Cold-set gelation of whey protein isolate and low-methoxyl pectin at low pH

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We report the fabrication of homogenous aqueous gels formed by a heat-cool step of whey protein isolate (WPI) and low methoxyl pectin (LMP) mixtures at low pH (2.0). The low pH suppresses the electrostatic repulsion between LMP molecules, leading to stronger intermolecular interactions between WPI and LMP molecules. Response surface methodology was applied to optimize and to investigate the effects of WPI and LMP concentrations on the gelation temperature (Tsol-gel), melting temperature (Tgel-sol), storage modulus (G'), critical stress (σc), and dynamic yield stress (σy) of the gels. The obtained results revealed that most of the characteristics evaluated were affected by the process variables, while there was also a significant interaction effect of WPI and LMP concentrations on the gel storage modulus as the main indicator of gel formation. A higher concentration of WPI and LMP resulted in a gel with lower porosity and almost frequency independent moduli. The gel microstructure showed the role of WPI to stabilize and develop the pectin network as crosslinking agent. The WPI-LMP gels showed pH-responsive behavior at higher pH values and stability over a wide range of pH values depending on the total biopolymer concentrations studied, respectively, which can make them very attractive for applications in bio-related fields.

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1. Introduction

Owing to their high biocompatibility, the gel forming properties of biopolymers are of great interest for production of functional biomaterials for a large variety of applications, particularly in biomedical and food industries (Avnir, Coradin, Lev, & Livage, 2006; Patel, Cludts, et al., 2014). Biopolymers, such as proteins and polysaccharides, are well-known to structure fluids through their gelation properties. Gelation of the aqueous phase by proteins can be induced by a simple heating process, which involves three distinct steps: denaturation, aggregation, and gelation (Totosaus, Montejano, Salazar, & Guerrero, 2002). Polysaccharide gelation usually involves the association of hydrated polysaccharide chains to form transient junction zones above a critical concentration (known as critical gelling concentration or Cc). Some polysaccharides require mono or divalent salt to form gels (De Kerchove & Elimelech, 2007). The gelation behavior varies depending on the chemical structure and the nature of inter-chain binding in different types of polysaccharides (Morris, 1998).

In recent years, mixed gels of proteins-polysaccharides have been investigated to achieve greater flexibility with respect to mechanical and structural properties compared to that of monocomponent gels (Eleya & Turgeon, 2000). It is important to understand how proteins and polysaccharides interact to affect the structure and how a specific structure contributes to the functional properties of the mixed gel (Renard, van de Velde, & Visschers, 2006).

Gelation of mixed gels has usually been associated with interpenetrating (Donato, Garnier, Novales, Durand, & Doublier, 2005) phase-separated (Firoozmand, Murray, & Dickinson, 2007) and coupled or complex network formation (Tsitsilianis, 2010). Interpenetrating networks are formed when both biopolymers associate independently to form separate networks. The gel network formed by one biopolymer is interpenetrated by the gel network from...
another biopolymer (Donato et al., 2005). Phase-separated gels are formed by incompatible polymers, which leads to the ‘arrested’ demixing of phases prior to gelation (Laneuville, Turgeon, Sanchez, & Paquin, 2006). Coupled or complex coacervate networks are developed when both biopolymers are linked together to form junction zones under associative conditions (Turgeon, Schmitt, & Sanchez, 2007).

The most important formulation factors that affect electrostatic-driven interactions between a specific combination of proteins and polysaccharides are pH, ionic strength, biopolymer ratio and total biopolymer concentration (Hosseini et al., 2013a; Neirynck et al., 2007; Schmitt & Turgeon, 2011). Through its role in the ionization of proteins and polysaccharides, pH is the most significant factor that determines protein-polysaccharide interaction in mixed gels. Optimum complexation and resultant particle formation occur at pH values where strong associative conditions of protein and polysaccharide are established (i.e. protein and polysaccharide are oppositely charged) (Weinbreck, Nieuwenhuijsen, Robijn, & de Kruif, 2003; Tavernier, Wijaya, Van de Meeren, Dewettinck, & Patel, 2016). However, these conditions can also deteriorate the solubility of complexes and their gelling ability because the system has a lower hydration capacity (De Kruif, Weinbreck & de Vries, 2004). At pH conditions where both biopolymers carry similar net charges, the interactions are minimal and thus, it is prerequisite to use a high concentration of total biopolymers to induce gelation via heat-cool step aggregation. The cold-set mixed gels produced at different pH values may show variation in micro- and macrostructure which can have an effect on the properties and eventual applications of gels. Therefore, it is of significant interest to have a comprehensive understanding of protein-polysaccharide mixed gels fabricated under different gelation conditions in order to develop functional soft biomaterials.

In this work, whey protein isolate (WPI) and low methoxyl pectin (LMP) were selected as model for a protein and an anionic polysaccharide. WPI is a mixture of globular proteins including β-lactoglobulin, α-lactalbumin, and lesser amounts of bovine serum albumin, and immunoglobulins (de Wit & Klarenbeek, 1981). β-lactoglobulin, the dominant compound of WPI, has an isoelectric point (pI) at about pH 5.1 (Bromley, Krebs, & Donald, 2005) and dominates the overall gelation properties of WPI (Mulvihill & Kinsella, 1987). On the other hand, LMP is an example of anionic polysaccharides obtained by acid de-esterification of high methoxyl pectin (Fishman et al., 2015).

Few studies related to cold-set of aggregated mixed gels of WPI and pectin at low pH have recently been investigated (Li & Zhong, 2016; Zhang, Hsieh, & Vardhanabhuti, 2014). In these two papers, the gelation of WPI-pectin mixtures can be achieved either by slow acidification (using glucono delta-lactone or GDL) or quick acidification (using HCl) from preheated mixtures at neutral pH. It is a prerequisite to preheat WPI at a concentration of >5% (w/v) at neutral pH to induce soluble aggregates. Then, as the pH decreases below the pI of WPI, the aggregation of protein becomes more extensive leading to gelation at a sufficiently high concentration of protein (Alting, de Jongh, Visschers, & Simons, 2002). In these mixed gels, the gelation at a sufficiently high concentration of protein occurs due to protein aggregation during acidification and with the help of pectin that acts as aggregates-binder via electrostatic interaction. Furthermore, the mixed gels produced by slow acidification result in a higher ordered structure and a stronger gel compared to those formed by quick acidification, attributed to controlled aggregation of preheated proteins during a gradual pH decrease (Li & Zhong, 2016).

In contrast with previous work, our goal was to study the synergistic cold gelation of WPI-LMP mixtures through a heat-cool step at a specific pH and to get insight in the optimization of gel properties by tuning concentrations and ratios of WPI and LMP (through response surface methodology). In addition, the pH stability of representative gel samples was investigated to evaluate possible applications of the gel for bioactive delivery or biomedical application.

2. Materials and method

2.1. Material

A minimally heat-treated WPI, enriched in β-lactoglobulin (approx. 85% of total protein), was obtained from Davisco Foods International, Inc., Le Sueur, MN, USA. Unipectine OB700 (low methoxyl apple pectin, with a degree of esterification between 33 and 38%) was received as a gift from Cargill, Belgium. Sodium azide, sodium chloride, and hydrochloric acid were obtained from Sigma Aldrich, USA, NaOH from VWR International, USA, and milli-Q water was used.

2.2. Preparation of stock biopolymer solutions

WPI and LMP powders were weighed into separate beakers at 2% (w/w) in 200 mL 10 mM Na-acetate buffer pH 7.0 with 0.02% (w/w) sodium azide. Then, the dispersions were stirred constantly at room temperature (20–25 °C) for 2 h (WPI) and 6 h (LMP). The dispersions were stored overnight at 4 °C and used for gel preparation in the following day.

2.3. Charge titration

WPI (0.5% w/w) and WPI-LMP mixture (0.5:0.5% w/w) in 200 mL 10 mM Na-acetate buffer pH 7.0 was each titrated by 1 N HCl. The WPI, LMP dispersion and WPI-LMP mixtures were titrated from pH 7.0 down to pH 1.5. The dispersion was connected to a pH electrode of a pH meter to monitor the pH change. The streaming potential signal was recorded by a Charge Analyser II (Rank Brothers Ltd, England) equipped with streaming potential cell.

2.4. Cold-set gelation at specific pH

To investigate the gelation pH of WPI-LMP mixture, WPI-LMP mixtures at a concentration of 0.5:0.5% (w/w) were heated at 4 different pH values (pH 5.5, 3.5, 2.5 and 2.0). WPI and LMP stock dispersions were mixed to achieve a final concentration of 0.5:0.5% (w/w). Control samples of WPI (0.5% w/w) and LMP (0.5% w/w) dispersion were also prepared. Each solution was then adjusted to pH 5.5, 3.5, 2.5, and 2.0 with 1 N NaOH and 0.1 N HCl. The solutions were stirred for 30 min at room temperature before 10 mL of the mixtures were poured into 15 mL capacity glass tubes with plastic screw caps. The solutions were then heated at 80 °C for 10 min, as 3 min were needed to reach a temperature above 74 °C, a total heating time of 13 min was used. Afterwards, the heated solutions were immediately immersed in a cold water bath.

2.5. Microstructure studies

Cryo-SEM was utilized to visualize the WPI (0.5% w/w), LMP (0.5% w/w) dispersion, and selected WPI-LMP mixtures (0.5:0.5% and 0.5:1.0% w/w). The samples were placed in the slots of a stub, plunge-frozen in slush nitrogen and transferred into the cryo-preparation chamber (PP3010T Cryo-SEM Preparation System, Quorum Technologies, UK), where they were freeze-fractured, sublimated for 15 min and subsequently sputter-coated with Pt and examined in a JEOL JSM 7100F SEM (JEOL Ltd, Tokyo, Japan).
2.6. Rheological measurements

For further characterization of WPI-LMP gels, rheological measurements were carried out on the gel samples prepared by different concentration combinations of WPI (0.5, 0.75, 1.0% w/w) and LMP (0.5, 0.75 and 1.0% w/w) solutions following the experimental design in section 2.8. Each solution mixture was adjusted to pH 2.0 with 1 N and 0.1 N HCl. Afterwards, the mixtures were subject to temperature ramps to understand the gelation temperature ($T_{gel}$) and melting temperature ($T_{gel-sol}$), and the linear viscoelastic region ($G'_LVR$), critical stress ($\sigma^*$), dynamic yield stress ($\sigma_y$) and frequency dependency were characterized by amplitude and frequency sweeps.

The rheological measurements of gel samples were carried out on an advanced rheometer AR 2000ex (TA Instruments, USA) equipped with a starch pasting cell system for temperature control and a starch pasting impeller geometry. The WPI-LMP mixtures (30 g) for rheology measurements were filled into the starch pasting cell. To identify the gelation and melting temperature of WPI-LMP mixtures, a range of experiments was carried out by first performing a temperature ramp (heating) from 25 °C to 80 °C at a rate of 5 °C/min and a time sweep test at 80 °C for 10 min, followed by a temperature ramp (cooling) from 80 to 5 °C, and a time sweep at 5 °C for 10 min. The whole cycle was repeated once to evaluate the reversibility of the gel. In the amplitude sweep tests to determine $G'_LVR$, $\sigma^*$, $\sigma_y$ of the gel samples, and the frequency sweep tests to understand the structural dependency of the gel samples to the frequency, WPI-LMP mixtures were subject to the same procedure of heating-holding-cooling as described in the procedure of temperature ramps (for one cycle) before amplitude or frequency sweeps was performed. Preliminary and separate oscillatory tests were carried out to determine the ‘safe’ strain as well as ‘safe’ frequency values using amplitude and frequency sweeps, respectively. The fixed strain (0.01) and fixed frequency (1 Hz) which were within the linear response region were further used in the oscillatory tests of the gel samples. Amplitude sweeps (0.1–100 Pa) and frequency sweeps (0.01–10 Hz) at a fixed frequency of 1 Hz and at a fixed strain of 0.01 were performed at 5 °C, respectively. Separate WPI-LMP mixtures were used in each rheological measurement.

2.7. pH stability

Selected WPI-LMP gel sample (1 g) at a total concentration of 0.5:0.5% and 1.0:1.0% (w/w) was weighed and placed into a series of 10 mL 10 mM Na-acetate solutions adjusted to different pH values (1–10) by 1 N and 0.1 N HCl and NaOH. The solutions containing WPI-LMP gels were then stored at 37 °C for 24 h.

2.8. Experimental design

A face-centered central composite design was used to perform the tests for the gel rheological properties, considering two factors (independent variables): the concentration of WPI (0.5, 0.75, 1.0% w/w) and the concentration of LMP (0.5, 0.75, 1.0% w/w). The experimental plan consisted of 13 trials (5 for the central point replicated for the estimation of error) and the independent variables were studied at three different levels. Central point conditions were 0.75:0.75% (w/w). The following polynomial equation was fitted to the data:

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2$$

where $\beta_0, \beta_1, \beta_2, \beta_{11}, \beta_{22}, \beta_{12}$ are constant regression coefficients, $y$ is the response variable ($T_{gel}$, $T_{gel-sol}$, $G'_LVR$, $LVR$, $\sigma^*$), and $X_1$ and $X_2$ are the coded independent variables (which were assigned a value of $-1, 0 + 1$) corresponding to WPI and LMP concentration (Table S1, Supplementary Information). The significance of each of the coefficients in the empirical model were selected or rejected based on the selected $p$-value. The terms statistically found non-significant (at $p > 0.1$) were removed from the initial models and the experimental data were refitted to produce the final reduced models. The fitness quality of the polynomial models was expressed by the coefficient of determination ($R^2$), the adjusted-$R^2$, the standard error of regression (S), the “lack-of-fit”, and the random distribution of the residuals (Ladjevardi, Gharibzahedi, & Mousavi, 2015). The statistical analysis of the data was performed by using MINITAB™ 16.0 Statistical Software (Minitab Inc., State College, PA, USA).

3. Results and discussion

3.1. Gelation of WPI-LMP mixture

3.1.1. Gelation at specific pH

During preliminary experiments, it was observed that WPI-LMP mixtures could only form a gel by a heat-cool step of the mixture at a specific pH. To show this effect, four different pH values (i.e. pH 5.5, 3.5, 2.5, 2.0) within the pH range of WPI-LMP complexation, were selected. These pH values also represented pH values below and above the zero potential of WPI-LMP complexes based on charge titration analysis of WPI-LMP (Fig. S1, Supplementary Information). In general, WPI-LMP mixtures (0.5:0.5% w/w) were heated and cooled at these 4 different pH values (pH 5.5–2.0). The heating of the mixture was performed at 80 °C for 10 min, followed by immediately cooling to 5 °C using a cold water bath. Fig. 1 shows the appearance of the WPI-LMP mixtures at four different pH values, both before and after heating-cooling step. At pH 5.5, the cold-set mixture turned only slightly turbid due to the formation of soluble complexes between heated protein and pectin, while the cold-set mixture at pH 3.5 and 2.5 showed turbidity and large aggregate formation, probably because heating the mixtures at these pH values could induce particles-particles aggregation due to strong intermolecular interactions between protein and pectin resulting in insoluble complexes. On the other hand, a sol to gel transformation was observed at pH 2.0 after the heated mixture was cooled to 5 °C. However, the gelation of the mixture after heating could also occur during cooling at room temperature (20–25 °C) (the gelation temperature was discussed in the section 3.2.2).

It has been reported that pectin can stabilize whey protein at a certain pH, by suppressing the heat-induced aggregation of whey protein (Zhang et al., 2014). At pH 5.5, WPI and LMP formed soluble complexes by heating as seen from the absence of aggregation of
the WPI-LMP mixture. As reported elsewhere, such complex formation is promoted by molecular interactions as the unfolded protein exposes positively charged local domains, further allowing interactions with negative charges on anionic polysaccharides (Vardhanabhuti, Yucel, Coupland, & Foegeding, 2009). In addition, soluble complexes formed at pH values which were around the pI of WPI (pH 5.3–4.9) could reduce the aggregation of the globular protein near its isoelectric point (Jones & McClements, 2008; Jones, Decker, & McClements, 2009). At pH 3.5, denatured WPI-LMP complexes contributed to the formation of micron-size particles as a result of intense protein aggregation resulting in the formation of relatively dense structures (Salminen & Weiss, 2014). Moreover, further acidification to pH 2.5 caused intense protein aggregation with the formation of precipitates as the pH value is still close to the pI of WPI-LMP complexes. At pH 2.0, the unheated mixture formed soluble complexes because of the weak attractive electrostatic interactions between both polymers as suggested by less cloudy WPI-LMP mixture compared to that at pH 2.5 and 3.5 (Fig. 1). Weakening of electrostatic attractions between β-lactoglobulin and anionic polysaccharides (e.g. κ-carrageenan or pectin) at low acidic pH has been previously proven based on the formed soluble complexes in the mixture (Hosseini et al., 2013b; Jones & McClements, 2011).

3.1.2. Synergistic gelation at pH 2.0

After investigating the pH of WPI-LMP gelation, we proved that the individual biopolymers could not gel the aqueous phase on their own by heating, as seen in Fig. 2a, b (inset picture). WPI (0.5% w/w) or LMP (0.5% w/w) subjected to a heat-cool step at pH 2.0 did not induce any gel formation as indicated by very low complex modulus (G*) (<1 Pa) and no viscoelastic behavior (Fig. S1, supplementary information), although LMP (0.5% w/w) showed a continuous network (Fig. 2b). Likewise, the highest LMP concentration (1.0% w/w) also showed no evidence of viscoelastic behavior (Fig. S2, supplementary information). However, WPI-LMP mixtures prepared at a same less or a higher biopolymer concentration (0.5:0.5% and 0.5:1.0% w/w) resulted in a viscoelastic gel. The cryo-SEM images further confirm a well-developed gel network in case of the WPI-LMP mixtures (Fig. 2c, d).

The molecular assembly of WPI and LMP that leads to gelation of the aqueous phase is largely affected by the thermal treatment. It has been reported that heating could promote binding of WPI and LMP in complexes (Krzeminski, Prell, Weiss, & Hinrichs, 2014). These interactions between protein and polysaccharide are non-specific low energy interactions such as hydrogen bonding or hydrophobic interactions (Turgeon, Beaulieu, Schmitt, & Sanchez, 2003). As it is known that hydrogen bonding becomes less important with increasing temperature, while hydrophobic forces increase in strength (Dickinson, 1998), it can be assumed that the hydrophobic interactions between WPI and LMP are mainly responsible for the highly-ordered protein-pectin gel network formation on acidification. Moreover, stabilization of heated WPI-LMP complexes by intermolecular disulfide (S-S) and sulfhydryl (SH/S-S) bonds resulting in a compact structure and a reduction in particle size has also been reported (Krzeminski et al., 2014). Fig. 2c, d clearly shows that the WPI-LMP mixtures can produce new structures with high network density resulting in self-standing gels (inset pictures). This new structural arrangement is due to the intermolecular interaction between WPI and LMP chains where WPI can attach and stabilize intermolecular junction zones of LMP chains, contributing to a denser network structure in cold-set WPI-LMP gels at low pH. Usually, under acidic conditions there is a reduction in the charge-density of LMP which may contribute to

![Fig. 2. Cryo-SEM images of the microstructure for cold-set WPI solution (0.5% w/w) (a), cold-set LMP solution (0.5% w/w) (b), WPI-LMP gel (0.5:0.5% w/w) (c), WPI-LMP gel (0.5:1.0% w/w) (d) at pH 2.0. Bars are 1 μm.](image)
extensive aggregation between pectin chains, i.e. the polymer-polymer hydrophobic interactions are dominant due to suppression of electrostatic repulsion (Gilsenan, Richardson, & Morris, 2000). In the presence of WPI, the extensive interactions between pectin chains at low pH are minimized (Le & Turgeon, 2013) and the resultant new structural arrangement leads to the formation of a compact and highly ordered network that can provide a framework for a viscoelastic gel structure. In addition, the presence of LMP at higher concentration (1.0% w/w, Fig. 2d) could prevent the WPI from heat-aggregation and accommodate the mixture of WPI to be spread more evenly through the junction zones than at lower concentration of LMP (0.5% w/w, Fig. 2c).

In addition to hydrophobic forces, electrostatic interactions among unlike charges could also contribute to the binding of WPI-LMP. However, it should be noted that at very low pH (like the one used in our study), the number of dissociated carboxyl groups is significantly reduced (Axelos & Thibault, 1991) and thus, the likelihood of electrostatic binding with WPI is rather limited. Hence, hydrophobic interactions are thought to play a dominant role. As reported previously, complexes of whey protein-xanthan gum could be stabilized through moderate heating, indicating a possible role of hydrophobic interactions to stabilize electrostatic complexes between polyelectrolytes and proteins (Turgeon et al., 2003).

3.2. Gel rheology

3.2.1. Gel behavior during heating and cooling (temperature ramps)

The behavior of WPI-LMP mixtures (pH 2.0) during heating and cooling was evaluated by subjecting the mixtures to temperature-controlled rheometry. Four phases of $G^*$ (complex modulus) transitions were observed in all treatments: 1) heating and holding at 80 °C (Fig. 2d) could prevent the WPI from heat-aggregation and accommodate the mixture of WPI to be spread more evenly through the junction zones than at lower concentration of LMP (0.5% w/w, Fig. 2c).

In addition to hydrophobic forces, electrostatic interactions among unlike charges could also contribute to the binding of WPI-LMP. However, it should be noted that at very low pH (like the one used in our study), the number of dissociated carboxyl groups is significantly reduced (Axelos & Thibault, 1991) and thus, the likelihood of electrostatic binding with WPI is rather limited. Hence, hydrophobic interactions are thought to play a dominant role. As reported previously, complexes of whey protein-xanthan gum could be stabilized through moderate heating, indicating a possible role of hydrophobic interactions to stabilize electrostatic complexes between polyelectrolytes and proteins (Turgeon et al., 2003).

According to Table 1, all the models were significant (at $p < 0.05$), with satisfactory determination coefficients ($R^2$). All main effects, linear and quadratic, and interaction effects were calculated for each model. The $p$ value was used as a tool to check the significance of each coefficient and also an indication of the pattern of interactions between variables (Sun, Liu, & Kennedy, 2010).

In this study, the coefficient of determination for all responses confirmed the desirability of the model to elucidate the relationship between the variables and thus allowing an acceptable fitness of response surface models to the experimental data. The relatively high adjusted-$R^2$ (as an unbiased estimator of the regression), low standard error of the regression (S) (Table S2, Supplementary Information), and randomly distributed residual plots (data not shown) showed that the model fitted the experimental data very well and could explain the relationship of the responses and the tested variables. Furthermore, as statistical analysis revealed, a significant “lack-of-fit” was only found in $\sigma_3$ and there was no evidence of lack-of-fit (at $p > 0.05$) for the four remaining responses (Table 1). The lack-of-fit test was used to compare the variation of the replications around their mean value (the “pure error”) and the variation of the mean values around the model prediction (the “bias error”). If the bias error was much larger than pure error, this means that there was a significant lack-of-fit (Charibzahedi, Razavi, Mousavi, & Moayedi, 2012). In some cases, a significant lack-of-fit can still be used if the determination coefficient ($R^2$) is highly significant by looking carefully to the other evaluation criteria of the goodness-of-fit (Kittisuan, Ritthiruangdej, & Suphantharika, 2014). The above results clearly show that the chosen model can satisfactorily explain the effect of the two factors, i.e. WPI and LMP concentration on all responses.

3.2.2.1. Gelation ($T_{gel-sol}$) and melting ($T_{sol-gel}$) temperatures. The values of the $T_{gel-sol}$ and the $T_{sol-gel}$, determined from the thirteen experimental runs generated by the central composite design, ranged from 27.3 to 36.3 °C and from 42.3 to 53.1 °C, respectively (Table S1, Supplementary Information). These values of the gel-to-sol and sol-to-gel transformation points are shown in Fig. 5 as two distinct crossover points ($G^* > G_c$ and $G_c > G^*$ indicated on the graphs with red and blue lines respectively) corresponding to the melting and the gelling points. According to the regression equation (Table 1), the $T_{gel-sol}$ and $T_{sol-gel}$ were significantly affected by the independent variables WPI (at $p < 0.05$) and LMP (at $p < 0.05$), with both variables exhibiting a positive linear effect. In addition,
only a quadratic effect of LMP was significant (at \( p \leq 0.05 \)) for \( T_{\text{gel-sol}} \) response. In order to better understand the correlation between the responses \( T_{\text{sol-gel}} \) or \( T_{\text{gel-sol}} \) and the experimental levels of each factor (WPI, LMP), response surface plots were created from the model. The 3D graphs that are illustrated in Fig. 4a, b, are plotted as a function of WPI and LMP concentrations. Fig. 4a, b depict that the \( T_{\text{sol-gel}} \) and the \( T_{\text{gel-sol}} \) points were optimum when the LMP concentration reached a maximum and the WPI concentration was in the range 0.90—1.00% (w/w). As seen in Fig. 5, an increase in the WPI and LMP concentration in all WPI-LMP combinations led to a

![Fig. 4. Response surface 3D plots for the effect of WPI and LMP concentrations on \( T_{\text{sol-gel}} \) (a), \( T_{\text{gel-sol}} \) (b), \( G'_{\text{LVR}} \) (c), \( \sigma^* \) (d), and \( \sigma_y \) (e).](image)

**Table 1**

<table>
<thead>
<tr>
<th>Equations</th>
<th>( r^2 )</th>
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<tr>
<td>( T_{\text{sol-gel}} = 31.82 + 2.60 \text{WPI} + 1.75 \text{LMP} - 1.37 \text{WPI}^2 + 1.28 \text{LMP}^2 )</td>
<td>0.8604</td>
</tr>
<tr>
<td>( T_{\text{gel-sol}} = 44 + 1.37 \text{WPI} + 4.32 \text{LMP} + 3.32 \text{LMP}^2 )</td>
<td>0.9257</td>
</tr>
<tr>
<td>( G'_{\text{LVR}} = 129.07 + 30.52 \text{WPI} + 40.97 \text{LMP} - 12.60 \text{WPI}^2 - 22.83 \text{LMP}^2 + 14.27 \text{WPI} \text{LMP} )</td>
<td>0.9753</td>
</tr>
<tr>
<td>( \sigma^* = 49.42 + 9.63 \text{WPI} + 19.42 \text{LMP} - 4.64 \text{LMP}^2 + 6.04 \text{WPI} \text{LMP} )</td>
<td>0.9715</td>
</tr>
<tr>
<td>( \sigma_y = 98.40 + 4.89 \text{WPI} + 28.83 \text{LMP} - 6.37 \text{WPI}^2 - 23.34 \text{LMP}^2 )</td>
<td>0.9797</td>
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All regression terms were significant at \( p \leq 0.1 \), regression terms in **bold** was significant at \( p \leq 0.05 \).
significant increase in both the $T_{\text{sol-gel}}$ and $T_{\text{gel-sol}}$ points, while the effect of an increase in the WPI concentration in increasing $T_{\text{sol-gel}}$ and $T_{\text{gel-sol}}$ points was found to be significant at higher LMP concentration. Moreover, WPI-LMP gels with a higher proportion of LMP in the same total concentration (1.5 and 1.75% w/w) showed higher $T_{\text{sol-gel}}$ and $T_{\text{gel-sol}}$ values compared to that with a higher proportion of WPI, suggesting the dominant role of LMP in the gel structure formation.

The resultant effect of the proportions and concentrations of WPI-LMP on the macrostructure of WPI-LMP gels is shown in Fig. 6. We found that to produce a gel with all of the liquid entrapped within the gel network, a minimum total biopolymer concentration (≥1.5% w/w) with certain ratio of WPI-LMP is necessary. As seen with the melting and gelation points, an increase in WPI concentration at a constant concentration of LMP (0.5% w/w) did not increase the gel stiffness and consequently these weak gels were prone to leakage of the liquid phase. On the other hand, when LMP was used at 1.0% (w/w), even the sample containing the minimum WPI concentration (0.5% w/w) produced a strong and stable gel.

In general, the internal structure of the gels was considerably influenced by LMP concentration: at 0.5% (w/w) LMP, the microstructure presents larger pores resulting in a weaker gel compared to the gel produced with a higher concentration of LMP (1.0% w/w) (Fig. 2c, d). It can be suggested that the higher LMP content may play a role in increasing the polymer network which in turn results in a more dense and homogeneous network. This is in agreement with the melting-gelation temperatures discussed in Fig. 5. The molecular weight of polysaccharides in general is more than 10 times higher than of proteins and this explains the dominant role played by LMP influencing the microstructure and resulting in a stronger gel compared to WPI.

3.2.2.2. $G'_{\text{LVR}},$ critical stress ($\sigma^*)$ and dynamic yield stress ($\sigma_y$). The responses of $G'_{\text{LVR}},$ $\sigma^*$ and $\sigma_y$ from amplitude (stress) sweeps are shown in Fig. 7. All the samples behaved as viscoelastic gels as indicated by higher values of storage modulus, $G'$ over loss modulus, $G''$ in the entire range of stress used within the linear viscoelastic region (LVR). Within the LVR, the average of y-axis values can be calculated as $G'_{\text{LVR}}$. The $\sigma^*$ is defined as the critical stress point where the LVR ends, which was taken as the point where the elastic modulus falls below 90% of $G'_{\text{LVR}}$. Moreover, the crossover point between $G''$ and $G'$ is defined as $\sigma_y$ observed when structured viscoelastic samples undergo a gel-sol transition ($\tan \delta = 1$) under increasing oscillatory shear (Patel et al., 2015).
Fig. 6. A series of WPI-LMP gel formed by heating WPI-LMP mixtures at pH 2.0.

Fig. 7. Plots of $G'$ (●) and $G''$ (○) against oscillatory stress (at a frequency of 1 Hz) for 4 selected combinations of WPI-LMP concentrations (0.5:0.5%, 0.5:1.0%, 1.0:0.5%, 1.0:1.0% w/w). The LVR is marked in the graph by the red dashed line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
The values of $G'_\text{LVR}$ and $\sigma^*$ ranged from 42.18 to 172.08 Pa and from 19.93 to 79.42 Pa (Table S1, Supplementary Information). It is apparent that the $G'_\text{LVR}$ and LVR were significantly influenced by the positive linear effect (at $p \leq 0.05$) of both factors, as shown in Fig. 7 that the $G'_\text{LVR}$ and $\sigma^*$ showed an increase as WPI and LMP concentration was increased. $G'_\text{LVR}$ increased approximately 4 fold from 42.18 ± 0.29 Pa to 172.1 ± 5.03 Pa as the total concentration of polymers was increased (WPI-LMP, 0.5:0.5% to 1.0:1.0% w/w), suggesting a strong effect of biopolymer concentration on the consistency of the gels as also illustrated by significantly positive coefficients in Table 1. Fig. 4c, d show that the optimum values for $G'_\text{LVR}$ and $\sigma^*$ were obtained at the highest WPI and LMP concentration.

In the case of $G'_\text{LVR}$, the negative quadratic effect of WPI and LMP was also considered as significant at $p \leq 0.05$, respectively. This quadratic effect of WPI and LMP can be seen by the characteristic curvature of the response surface, especially at values near the middle level of both factors (Fig. 3c). The highly significant quadratic effect of LMP suggests that a higher LMP ratio than WPI ratio contributed to a dramatic increase the storage modulus ($G'_\text{LVR}$). For instance, an increase in WPI concentration at a fixed LMP concentration (1.0% w/w) resulted in a higher increase of the $G'_\text{LVR}$ (2.5 fold) than the $G'_\text{LVR}$ produced by an increase in LMP concentration at a fixed WPI concentration (1.0% w/w) (1.8 fold).

The interaction effect of WPI and LMP on $G'_\text{LVR}$ and $\sigma^*$ (as the main indicator of gel formation) in the model was considered as statistically significant, respectively, indicating the synergistic effect of WPI and LMP concentration on the WPI-LMP gelation. As observed, an increased WPI and LMP concentration significantly increased the $G'_\text{LVR}$ and LVR of all gel samples. This interaction effect also indicated the dependency of the WPI-LMP gelation on the monocomponents. For instance, although WPI didn’t form a continuous networks (Fig. 2a), WPI could associate with the LMP network and develop junction zones of the gel network. On the other hand, LMP couldn’t form the network connections or junction zones without the presence of WPI. The increase of WPI concentration also increased the junction zones resulting into a denser structure of the gel network (Le & Turgeon, 2013). The stronger gel obtained at a higher LMP concentration, suggests that LMP provides a higher number of pectin chains in the formation of the gel network (Zhang et al., 2014). This synergistic gelation effect was previously discussed in the section 3.1.2 based on the microstructure analysis (by cryo-SEM).

All gel samples also showed a clear yielding behavior with $\sigma_y$ ranging between 36.2 and 99.7 Pa. As seen from the regression equation (Table 1), the $\sigma_y$ was significantly influenced by positive linear effect of WPI and LMP (at $p \leq 0.05$) and negative quadratic effect of WPI and LMP (at $p \leq 0.05$). As the gel stiffness increased due to an increase of WPI and LMP concentration, it also increased the $\sigma_y$ to undergo the gel-sol transition ($\tan \delta = 1$). Furthermore, the LMP plays a dominant role in the yielding behavior of WPI-LMP gels prepared at different concentration combinations as
statistically revealed. The $\sigma_v$ reached an optimum at the highest LMP concentration and at a WPI concentration which was in the range 0.75–1.00% (w/w) (Fig. 4e).

### 3.2.3. Gels structure by frequency sweep

Frequency sweeps were also performed to study the gel structure under the influence of the applied rate of deformation or frequency (Fig. 8). Unlike amplitude sweeps, when analyzing the frequency scans, more emphasis is laid on looking at the trends and changes in the data rather than at the specific peaks or transitions (Menard, 2008; Patel et al., 2015). For example, an entangled network shows frequency dependence, while a covalent strong gel shows frequency independence (Clark & Ross-Murphy, 1987). The frequency sweep was performed at 5 °C after the holding period (10 min) on WPI-LMP samples and showed a slight dependency of $G'$ over the decades of frequency range used. Moreover, the $G'$ was higher than $G''$ over the entire frequency range further confirming a well-formed physical gel structure (Malkin, Malkin, & Isayev, 2006; Patel, Rajarethinem, et al., 2014).

### 3.3. pH stability

The effect of pH on the morphology of WPI-LMP gels (formed at pH 2.0) was evaluated on samples prepared at the lowest and highest total polymer concentrations (WPI-LMP, 0.5:0.5% and 1.0:1.0% w/w). The weighted pieces of gels (1 g) were immersed in 10 mL of pH-adjusted Na-acetate buffer solutions and stored at 37 °C (selected for future applications of the gel at body temperature) for 24 h. We hypothesized that heat-induced crosslinking of WPI molecules within LMP chain networks at pH 2.0 might improve their stability to pH changes. The general appearance of the WPI-LMP gels was recorded as a function of pH (Fig. 9). At pre-storage, the WPI-LMP gel (0.5:0.5% w/w) showed a complete collapse and partial solubilization at pH 1.0. At all other pH values, there was no evidence of dissolution and the 1.0:1.0% (w/w) gel showed a high stability at all pH values, as expected. At post-storage, dissolution and sedimentation occurred at pH 1.0, 7.0 and 10.0. In contrast, the gels prepared at a higher total biopolymer concentration (1.0:1.0% w/w) did not show complete dissolution at any of the pH values as indicated from the few pieces of gel that still remained intact. The relative stability of 1.0:1.0% (w/w) gels over the entire pH range is attributed to the thermal crosslinking (e.g. hydrogen and hydrophobic bonds) between the WPI-LMP molecules at higher concentrations. These cold-set gels showed a better tolerance to pH than acid-induced protein-polysaccharide gels (which is mainly driven by electrostatic interaction) as they tend to dissociate at a pH which is away from their complexation pH (Le & Turgeon, 2013). Interestingly, at pH 4.0, i.e. at $p\text{I}_{\text{LMP}} < \text{pH} < p\text{I}_{\text{WPI}}$, both 0.5:0.5% (w/w) and 1.0:1.0% (w/w) gels maintained their original forms confirming their stability. As reported in literature, at pH values characterized by strong protein-polysaccharide complexation, interpolymeric complexes contribute to the strengthening of the network (Li & Zhong, 2016).

Overall, the gels formed in this study are stable to a wide pH range at high polymer concentrations (Fig. 9b, d) and show a pH-responsive behavior at low polymer concentrations (Fig. 9a, c). The pH responsiveness together with stability at 37 °C could be utilized to create gel matrices with controlled release properties, which could be attractive for design and formulation of physical gels geared towards bioactive delivery or biomedical applications.

### 4. Conclusion

In conclusion, this study demonstrated that gelation of WPI and LMP mixed systems could be performed by heating soluble WPI-LMP complexes at low pH where electrostatic repulsion of LMP chains is suppressed. Heating whey protein and pectin together at low specific pH promoted the interactions between the two bio-polymers, which resulted in dense and homogenous network formation (with protein particles incorporated in the pectin network) leading to gelation of the aqueous phase. The extent of the interactions between protein and pectin was greatly influenced by the concentrations and the proportions of the individual components. According to the statistical analysis, the interaction effects were only significant (at $p \leq 0.05$) for $C_{\text{LMP}}$ and LVR response as the main indicator of gel formation. Furthermore, this significant interaction between WPI and LMP showed a synergistic effect of WPI and LMP on the gel formation, where LMP contributes to the building of the network that acts as the structural framework to support the gel system and WPI provides the stabilization of the networks through connecting the junction zones. The WPI-LMP gels prepared at lower and higher total polymer concentration also showed a pH-responsive behavior at higher pH values and stability over a wide pH range at body temperature, respectively, which warrants further investigation of these gels as attractive candidates for bioactive delivery and biomedical applications.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodhyd.2016.10.037.

References


