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Emulsion gels loaded with pancreatic lipase: Preparation from spontaneously made emulsions and assessment of the rheological, microscopic and cargo release properties

Saina Moayedzadeh [a], Asghar Khosrowshahi asl [a], Sundaram Gunasekaran [b], Ashkan Madadlou [c,*]

[a] Department of Food Science and Technology, Faculty of Agriculture, Urmia University, Urmia, Iran
[b] Department of Biological Systems Engineering, University of Wisconsin-Madison, 460 Henry Mall, Madison, WI 53706, USA
[c] Department of Biotechnology and Food Science, Norwegian University of Science and Technology (NTNU), Trondheim, Norway

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ABSTRACT

Emulsion gels are solidified emulsions, which can be used for delivery of both hydrophilic and lipophilic substances. In this research, at first fish oil-in-water (O/W; 10% w/w) emulsions were prepared through the spontaneous emulsification technique. As emulsifier, a blend of the small-molecule surfactant tween 80, and either low-acyl (LaG) or high-acyl (HaG) gellan was used. For making fully stable (100% stability index) emulsions, a 10-fold higher concentration of LaG than HaG in the emulsion aqueous phase was required. The difference in gellan concentration resulted in a bigger mean drop size, as well as lesser consistency coefficient and yield stress for HaG emulsion than LaG emulsion.

Subsequently, the fully stable HaG and LaG emulsions were gelled by $CaCl_2$ addition. LaG emulsion gel was self-supporting and had a dense microstructure (as observed by electron microscopy), whereas HaG emulsion gel was not self-supporting. Loading lipase into the emulsions before ionotropic gelation did not lead to unacceptable acid values for fish oil during the emulsion gels storage.

When the lipase-loaded fish O/W emulsion gels were immersed in an acid solution which imitated the gastric fluid (yet without digestive enzymes) oil droplets flocculated (as observed by confocal microscopy). The acid immersion also increased the dynamic moduli of the gels. Lipase was not released into the surrounding acid solution from LaG emulsion gel. A subsequent immersion within an alkaline solution imitating the small intestine fluid (yet without digestive enzymes) reduced the dynamic moduli of both kinds of emulsion gels. The alkaline immersion also caused extensive crack propagation in LaG emulsion gel network, which was found associated with diminished value of tan δ (G"/G') as an index of gel energy dissipation. Lipase was released form LaG emulsion gel into the alkaline solution, however, it took a remarkable period of time to begin.

1. Introduction

Emulsion gels (emulsion-filled hydrogels) are increasingly used as delivery systems in food, cosmetic, pharmaceutical (Lorenzo et al., 2013), and biomedicine sectors. The solid-like, yet soft structure of emulsion gels grants superior features such as enhanced colloidal stability and improved oxidative stability of lipids compared to emulsions. Emulsion gels combine the advantages of emulsions and hydrogels i.e., dissolution of both hydrophobic and hydrophilic components and also good thermodynamic stability (Farjami & Madadlou, 2019), as well as controlled gastric and/or intestinal cargo release properties (Corstens et al., 2017). Various gelling agents such as pectin, sodium alginate, and gellan are used to form emulsion gels. The latter is extensively used to make gels for the entrapment of drugs, enzymes, cells, and microorganisms (Das & Giri, 2020).

Gellan is an anionic heteropolysaccharide secreted by the bacterium Sphingomonas elodea. It has a linear tetra-saccharide repeating unit of (→3)-β-D-glucose residue preceding D-glucuronic acid, D-glucose, and L-rhamnose residues. The gelation mechanism of gellan in aqueous solutions has a two-step process including conformational change from

* Corresponding author.
E-mail address: Ashkan.madadlou@ntnu.no (A. Madadlou).

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random coil to double helix followed by aggregation of the double helices to form a three-dimensional network structure (Picone & Cunha, 2011). Gellan is commercially available in high and low acyl forms. High-acyl gellan (HaG) is rich in two acyl substituents, namely L-glyceryl and acetyl. The glyceryl groups increase the double helices stability by forming additional hydrogen bonds. Nonetheless, they as well change the orientation of adjacent carboxyl groups, destroying the biding sites for metal cations. In comparison to low-acyl gellan (LaG) gels, the gels formed by HaG are softer but less prone to syneresis (Morris et al., 2012). At low pHs, gellan gels strengthen, whereas the gels formed by many other polysaccharides such as agarose and carrageenan show reduced gel capacity (Lorenzo et al., 2013). Moreover, the advantageous properties such as thermal stability, adjustable gel elasticity and rigidity, and good cargo (e.g. flavor) release (Taylor et al., 2012), all confer the potential of gellan for making food systems that pass labile cargos like enzymes through the stomach.

The gastrointestinal digestion and the subsequent release of cargo from (gellan emulsion) gels can be well governed via tuning the gel rheology. Gellan addition into quercetin-loaded caseinate-stabilized emulsions and the successive supplementation of the emulsions with glucono-δ-lactone yielded emulsion gels with decreased digestibility in simulated gastrointestinal fluids. The higher the gellan content in emulsion gels, the lower was proteolysis, lipolysis and quercetin bioaccessibility (Chen et al., 2018). Besides the initial attributes, the developments/alterations that occur in gel rheology within the gastrointestinal tract influence the loaded cargo release. When assessing fluid (sheared) gels as oral liquids for administration of ibuprofen, it was found that the characteristic acid-induced gelation property of gellan prevents ibuprofen release in simulated gastric fluid (SGF). Moreover, the subsequent release of the drug in simulated intestinal fluid (SIF) was delayed because of increase in gel stiffness during the preceding acidic exposure in SGF (Mahdi et al., 2014).

In a previous study (Moayedzadeh et al., 2018), we used LaG gum to spontaneously emulsify fish oil without requiring a co-solvent and at a substantially low small-molecule surfactant-to-oil ratio (SOR). In the present work, both HaG and LaG were applied for the spontaneous emulsification of fish oil. Then, lipase-loaded emulsion gels were produced via ionotropic gelation of the emulsions. The objective of the present study was to develop emulsion gels which can concurrently be used as reservoirs of fish oil at ambient condition and as delivery vehicles of lipase into the gastrointestinal tract. Delivery of pancreatic lipase into the small intestine is an effective approach to treat elderly people and individuals with exocrine pancreatic insufficiency (Perbtani & Forsmark, 2019). Lipase-loaded emulsion gels for such treatments ought to protect the enzyme at the stomach and release it into the upper small intestine. Individuals with EPI are additionally recommended to ingest high-fat foods to attain optimal growth and prevent nutritional deficiencies (Sankararaman et al., 2019). Therefore, lipase-loaded fish oil-in-water emulsions can be proficient nominees for EPI treatment. The emulsions were made using different concentrations of HaG or LaG together with tween 80, and the formulations which brought about full stability (i.e., 100% stability index) for $\geq$ 60 days were selected for lipase supplementation and emulsion gel formation. As a primitive assessment, the rheology, microstructure, and cargo (i.e., lipase) release properties of the lipase-loaded emulsion gels upon sequential exposure into acidic and basic media that simulate the conditions found in the stomach and intestine were examined.

2. Materials and methods

2.1. Materials

CP Kelco (San Diego, CA, USA) kindly donated low-acyl (LaG) and high-acyl (HaG) gellan gums. According to the manufacturer, the average molecular weights of LaG and HaG were 2–3 $\times$ 10^5 and 1–2 $\times$ 10^6 Da, respectively. Fish oil (Ho307) was a kind gift from LYSI (Reykjavik, Iceland). The oil contained 351 mg g^{-1} total ω-3 fatty acids, 171 mg g^{-1} eicosapentaenoic acid (EPA), and 112 mg g^{-1} docosahexaenoic acid (DHA). The emulsifier polyoxyethylene sorbitan monooleate (tween 80), sodium azide, lipase from porcine pancrease, *p*-Nitrophenyl laurate (*p*-NPL), and Triton X-100 were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). The Quick Start™ Bradford Protein Assay kit was purchased from Bio-Rad (Hercules, CA, USA). All other reagents were of analytical grade.

2.2. Emulsion preparation and characterization

2.2.1. Emulsion preparation

Fish oil-in-water (O/W) emulsions were prepared via the spontaneous emulsification method at 25 °C as described by Moayedzadeh et al. (2018). For this purpose, aqueous solutions of different concentrations of either LaG (0.0, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mg g^{-1}) or HaG (0.0, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg g^{-1}) were heated separately at 90 °C for 30 min at a constant stirring speed (500 RPM), cooled to 25 °C and supplemented with sodium azide (50 μg g^{-1}) as the antimicrobial agent. The pH of the gum solutions (or distilled water in the case of gellan-free samples) was adjusted to 7.0 with 0.1 N NaOH. Tween 80 was mixed with fish oil at a constant SOR of 0.1 by a magnetic stirrer at 500 RPM and 25 °C for 30 min. Subsequently, the aqueous phase was titrated with the oil phase (fish oil and tween) for 10 min at 25 °C and 500 RPM. Fish oil proportion of samples was kept constant (10% w/w). Samples were mixed for an additional 10 min, poured into glass tubes, and transferred to 4 °C. An emulsion sample with a higher SOR, i.e., 1.0 but free of gellan, as well as two tween-free emulsion samples with 6.0 mg g^{-1} LaG aqueous phase and 0.6 mg g^{-1} HaG aqueous phase were prepared for comparison (Fig. 1).

Lipase powder was added (10 mg mL^{-1}) to selected emulsions (100% stability for > 2 months) while being stirred at 100 RPM for 10 min. The emulsions loaded with lipase were stirred at 100 RPM for an additional 30 min and stored at 4 °C for 2 h to ensure complete hydration. The measured lipase activity was 5.35 U g^{-1} protein-specific activity.

2.2.2. Determination of emulsion stability

Immediately after preparation, samples were monitored for their creaming stability. The initial height of the emulsion (H_E) and the height of the cream layer (H_C) were measured and used for calculating the emulsion stability index (ESI) as follows:

$$\mathrm{ESI} = \left(\frac{H_E - H_C}{H_E}\right)*100 \quad (1)$$

Consequently, two emulsion samples with 100% ESI (no creaming) after storage for two months were selected and subjected to further analysis.

2.2.3. Droplet size distribution and mean diameter

The droplet size distribution of the freshly made emulsions was determined by the laser light scattering technique using a Malvern Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK). The refractive index of the aqueous medium was 1.33 and the refractive index of the dispersed phase (fish oil) was 1.47. Absorbance was set at 0.01. Light scattering data are reported as the volume moment mean (D [43]). Samples were diluted ten-fold with buffer solutions (same pH as sample) before analysis, to prevent multiple scattering effects. Two replicates of each emulsion were prepared, and the droplet size was measured two times per replicate.

2.2.4. ζ-potential of emulsion droplets

A Zeta-sizer (Zeta PALS, Brookhaven Instruments Corp., Holtsville, NY, USA) was used for the measurement of emulsion drops ζ-potential. Samples were diluted 10 folds with distilled water (same pH as sample) before analysis to prevent multiple scattering effects.

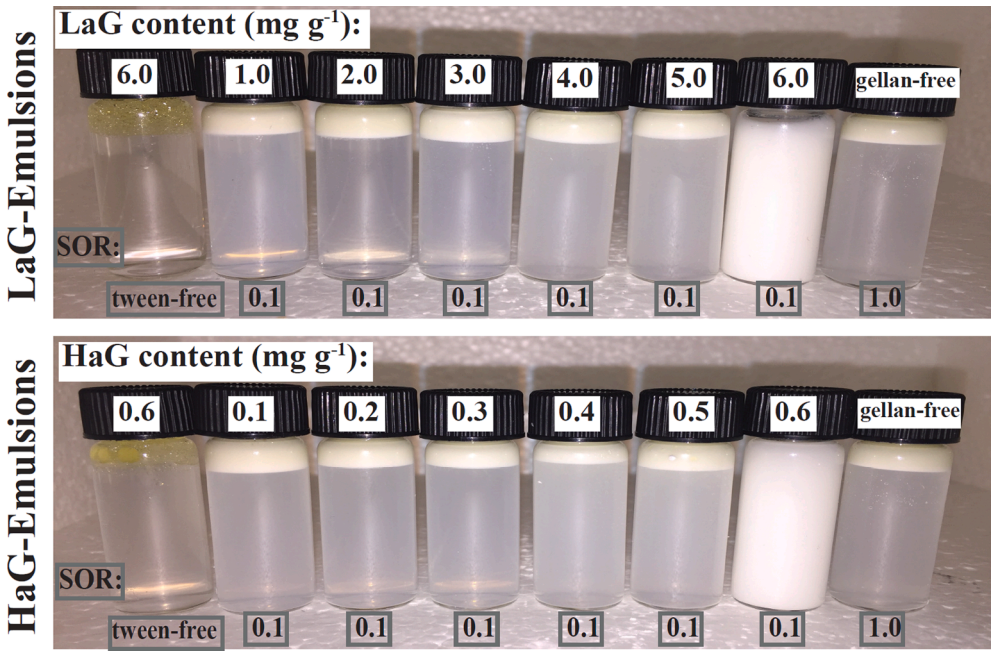

Fig. 1. The visual appearance of spontaneously formed emulsions having different contents of low-acyl gellan (LaG) and high-acyl gellan (HaG) and surfactant-to-oil ratio (SOR). The fish oil concentration was constant at 10 wt%.

2.2.5. Viscosity measurement

The viscosity of gellan solutions and freshly made emulsions was measured using a Discovery Hybrid Rheometer 2 (TA Instrument, New Castle, DE, USA). A concentric cylinder geometry attachment with a cup radius of 15 mm, a bob radius of 14 mm, and a bob height of 42 mm was used. Shear stress was measured as a function of shear rate from 0.1 to 100 s^{-1} at 25 °C. The obtained data were fitted to the Power-law ($\sigma = K\gamma^n$) and Herschel-Bulkley ($\sigma = \sigma_0 + K\gamma^n$) models by TRIOS software. and, where σ is the shear stress (Pa), σ_0 is the yield stress (Pa), K is the consistency coefficient (Pa s^n), γ is the shear rate (s^{-1}) and n is the flow behavior index (dimensionless).

2.3. Fabrication and characterization of emulsion gels

2.3.1. Fabrication

The emulsions loaded with lipase were supplemented with Ca^{2+} to undergo gelation. The selected two emulsions (with 100% ESI) were transferred into clear plastic containers (25 mm height and 15 mm inner diameter) and cold gelation was induced by mixing the samples with an appropriate volume of 4 M $CaCl_2$ solution to a final Ca^{2+} concentration of 20 mM in the aqueous phase. Then, samples were stored for one day at 4 °C for transforming into emulsion gels. Gel samples were subjected to further analysis.

2.3.2. Microstructure

The microstructural features of the emulsion gels were imaged by a scanning electron microscope (SEM) using LEO 1530 Gemini FE-SEM (Carl Zeiss, USA). For this purpose, small pieces of freeze-dried (Labconco FreeZone Freeze Dry System, Labconco Corp., Kansas City, MO) gels were gently broken at liquid nitrogen, then mounted on aluminum stubs and coated with a thin layer of gold by a DESK II gold sputter machine (Denton Vacuum, USA) for 30 s before imaging. At least three images of typical structures were recorded at an acceleration voltage of 10 kV.

2.3.3. Hydrolysis of emulsified oil during storage

Emulsion gels were stored for 60 days at 25 °C and the acid value (AV) of the emulsified oil as an index of triacylglycerol hydrolysis was continuously measured. At certain time points, oil was extracted from the O/W emulsion gels according to the procedure described by Maltais et al. (Maltais et al., 2009) with slight modifications. Briefly, chloroform and methanol (2:1, v/v) were added into the samples, vortexed three times for 10 s each and centrifuged for 30 min at 3000 × g. Following centrifugation, the chloroform–methanol extract was collected, and the solvent was evaporated under vacuum in a rotary evaporator. After solvent removal, the oil was weighed and the amount of free fatty acids as an index of oil hydrolysis was measured by the AOCS Official Method Cd 3d-63 (AOCS., 1997). Each oil sample was analyzed in triplicate and the mean values are presented.

2.3.4. Influence of the gastrointestinal pH profile

Emulsion gels loaded with lipase were sequentially immersed in acidic and slightly alkaline solutions, simply mimicking the fluids found in the stomach and intestine. The immersion time in the acid and alkaline solutions was up to 1 h and up to 2.5 h, respectively. The acid solution consisted of 2.0 g of sodium chloride, 7.0 mL of 37% hydrochloric acid and 1000 mL of double-distilled water. The final pH was 1.2. The slightly alkaline solution consisted of 6.8 g of monobasic potassium phosphate dissolved in 250 mL of double-distilled water plus 190 mL of 0.2 N sodium hydroxide and 400 mL of double-distilled water. The pH was adjusted to 7.4 using 0.2 N sodium hydroxide and the final volume was brought 1000 mL with double-distilled water (The United States Pharmacopoeial Convention, 2004). First, the acid solution (pH = 1.2) was preheated to 37 °C and added into beakers containing emulsion gel samples. The ratio of acid solution to emulsion gel was 1:1 (w/w). The mixture was incubated in an incubator shaker at a rotation speed of 100 RPM at 37 °C for 60 min to mimic agitation in the stomach. Then, the preheated alkaline solution (pH = 7.4) was added to the reaction beakers at a ratio of 1:1 (w/w). The mixture was further incubated at 100 RPM for 150 min at 37 °C to mimic the intestinal condition. Several experiments (confocal laser scanning microscopy (CLSM), rheological properties, lipase activity, and Bradford protein assay) were carried out during exposure into acidic and alkaline media at specific time intervals.

The CLSM imaging of the emulsion gels was performed before and after immersion within the acidic and alkaline media. Before gelation, the emulsions were supplemented (≈10 μl/mL) with a mixed solution

containing the fat specific dye (to label the oil phase) Nile red (0.15%, w/v, in 1, 2-propylene glycol), and the protein specific dye (to label lipase) Nile blue (0.2%, w/v, in distilled water) at a ratio of 3:1 (Abhyankar et al., 2011). Then the samples were gelled as described above. CLSM observations were performed using an inverted Zeiss 710 confocal microscope (Carl Zeiss AG, Germany) housed at the Newcomb Image Center at the University of Wisconsin-Madison. Nile red and Nile blue were excited at 488 nm and 633 nm, respectively and the emissions were collected in two separate channels, in the wavelength ranges of 503–613 nm (for Nile red) and 653–750 nm (for Nile blue). The images were analyzed by the ImageJ software (1.53c, Wayne Rasband, National Institutes of Health, USA). Nile Red fluorescence intensity was quantitated from the CLSM images using FIJI-ImageJ software (Schindelin et al., 2012).

A Discovery Hybrid Rheometer 2 (TA Instrument, New Castle, DE, USA) equipped with TRIOS software was used to measure the rheological properties of emulsion gels. Storage (G'), loss (G"), and complex (G*) moduli, loss tangent (tan δ), and critical strain were measured after strain sweep (0.01–10000%, 1 Hz) tests. A parallel plate measuring geometry (PP 998558) with a diameter of 20 mm was used.

The release of lipase from emulsion gels into the acidic and alkaline solutions was determined by measuring the protein content by the Bradford method in digestion media. Quick Start™ Bradford Protein Assay reagent and bovine serum albumin as the standard were used. All experiments were carried out in triplicate.

The activity of the released lipase from emulsion gels was measured using an assay based on the hydrolysis of *p*-NPL. In this enzymatic reaction, the cleavage of the ester bond of *p*-NPL produces *p*-nitrophenol (*p*-NP). The amount of the released *p*-NP was measured at 410 nm by a UV–vis spectrophotometer (Perkin-Elmer lambda 25). The activity of lipase was calculated using the standard calibration curve of *p*-NP. One unit (1 U) of enzyme activity was defined as the amount of enzyme that liberates 1 μmol min^{-1} of *p*-NP. Non-protected lipase was also studied under similar conditions as the control for comparison. The relative activity (%) was expressed as the ratio between the activity of released lipase from emulsion gels and the activity of the loaded amount of lipase into emulsion gels. All experiments were carried out in triplicate.

2.4. Statistical analysis

The results reported as mean ± standard deviation were analyzed by one-way ANOVA and significant differences between samples were evaluated using Duncan's test with IBM SPSS Statistics 20 software. The level of significance used was $p < 0.05$.

3. Results and discussion

3.1. Characterization of emulsion samples

3.1.1. Formation, stability, and droplet size

Fig. 1 shows exemplar photographs of the emulsions prepared by the spontaneous emulsification technique. The time period that the emulsions remained fully stable, i.e., 100% ESI was recorded, and the results are reported in Table 1. Samples prepared without surfactant (tween) were tremendously unstable and an oil layer quickly formed. Similarly, gellan-free emulsions even with the highest SOR (i.e., 1.0) were unstable and underwent creaming (Fig. 1). Using a combination of gellan and tween, the stability of the emulsions increased. The emulsion samples prepared by LaG at 6 mg g^{-1} aqueous phase or HaG at 0.6 mg g^{-1} aqueous phase and at a SOR of 0.1 remained fully stable (i.e., 100% ESI) for more than two months (Table 1). The emulsion stabilization is ascribed to the high viscosity of the emulsion aqueous phase and a Pickering-type functionality of gellan aggregates (Vilela & da Cunha, 2016). Neither HaG, nor LaG are surface active; they do not decrease the interfacial tension (Vilela & da Cunha, 2016, 2017). However, it is known that HaG molecules form micellar aggregates in aqueous solutions at concentrations as low as 0.5 mg mL-1 (Yang et al., 2019). Likewise, LaG forms irregular sphere-like aggregates in solution (Sow et al., 2019). The accumulation of gellan aggregates at the oil–water interface, i.e., a Pickering-type mechanism, has been appointed as a stabilization mechanism of O/W emulsions when gellan is added into the emulsion aqueous phase (Vilela & da Cunha, 2016; Vilela & da Cunha, 2017). It is noteworthy that HaG at concentrations ≥ 0.7 mg g^{-1} was hardly soluble, and clumps were observed during dissolution by the naked eye. Therefore, it was not feasible to prepare emulsions using HaG at concentrations ≥ 0.7 mg g^{-1} aqueous phase. Likewise, large clumps were formed by LaG at concentrations ≥ 7 mg g^{-1}.

Table 1
Stability duration and droplet characteristics of O/W emulsions (oil content 10% wt) with different concentrations of low-acyl (LaG) or high-acyl (HaG) gellan in the aqueous phase and surfactant-to-oil ratios (SOR).

Emulsion samples	SOR	100% ESI[1]	Droplet diameter (μm)[2]	ζ-potential (mV)
Gellan-free	1.0	<1 min	–	–
LaG (mg g^{-1})				
1.0–5.0	0.1	<1 h	–	–
6.0	tween-free	<5 min	–	–
6.0	0.1	>60 days	1.52 ± 0.09	−14.38 ± 0.05
HaG (mg g^{-1})				
0.1–0.5	0.1	<1 h	–	–
0.6	tween-free	<5 min	–	–
0.6	0.1	>60 days	3.28 ± 0.1	−17.07 ± 0.28

[1] ESI: Emulsion stability index.
[2] Freshly prepared samples.

The emulsion samples with an ESI of 100% for > 2 months were subjected to additional analysis. The droplet size of freshly made emulsions are reported in Table 1. The LaG emulsion had a smaller droplet size than that prepared by HaG. Differences between the two kinds of gellan regarding their concentration in the emulsion aqueous phase, molecular weight, and hydrophobicity (acetyl groups count) could result in the observed dissimilarity in droplet sizes.

Zeta-potentials of LaG and HaG were not expected to be significantly different (Buldo et al., 2016) because removal of acetyl groups during the deacylation process co-occurs with the removal of glyceryl moieties. The former (i.e., deacetylation) causes the introduction of a carboxyl group (glucuronic acid formation) to the molecule backbone, whereas the latter removes a carboxyl group. Hence, the difference between the ζ-potential values of LaG and HaG emulsion samples (Table 1) is attributed to difference in the amount of gellan aggregates accumulated at the oil–water interface in the two emulsion samples. Compared to LaG emulsion, HaG emulsion droplets had a larger absolute value of ζ-potential. We presume that the micellar aggregates of HaG (Yang et al., 2019) made a more crowded (denser) particle layer around oil droplets, in comparison to the irregular aggregates of LaG (Sow et al., 2019). The denser layer resulted in a more negatively charged (i.e., larger absolute value of ζ-potential) interface.

3.1.2. Viscosity and flow behavior of solutions and emulsions

The viscosity of LaG and HaG solutions at different concentrations, as well as that of the stable emulsions was measured and the data of shear stress as a function of shear rate were fitted to either Power-law (for solutions) or Herschel-Bulkley (for emulsions) model. The consistency coefficient (*K*) of HaG solution was significantly higher than that of LaG solution at a comparable concentration, i.e., 0.6 mg g^{-1} (Table 2). The higher number of acyl groups could cause extensive interactions between HaG molecules (Taylor et al., 2012), increasing the *K* value. Moreover, the HaG solution showed a higher shear-thinning (i.e., smaller *n* value, Table 2) behavior. As expected, when the concentration of LaG solution increased from 0.6 mg g^{-1} to 6 mg g^{-1} (10-fold increase), its *K* value significantly increased (roughly 36 folds). As well,

Table 2
Rheological characteristics of different gellan solutions (data were fitted to the power law model) and emulsions (data were fitted to the Herschel-Bulkly model).

Samples	Model	K (mPa s^n)	n	σ_0(Pa)
Solutions: *Gellan content (mg g^{-1})*				
HaG, 0.6	Power law	75.48 ± 0.02^{Bc}	0.54 ± 0.002^{Cc}	–
LaG, 0.6	Power law	4.06 ± 0.001^{Cd}	0.82 ± 0.03^{Aa}	–
HaG, 6.0	Gel-like structure	–	–	–
LaG, 6.0	Power law	145.2 ± 0.07^{Ab}	0.66 ± 0.001^{Bb}	–
Emulsions: *Gellan content (mg g^{-1})*				
HaG, 0.6	Herschel-Bulkley	144.6 ± 0.01^{Bb}	0.50 ± 0.02^{Ad}	0.48 ± 0.025^{B}
LaG, 0.6	Unstable emulsion	–	–	–
HaG, 6.0	Gel-like structure	–	–	–
LaG, 6.0	Herschel-Bulkley	1034.1 ± 0.6^{Aa}	0.46 ± 0.018^{Be}	1.67 ± 0.166^{A}

LaG: low-acyl gellan; HaG: high-acyl gellan.
Different uppercase superscript letters indicate significant differences among different solutions and different emulsions separately; different lowercase superscript letters indicate significant differences between all samples together (solutions and emulsions) ($P < 0.05$).

the solution became more shear-thinning (Table 2).

Emulsification of fish oil increased the consistency coefficient (K) of both LaG (at 6 mg g^{-1}) and HaG (at 0.6 mg g^{-1}) solutions. However, the LaG solution underwent a more remarkable increase in consistency coefficient (Table 2). Furthermore, the LaG emulsion had a higher yield stress (σ_0) than HaG emulsion. The higher consistency and yield stress of LaG emulsion is attributed to its smaller droplet size. Fine emulsions are known to have much higher viscosities than coarse emulsions (Pal, 1996). Both kinds of the emulsions were shear-thinning (Table 2), indicating progressive orientation of oil droplets in direction of shear field with increasing shear rate (Moayedzadeh et al., 2018).

3.2. Characterization of emulsion gels

3.2.1. Scanning electron microscopy

The spontaneously formed fish O/W emulsions prepared using LaG (6 mg g^{-1} aqueous phase) or HaG (0.6 mg g^{-1} aqueous phase) were gelled by $CaCl_2$ addition and subjected to analysis. In agreement with the literature (Lu et al., 2019); entrapped fat particles within the gel network were observed for both kinds of the emulsion gels (Fig. 2). The matrix of LaG emulsion gel was dense and included small particles, whereas that of HaG emulsion gel included larger particles with some interspaces. The presence of interparticle gaps weakens gel texture (Lu et al., 2019). In accordance with the microstructural features, LaG emulsion gel was self-supporting, whereas HaG emulsion gel could not hold its weight when put upside down (Fig. 2).

3.2.2. Oil hydrolysis during the emulsion gels storage

Fish O/W emulsion gels were stored for two months at 25 °C and the acid value (AV) of the oil phase was measured after extraction. The results are indicated in Fig. 3. AV is associated with the content of free fatty acids and depends on the hydrolysis degree of oil. According to the Codex Committee on Fats and Oils (2017) the maximum acceptable AV for fish oil is 3 mg KOH g^{-1}. AV of the fish oil before emulsification was low (0.14 mg KOH g^{-1} oil) and met the Codex specification. However, it significantly increased for the non-emulsified oil (control sample) during storage and reached ≈8.5 mg KOH g^{-1} after 60 days. On the contrary, AV of fish oil remained below 3 mg KOH g^{-1} throughout the whole storage period of both kinds (HaG and LaG) of emulsion gels. Therefore, emulsification of fish oil and gelation of the emulsion was an efficient way to prevent the oil hydrolysis.

A closer inspection of the AV data indicated that LaG emulsion gel had a stronger preventive effect on fish oil hydrolysis than HaG emulsion gel (Fig. 3A). The emulsion gels protective effect on fish oil correlates with their microstructural attributes as observed by SEM (Fig. 2B). The presence of cracks and gaps between (fat) particles in HaG emulsion gel microstructure resulted in higher oil hydrolysis.

For assessing the influence of lipase on fish oil AV in emulsion gels, lipase was added into freshly made emulsions and the emulsions were gelled. Loading lipase significantly increased the AV of entrapped fish oil in both types of the emulsion gels (Fig. 3). The distribution of lipase within the gel network was even and is discussed in a subsequent section (by CLSM images). However, lipase is also known to adsorb at the oil–water interface. Loading lipase increased the emulsified fish oil hydrolysis, i.e., increased the AV. Yet, the AV of oil in lipase-loaded

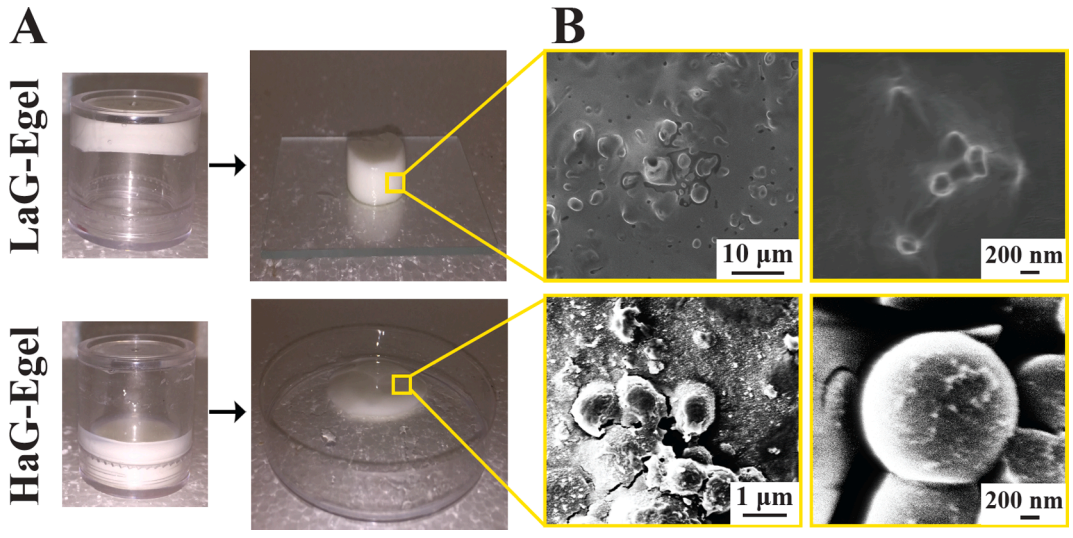

Fig. 2. (A) Photographs and (B) scanning electron micrographs of low and high- acyl gellan emulsion gels (LaG-Egel and HaG-Egel, respectively). The concentration of LaG and HaG in the emulsion gels were 6 and 0.6 mg g^{-1} aqueous phase, respectively.

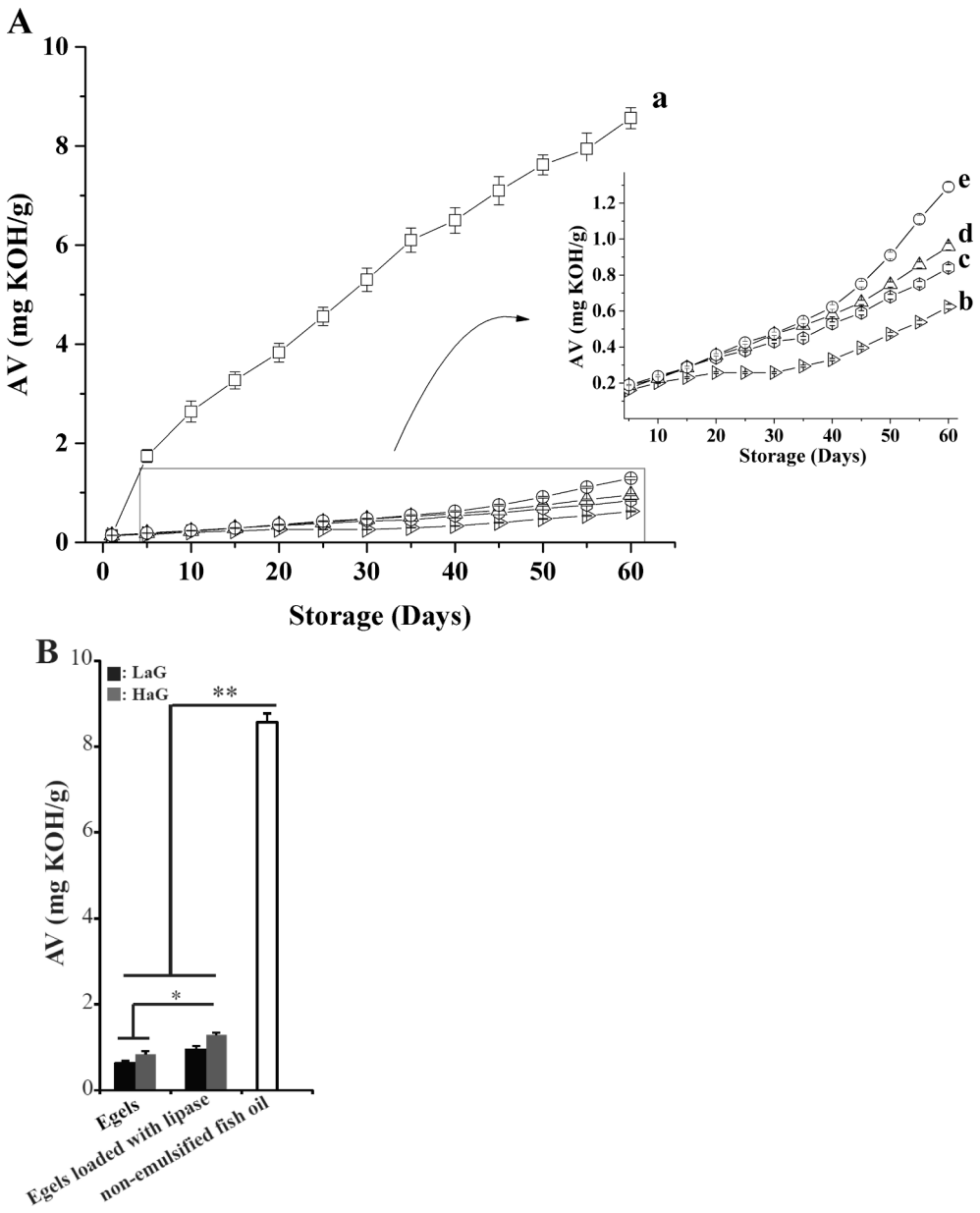

Fig. 3. (A) Acid values (AV) of fish oil at 25 °C: (a) non-emulsified, (b) entrapped in LaG emulsion gel, (c) entrapped in HaG emulsion gel, (d) entrapped in lipase-loaded LaG emulsion gel, (e) entrapped in lipase-loaded HaG emulsion gel. (B) Quantitative analysis of the AVs at day 60. Data are mean ± SD. * represents $p < 0.05$ and ** represents $p < 0.001$.

emulsion gels did not exceed the Codex specification (i.e., 3 mg KOH g^{-1}) and was remarkably lesser than that of the non-emulsified fish oil at the end of storage. The results indicate that the emulsion gels were quite efficient at preventing fish oil hydrolysis in presence of the co-loaded lipase.

3.2.3. *Influence of the gastrointestinal pH profile*

3.2.3.1. Microstructure of emulsion gels. Lipase-loaded fish O/W emulsion gels were immersed sequentially within acidic (imitating stomach) and alkaline (imitating small intestine) solutions. The samples were imaged by confocal microscopy before and after the treatments. Oil and lipase were dyed green (using Nile Red) and red (using Nile Blue), respectively (Fig. 4A) and the fluorescence intensity of the oil-soluble fluorescent dye, i.e., Nile red was measured (Fig. 4B). Initially, the oil droplets and lipase were evenly distributed in the emulsion gels matrix. However, immersion of the gels within the acid solution caused flocculation of oil droplets with a consequent increase in drops mean size (Fig. 4C). Ionotropic gelation of gellan is imperfect, leaving free anionic sites to be filled by hydrogen ions in the gastric solution. This leads to excessive aggregation of the polysaccharide helix pairs (Santos & Cunha, 2018). Accordingly, when immersed in the acid solution, the emulsion gels changed from entirely ionotropic to ionotropic/acid-set gels. Atomic force microscopy imaging has demonstrated that gellan gel network density is higher in acid-set samples than cation-induced counterparts (Ikeda et al., 2004). Development of a denser network due to transformation of entirely ionotropic gel to ionotropic/acid-set could shrink the network, causing the oil droplets flocculation. It is noteworthy that the color (Fig. 4A) and fluorescence intensity (Fig. 4B) of oil droplets were indifferent before and after immersion within the acidic solution.

When the emulsion gels (either with LaG or HaG) were taken from the acid solution and stored in the alkaline solution, the mean size of oil droplets significantly decreased (Fig. 4C). The treatment in the alkaline

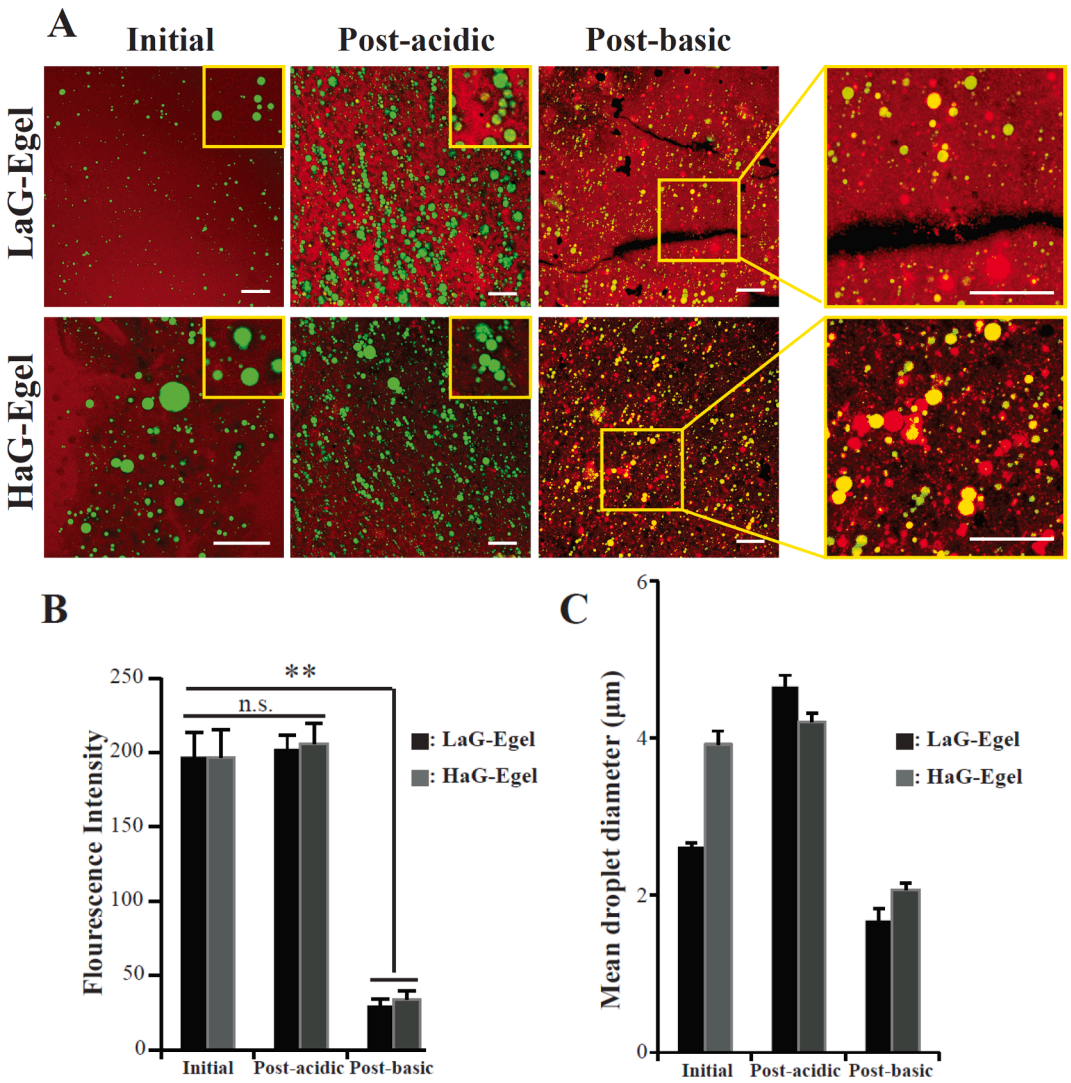

Fig. 4. (A) Confocal micrographs of low- and high-acyl emulsion gels (LaG-Egel and HaG-Egel, respectively) upon sequential immersion within the acidic and basic media. The oil phase stained with Nile Red, is shown in green and the protein (pancreatic lipase) stained with Nile Blue is shown in red. Quantitative analysis of (B) fluorescence intensity of Nile Red and (C) Mean droplet size of oil drops obtained in (A). Data are mean ± SD. ** represents $p < 0.001$. Scale bars represent 50 µm.

solution also changed oil droplets color from green to yellow and red (Fig. 4A), as well as substantially reduced the droplets fluorescent intensity (Fig. 4B). Nile Red is a hydrophobic and metachromatic dye with color emission ranging from green to yellow, orange-red, and red depending on the surrounding environment's polarity (Bhatla et al., 2009). When polar lipids such as phospholipids, free fatty acids, and glycerol are present, the emission of the dye red-shifts (Greenspan & Fowler, 1985). We believe that the observed red-shift (i.e., change of oil droplets color form green to yellow/red) indicates oil hydrolysis and generation of polar lipids in the oil droplets. The alkaline solution used in the present study did not contain lipase. Therefore, only the lipase loaded into the emulsion gels could cause hydrolysis, increasing the polarity of oil droplets.

It is observed in Fig. 4A that immersion within the alkaline solution (high pH) caused formation of apertures, and cracks throughout the emulsion gels microstructure. Cracking was much more extensive in LaG emulsion gel than HaG emulsion gel (Fig. 4A). Crack propagation in the samples is associated with their energy dissipation and will be discussed in the next section.

3.2.3.2. Rheological characterization of emulsion gels. The rheology of lipase-loaded emulsion gels was assessed, and the results are tabulated in Table 3. The storage modulus (G') of the samples was always larger than their loss modulus (G"), which indicates that the emulsion gels were predominantly elastic, regardless of gellan type and immersion media. As expected from the gellan concentration difference, LaG emulsion gel had higher G', and complex modulus (G*) than HaG emulsion gel.

Immersion within the acid solution significantly increased all the dynamic moduli (G', G", and G*) of both LaG and HaG emulsion gels. The increase accords with the oil droplets flocculation (Fig. 4A) and the transformation from entirely ionotropic to ionotropic/acid-set gel. Acid-set gellan gels are known to be rigid and have higher frequency-dependent dynamic moduli than ion-induced counterparts at identical concentrations (Gunning et al., 1996).

At first, HaG and LaG emulsion gels had comparable tan δ values (Table 3). *Tan* δ indicates how efficiently a material dissipates energy and is a measure of the ratio between G" to G'. Therefore, gellan type did not influence the energy dissipation of entirely ionotropic emulsion gels. Immersion within the acid solution decreased the emulsions gels tan δ, which suggests that transformation of the emulsion gel samples from entirely inotropic to ionotropic/acid-set decreased the energy dissipation. Compared with LaG emulsion gel, the reduction in tan δ was much more pronounced for HaG emulsion gel following immersion in the

Table 3
Rheological properties of lipase-loaded LaG and HaG emulsion gels upon sequential immersion within the acidic and alkaline media.

Samples	G'_{LVR}(Pa)	G''_{LVR}(Pa)	G^*_{LVR}(Pa)	tan δ
HaG-Egel	3.1 ± 0.14^{Db}	0.72 ± 0.06^{Eb}	3.16 ± 0.37^{Db}	0.2329 ± 0.0288^{Aa}
HaG-$Egel_{30min}$ acidic medium	91.49 ± 5.98^{Ab}	9.96 ± 0.49^{Bb}	92.03 ± 7.49^{Ab}	0.1089 ± 0.0017^{Db}
HaG-$Egel_{60min}$ acidic medium	96.82 ± 6.84^{Ab}	11.48 ± 0.88^{Ab}	97.50 ± 8.69^{Ab}	0.1185 ± 0.0006^{Db}
HaG-$Egel_{60min}$ alkaline medium	48.89 ± 2.89^{Bb}	8.63 ± 0.47^{Cb}	49.65 ± 2.78^{Bb}	0.1765 ± 0.0009^{Ba}
HaG-$Egel_{150min}$ alkaline medium	24.79 ± 2.17^{Cb}	3.74 ± 0.28^{Db}	25.07 ± 3.3^{Cb}	0.1509 ± 0.0018^{Ca}
LaG-Egel	30.13 ± 2.29 Da	7.21 ± 0.35 Da	30.98 ± 2.47 Da	0.2395 ± 0.0064^{Aa}
LaG-$Egel_{30min}$ acidic medium	10201.7 ± 739.75^{Ba}	2161.13 ± 179.675^{Ba}	10428.1 ± 798.18^{Ba}	0.2118 ± 0.0022^{Ba}
LaG-$Egel_{60min}$ acidic medium	18613.2 ± 1341.61^{Aa}	4057.23 ± 343.45^{Aa}	19050.3 ± 1378.16^{Aa}	0.2179 ± 0.0027^{Ba}
LaG-$Egel_{60min}$ alkaline medium	1405.46 ± 106.45^{Ca}	178.20 ± 13.16^{Ca}	1416.72 ± 98.76^{Ca}	0.1268 ± 0.0002^{Cb}
LaG-$Egel_{150min}$ alkaline medium	1335.36 ± 88.75^{Ca}	133.54 ± 12.31^{Ca}	1342.02 ± 94.89^{Ca}	0.0999 ± 0.0025^{Db}

LaG: low-acyl gellan; HaG: high-acyl gellan.
Lower case letters indicate significant differences among different type of emulsion filled-hydrogels at a given time; Superscript uppercase letters indicate significant differences at different times for one type of emulsion filled-hydrogels ($P < 0.05$).
LVR: linear viscoelastic range.

acidic solution (Table 3). Indeed, HaG slightly aggregates via direct interaction with cations (Bradbeer et al., 2014). Hence, a plenty of carboxyl groups became protonated when HaG emulsion gel was immersed in the acid solution. Together with the glyceryl groups, protonated carboxyl groups caused massive hydrogen bonding between gellan double helices, leading to less energy dissipation.

The hydrogen-filled (i.e., protonated) carboxyl groups did not completely deprotonate upon the subsequent immersion of HaG emulsion gel in the alkaline solution. Thus, tan δ of HaG emulsion gel did not recover after the alkaline treatment to the value that happened in the entirely ionotropic sample (before acid and alkaline immersions). An opposite change in tan δ was observed when LaG emulsion gel was placed form the acid solution into the alkaline solution. On the contrary to HaG emulsion gel, LaG emulsion gel sample became lesser energy dissipating, i.e., tan δ decreased consequent to the alkaline immersion. Energy dissipation and crack propagation in gel network are inversely related; effective energy dissipation hinders crack propagation (Narasimhan et al., 2021). LaG emulsion gel was significantly less energy dissipating than HaG emulsion gel consequent to immersion in the alkaline solution. This accords with the remarkable crack propagation in LaG emulsion gel following immersion in the alkaline solution as observed by CLSM (Fig. 4A).

3.2.3.3. Lipase release and activity. The release of lipase from emulsion gels into the acidic solution (for 60 min) and then into the alkaline solution (over the next 150 min) was measured and the results are shown in Fig. 5A. Non-emulsified (i.e., added freely into the acidic and basic media) lipase was used for comparison purposes. The non-emulsified lipase had no enzymatic activity in the acidic solution. Furthermore, it did not show any activity in the alkaline solution after pre-immersion in the acidic medium. This indicates that the enzyme was irreversibly inactivated in acid. It is known that lipase is irreversibly inactivated at a pH of 4 or less {Trang, 2014 #46} (Trang et al., 2014).

The release profile of lipase from the two kinds of emulsion gels was substantially different (Fig. 5A). Roughly 20% of the loaded lipase progressively released from the HaG emulsion gel into the acidic medium. Within the alkaline medium, the release of lipase from HaG

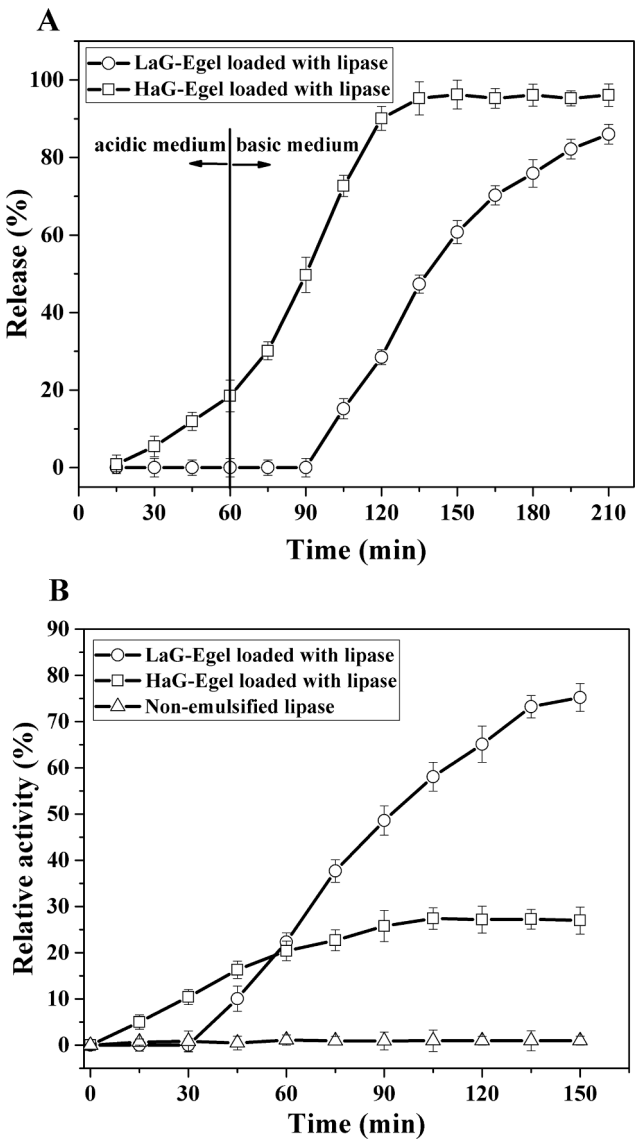

Fig. 5. (A) The release of lipase from the low-acyl and high-acyl gellan emulsion gels (LaG-Egel and HaG-Egel, respectively) upon immersion within the acidic medium (pH, 1.2) for 60 min and then, within the alkaline medium (pH, 7.4) for the next 150 min and (B) relative activity of the released lipase from the emulsion gels during immersion within the basic medium for 150 min.

emulsion gel continued to progressively take place until a plateau was reached after ≈75 min (a total duration of ≈135 min upon sequentially immersing the gel samples within the acidic and alkaline media). In contrast, lipase did not release into the acidic medium from the LaG emulsion gel. This indicates that the remarkable increase in G' of LaG emulsion gel consequent to acid immersion (Table 3) prevented lipase permeation out of the gel structure. Likewise, the release of the loaded lipase from the LaG emulsion gel into the basic medium was retarded for 30 min. This is an issue to be resolved in subsequent studies; it is known that the small intestine transit in both fasting and postprandial states occurs through very fast movements (>15 cm min^{-1}), accounting for median 60% of the distance in median 3% of the time (Worsøe et al., 2011). The loaded lipase must be soon released into the duodenum, where most of the digestion happens.

The lipase released into the alkaline medium from LaG emulsion gel had 2–3 times higher activity than that released from the HaG emulsion gel (Fig. 5B). This indicates that LaG emulsion gel could preserve the activity of the loaded enzyme within the acidic solution.

4. Conclusion

Spontaneous emulsification is a low-energy emulsion formation method, which is usually carried out at high SORs. Using gellan, either HaG (at 0.6 mg g^{-1} aqueous phase) or LaG (at 6 mg g^{-1} aqueous phase), together with the small molecule surfactant tween 80, we could decrease the SOR to 0.1 in the formulation of fully stable fish O/W emulsions. The addition of gellan into emulsions allowed gelling emulsions by $CaCl_2$ addition.

Although HaG could be used at a significantly lower concentration than LaG for making stable emulsions, it did not yield self-supporting emulsion gels. Despite this difference, both LaG and HaG emulsion gels efficiently protected the entrapped (emulsified) fish oil against lipolysis, also when lipase was co-loaded into the emulsion gels.

The emulsion gel samples underwent microstructural and rheological changes after immersion in the acidic and alkaline solutions, which imitated the gastric and intestinal fluids. The oil droplets flocculated consequent to acidic immersion and concurrently, all the dynamic moduli (G', G", and G*) of both kinds of the emulsion gels increased. The increase was more pronounced for LaG emulsion gel. Correspondingly, this sample did not release the loaded lipase into the acidic medium, which is promising for the protection of lipase in the stomach. However, lipase release from the LaG emulsion gel into the alkaline solution was also delayed for 30 min, which is a drawback for *in vivo* gastrointestinal applications and should be properly addressed in future studies.

CRediT authorship contribution statement

Saina Moayedzadeh: Investigation, Resources, Formal analysis. **Asghar Khosrowshahi asl:** Funding acquisition, Supervision, Project administration. **Sundaram Gunasekaran:** Supervision, Writing – review & editing. **Ashkan Madadlou:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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