Salt and pH-Induced Attractive Interactions on the Rheology of Food Protein-Stabilized Nanoemulsions

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Supporting Information

ABSTRACT: This research aimed to investigate the possibility of forming gelled nanoemulsions (NEs) by inducing attractive interactions among the nanodroplets. The effect of salt concentration and changes in pH on the stability and gelation behavior of 2, 4, and 5% sodium caseinate (SC) and whey protein isolate (WPI)-stabilized 40% canola oil-in-water NEs were investigated. For the effect of salt, sodium chloride was added in a concentration of 0.1, 0.5, and 1 M in the continuous phase of the NEs at neutral pH, whereas to study the effect of acidification, the pH of the NEs was adjusted to the isoelectric point (pI) of the proteins. The addition of salt led to attractive gelation in WPI NEs because of a screening of charge. In contrast, the gel strength of SC-stabilized NEs was reduced with salt, which was attributed to the loss of close packing of droplets and their surrounding repulsive barriers because of charge screening and to the steric barrier of interfacial SC preventing droplet aggregation. All the NEs with pH at the pI of proteins transformed into strong attractive gels made of droplet aggregates irrespective of the type or concentration of protein because of the complete charge neutralization. The strength of the acidified NE gels increased with a decrease in droplet size and the type of protein used. Overall, research on the effect of different environmental factors on the stability and gelation behavior of protein-stabilized NEs could be useful for possible applications of these nanoscale materials in various food systems.

INTRODUCTION

The emulsion is an integral part of many foods and related soft materials. Milk, coffee creamer, and mayonnaise are some of the typical examples of oil-in-water (O/W) emulsions that we use in daily life. In conventional food emulsions, dispersed phase droplet sizes are generally in the range of 1–100 μm. Nanoemulsions (NEs), whose average droplet size is less than 200 nm (0.2 μm), have recently caught the attention of researchers because of their advantages over conventional emulsions.1,2 NEs are unique because of their significantly higher stability and decreased opacity. Researchers have also shown that merely a reduction of average droplet size of emulsions to the nanoscale (<200 nm) converts them into NE gels. Gelation in NEs happens at a lower oil droplet volume fraction than that of conventional emulsions.3,4 Wilking & Mason showed that a decrease in droplet radius below ~75 nm could transform a sodium dodecyl sulfate (SDS)-stabilized 40% silicone oil monodisperse NE into a strong NE gel. The authors attributed the gelation to an increase in effective droplet volume fraction (ϕeff) owing to the surrounding repulsive charge cloud leading to a close-packed structure.5 Erramreddy & Ghosh5 have also reported similar findings for polydisperse SDS-stabilized canola oil-in-water NEs made, which showed an increase in viscosity and gel strength when the surface average droplet size (d32) was reduced to less than 200 nm. Depending on SDS concentration, these were repulsive nanogels formed because of droplet random jamming or attractive nanogels formed by micelle-induced depletion attraction.6

Recently, we have shown that even protein can be used to form repulsive NE gels. For example, sodium caseinate (SC)-stabilized emulsions have shown an increase in storage moduli (G′) with a decrease in average droplet size.5,7 SC-stabilized NEs (SC NEs) prepared with 5% SC and 40% oil transformed into a nanogel at an average droplet size of ~150 nm. The gelation at 40% dispersed phase is significantly lower than 64% needed for close packing in monodisperse emulsions.8 The gelation in this SC NE at a lower oil volume fraction was attributed to the combined contribution of the thickness of the protein steric layer and the repulsive charge cloud around them leading to an increase in ϕeff to more than 0.7, which was higher than the random jamming volume fraction (ϕRJ). However, when the NEs were formed with whey protein isolate (WPI), no gelation was observed with 40% oil and 5% WPI, even if the average droplet size was less than 200 nm.7 The lack of gelation in WPI-stabilized NEs (WPI NEs) was

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ascribed to the significantly shorter steric layer (∼2 nm) owing to the compact globular structure of WPI compared to a much longer steric layer of the flexible casein molecule (∼10 nm) leading to a low \( \phi_{\text{eff}} \). The lower \( \phi_{\text{eff}} \) was responsible for the lack of droplet random jamming and resulted in flowable liquid-like to weak gel behavior in most of the NEs. This led us to the aim of this study, to investigate the possibility of forming NE gels by inducing attractive interactions among the protein-coated nanodroplets. Two different approaches were used to induce attractive interactions among the nanodroplets: addition of salt to screen the repulsive charge cloud around the droplets and altering the pH to produce an attractive interaction at the protein isoelectric point (pI). The gelation behavior of the attractive NEs was investigated as a function of protein type (SC and WPI) and concentration.

■ RESULTS AND DISCUSSION

Effect of Salt Concentration on the Gelation in NEs. Influence of Salt Concentration on Droplet Size, Charge, and Interdroplet Interactions. Figure 1 shows the average droplet size of all NEs at different salt concentrations in the aqueous phase. When no salt was present, the average droplet size was smaller for 4% protein-stabilized emulsions compared to 2% protein, which could be due to the greater lowering of interfacial tension at higher emulsifier concentration and availability of more protein to cover the increased surface area of smaller droplets. A very different effect of salt...
on SC and WPI NEs can be seen, where a noticeable increase in the average droplet size was observed with the addition of salt for the WPI NEs but not for SC NEs. For example, for 3% WPI NEs, the droplet size increased from 0.16 ± 0.02 μm for no salt to 22.8 ± 0.3 μm with 1 M salt. However, SC NEs did not show much change with the increase in salt concentration beyond 0.1 M. For example, for 5% SC NEs, from no salt to 0.1 M salt, droplet size increased from 0.15 ± 0.01 to 0.21 ± 0.01 μm; however, thereafter it only increased to 0.24 ± 0.04 μm when 1 M salt was added. It should be mentioned here that the average droplet size of salt-added NEs reported here are not the true size of the oil droplets, rather the size of polydisperse aggregates of droplets with various shapes. This is a drawback of laser diffraction analysis, which measures the size of the whole aggregate diffracting laser. Nevertheless, these results indicate that the addition of salt has a profound effect on WPI NEs, whereas it is virtually ineffective on SC NEs in the present concentration range. This variation of droplet size also resonates with the droplet size distributions of the salt-added NEs (Figure 2). In SC NEs, the addition of salt led to a slight increase in the peak above 1 μm, whereas the peak below 1 μm showed a slight decrease (Figure 2A,C,E). For 5% SC, however, a significant increase in the large droplet size peak was observed (Figure 2E). For WPI NEs with 2% protein, the droplet size distribution moved toward a bigger size range even upon addition of 0.1 M salt (Figure 2B). For NEs with 4% WPI, addition of 0.1 M salt did not significantly change the droplet size distribution, but 0.5 and 1 M salt led to multimodal droplet size distribution with a couple of large peaks at higher droplet sizes (1–500 μm), whereas the peak below 1 μm was significantly reduced (Figure 2D). For 5% WPI, the changes were even more significant, with the large droplet size peak reaching beyond 600 μm when 1 M salt was added (Figure 2F). Such an increase in size distribution could be due to coalescence or aggregation, as aggregated droplets act as one large droplet in the laser diffraction particle size analyzer. To determine the exact mechanism of the increase in droplet size, 0.5% SDS solution was gently mixed with the emulsion in a 1:5 ratio to break any floccs before droplet size analysis (see Supporting Information Figure S1). The results demonstrated a significant shift in droplet size distribution to smaller sizes upon SDS addition, which indicates that the increase in droplet size was mostly due to extensive droplet aggregation upon addition of salt.

The effect of salt addition on the screening of the droplet charge was confirmed by measuring the zeta potential (a measure of charge on droplets) of the NEs. The results shown in Figure 3 support the hypothesis about the decrease in zeta potential of emulsions with the addition of salt irrespective of the protein type and concentration. The control NEs (without any salt) had a zeta potential ranging from −50 to −70 mV. With the addition of only 0.1 M salt, the zeta potential decreased to values ranging from −10 to −30 mV. With further increase in salt concentration up to 1 M, only a slight decrease in zeta potential was observed, and the minimum zeta potential was reported to be about −10 mV. From Figure 3, almost similar values of zeta potential for SC and WPI-stabilized NEs were observed at high salt concentrations, although extensive droplet aggregation was only observed for WPI NEs. This could be due to the difference in the structure of the proteins. As for SC, even after screening of charge by the addition of salt, the strong steric barrier by the hydrophilic tail of SC (which extends toward the aqueous phase for about 10 nm) would create enough steric repulsion between the droplets to stop them from causing attractive aggregation. The stability of SC-stabilized emulsions against salt-induced aggregation has also been reported and explained by Dickinson in the late 1990s. Casanova and Dickinson showed the excellent stability of mixed αs1-casein- and β-casein-stabilized emulsion against flocculation at an ionic strength as high as 2 M NaCl when about 40% of the adsorbed protein consisted of β-casein and up to 1 M NaCl (highest salt conc. in our samples) when 30% of the adsorbed protein consisted of β-casein. It was also shown that the emulsion stabilized with only αs1-casein was extensively flocculated above 0.1 M NaCl, whereas β-casein-stabilized droplets were not affected. It was proposed that the emulsions prepared with αs1-casein were stabilized by high surface charge density, whereas the main stabilization mechanism of β-casein was steric repulsion. Hence, in the presence of salt, a net attraction prevails for αs1-casein-stabilized emulsions, whereas strong steric repulsion prevented flocculation among the β-casein-stabilized droplets. Sodium caseinate is known to contain a roughly equal amount of both αs1 and β-casein; hence, the NEs in the present research were stable against salt-induced flocculation. WPI, on the other hand, forms a compact globular structure at the oil droplet surface, extending only about 2 nm in the aqueous phase, which is probably not enough to prevent them from aggregation. Increase in the average droplet size of WPI-stabilized conventional emulsions owing to the addition of salt at neutral pH has also been reported by many researchers and was attributed to the inability of WPI to prevent the close approach of the droplet because of salt-induced charge screening. DOI: 10.1021/acsomega.8b03360

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A better understanding of the effect of salt concentration on the droplet aggregation behavior can be obtained from their DLVO interdroplet pair potential. Details of the calculation and the plots of overall interaction potential as a function of salt concentration (0–1 M) on the storage ($G'$, filled) and loss ($G''$, open) moduli as a function of % strain at a constant 1 Hz frequency for SC-stabilized NEs with 2% (circle, A–D), 4% (triangle, E–H), and 5% (square, I–L) protein.

Figure 4. Effect of salt concentration (0–1 M) on the storage ($G'$, filled) and loss ($G''$, open) moduli as a function of % strain at a constant 1 Hz frequency for SC-stabilized NEs with 2% (circle, A–D), 4% (triangle, E–H), and 5% (square, I–L) protein.

Figure 5. Effect of salt concentration (0–1 M) on the storage ($G'$, filled) and loss ($G''$, open) moduli as a function of % strain at a constant 1 Hz frequency for WPI-stabilized NEs with 2% (circle, A–D), 4% (triangle, E–H), and 5% (square, I–L) protein.
interdroplet distance is given in the Supporting Information (Figure S3). The major findings from the interdroplet pair potential are that the addition of just 0.1 M salt significantly shifted the interdroplet potential from strongly repulsive to weakly attractive with the curve going through secondary minima in an attractive interaction. The depth of the minima and the strength of the attractive interaction increased with increase in salt concentration, which could lead to droplet aggregation. Beyond the secondary minima, on further approach, the droplets face a strong electrostatic repulsion but at a much shorter interdroplet separation compared to no-salt-added emulsions. Of all the NEs at all salt concentrations, attractive interaction was maximum for 2% WPI NEs (Figure S3B) followed by 2% SC NEs (Figure S3A), which could be due to their larger initial droplet size compared to NEs with 4 and 5% protein (Figure 2). Among the three WPI NEs, 2% WPI showed a substantial increase in droplet aggregation upon addition of just 0.1 M salt (Figure 2B), which matches with their higher attractive secondary minima as shown in Figure S3B. The initial droplet size of the NEs plays a vital role in salt-induced aggregation where larger droplets (NEs with 2% proteins) led to more depth in the secondary minima and a higher probability of droplet aggregation. Salt-induced attractive interaction decreased with an increase in protein concentration because of the lowering of oil droplets’ size. However, no significant difference in attractive interactions can be seen between the SC and WPI-stabilized NEs with 4 and 5% protein concentrations (Figure S3C–F), despite the significantly higher droplet aggregation seen in WPI NEs (Figure 2D,F) compared to SC NEs (Figure 2C,E). It should be noted that in the calculation of the DLVO interaction, the effective droplet size was considered as the actual droplet size plus the steric layer of interfacial protein. Hence, although the DLVO attractive interaction appeared similar for SC and WPI NEs, because of the more extended steric layer of SC-coated droplets, SC NEs were stable against droplet aggregation. In contrast, a small steric layer of WPI could not prevent the close approach of the droplets and subsequent aggregation.

Visual Observation of Flow Behavior. Photographs of visual representation of the NEs in a glass beaker are provided in the Supporting Information (Figure S2). The SC NEs with 2 and 4% protein were flowable, and the addition of salt did not change their flow behavior. SC NEs with 5% protein and without salt were a strong gel and did not flow under gravity. However, addition of just 0.1 M salt broke the gel into a flowable liquid. In contrast, all WPI NEs without salt were flowable liquids; however, with the addition of salt, they form a nonflowable gel with a self-supporting structure.

Influence of Salt Concentration on Viscoelasticity. The viscoelasticity of the NEs was measured as a function of strain at a constant frequency to understand the effect of salt concentration on their gel strength. Figure 4 shows the G’ (storage modulus) and G” (loss modulus) of SC NEs with 2, 4, and 5% protein at a different concentration of salt. Figure 5 shows similar data for WPI NEs. A higher G’ compared to G” in the low-strain regime indicates the presence of a gel structure (both weak and strong gelation), whereas the appearance of a linear viscoelastic region (LVR), where G’ remain unchanged as a function of strain, indicates strong gelation. For all SC NEs, G’ > G” in the low-strain regime; however, except for 4 and 5% SC NEs without salt, none of them showed any LVR, indicating weak gelation. For 5% SC NE without salt (Figure 4I) the presence of a firm LVR and the values of G’ ≈ 650 Pa indicates a strong NE gel, which also matches well with their restricted flow behavior (no flow according to Figure S2). For 4% SC NE (Figure 4E), although an LVR was observed, the G’ values were ~77 Pa, indicating weak gelation (flow under gravity according to Figure S2). The weak versus strong gelation behavior was confirmed by frequency sweep analysis at a constant strain (0.1%) within the LVR (data not shown). Beyond the low-strain regime (until 1% strain), the values of G’ dropped at a critical yield strain, followed by a crossover of G’ and G”. The crossover indicates
Isoelectric point of protein (pI) was considered as 4.6 for SC and 5.0 for WPI NEs.

From the strain sweep data from Figures 4 and 6 were re-plotted against the salt concentration (Figure 6A,B). For the SC NEs with 4 and 5% protein, a drop in G’ increased drastically with salt concentration till 0.5 M salt followed by a plateau to 1 M. The values of gel strength with 0.5 M salt and higher were much higher for WPI NEs compared to SC NEs. This behavior indicates that the addition of salt affects WPI NEs more profoundly and leads to stronger gel formation at a higher salt concentration, whereas SC NEs showed a drop in the gel strength at low salt concentrations and are quite unaffected by the presence of a high level of salt in the aqueous phase.

Figure 7. Average droplet size ($d_{z}$) of (A) SC and (B) WPI NEs containing different protein concentrations at pH 7.0 and pH = pI of protein. Isoelectric point of protein (pI) was considered as 4.6 for SC and 5.0 for WPI NEs.

A breakdown of the gel structure, beyond which both the $G'$ and $G''$ dropped linearly and $G''$ remain higher than $G'$, indicating a liquid-like behavior. For the samples without any salt, $G''$ showed a peak at the crossover, which indicates structural relaxation of the repulsive gel where the close-packed nanodroplets break away from the cage. However, with the addition of salt, specifically with 0.5 M salt and higher, the peak in $G''$ disappeared, which could be attributed to the reduction of the packed structure because of the salt-induced lowering of repulsive electrostatic interactions among the nanodroplets.

For the NE with 4 and 5% SC without salt (Figure 4E,I), the LVR in the low strain regime disappeared upon addition of just 0.1 M salt (Figure 4F,J), indicating a weakening of the gel structure with salt-induced lowering of Debye screening length and effective oil volume fraction. The strong to weak gel conversion upon addition of salt was also confirmed by frequency sweep analysis (data not shown). This phenomenon of the decrease in gel strength with the addition of salt was called “melting” of the gel structure and observed in the case of SDS-stabilized nanogels (Fryd & Mason 2012). This effect can be attributed to the weakening of the repulsive gel structure by the screening of droplet charge, which led to a reduced effective volume fraction and elimination of close-packed structure. With the increase in salt concentration, the repulsive interaction further decreased, but as SC-stabilized droplets were stable against aggregation because of its more extended steric barrier, no significant change in viscoelasticity was observed even with 1 M salt (Figure 4H,L).

Contrary to the SC NEs, WPI NEs showed an opposite behavior with the addition of salt (Figure 5). The control WPI NEs without salt were either weak gel (2% WPI NE, Figure 5A) or liquid-like (4 and 5% WPI NEs, Figure 5E,I). However, with the addition of salt, the gel strength increased and at 0.5 M salt concentration, a clear LVR appeared for all WPI NEs, thereby converting a weak gel and liquid-like NEs into a strong gel (Figure 5C,G,K). Salt-induced gelation because of a screening of electrostatic repulsion is a well-known phenomenon, and in this case, unlike SC, the lower steric barrier of WPI could not prevent the droplets against aggregation.

In order to better compare the effect of salt concentration on the gel strength of the various SC and WPI NEs, their plateau storage moduli ($G''$) values at 0.15% strain (within the LVR) from the strain sweep data from Figures 4 and 6 were re-plotted against the salt concentration (Figure 6A,B). For the SC NEs with 4 and 5% protein, a drop in $G''$ was observed with the addition of 0.1 M salt. For example, 4% SC NE showed a decrease in $G''$ from 77.6 ± 24.8 with no salt to 23.7 ± 6.2 Pa upon addition of 0.1 M salt ($p > 0.05$) and 5% SC NE showed a drop in $G''$ from 624.7 ± 7.7 with no salt to 112.4 ± 11.8 Pa upon addition of 0.1 M salt, both of which indicate the gel-melting behavior as discussed before. In contrast, for WPI NEs, $G''$ increased drastically with salt concentration till 0.5 M salt followed by a plateau to 1 M. The values of gel strength with 0.5 M salt and higher were much higher for WPI NEs compared to SC NEs. This behavior indicates that the addition of salt affects WPI NEs more profoundly and leads to stronger gel formation at a higher salt concentration, whereas SC NEs showed a drop in the gel strength at low salt concentrations and are quite unaffected by the presence of a high level of salt in the aqueous phase.

From zeta potential measurement, we have seen that the reduction in droplet charge with the addition of salt was very similar for all NEs (Figure 3). Therefore, it is not due to the charge, rather the stronger steric repulsion from the SC molecules on the oil droplet surface, which extends toward the aqueous phase for about 10 nm, which prevented aggregation of the oil droplets. For WPI, the steric barrier is much smaller, reported to be about 2 nm in length; hence the droplets, in the absence of a strong electrostatic repulsion (as shown in Figure 4) were able to form stronger aggregates, as evident from their droplet size distribution reported in Figure 2. The strong attractive aggregates of WPI-stabilized nanodroplets in the presence of salt led to a substantial increase in gel strength compared to the SC NEs. Cold gelation in WPI-stabilized conventional emulsion (average droplet size > 1 μm) has previously been reported by many authors. However, in most cases, a heat-denatured protein was used to make emulsions, followed by the addition of salt to induce gelation. Heat denaturation opened up whey protein molecules and improved the formation of intermolecular and interdroplet interactions which led to strong gelation. An excess amount of proteins (9.5 wt%) was also used, which facilitated network formation in the continuous phase, and the protein-covered oil droplets acted as an active filler, thereby increasing the overall gel strength. In the present case, no heat treatment was used, and the formation of extremely small nanodroplets (and the corresponding high surface area) led to the lower amount of excess WPI in the continuous phase. Together, these two factors led to lower values of gel strength compared to the Line et al. and Rosa et al. Nevertheless, formation of self-supporting nanogels from WPI NEs in the presence of salt and without using any heat treatment and acidification could be a novel way to utilize these materials in food and related applications.

The $G'$ and $G''$ crossover strain is the force required to break a gel and hence gives further indication of the strength of a gel...
to withstand the applied force before breakdown. For comparison, the crossover strain of all NEs was also plotted from Figures 4 and 5 into Figures 6C and 7D as a function of the salt concentration. The crossover strain mostly follows a similar pattern to the $G_s'$ values. A large increase in crossover strain was observed for 5% SC NE with 1 M salt compared to the other NEs, whereas for WPI NEs with 5% protein, a substantial increase in crossover strain was observed with an increase in salt concentration to 0.5 M. Without any salt, no crossover was observed for 5% WPI NE. A high value of crossover strain indicates that the gels were stretchy under applied shear, which could be due to the presence of stronger droplet aggregates or the presence of smaller droplets with excess proteins in the emulsion continuous phase.

**pH-Induced Attractive Gelation in NEs. Influence of the Protein Isoelectric Point on NE Droplet Size and Charge.**

Attractive gelation in the SC and WPI NEs was induced by changing the pH to the protein’s isoelectric point ($pI = pI$), where the charge on the droplets would be neutralized. The PI used for SC and WPI NEs were pH 4.6 and pH 5.0, respectively. The droplet size analysis of NEs was carried out at $pH = pI$. As shown in Figure 7, for all samples, a steep increase in the surface average droplet diameter ($d_{av}$) was observed at $pH = pI$ of the proteins compared to $pH > pI$. The droplet size distribution is also essential to understand the shift in droplet size upon a change in pH (see Supporting Information Figure S3). For the NEs at $pH = pI$, droplet distribution shifted to significantly larger multimodal peaks at droplet size ranging from 10 to more than 1000 μm. This change in size distribution justifies the difference in average droplet size upon changing the pH to $pI$, where the droplets lost their charge and repulsive interaction. The zeta potential of the NEs at $pH = pI$ was also measured to confirm the change in droplet charge and understand the mechanism of droplet aggregation or gelation in the NEs. As reported in Figure 3, all NEs had a high negative zeta potential at $pH = pI$ between $-50$ and $-65$ mV for SC NEs and between $-47$ and $-60$ mV for WPI NEs. The zeta potential reached near zero for NEs with $pH = pI$ (ranging from 0.06 to 0.5 mV for all NEs), indicating cancellation of charge upon bringing the pH to the PI of the protein. Therefore, significant electrostatic repulsive interaction among the droplets at a pH away from $pI$ was cancelled at $pH = pI$. The cancellation of the repulsive charge over the droplet makes them susceptible to aggregation owing to hydrophobic interactions leading to attractive gelation in the NEs. Extensive droplet aggregation in acidified protein-stabilized emulsions near the $pI$ was also reported by others.²³,2⁴

**Visual Observation of the NEs at $pH = pI$.** When the pH of the NEs reached the $pI$ of the proteins, strong gels were formed, which did not flow upon tilting the beakers (see Supporting Information Figure S4). All SC NEs at $pH = pI$ showed a grainy and coarse texture, whereas the samples at $pH$ away from $pI$ showed a viscous liquid-like flow behavior, except 5% SC NE, which formed a strong repulsive gel with a soft and smooth texture (Figure 8). In contrast, all WPI NEs at $pH 7$ were liquid and when the pH reached the $pI$ of WPI, they formed strong gels, but with a smooth texture, unlike the grainy texture of SC NEs.

**Compression Analysis of the NEs at the Protein’s Isoelectric Point.** The viscoelasticity of the NEs at $pH = pI$ could not be measured as the strongly aggregated attractive gels could not be uniformly compressed into the required gap between the two plates in the rheometer. The acidified gels were hard, self-standing, and nonflowing, which made it difficult to achieve the desired gap for the rheological measurement. Therefore, we have performed compression analysis for these samples using a texture analyzer to compare the firmness of the aggregated gels made by both the SC and WPI NEs at pH values equal to the $pI$ of the protein (Figure 9). The results of the compression analysis report the maximum force needed to compress the aggregated gels up to a certain distance, thereby indicating the firmness of the NE gels. For 2 and 4% protein containing NEs (which were liquid to weak gel at $pH 7$), the peak force of compression was significantly higher for WPI NEs than their SC counterparts ($p < 0.05$). However, at 5% protein, the peak force for SC NE (180.1 ± 8.3 g) (which formed a strong repulsive gel at $pH 7$) was significantly higher than that of the WPI NE (112.1 ± 18.5 g) ($p < 0.05$). Among the SC NEs, no significant difference ($p > 0.05$) in the peak force was observed for 2 and 4% SC NEs, but it was much higher for 5% SC NEs. However, for WPI NEs, the peak force of compression increased with increase in the WPI concentration from 2 to 4%, after that no significant change was observed for 5% WPI NE ($p > 0.05$).

For SC NEs, attractive gels induced by acidification were much stronger compared to the repulsive NE gel developed with 5% SC NE at $pH 7$. For comparison, the peak force for
the repulsive SC NE gel was also determined and is shown in Figure 9. The peak compression force for the repulsive gel was only 8.9 ± 3.6 g, which was about 20 times lower than that of the acidified attractive gel. Such a substantial change in gel strength could be attributed to the 3D network of strongly attractive droplets compared to the random jamming of repulsive droplets. In the acidified gels, the charged amino acid side chain of casein molecules are neutralized, and the steric barrier collapsed, leading to stronger attractive interactions among the SC-stabilized droplets and a stronger ability to hold the aqueous phase within the aggregated droplet structure.\(^{24}\)

Compression analysis of attractive WPI emulsion gel has been studied before.\(^{22,25}\) Rosa et al.\(^{22}\) investigated the formation and rheology of emulsion-filled gels by first preparing heat-induced whey protein aggregates followed by mixing with native whey protein-stabilized emulsions and finally acidification to induce gelation at the protein isoelectric point. The authors proposed that heating the whey protein solution was essential to form a self-supporting gel. Recently, Mantovani et al.\(^{25}\) reported that acidification of a 5% WPI-stabilized 30% oil-in-water emulsion did not form a gel, whereas mixing with a heat-treated 5% WPI before acidification led to a self-supporting gel. In contrast, Ye and Taylor\(^{26}\) were able to form whey protein-stabilized emulsion gel by heating an emulsion followed by acidification, without the need for adding additional heat-treated protein to form an emulsion-filled gel. In the present work, no heat treatment was used; still, a strong self-supporting gel was formed by acidification of WPI NEs to pH 5. The improvement in firmness of the gel could be attributed to the nanoscale droplet size of our NEs compared to microscale droplets of both Rosa et al.\(^{22}\) and Mantovani et al.'s\(^{25}\) emulsions. Ye and Taylor\(^{26}\) also showed that the storage modulus of a WPI emulsion gel, made by acidification of a preheated WPI emulsion, increased with a decrease in the average droplet size. Similarly, in the present case, the higher gel strength for 4 and 5% WPI NEs could be attributed to their significantly lower initial droplet size (recorded at pH 7) compared to 2% WPI NEs (Figure 7). Formation of many smaller droplets would lead to a better aggregated network of three-dimensional structure necessary for gelation compared to a lesser number of large oil droplets.\(^{25}\) The presence of higher interfacial WPI could also be responsible for more attractive interactions between the droplets, leading to a stronger gel.\(^{23}\)

One of the major differences between the acidified SC versus WPI NE gel was their appearance (Figure 8) and gel compression strength (Figure 9). At 2 and 4% protein concentration, WPI NE gels were stronger than the SC NE gels. However, it was opposite for 5% protein. It is possible that a compact interfacial structure of WPI compared to SC could lead to stronger attractive interactions among the nanodroplets and higher gel strength. However, at 5% protein concentration and pH 7, the SC NE formed repulsive nanogels where the droplets randomly jammed owing to the combined effect of nanoscale droplet size and interfacial shell layer thickness. In contrast, 5% WPI NE at pH 7 was liquid-like owing to a lower interfacial shell layer thickness. During acidification, perhaps the initial jammed structure of the SC NE formed a more space-filling network, efficiently trapping more aqueous phase compared to the corresponding WPI NE. However, more research is needed to better understand the mechanisms and nanostructure of these two attractive NE gels.

### CONCLUSIONS

Salt and pH-induced attractive interactions in SC and WPI-NEs were investigated with the aim to convert repulsive NEs into attractive NE gels. The addition of salt (up to 1 M) showed repulsive gel “melting” for 4 and 5% SC NEs, whereas no significant effect was observed for 2% SC NEs. The gel breakdown was ascribed to the screening of surface charge that caused a decrease in effective droplet volume fraction and close packing. In contrast, WPI NEs showed gelation due to droplet aggregation upon addition of salt and increasing gel strength with increase in salt concentration. In contrast, SC NEs did not show the aggregation or formation of attractive gels despite similar charge screening owing to the stronger steric repulsion among the SC-coated droplets.

Changing the pH to the pI of the protein resulted in the formation of aggregated attractive gels for both SC and WPI NEs owing to a loss of repulsive interaction among the droplets (both electrostatic and steric). The acidified NE gels were much stronger than the repulsive 5% SC NE gel and salt-induced attractive gels. Acidified SC gels formed a grainy and coarse texture, whereas the acidified WPI gels had a smoother texture. The strength of the acidified gels increased with a decrease in oil droplet size and is dependent on the type of protein used to stabilize them. For 2 and 4% proteins, WPI gels were stronger than the SC gels; however, the opposite was true for the NE with 5% protein, which could be due to the pre-formed jammed structure of the SC-stabilized droplets at pH 7. Similar to salt, pH-induced gelation without the aid of any heat treatment could be attributed to the nanoscale droplets responsible for forming a stronger three-dimensional structure. Overall, research on the effect of different environmental factors on the stability and gelation behavior of protein-stabilized NEs could be useful in food and related soft material applications as changes in these conditions are frequently observed during processing, storage, and consumption of these materials.

### MATERIALS AND METHODS

#### Materials

Canola oil (No Name brand) was purchased from a local grocery store. Milli-Q water (Millipore Corporation, MA, USA) was used for the preparation of the continuous aqueous phase. SC was purchased from Sigma-Aldrich, ON, Canada. WPI was a gift from Fonterra (USA) Inc., IL, USA. SDS was purchased from Fisher Scientific (Nepean, ON, Canada). All the other chemicals were purchased from Sigma-Aldrich (ON, Canada).

#### Preparation and Treatment of NEs

Protein solutions with three different concentrations (2, 4, and 5% w/w) of either SC or WPI were prepared by mixing overnight on a benchtop magnetic stirrer. Sodium azide (0.02% w/w) was added to the aqueous phase to inhibit microbial growth. The protein solutions were mixed with 40% canola oil, and a coarse oil-in-water emulsion was prepared using a rotor/stator mixer (Polytron, Brinkmann Instruments, ON, Canada) for 1 min at 20,000 rpm. NEs were then prepared by passing these coarse emulsions through a high-pressure homogenizer (Emulsiflex-C3, Avestin Inc., Ottawa, ON, Canada) at 20 000 psi (137.9 MPa) for eight cycles. Homogenization was carried out at room temperature (24 ± 1 °C); however, during the process, the temperature of emulsions reached 55–60 ºC toward the final cycle. Emulsions were stored at room temperature. All
quantities discussed in the paper are by weight basis and was termed with "%" instead of "% w/w".

Preparation of Samples to Study the Effect of Salt. NEs (10 g) were transferred to plastic centrifuge tubes. Different quantities of salt (NaCl) were directly added to the NEs to obtain a final salt concentration of 0.1, 0.5, or 1 M in the aqueous phase of the NEs. The two lower levels of salt concentration can be typically seen in many different food applications, for example, in various types of mayonnaise and salad dressings. Samples were stirred with a vortex mixer to properly mix salt in the NEs. The samples were analyzed for droplet size, zeta potential, rheology, and visual observation for flowability after overnight storage (∼15 h) at room temperature.

Preparation of Samples to Study the Effect of Change in pH. NEs (10 g) were transferred to centrifuge tubes, and different quantities of HCl solutions (1 M) were added to the vials to obtain NEs with pH at the isoelectric points (pI) of the proteins used (pH 4.6 for SC and pH 5.0 for WPI). The original NEs at pH 7 were also equally diluted with deionized water for comparison. The final oil concentration after dilution of 40% oil containing NEs was 39%. The samples were analyzed for droplet size, zeta potential, texture, and visual observation for flowability after overnight storage (∼15 h) at room temperature.

Droplet Size Distribution. The droplet size distribution and the surface mean diameter ($d_{32}$) of the NEs were determined using a static laser diffraction particle size analyzer (Mastersizer 2000, Malvern Instruments, Montreal, QC, Canada) with a relative refractive index of 1.465 for canola oil and 1.33 for water. The samples were gently mixed before their addition to the sampling cell to obtain a uniform sample, and few drops were added to the measuring cell, mixed to get the desired dilution for proper laser diffraction before starting the analysis.

Zeta Potential. Zeta potential measurements were carried out by Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA, USA) by determining the electrophoretic mobility of the protein-coated droplets under a particular electric field. The samples were diluted (2 drops of sample in 50 mL DI water/salt solution/pH buffer) and filled in the electrode cell, which was loaded into the Zetasizer for analysis. For salt-added NEs, the samples were diluted with respective salt solutions. For the NEs at different pH values, dilution was done with specific pH buffer solutions.

Visual Observation of Gelation. For visual inspection, samples of NEs with different ionic strengths and pHs were stored in 30 mL glass beakers. After overnight storage (∼15 h), the beakers were tilted at a 45° angle, and the flow behavior of the samples was recorded with a digital camera.

Determination of Viscoelasticity. The viscoelastic behavior of the NEs was determined using an AR-G2 rheometer (TA Instruments, Montreal, QC, Canada). A 40 mm cross-hatched parallel plate geometry was used to avoid wall-slip. The viscoelasticity was determined by applying an oscillatory strain sweep from 0.01 to 100% strain at a constant frequency of 1 Hz at 25 °C. The analysis provided storage modulus ($G'$) and loss modulus ($G''$) as functions of the applied strain. To eliminate any effect of sample history and loading, a pre-shear was applied at a 2 s$^{-1}$ shear rate for 10 s before the strain sweep analysis.

Large Deformation Compression Analysis. The pH-adjusted NEs at the pI of the protein had a too firm gel texture to analyze using the rheometer. For these samples, compression analysis was carried out by a TA-XT plus Texture Analyzer (Stable Micro System, England) at room temperature with a 1/2 in. diameter cylindrical probe. Samples were placed in a beaker (20 mm in height and 15 mm in diameter), and the probe was set to penetrate 5 mm from the surface of the gel at a rate of 10 mm/s to measure the peak force of gel compression.

Statistics. All the samples were prepared, and the experiments were performed with at least three replicates ($n \geq 3$). Statistical significance of the data was analyzed at a 95% confidence level using a single factor ANOVA function available in Microsoft Excel (Microsoft Canada Co, Mississauga, ON, Canada).

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b03360.

Effect of SDS addition on droplet size distribution of WPI NEs; visual observation of flow behavior of SC and WPI NEs at different salt and protein concentrations; DLVO interdroplet pair potential for all NEs; droplet size distribution of SC WPI NEs at pH 7.0 and pH = pI of the protein; and visual observation of flow behavior of SC and WPI NEs at pH 7 and pH = pI of the proteins

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### Notes

The authors declare no competing financial interest.

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