for calculations. Tocopherol concentration in each lipid extract was determined by integrating chromatogram peak areas. Each oil extract was measured twice for an average value in µg/g sample.

2.12 | Analysis of Secondary Volatile Oxidation Compounds

Volatile compounds were extracted by purging the mixture of emulsion (4 g) and an internal standard (4-methyl-1-pentanol, 30 mg) with N₂ at 150 mL/min and heated at 45°C for 30 min. Volatile compounds released from the mixture were passed through potassium hydroxide powder packed in S-shape glass tube before they were trapped into Tenax GR tubes [35]. These tubes were purged with N₂ for 20 min before they were transferred into a TurboMatrix 650 Automatic Thermal Desorber (Perkin Elmer, Norwalk, CN, USA) connected to a gas chromatograph (GC Agilent 6890 N, Palo Alto, CA, USA). Volatile compounds were released from Tenax GR tubes by thermal desorption at 200°C and flow of helium (80 mL/min). The desorbed volatile compounds were separated through a capillary GC column (DB-1701, 30 m × 0.25 mm × 1.0 µm). The temperature program for the GC oven was initially set at 45°C for 5 min, then increased to 55°C at an interval of 1.5 °C/min, then to 90°C at 2.5 °C/min, and finally to 220°C at 12 °C/min and held for 4 min. The MS parameters were set to scan mass to charge ratio between 30 and 250 amu and electron ionization mode at 70 eV. The compounds were identified based on retention time and mass spectrum by using MS library (Wiley 138 K, John Wiley and Sons, HewlettPackard).

For the calibration curve, eleven volatile compounds (1-penten-3-one, pentanal, 1-penten-3-ol, hexanal, 2-hexenal, heptanal, 4-heptenal, 2-pentyl furan, 2,4-heptadienal, 2,6-nonadienal, and 2,4-decadienal) were prepared with absolute ethanol and diluted to different concentrations from 0 to 100 µg/g solution. Each dilution of the standard solution (30 mg) was added to a control emulsion stabilized with sodium caseinate. This ensures that the standards of volatile compounds were released in a comparable way to the volatile compounds released in all other emulsions. All analyses were conducted in triplicate for average results.

2.13 | Statistical Analysis

Statistical analysis was performed with two-way ANOVA test to determine the significant differences ($p < 0.05$) with Origin Pro through Tukey's post hoc test.

3 | Results

3.1 | Characterizations of Particles

To address the question whether EHD co-jetting technique can be used to fabricate anisotropic particles from zein and caseinate, we loaded jetting solutions with two different dyes, zein with Nile red and sodium caseinate with fluorescein-isothiocyanate (FITC). CLSM was employed to identify Nile-red (red fluorescence) and FITC (green fluorescence) based on their distinct fluorescence emissions. Micrographs from CLSM suggest that two compartments were present within the particles, red from zein composition with Nile red and green from sodium caseinate with FITC (Figure 1a–c). The overlapping micrograph (Figure 1a) revealed that particles consist of two distinct regions, with a yellow interface separating between the red and green fluorescence in the confocal micrographs (Figure 1b and c). The particles after electrospraying on the collector were characterized with SEM. The particle sizes ranged from 0.2 to 2 µm, with up to 95% of the particles below 1.0 µm and an average diameter of 0.6 µm (Figures 1d and S1). Thermal analysis for caseinate, zein, and zein-caseinate particles was characterized by using DSC (Figure 1e). The glass transition temperature (T_g) was observed at 58°C and 67°C for caseinate and zein, respectively. The T_g of zein-caseinate particles was at 64°C. Major endothermic peaks for zein, caseinate, and zein-caseinate particles were observed at 124°C, 128°C, and 133°C, respectively. These peaks are very close to each other, but the zein-caseinate particles exhibited slightly higher thermal stability. The zeta-potential as a function of pH for zein-caseinate particles (Figure 1f) shows pI at 5.2, which is between those of sodium caseinate 4.5 and of zein at 6.5 [10, 36].

To elaborate the interfacial behaviors of zein-caseinate particles, the surface activity of zein, caseinate, and zein-caseinate particles was measured. As shown in Figure S2, the IFT between water and MCT oil was maintained at 26 mN/m. It was followed by the IFT of zein particles, at 24 mN/m, and maintained till the end of measurement. The IFT of sodium caseinate quickly decreased from 26 to 15 mN/m and slightly decreased to 14 mN/m at the end of measurement while zein-caseinate particles led to a gradual decrease of IFT from 26 to 18 mN/m over the same period.

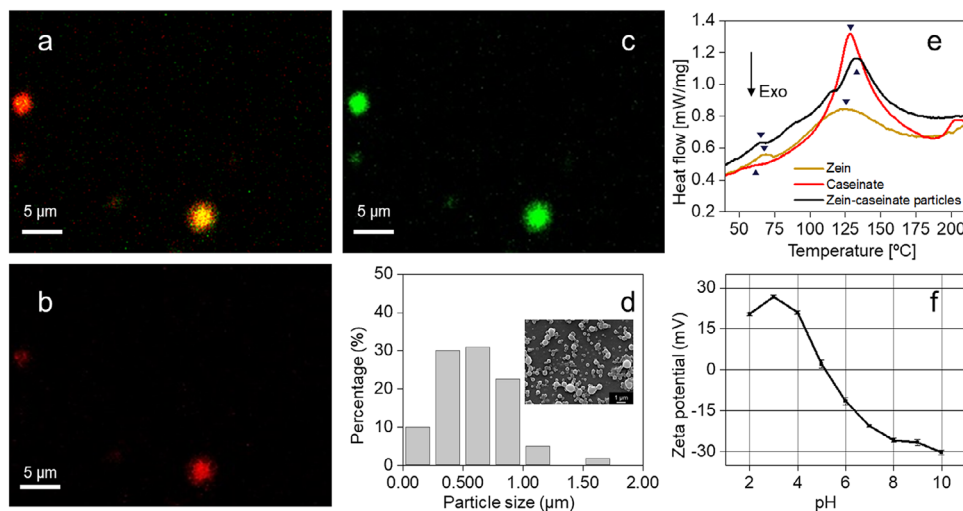


FIGURE 1 | CLSM images of bicompartmental zein-caseinate particles with Nile red for zein (b), FITC for sodium caseinate (c), and overlay of these two compartments (a). (d) Size distributions of zein-caseinate particles measured from SEM images. (e) DSC curves (exo down) and (f) zeta potential of zein-caseinate particles as function of pH from 2 to 10.

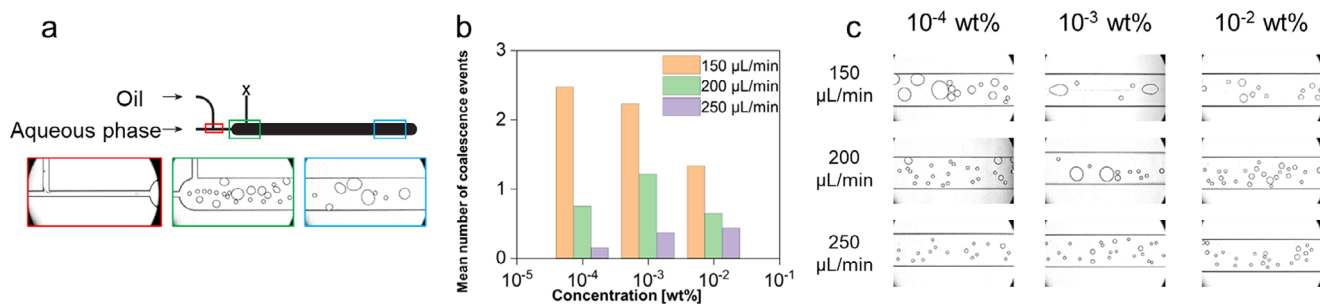


FIGURE 2 | Coalescence stability of Pickering emulsions stabilized with zein-caseinate particles. (a) Schematic diagrams for microfluidic coalescence cell with three positions, T-junction (red rectangle), position A at the beginning (green rectangle) and position B at the end of coalescence chamber (blue rectangle). (b) Mean number of coalescence events of Pickering emulsions stabilized with zein-caseinate particles. (c) Snapshot at position B at different concentrations and flow rate of emulsifier.

3.2 | Coalescence Stability With Microfluidic Device

The microfluidic coalescence cell was used to evaluate the coalescence stability of oil droplets produced under different conditions. Unlike surfactants, particles need to overcome an energy barrier before they can nest in the interface; hence, the flow rate of the continuous phase was varied. Because some of the droplets were very stable, we decided to investigate their stability at the end of the coalescence chamber (position B; Blue rectangle, Figure 2a).

The mean number of coalescence events and snapshots at position B are shown for three concentrations and three flow rates of zein-caseinate Janus particles (Figure 2b and c). Emulsions produced at a flow rate of 150 $\mu\text{L}/\text{min}$ were unstable with mean numbers of coalescence events at 2.5, 2.2, and 1.3 at concentrations of 10^{-4} , 10^{-3} , and 10^{-2} wt%, respectively. This is an indication that the shear was not sufficient to bring the particles effectively into the oil-water interface, or that bridging occurred. This was previously shown to be the case when using caseinate-covered solid lipid nanoparticles in the same coalescence cell [8]. There, the concentration of particles was much higher (5%),

and bridging occurred up till a continuous phase flow rate of 70 $\mu\text{L}/\text{min}$. Given the fact that our concentrations were much lower, it is not surprising that the transition to stable droplets occurred at a higher flow rate, and hence a higher number of particles available for interface stabilization. For a flow rate of 200 $\mu\text{L}/\text{min}$, the coalescence rate was considerably reduced for all concentrations, and at the highest flow rate of the aqueous phase (250 $\mu\text{L}/\text{min}$), the coalescence rate was even lower, albeit not zero. In previous studies, highly stable oil droplets with a very low number of coalescence events (almost zero) could be achieved with a higher concentration of emulsifiers [37]. More strongly even, the coalescence rate increased with the number of particles used, and that is typical for higher energy collision events at higher flow rate that may induce coalescence.

Coalescence stability of oil droplets stabilized with sodium caseinate under the same conditions as used for the zein-caseinate particles was also evaluated. In this experiment, the mean numbers of coalescence events were considerably reduced at increasing concentration of sodium caseinate (Figure S3). This aligns with the results from experiments with zein-caseinate particles and other previous studies using protein emulsifiers, where an increase in protein concentration led to a reduction in

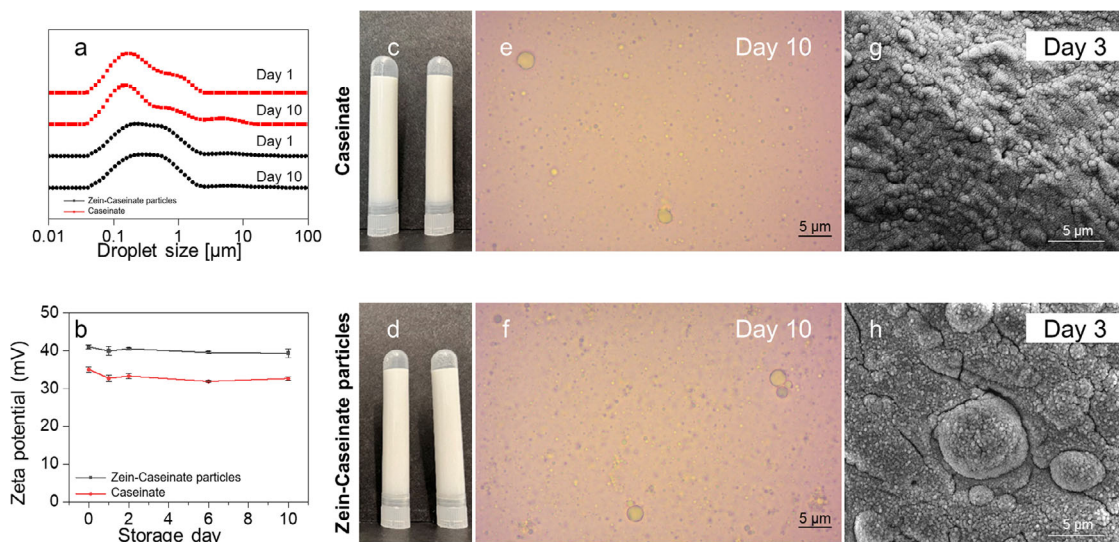


FIGURE 3 | (a) Size distributions, (b) zeta-potential, (c, d) photographs, (e, f) light microscopic images, and (g, h) Cryo-SEM image of emulsions stabilized with caseinate and zein-caseinate particles.

the mean number of coalescence events (over 0.5 g/L for non-oxidized pea protein and 0.1 g/L for whey protein isolate) [37, 38]. The effect of the flow rate on coalescence is most probably caused by the reduced number of droplets, and not by the properties of the droplets as such. The observation that oil droplets get stuck is an indication of a wettability change created by the protein on the wall of the microfluidic device. This has also been reported to be relevant for other devices [39, 40]. The fact that this is not observed when using Janus particles is an indication that wettability changes do not occur.

From the microfluidic observations, it is clear that there is a complex relationship between particle, coalescence dynamics, concentration, flow rate, and coalescence stability. Besides, it is good to point out that the volume fraction of oil is expected to play a role in this, although for droplets stabilized with regular surfactants this was shown not to have a very big effect, unless the concentrations are very high [41–43]. In conclusion, even for particle concentrations as low as used here, droplets gained considerable stability, which illustrates their efficacy in stabilizing droplets, which is next tested in emulsions for which both physical and chemical stability is investigated.

3.3 | Characterizations of Emulsions

Emulsions (2.5 wt% oil and 1 wt% protein) were prepared by using sodium caseinate or zein-caseinate particles. These emulsions were then evaluated for physical stability (zeta potential, droplet size, morphology) and oxidative stability (peroxide value, content of tocopherol homologues, content of secondary volatile products) over 10 days at room temperature.

3.4 | Physical Stability

Physical stability of emulsion refers to the ability of oil droplets to maintain their size over time. Emulsions stabilized with both types emulsifiers were observed without any gravitational

separation (Figure 3c and d). Their size distribution was analyzed (Figure 3a). On day 1, emulsions stabilized with caseinate exhibited bimodal droplet size distribution at 0.1 μm and 2.0 μm, which may have been aggregated droplets. On day 10, a new population with a size of 5 μm emerged without flocculation as shown in Figure 3e. In emulsions stabilized with zein-caseinate particles the droplet size was quite monodisperse and remained unchanged during the entire storage time from 0.1 to 2.0 μm.

The zeta potential of emulsions was evaluated over 10 days (Figure 3b), with values for the zeta-potential of the emulsions stabilized with zein-caseinate particles and caseinate of 40.9 ± 0.5 and 35.0 ± 0.6 mV on day 0, respectively. A slight decrease from 35.0 ± 0.6 mV on day 0 to 32.7 ± 0.8 mV on day 1 was observed in emulsions stabilized with caseinate that next remained constant. The zeta-potential remained stable around 40.0 mV over 10 days for emulsions stabilized with zein-caseinate particles.

Small oil droplets were observed in microscopy images of emulsions produced with caseinate and relatively big droplets in emulsions with zein-caseinate particles (Figure 3e and f). Oil droplets stabilized with zein-caseinate particles displayed a unique surface morphology characterized by the presence of small particles (Figure 3h). This feature was notably absent in caseinate-stabilized emulsions (Figure 3g).

3.5 | Oxidative Stability

In this experiment, the effect of caseinate and zein-caseinate particles on the oxidative stability of the emulsions was evaluated by tracking changes in the peroxide values (PV) and volatile compounds during 10-day storage period at 20°C. For oil content, PV, and tocopherols analysis, they were analyzed twice, and reproducible results were obtained. The oil content, from Bligh and Dye extractions varied from 2.2 to 2.3 wt%, compared to theoretical value at 2.5 wt% oil. The formation of lipid hydroperoxides in both emulsions was similar, with oxidation progressing rapidly over 10 days without showing a lag phase (Figure 4a). The

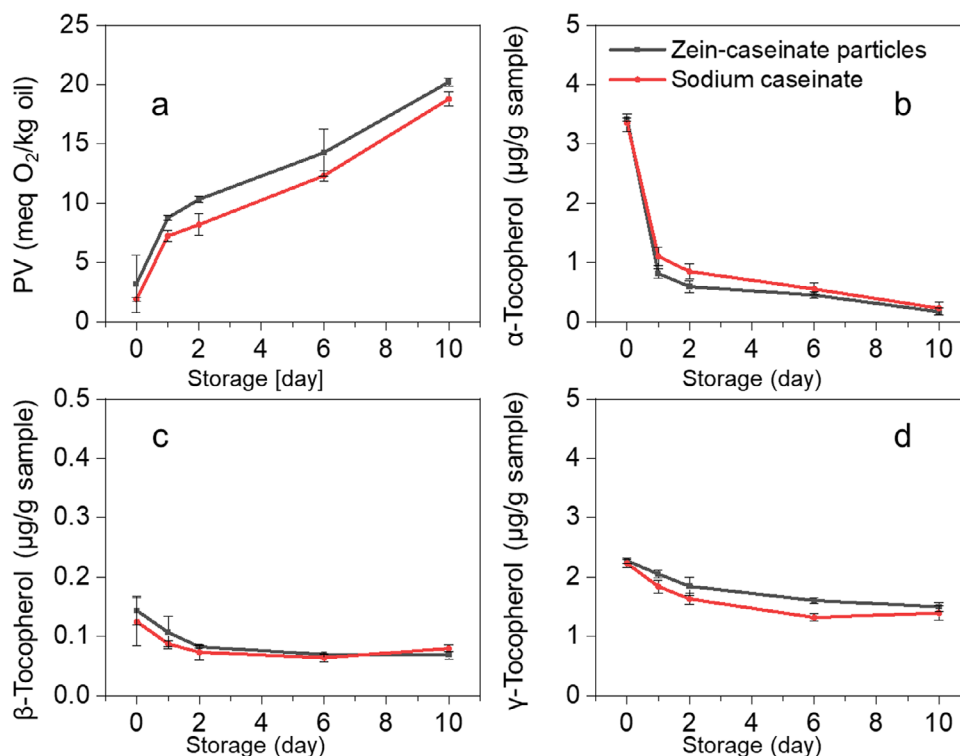


FIGURE 4 | (a) Peroxide values (PV) and contents of α -tocopherol (b), β -tocopherol (c), and γ -tocopherol (d) in emulsions stabilized with zein-caseinate particles (black) and caseinate (red) over 10 storage days.

peroxide value of caseinate was 1.89 ± 0.15 meq O₂/kg oil on day 0 and increased to 12.46 ± 0.63 and 14.76 ± 0.17 meq O₂/kg oil on days 6 and 10, respectively. A similar trend was observed with zein-caseinate particles, in which the PV on day 0 were 3.20 ± 2.42 meq O₂/kg oil and rose to 20.21 ± 0.32 meq O₂/kg oil by day 10, clearly showing PV increased over time and slightly higher than that of emulsions stabilized with caseinate.

The consumption of four tocopherol homologues in all emulsions was monitored and illustrated in Figures 4 and S4. While α -, β -, and γ -tocopherol levels decreased significantly, δ -tocopherol concentration fluctuated around $0.8 \mu\text{g/g}$ sample for all emulsions during the entire storage period (Figure S4). From day 0 to day 1, α -tocopherol was quickly consumed, decreasing drastically from 3.41 ± 0.03 to $0.81 \pm 0.08 \mu\text{g/g}$ sample for zein-caseinate particles and from 3.35 ± 0.15 to $1.10 \pm 0.15 \mu\text{g/g}$ sample for caseinate. After this, the decrease slowed down with values at day 10 being rather similar. A decrease in concentration of β - and γ -tocopherol was also observed in all emulsions during 10-day storage experiments (Figure 4c and d).

Formation of six volatile compounds in emulsions stabilized with zein-caseinate particles and caseinate is reported in Figure 5. The oxidative stability was considerably higher in emulsions stabilized with zein-caseinate particles than with conventional emulsifier, caseinate, unlike the PV values that were similar. The formation of 2,4-heptadienal was not observed at day 0. For emulsions made with zein-caseinate particles, 2,4-heptadienal increased to 10.92 ± 0.14 ng/g on day 1 and to 33.76 ± 16.67 ng/g on day 10, which is considerably less than in the caseinate emulsion, 102.61 ± 6.95 ng/g. A similar trend was observed in the formation of other volatile compounds, although hexanal had

already formed at a concentration of approximately 10.00 ng/g at day 0 in both emulsions.

4 | Discussion

Anisotropic Janus particles were fabricated by using EHD co-jetting processes and these particles were characterized with CLSM to distinguish two compartments with specific fluorescent dyes [30, 44]. In the present study, zein was stained with Nile red and sodium caseinate was mixed with FITC. Two jetting solutions were pumped through two needles in a side-by-side configuration. Two jetting solutions came into contact only at the tips of the needles. A collector connected to the counter-electrode was placed 15 cm away from the tips of the needles to gather particles. When an electrical potential, 25 kV, was applied, a single thread at the interface of two jetting solutions formed toward the collector. The liquid thread stretched quickly, causing its diameter to decrease significantly and forming tiny droplets. This formation of tiny droplets was accompanied by a significant increase in surface area, leading to the rapid evaporation of the solvent and the solidification of the protein particles. The solidification process took place more quickly than the mixing process between two compartments [45]. This rapid solidification preserved the original two-compartment geometry of the droplets and prevented the fluorescent dyes from mixing between compartments during the EHD co-jetting process. As a result, protein particles with two distinct compartments embedded with two fluorescent dyes were electrosprayed on the collector and visualized with CLSM. In line with previous studies, our results confirmed the formation of Janus particles with two compartments detected with CLSM. In the CLSM

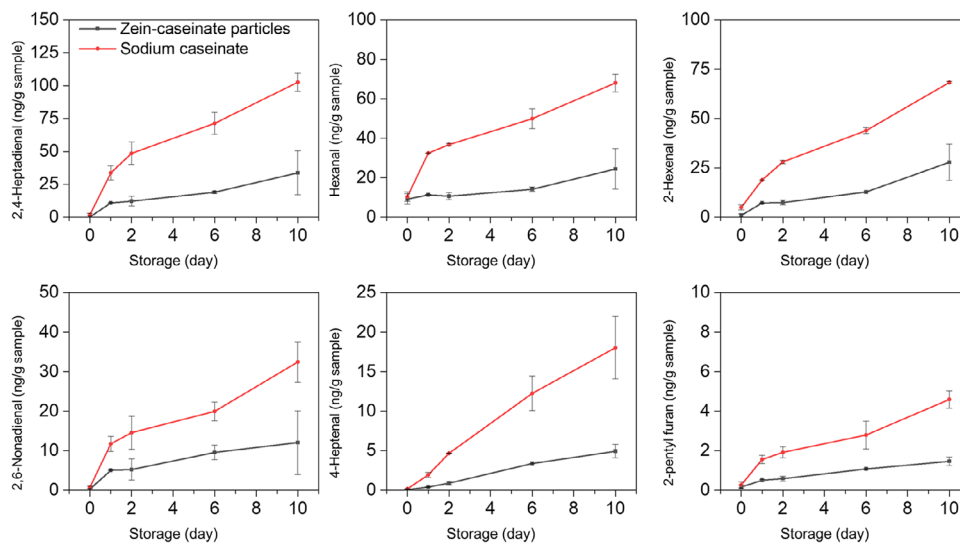


FIGURE 5 | Evolution of volatile compounds in emulsions stabilized with zein-caseinate particles (black) and caseinate (black) during 10 storage days.

micrographs, the yellow interface between the two compartments is a minor difference worth discussing compared to previous studies. In previous studies, using the same polymer matrix stained with two different dyes resulted in more uniform labeling and clearer distinction between compartments [30, 46]. In this study, however, the use of two different jetting solutions, zein with Nile Red and caseinate with FITC, might introduce a minor variation, leading to less distinct boundaries between the compartments. SEM images revealed that these particles consist of two distinct but perfectly joined compartments within a single particle. One compartment is smooth, spherical, and compact, while the other has an irregular structure with rough surface. Typically, the resulting zein nanoparticles, produced with various fabricating methods in previous studies, are smooth-surfaced nanospheres with sizes ranging from nanoscale to microscale [47, 48]. Another study found that coating zein particles with sodium caseinate altered the surface roughness [10]. This suggests that the smooth compartment originates from zein, while the irregular compartment is likely from caseinate. SEM analysis showed zein-caseinate Janus particles with a size distribution from 0.2 to 2 μm , about 0.6 μm for average size (Figures 1d and S1). This also aligns with dynamic light scattering measurements showing a size distribution of 200 to 700 nm for these Janus particles (Figure S5). In this study, the results indicate that the EHD co-jetting technique is suitable for fabricating anisotropic zein-caseinate Janus nanoparticles with two distinct compartments. This process results in an anisotropic surface structure, as evidenced by CLSM and SEM.

The formation of zein-caseinate particles in various fabricating methods is driven by different mechanisms and interactions, including electrostatic interactions, hydrophobic interactions, hydrogen bonds, and van der Waals forces. These driving forces significantly influence the physicochemical and structural properties of the nanoparticles [49]. This may enhance thermal stability and lead to the difference of these major endothermal peaks between zein, caseinate, and zein-caseinate Janus particles (Figure 1e). Regarding the shift of pI, it is similar to those reported in previous studies [10, 36]. The similarity of pI between zein-

caseinate Janus particles and other zein-caseinate particles may have been caused by similar composition, both have zein and caseinate and they can interact in comparable ways, leading to similar pI.

The interfacial behaviors of zein, caseinate, and zein-caseinate Janus particles were different. Sodium caseinate resulted in the lowest IFT. This result confirms that sodium caseinate molecules, as a conventional emulsifier, quickly diffuse to the oil-water surface and adsorb to the oil-water interface, where they rapidly decrease the interfacial tension until the IFT was maintained at 14 mN/m. This was not the case with zein particles, which only resulted in a slight decrease of the IFT. One interesting finding is that a gradual decrease of the IFT was observed in the case of zein-caseinate Janus particles. This implies that these Janus particles can improve the interfacial activity in comparison to homogenous zein particles. This decrease could be attributed to the composition or the geometry of zein-caseinate Janus particles. Janus particles were theoretically predicted to possess good stabilizing ability for Pickering emulsions [26]. In another research, the prediction was confirmed with Janus particles made of iron oxide nanoparticles and dodecanethiol when the IFT of Janus particles was lower than that of homogenous iron oxide nanoparticles [25]. Furthermore, the shape of Janus particles was observed to reduce the IFT, a finding corroborated by evaluations of Janus particles in disc, spherical, and cylindrical forms [50, 51]. This finding suggests that zein-caseinate Janus particles could slowly diffuse to the interfacial layer and adsorb to the oil-water interfaces, where they lower the IFT. Thus, the IFT results for zein-caseinate Janus particles suggest that they can considerably enhance the physical stability of Pickering emulsions. However, the decrease of IFT could also be due to the presence of free sodium caseinate, which is difficult to completely remove from zein-caseinate Janus particles.

In agreement with the promising IFT results obtained with Janus particles, emulsions stabilized with protein-based Janus particles were stable during 10-day storage in terms of physical stability in this study. As observed in previous research, when surface of zein

nanoparticles were modified with caseinate, physical stability of Pickering emulsions was enhanced significantly. In this study, size distribution of oil droplets is similar between two emulsifiers, ranging from 0.1 to 2.0 μm on day 1, and Pickering emulsions stabilized with Janus particles were observed without formation of big oil droplets on day 10. It may highlight the advantage of Pickering emulsions over conventional emulsions in terms of coalescence stability. It was also observed in a previous study with emulsions stabilized with starch and other surfactants. Size distribution of droplets stabilized with surfactant is comparable to those stabilized with mixture of starch particles and surfactant [52]. Droplet sizes of Pickering emulsions stabilized with zein-caseinate Janus particles were found to be from 0.1 to 2.0 μm during storage experiment. This is somewhat surprising given the fact that the particle size of zein-caseinate Janus particles was around 0.6 μm . A possible explanation for this might be a combined effect of abundant protein particles and the homogenization process applied. As Janus particles were abundant at high concentrations, oil droplet size significantly decreased with high-energy emulsification. Droplet size converged toward the particle size, as observed in previous studies Pickering emulsions stabilized with soy protein isolates [53]. Another explanation could be that the high-pressure emulsification affected the size of the protein particles; a small portion of particles below 100 nm was detected after treating the zein-caseinate particle suspension with the emulsification process (Figure S2). This suggests that some of the Janus particles were disintegrated into smaller fragments. The formation of small oil droplets by these smaller fragments may occur, with these droplets subsequently being stabilized by abundant protein particles in the continuous phase. Similar disintegration of Pickering particles has been observed in other studies when high-pressure homogenization methods were employed [1, 54].

Results from the IFT measurements and effects of emulsification process on zein-caseinate Janus particles suggest that there is possibility of surface-active component, sodium caseinate in this case, participate in stabilizing Pickering emulsions together with zein-caseinate Janus particles. Thus, they form mixed interfaces that are usually observed in food Pickering emulsions [3]. This characteristic makes food Pickering emulsions stand out from true Pickering emulsions that are solely stabilized with particles [14].

In this study, emulsions stabilized either with sodium caseinate or zein-caseinate Janus particles exhibited a positive surface charge at pH 3 (Figure 3b). It correlates with the zeta-potential of the proteins used (+30 mV) at pH 3 (Figure 1f). It aligns well with other studies using zein nanoparticles as Pickering particles at acidic conditions, Pickering emulsions were also observed with positive surface charge in these studies [2, 9]. The high surface charge plays a key role in preventing oil droplets from coming together to flocculate and destabilize. A high protein concentration in the bulk aqueous phase is also another factor contributing to preventing them from destabilizing. Pickering emulsions stabilized with sodium caseinate-coated zein nanoparticles also showed an enhanced physical stability at pH 3 and 9 [10, 18, 55]. Cryo-SEM analysis of emulsions stabilized with sodium caseinate and Pickering emulsions stabilized with zein-caseinate Janus particles showed a notable difference in the morphology of oil droplets between these two emulsions. Careful observation

shows that oil droplets were covered with small particles, and they were kept at a distance from each other due to a network of unadsorbed protein particles in the continuous phase (Figure 3h). The results of this observation from Cryo-SEM analysis suggest that zein-caseinate Janus particles can contribute to the physical stability of Pickering emulsions via two main mechanisms. Zein-caseinate Janus particles adsorb to the oil-water interfaces and form a thick layer of Janus particles covering oil droplets. Oil droplets are also surrounded by a network of unadsorbed protein particles. The network hinders the movement of oil droplets, thus limiting their interactions. A similar mechanism of Pickering stabilization was reported with zein and cellulose nanomaterials [56]. Despite these interesting findings, Cryo-SEM analysis did not reveal how zein-caseinate Janus particles orient at the oil-water interface. Further studies working with CLSM analysis may help to address the remaining question [46].

On the question of chemical stability, this study found that zein-caseinate Janus particles did not prevent the formation of primary products, lipid hydroperoxides, but the secondary ones, volatile compounds. A similar increase in PV and decrease of tocopherol homologues were illustrated in Figure 4. In Figure 5, a slow formation of various volatile compounds was observed in emulsions stabilized with zein-caseinate Janus particles, in comparison to those stabilized with sodium caseinate. Several factors can affect lipid oxidation, such as tocopherol content in the lipid phase, emulsification process, droplet size, surface charge, and non-adsorbed protein emulsifiers in the continuous phase.

Tocopherol plays a key role in preventing the formation of peroxide compounds in this system. As tocopherol homologues were depleted on day 1 and maintained at a low level in the following days, the PV increased linearly till the end of storage experiments (Figure 4), with no distinction between the emulsions. Increase of PV and depletion of tocopherol homologues in this research are in line with other studies [57, 58]. In this study, the most consumed homologue is α -tocopherol due to its superior hydrogen-donating ability, attributed to its two ortho-methyl substituents [59].

The emulsification process had an effect on PV since the initial values in the emulsions on day 0 were higher than in fish oil (Figure 4a). This indicates that lipid hydroperoxides were formed during the emulsification process. Both fish oil and emulsions were exposed to the ambient environment during the process, and this can initiate lipid oxidation.

Lipid oxidation can be affected by droplet size because smaller droplets have a larger specific surface area and thus more chances for lipid-aqueous phase interactions. In this study, although the size distributions between the emulsions with the two different emulsifiers were similar, the differences in formation of secondary volatile compounds between emulsions stabilized with sodium caseinate and food Pickering emulsions indicate that droplet size was not the only significant factor affecting lipid oxidation in fish oil-in-water emulsions. This finding broadly supports the previous research working with fish oil-enriched milk [60]. The research also pointed out the importance of interfacial composition.

As mentioned in the introduction, lipid oxidation of emulsions takes place mainly at the oil-water interface. Thus, modification

of the interface with an emulsifier that can form a strong physical barrier and has antioxidant activity would delay the lipid oxidation. A slow formation of secondary products of lipid oxidation was observed in Pickering emulsions stabilized with zein-caseinate Janus particles. As indicated by Cryo-SEM analysis, oil droplets were covered with small particles. At the interface, zein-caseinate Janus particles offer a synergistic dual functionality, where the zein compartment may form a robust barrier and the caseinate compartment may exhibit antioxidant activity. This underscores the critical role of their combined properties. This result corroborates the findings of previous work with Pickering emulsions stabilized by zein-gallic acid nanoparticles [17]. When gallic acid and zein are combined via covalent bonding, Pickering emulsions stabilized these nanoparticles exhibited superior oxidative stability, and this can be attributed to the co-existence of zein and gallic acid at the interface [17].

Oil droplet surface charge has also been suggested as an important factor limiting the interaction between trace metal ions and lipid hydroperoxides. Positively charged oil droplets can repel trace metal ions away from the oil-water interface. The order of magnitude of the surface charge was quite similar for both emulsions (both positive), which may have led to repulsion of the positively charged pro-oxidant ferrous ions. Besides, the emulsifier present in the aqueous phase may have bound ferrous ions. Our emulsions were produced with a considerable excess amount of protein, and the estimation of non-adsorbed protein in emulsions stabilized with caseinate was 9.420 g/L with a theoretical surface load at 3 mg/m² (Table S1). As mentioned in the former review, the surface load of Pickering particles is closely linked to the size and density of the adsorbed particles and can greatly exceed that of biopolymers by several orders of magnitude [14]. In this case of zein-caseinate Janus particles, the value of theoretical surface load may be considerably larger than the value for caseinate, leading to lower non-adsorbed protein concentration in the aqueous phase. Together with positive surface charge at the interface, this may have helped to keep ferrous ions away from the oil-water interface and improve oxidative stability because the concentration of adsorbed protein was higher than the non-adsorbed protein concentration [20]. Furthermore, a network of unadsorbed protein particles and small fragments of disintegrated Janus particles also hinders the mobility of metal ions; thus, it lowers the interaction between these ions and lipid hydroperoxides.

5 | Conclusions

EHD co-jetting of zein and caseinate was successfully used to produce Janus particles with two compartments, as confirmed with CLSM and SEM. Droplets stabilized with zein-caseinate particles showed excellent coalescence stability as shown by microfluidic analysis. Stable fish oil-in-water emulsions can be produced with caseinate and zein-caseinate Janus particles at pH 3. The use of the Janus particles improved the coalescence stability and reduced the formation of secondary oxidation products considerably. Still, some limitations that were not addressed in this study are how zein-caseinate Janus particles orient at the oil-water interface and to what extent the emulsification process has effects on zein-caseinate Janus particles. A greater focus on the orientation of zein-caseinate Janus particles at the oil-water

interface would provide more insights into the physicochemical properties of these Pickering emulsions. Further study could assess the effects of homogenization on zein-caseinate Janus particles. Notwithstanding these limitations, this study confirms the ability of the EHD co-jetting technique to fabricate zein-caseinate Janus particles and that the Janus particles exhibited dual stability effects to provide physically and oxidatively stable food Pickering emulsions.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File 1: ejlt70065-sup-0001-SuppMat.docx