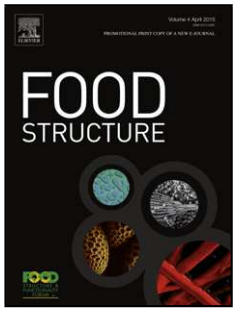

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Insignificant impact of the presence of lactose impurity on formation and colloid stabilizing properties of whey protein-maltodextrin conjugates prepared via Millard reactions

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Keywords

Whey protein-polysaccharide conjugates; maltodextrin; lactose impurity; Maillard reaction; Emulsion stability

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Abstract

We investigate the sensitivity of steric stabilising properties of Protein-polysaccharide conjugates, prepared via the Maillard reaction, to the presence of sugar impurity during synthesis. The sugar can also react with protein, thus rendering potential sites on protein unavailable for linkage with polysaccharide and severely reducing the efficiency of producing these types of food dispersants. We demonstrate that despite the presence of a relatively high molar ratio of lactose contaminant to maltodextrin (10:1), the covalent complexes between maltodextrin DE19 (MD19) and Whey Protein Isolate (WPI) are still formed and continue to show superior emulsifying and colloid stabilising properties compared to native protein. The improvement was particularly marked under unfavourable environmental conditions, such as pH ~ pI of protein, up to a storage time of 21 day. In contrast, the covalent complexes of lactose + WPI were found to have rather poor emulsion stabilising characteristics, under the same conditions. We also confirm this result by performing theoretical Self Consistent Field type calculations. The stability of emulsions was monitored using a variety of measures including the average droplet size (ADS), droplet-size distribution (DSD), rheological flow behaviours and confocal laser imaging microscopy. The suggestion that the WPI-MD19 (1:2 w/w) system is quite tolerant to the presence of lactose is of significance in future large scale industrial manufacturing of such food dispersants, due to less stringent requirements for the purity of raw material (WPI).

1. Introduction

Food proteins have been emulsifiers and colloid stabilisers of choice in many food colloid formulations. Proteins are surface active by the virtue of their amphiphilic nature. When proteins are adsorbed at the oil-water interface they contribute to both of the two major means of protecting emulsion droplets against flocculation and coalescence. The two mechanisms in mind are the electrostatic and steric stabilisations of colloids (Dickinson, 2010). However, these stabilising properties can be detrimentally affected, or even completely eliminated, under the influence of certain environmental conditions, such as pH, being too close to the pI of proteins, or at high ionic strength (McClements, 2015; Walstra, 2003). In order to maintain and improve the emulsifying and stabilising properties of proteins under these unfavourable conditions, native proteins are covalently bonded to polysaccharide molecules (Akhtar & Dickinson, 2007; Dickinson & Semenova, 1992). The fundamental concept underlying this modification is to synthesise a conjugate which can behave as a stabilising agent with considerably enhanced steric stabilising property, not overtly sensitive to changes in pH or to background electrolyte concentration. The hydrophobic segments of protein can strongly adsorb at the oil-water interface, while the hydrophilic regions (i.e. now mainly polysaccharides) protrude away from the interface, thus providing a thick surface layer and improved steric stabilisation (Dickinson, 2015).

One of the simple methods to prepare the conjugates is by heat treatment to induce the necessary Maillard reactions between the protein and polysaccharide. This has to be achieved under careful controlled conditions, such as relative humidity, temperature and the processing time. This can be done either in powdered state or in an aqueous solution (Aoki et al., 1999; Dickinson & Galazka, 1991; Kato & Kobayashi, 1991; Kim & Shin, 2015; Qi, Liao, Yin, Zhu, & Yang, 2010; Zhu, Damodaran, & Lucey, 2010). Moreover, the Maillard-type conjugates can be prepared under high pressure (Xu et al., 2010), by microwave heating (Guan, Qiu, Liu, Hua, & Ma, 2006), or using pulsed electric fields (Sun, Yu, Zeng, Yang, & Jia, 2011). Almost all of the previous studies in literature have suggested that the emulsifying and stabilising properties of various food proteins are improved significantly through such Maillard reactions with a polysaccharide. Furthermore, these conjugates also exhibit other potential

applications as foaming agents and heat-set gelling agents (Martinez-Alvarenga et al., 2014; Spotti et al., 2013a, 2013b). The overall conclusion of these studies has been that the conjugates, prepared in this manner, are excellent emulsifiers and stabilisers with significant possible potential for use in food industry. This is especially the case as their synthesis involves no additional chemicals and due to their relatively simple, if not exactly cheap, preparation method (Kato, 2002).

Before conjugates can be produced on an industrial scale, it is necessary to investigate a number of crucial factors which can significantly influence the required functionalities of these complexes. Examples are the ratio of protein to polysaccharide, processing conditions, and the molecular weights of polysaccharides used, but to name a few (Oliver, 2011). It has been reported that the emulsifying and stabilising properties of conjugates have a positive correlation with the length of polysaccharides grafted on the protein (Shu, Sahara, Nakamura, & Kato, 1996). This finding supports the stabilising model of conjugates which was first proposed by Dickinson and Semenova (1992). Based on the evidence, it can be predicted that proteins attached to high-molecular-weight (HMW) polysaccharides such as maltodextrin should have better stabilising properties than those modified by low-molecular-weight (LMW) sugars, like lactose under the same processing conditions. Similar conclusions are also supported by theoretical considerations where it has been shown that the attachment of short polysaccharide chains, depending on the location of grafting, can result in conjugates with an inferior stabilising property when compared to protein on its own (Akinshina, Ettelaie, Dickinson, & Smyth, 2008). It may be expected then, that if both HMW and LMW polysaccharides exist during the Maillard reaction, the stabilising properties of the conjugates may lie somewhere between that of the complexes resulting from grafting by HMW polysaccharides and ones produced by the Maillard reaction involving LMW sugars. The aim of the current work is to find out at what level of lactose impurity, unavoidable in commercial whey protein, it is possible to produce conjugates with interfacial properties comparable to those prepared by the Maillard reaction between pure WPI and maltodextrin. To evaluate the critical molar ratio of lactose, we have chosen the whey protein isolate-maltodextrin DE19 (WPI-MD19) conjugate as a model system. This conjugate is known to

have excellent emulsifying and stabilising properties, as has been demonstrated in our previous studies (Akhtar & Dickinson, 2003; Akhtar & Dickinson, 2007).

The paper is organised as follows. In the next section we describe our method for preparation of conjugates, that of emulsions and the evaluation of the emulsion stability. The results are presented next and discussed in the light of our current understanding of the interfacial behaviour of covalently bonded protein + polysaccharides. We provide a few preliminary theoretical calculations based on Self Consistent Field theory (SCF), along the lines used in our previous work, to compare the emulsion steric stabilising properties of WPI+MD19 with those of WPI+Lactose (Akinshina et al., 2008; R. Ettelaie & Akinshina, 2014; Rammile Ettelaie, Akinshina, & Maurer, 2012). These calculations are useful in lending further support to conclusions drawn from the experimental observations.

2. Materials and Methods

2.1 Materials

The lactose-free whey protein isolate powder was offered by Davisco Foods International (USA). The lactose was purchased from Fisher Scientific Ltd., and maltodextrin DE19 (M_w = 8.7 kDa) was offered by Roquette (UK) Ltd. The sunflower oil was purchased from local supermarket Morrison (Leeds, UK). Other chemicals and reagents used in this project are of analytical grade.

2.2 Conjugates preparation

The whey protein isolate (WPI) and maltodextrin DE19 (MD19) were fully dissolved in 100ml distilled water with gentle stirring under room temperature. Various recipes involving different ratios of MD19 to lactose content, were prepared as shown in Table 1. The solutions were stored in the fridge (4 °C) overnight were frozen at – 30 °C for 3 hours. These were freeze dried for a period of 24 hours. After collection, the resulting powder of WPI and MD19 (and lactose were appropriate) was placed in a pre-

heated desiccator under 80 °C for 3 hours, with relative humidity controlled by saturated KBr solution. The complex of WPI and MD19 was stored in a dark and dry place for further application. The conjugates of WPI-MD19 (1:2 w/w) and WPI-Lactose (2:1 w/w) were similarly prepared as controls.

2.3 Degree of conjugation

The degree of conjugation (DC) of each protein-polysaccharide complex after the Maillard reaction was determined by o-phthalaldehyde (OPA) tests. The OPA reagent was prepared based on the previous literature (Nielsen, Petersen, & Dambmann, 2001). Each conjugate was dissolved into distilled water with gentle stirring at a concentration corresponding to a WPI content of 1.0 mg/ml. For each prepared solution, 0.4 ml of the sample was added to 3 ml OPA reagent mixing on a Topmix at 1600 rpm for 5 seconds. The mixture was allowed to stand for exactly 2 mins at room temperature before its absorbance at a wavelength of 340 nm was measured using a spectrophotometer. The baseline was established by untreated pure WPI solution. The degree of conjugation for this complex can thus be calculated as follows:

$$\text{Degree of conjugation (DC) \%} = (C_{WPI} - C_{nConj}) \text{ X } 100\% / C_{WPI}$$

where C_{WPI} is the concentration of native WPI and C_{nConj} is the concentration of unreacted WPI in the conjugate sample. The analysis of each sample was carried out in triplicate.

2.4 O/W emulsion preparation

Before homogenization process, the aqueous buffer (500 ml) at ionic strength 0.1 M and pH of 2.9, was prepared by mixing citric acid (3.125 g) and sodium chloride (2.920 g) into distilled water. Sodium azide was also added to the aqueous buffer at the concentration 0.1 % (w/v) as a preservative. The appropriate amount of protein-polysaccharide conjugates were dissolved into the aqueous buffer

by gentle stirring at room temperature. The concentration was chosen so as to ensure a protein (WPI) concentration of 2 % (w/v), taken account of the fact that conjugated biopolymers have a considerably larger molecular weight. This then will give the same molar concentration for all conjugates. When dissolution process was completed, the clear solution and sunflower oil were passed through the jet homogenizer under 350 bar at a volume ratio of 80 : 20. After emulsification, the pH of the emulsions was adjusted to 4.6 by addition of a few drops of 6 mol dm^{-3} NaOH before they were stored quiescently at 30 °C. Protein stabilized emulsions are least colloidally stable at a pH corresponding to the pI of the stabilizing protein. Adjusting the pH of the emulsion to 4.6 here (iso-electric pH for WPI), insures that the electrostatic interactions are minimized and that the only contribution to repulsive forces are steric ones. WPI is known to form thin surface layers and thus not sufficient steric stability. Therefore, any advantages of conjugates as dispersants in comparison to WPI, are to be seen most clearly at this pH value.

2.5 Emulsion stability monitoring

The emulsion stability was assessed using several different measures, including average droplet size (*d[4,3]*) (ADS), droplet-size distributions (DSD), rheological flow properties, and images obtained from confocal laser scanning microscopy (CLSM). The measurements were conducted at various stages of storage. The particle sizing of emulsions was performed using a Malvern Mastersizer 3000. The average droplet size *d[4,3]* is defined as

$$d[4,3] = \frac{\sum_i n_i d_i^4}{\sum_i n_i d_i^3}$$

where n_i is the number of droplets with diameter d_i. The droplet-size distributions can also be obtained from the same Mastersizer. There is a major disadvantage in this kind of particle-sizing technique as it involves considerable dilution of the sample. This inevitably eliminates some of the possible instability mechanisms in the emulsion system, as for example the depletion flocculation. Therefore, other

techniques are necessary to complement and support the particle sizing results. Rheological assessments are quite useful to analyse the stability of emulsions which are not diluted during tests. In this work, the shear dependence behaviour of emulsions was investigated using a MCR102 Rheometer from Anton Paar. The results were analysed according to the power-law model (Dickinson, 1992):

$$\tau = k\dot{\gamma}^n$$

where τ (shear stress) is the function of $\dot{\gamma}$ (shear rate), k is the consistency index and n is the flow behaviour index, indicating a shear thinning fluid if n < 1, Newtonian behaviour where n = 1 and shear thickening (dialatant) one if n > 1. The *apparent viscosity* μ defined as (τ/γ) was also investigated under different shear rates.

We also took confocal laser scanning microscopy (CLSM) images of emulsions at freshly prepared and after 28 days of storage to provide visual assessments of the stability of droplets. The emulsion samples (2.5 g) were stained by Nile red (25 µl of 0.01% w/v dye in polyethylene glycol) and gently mixed with a glass rod at room temperature. Then the stained samples were placed in the plastic cell and covered with the cover slip. A Leica microsystems was utilised to observe and record digital images.

2.6 Statistical analysis

Data obtained from ADS, DSD and rheological measurements were analysed by using MS Excel© 2013 for the average values and their standard deviations.

3. Results and discussion

3.1 Appearance of conjugates

The pure white mixture of WPI and MD19/lactose turned to yellow with a milky smell after incubating in a desiccator under 80 °C for 3 hours. This indicates that the Maillard reaction occurred during the heating treatment (see Figure 1). Furthermore, the texture of conjugates altered significantly when the proportion of lactose was increased upwards to 1:6 and beyond to 1:10 (MD19 : lactose, molar ratio). The structure of resulting conjugates at relatively lower lactose ratio was loose and porous, whilst it became rigid and hard when the molar abundance ratio of lactose was increased to 1 : 6 and higher.

3.2 OPA analysis of conjugates

It can be seen from Figure 3 that the DC increases with the amount of lactose added to the WPI-MD19 system. As expected, with the much higher molecular weight of maltodextrin, the bigger the proportion of lactose the larger is the extent of conjugation due to there being a larger number of molecules available for reaction with protein. However, the overall DC increases only about 20%, from 60% to 80%, whilst the molar ratio of lactose is altered from no lactose impurity to 1:10 (MD19 : lactose). There are two possible explanations for this incomplete reaction: 1) the major component protein in WPI is β-lactoglobulin which has complicated secondary structure and a compact globular nature. In such a structure, not all the potential amino groups capable of reaction may be accessible to reducing sugars, even after partial denaturing due to heat treatment. 2) sugars can be polymerised under high temperature in the dry condition, in what is known as caramelisation process. This greatly reduces the number of sugar molecules available for covalent bonding with protein. When the amount of lactose increases in the system, the extra sugars can react with each other to form a rigid and hard

structure, which indeed is what has been found here (Figure 1 in section 3.1). Similar results have been reported from many other studies in the literature, even though different proteins and polysaccharides were adopted (Markman & Livney, 2012; Mu, Zhao, Zhao, Cui, & Liu, 2011; Spotti et al., 2013b; Sun, Yu, Yang, et al., 2011; Xu et al., 2010). Nevertheless, many researches focused on the positive relationship between the loss of free amino groups in proteins and the reaction time to confirm the attachment of polysaccharides (Mu et al., 2011; Spotti et al., 2013b; Xu et al., 2010). Compared to the results reported from other researchers, the DC (> 60%) of the conjugates in our project are significantly better than these other studies (typically < 50%) (Spotti et al., 2013b; Sun, Yu, Yang, et al., 2011). It must be noted that the processing conditions are different from those other investigations and this may explain the higher DC we obtain here in our work. We used a relatively higher temperature (80 °C) and shorter reaction time (3 hrs) in a dry conditions, while low temperatures (usually around 60 °C) and longer reaction times (often a couple of days) were adopted in many other reported experiments (Spotti et al., 2013b).

3.3 Stabilising properties of conjugates in emulsions

The stabilising properties of WPI-MD19 (1:2 w/w) complexes, synthesised in absence of lactose contaminant, and the conjugates with various levels of lactose present during the production, were studied by preparing a basic O/W emulsions (20 oil : 80 aqueous phase vol.) at pH 4.6, and a background ionic strength of 0.1 M. In parallel, the emulsion stabilised by glycoprotein WPI-lactose (2:1 w/w) made with no MD19 present, was also prepared. These latter represent a reference for the very highly contaminated systems, where one may assume that all available amino acid sites of WPI react with sugar as oppose to long maltodextrin chains. The stability of each stabiliser was assessed according to average droplet size, droplet size distribution, rheological flow properties and using CLSM images.

192 **3.3.1 Emulsion stability by visual assessment**

193 Figure 2 shows four emulsions prepared with different stabilisers at pH4.6 after a storage time
194 of 28 days at 30 °C. As can be seen from the picture, the sample 4 is the most unstable system amongst
195 the four emulsions because it is stabilised by the untreated mixture of WPI and MD19. When the pH of
196 the environment is close to the pI of WPI, the net charge of the protein nearly equals zero. In this case,
197 the electrostatic stabilising effect was almost eliminated at pH 4.6. However, sample 1 and sample 2 are
198 quite homogeneous after the observation period, and they are stabilised by WPI-MD19 (1:2 w/w) and
199 WPI-MD19 with lactose contamination at molar ratio of 1:10 (MD19 : lactose), respectively. This
200 observation indicates that the lactose impurity has insignificant influence on the emulsifying and
201 stabilising properties of conjugates during the Maillard reaction. In terms of sample 3, it is stabilised by
202 WPI-lactose (2:1 w/w) and exhibit certain degree of stability. It is better than sample 4 but not as good
203 as sample 1 and 2 due to the clear cream layer on the top. Further characterisation of these emulsions
204 by various techniques is following.

205

206 **3.3.2 Average droplet size (ADS)**

207 Figure 4 shows that the ADS of most emulsions is under 1 μm, except the emulsion stabilised by
208 WPI-lactose (2:1 w/w) where it was closer to 3 μm. The average droplet size (ADS) seems to decrease
209 slightly, in the period 7 to 21 days, for some of the samples and in particular the WPI-lactose stabilised
210 emulsions. In reality, there was no further effort put into the emulsions to reduce their droplet size,
211 post jet homogenisation. We believe that this slight decrease is predominantly due to the creaming of
212 the larger droplets, and the bias towards the smaller remaining droplets in the dispersion during
213 sampling. For such WPI-lactose samples the initial average size of droplet was larger than the WPI-
214 MD19 based emulsions and by the end of 7 days it is larger than 7 μm (see Figure 4), exasperating
215 possible creaming at later stages beyond 7 days. Nonetheless, the initial rapid increase of ADS from 3

μm to 7 μm is an indication that WPI-lactose complex is not an efficient emulsion stabiliser. The ADS of these emulsions remain around 7 μm for the rest of the monitoring period. In contrast, the emulsions stabilised by other conjugates are quite stable throughout the whole period of observation. As expected, WPI-MD19 (1:2 w/w) alone exhibits excellent emulsifying and stabilising properties, due to the enhanced steric stability under unfavourable environmental conditions. This is line with previous observations, including those of our own (Akhtar & Dickinson, 2007). Surprisingly, the WPI-MD19 systems, contaminated with different molar ratios of lactose, still presented acceptable stability comparable to the WPI-MD19 conjugate itself, synthesised in absence of sugar. This remains true even for a molar ratio of MD19 to lactose of 1:10. This result suggests that the WPI-MD19 conjugation system is quite tolerant to the existence of lactose from the stability point of view. However, other further assessments of emulsion stability are required to support this suggestion. These are presented next.

3.3.3 Droplet-size distribution (DSD)

The DSD of emulsions is another parameter to monitor the stability during storage, which has been illustrated in Figure 5. At day 0, both of WPI-MD19 and WPI-MD19 with lactose (MD19 : lactose = 1 : 10 molar) show excellent emulsifying properties because most of the droplets sampled in these emulsions are less than 1 μm whereas the droplets in the emulsion emulsified by WPI-lactose have a clear peak in the range from 2.27 to 22.60 μm. This suggests that the emulsifying properties of conjugates involving MD19 are better than those of glycoprotein produced through reaction of lactose + WPI, at a pH value of 4.6, close to the pI of WPI. After 28 days of storage, the DSD of emulsion with WPI-MD19 maintains almost the same size distribution as that on day 0, with a slight shift in peak from 0.1 μm to 0.23 μm. In contrast, there is a significant shift of the DSD for emulsions stabilised by WPI-lactose from smaller sizes to larger ones, following storage. However, no large shift of the DSD in the emulsion containing lactose (at the time of producing the conjugate) was observed, at MD19 to lactose molar ratio of 1:10. Indeed, the DSD of emulsions containing various molar ratios of lactose from 1:1 to 1:6 were all similar to that for 1:10 system. These results further support the same conclusions as those

arrived at from examining ADS (see section 3.3.2). This indicates that the presence of lactose has little influence on the emulsifying and stabilising properties of synthesised WPI-MD19 conjugate. On the other hand, in the absence of MD19, the resulting WPI-lactose glycoprotein is not capable of providing sufficient steric stabilisation, at least not at pH values close to pI of WPI.

3.3.4 Rheological properties of emulsions

Figure 6 and 7 show the rheological properties of emulsions stabilised by various conjugates, during the storage time from 0 to 28 days. All the emulsions thus prepared showed some degree of shear thinning behaviour over the entire range of shear rates considered. We fitted the equation for a power law fluids to our rheological data and obtained the values of consistency index *k* and the flow behaviour index *n* for each sample. The emulsions stabilised by WPI-MD19, or by WPI-MD19 synthesised in the presence of lactose (MD19 : lactose = 1 : 10), displayed relatively low values of *k* (< 0.1) during the entire storage period, apart from the emulsion with the conjugate (MD19 : lactose = 1 : 10) at day 28 which possessed a slightly larger consistency index. In contrast, the *k* value for emulsions stabilised by WPI-lactose was found to be much higher compared to other systems. Similarly, the value of *n* in emulsions prepared with WPI-MD19 stayed round 1 during the whole 4 weeks of observation time. This indicates that these emulsions exhibit an almost constant apparent viscosity in the range of studied shear rates and therefore a behaviour close to that of a Newtonian fluid. The emulsions stabilised by WPI-MD19 conjugates, containing lactose contamination during the synthesis of the complex, also had Newtonian flow properties during the initial 21 days, whilst this changed to a shear-thinning fluid like behaviour ($n < 0.4$) on day 28. Compared to these two systems, the samples prepared with WPI-lactose (i.e. no MD19) showed rather strong shear-thinning ($n < 0.6$) from the very onset, remaining so throughout the period of observation. The viscosity was also found to be higher for these samples, most likely due to flocculation and formation of clusters of emulsion droplets. This tends to slow down the creaming but does not prevent coalescence and the increase in the average droplet size

(figure 5). Examples of the detected rheological behaviour after 28 days of storage are shown for some of the studied samples in Figure7. The shear-thinning behaviours is clearly displayed, except for the emulsion stabilised by WPI-MD19. Furthermore, the emulsion with the lactose (MD19: lactose = 1:10) shows the strongest shear-thinning character. Nonetheless, recall that the ADS and DSD results for this emulsion suggests that there is no significant coalescence occurring after 28-days (see section 3.3.1 and 3.3.2). Therefore, the instability (indicated by shear-thinning behaviour) of this emulsion may be due to the flocculation, especially depletion flocculation, without coalescence. This may be due to covalently bonded WPI + lactose complex becoming too soluble. This reduces the adsorbed amount of the glycoprotein on the surface of droplets, leaving a large portion of the chains in the solution. The presence of such free biopolymers in bulk will induce depletion attraction between the droplets and may account for the formation of flocs in such systems. Upon a significant dilution, required during the ADS and DSD measurements, such flocs will most certainly fall apart, as the depletion effect is no longer be present. This then accounts for the small size of droplets measured even for WPI+lactose stabilised system. Yet, prior to any dilution, it is precisely the presence of these flocs that is responsible for the strong shear thinning behaviour, observed even at low shear rates.

The comparison of the rheological properties of the three type of emulsion samples, suggest that the emulsifying and stabilising capacity of conjugate WPI-MD19 with lactose, at a molar ratio of MD19 : lactose = 1:10, and that without any impurity are quite similar to each other, both providing stable well dispersed emulsions and a Newtonian type behaviour, at least over a reasonably period of storage. It is also interesting then to note that the lactose, existing in WPI during conjugate synthesis, does not seem to affect the emulsifying and stabilising properties of latter in any significantly way. However, the stabilising property of conjugates produced lactose as opposed to MD19, is clearly not as good as those of WPI-MD19, especially after 4 weeks.

The images from CLSM analysis can provide further evidence to support the conclusions drawn from rheological measurements. These will be presented next.

3.3.5 Images of emulsions from CLSM

There are three CLSM images in Figure 8 showing the emulsions stabilised by WPI-MD19 (1:2 w/w), those stabilised by WPI-MD19 (1:2 w/w) with lactose impurity present at a molar ratio 1:10 (MD19 : lactose), and finally WPI-lactose (2:1 w/w). All three images were taken after 28 days of storage at 30 °C. For the emulsion system stabilised by WPI-MD19, it is clearly seen that the droplets are well dispersed and still separated from each other after 4 weeks. Additionally, the diameter of the largest droplet in this image is less than 5 μm, with majority of droplets much smaller than this. This observation is in agreement with the ADS values for this emulsion system, where on day 28 $d[4,3] < 1$ μm (see section 3.3.1). Images taken at the same time indicate many flocs formed by droplets in the emulsion stabilised by WPI-MD19, when lactose was present at the time of conjugate synthesis. Such flocs, though were not seen at earlier times (images for earlier time not shown), did develop at later stages of storage. There are still some individual droplets seen in the image for this sample. More significantly, the diameter of the largest droplet is quite similar to that in the emulsion system stabilised by WPI-MD19 alone. The CLSM image of the emulsion stabilised by WPI-MD19 (1:2 w/w) with lactose (MD19 : lactose 1 : 10) give further support that the ADS of this emulsion system is close to that of the emulsion stabilised with WPI-MD19 only. However, the difference between the two is that the latter still exhibits clear shear-thinning character during the rheological analysis. When the samples are introduced in Mastersizer 3000, they are considerably diluted and this eliminates the depletion flocculation effect, present under original concentrations (Chang & McClements, 2015; Asakura & Oosawa, 1954).

Compared to the above two stable emulsions, the sample stabilised by WPI-lactose shows few individual droplets in the CLSM image very soon after homogenisation. However, even though most of the droplets stick to each other to form clusters, there is no oil layer creaming on the top of the emulsion after 28 days of storage. We believe this is due to the much enhanced viscosity seen for this

316 sample. The CLSM images support the explanation of shear-thinning character, arising from breakup of
317 aggregates, seen in section 3.3.4.

318

319 **3.4. Preliminary theoretical results**

320 Behaviour of protein + polysaccharide conjugates have also been studied using a number of
321 different theoretical techniques. In one of the earliest such studies, Dickinson and Euston carried out
322 Monte Carlo simulations of conjugates in presence of a hydrophobic-hydrophilic interface, performed
323 on a discretised lattice (Dickinson & Euston, 1992). Their results highlighted the existence of an
324 optimum number of attached polysaccharide chains per protein, where the maximum adsorption to
325 interface (and hence the thickest surfaces layers) were observed.

326 Akinshina et al (2008) used Self Consistant Field (SCF) numerical calculations, also represented
327 on a lattice model, to evaluate and compare the strength and the nature of steric interactions mediated
328 by adsorbed interfacial layers of various protein + polysaccharide conjugates. In particular, they showed
329 that for sufficiently large attached polysaccharide chains, the induced forces were always strongly
330 repulsive, irrespective of the position of covalent bond along the protein backbone. The picture alters
331 considerably when the size of polysaccharide is smaller than protein. Now, the numerical calculations
332 predicted that the position of attachment is critical. The steric repulsion continues to be strong for
333 conjugates with the linkage bond occurring at or near either ends of the protein. However, when the
334 attachment was more central, then the stabilising performance of conjugates was found to suffer. In
335 fact, in such cases it was found to be less effective than the unreacted protein. The protein model used
336 in these studies was based on primary structure of α_{s1}-casein, which itself had already been studied
337 using the same type of technique (Dickinson, Horne, Pinfield, & Leermakers, 1997; Dickinson, Pinfield,
338 Horne, & Leermakers, 1997). As compared to a globular protein such as β-lactoglobulin, the bovine milk

protein α_{s1}-casein is thought to be a more disordered and coil-like molecule. Thus as such, it is better suited to treatment by SCF type calculations used in these studies.

Given the above results, it is of some interest in the current work to extend the SCF calculations to cases involving reaction of protein with sugar moieties (i.e. lactose). In particular, we would like to explore the possibility that an improvement in steric stabilisation can arise from increased solubility of the protein, as caused by the attachment of lactose, without the necessity of grafting a long hydrophilic polysaccharide chain to the molecule. In order to make our results comparable to those earlier studies mentioned above, and also due to inherent limitation of SCF calculations in dealing with globular proteins, we continue to consider α_{s1}-casein, as oppose to β-lactoglobulin, as our model protein here. We shall refrain from providing a detailed account of SCF calculations and the model, as these can be found in our previous work (Ettelaie et al., 2012; Ettelaie, Dickinson, & Pugnaloni, 2014) and others (Dickinson, Horne, et al., 1997; Leermakers, Atkinson, Dickinson & Horne, 1996) and will also be provided in greater detail elsewhere. It suffices to say that we divide the amino acid residues making up our model α_{s1}-casein into six separate categories along the same line as Leermakers et al. (1996). The short range interactions between amino acids in different groups, as well as those with surface, ions and solvent molecules, are specified by a set of Flory-Huggins χ parameters. It is these parameters that reflect the hydrophobic, polar or charged nature of amino acid residues in each category. Attachment of sugar groups was made at proline or lysine sites, as these are the residues most vulnerable to Millard reaction. As well as the short range interactions, the charge carrying residues and free ions in the solution also interact with each other through the longer range Columbic forces, also accounted for in the model.

The SCF calculation involves an iterative procedure, performed numerically, in which the density profile of the biopolymer, solvent and ions in the gap between two approaching surfaces are altered systematically in a way that reduces the free energy of the system. Convergence is obtained when the set of density profiles with the lowest free energy is achieved. It is this set of density profiles, having

the lowest free energy that dominates the thermodynamic behaviour of the system. In particular, by repeating the calculations at different gap sizes, and by monitoring the resulting changes in the free energy of the system, one can evaluate the colloidal interactions between the two interfaces (Ettelaie & Akinshina, 2014; Ettelaie, Khandelwal, & Wilkinson, 2014), as induced by the overlap of adsorbed interfacial layers of biopolymer.

The results of the above calculations, performed for the reacted and unmodified model α_{s1}–casein, show that the addition of a single lactose moiety to any of the proline or lysine sites does not produce a drastic improvement beyond the stabilising capability of the protein itself. In many cases, depending on the location of the covalent bond between lactose and protein, one finds that there is actually a detrimental impact. The predicted interactions between two emulsion droplets of size 1 μm, stabilised by a variety of such α_{s1}-casein + lactose complexes are displayed for some of the studied cases in Fig. 9. The graphs also include the curves for the unmodified α_{s1}-casein, as well the protein + lactose complex that leads to the best result. This occurs when lactose is attached to a proline residue, situated at position 12 on the protein primary sequence. All data were obtained at a pH=4.5, close to pI of our model casein, and at a relatively high electrolyte concentration of 0.4 mol/l. We have also included the van der Waals component of the attraction, present independently of the adsorbed layers, in our interaction curves. For this latter, we took the Hamaker constant to be $1k_BT$ for emulsion droplets, where k_B denotes the Boltzmann constant and T the temperature. This value is typical of those reported for edible food oils (Dickinson, 1992).

It is clear from the graphs in Fig. 9 that all the interaction potentials possess deep minima. When lactose was covalently bonded to proline amino acid at either position 102 or 73, the depth of the minima in the mediated interaction potential increased, becoming $-20k_BT$ and $-27k_BT$, respectively. These values are to be compared with $-16k_BT$ for the unmodified α_{s1}-casein. Slight improvement was obtained when the location of the reacted proline was chosen closer to the N-terminus end of the protein, as can be seen from the graphs for pro-2 and pro-12 in Fig. 9. In particular, the attachment of

lactose to the proline residue, residing at position 12 along the protein backbone, produced the shallowest energy well, less deep than all the other possible modifications involving a single lactose moiety. Nonetheless, even in such a case, at a value of -9k_BT, the resulting potential is still attractive enough to cause aggregation and therefore possible coalescence of the emulsion droplets.

All lysine and proline residues of α_{s1}-casein are susceptible to Millard reaction with lactose. Hence, it is also useful to consider situations where a greater number of lactose molecules react with the protein. For the majority of cases, our SCF calculations seem to suggest that the steric stabilising power of the modified complex is reduced when further lactose moieties, in addition to that at location 12, are attached to α_{s1}-casein. This is especially the case when these additions are made at central locations on the chain. It was reported by Akinshina et al (2008) that the attachment of a short polysaccharide chain to α_{s1}-casein, made at positions away from the two ends of the protein, promoted a higher level of bridging attraction between two approaching surfaces covered by such conjugates. This also seems to be the true here. In Fig. 10 we show a selection of our results, involving conjugates with one or two more lactose molecules, beside the one already bonded to proline at position 12 (optimum location for a single connection), also attached to the protein. Once again, interactions curves for unmodified protein (dashed line) and the one with only a single lactose attached at position 12 (long dashed line) have been included for comparison. With one more bond made with the lysine residue at location 7, the depth of minimum in the interaction potential increases (dotted line compared to long dashed line). We believe that this is due to the fact that lysine possesses a positive charge. When a covalent bond with lactose is made, lysine loses its ability to carry charge. At a pH=4, slightly below pI of the protein, this further reduces the net positive charge of the adsorbed conjugates on the surface of the droplets. The subsequent reduction in the electrostatic repulsion manifests itself as a deeper energy well in the interaction potential. This is to be contrasted with the case when the additional lactose attachment is made on a proline residue at position 5 (dash-dotted line). Now there is a slight improvement with the depth of energy well calculated to be -8 k_BT. This of course is still deep enough to cause aggregation of droplets. It is also seen that the conjugate with three lactose molecules attached at Pro-5, Lys-7 and

Pro-12 (solid line), has almost an identical behaviour to one with single modification at position 12. It seems that the detrimental effect of lactose attachment to lysine at location 7 is compensated by the improvement of doing so with pro-5.

To summarise the overall conclusion of the SCF calculations in this section then, it is predicted that the attachment of small sugar molecules at various locations along the protein backbone, largely result in no improvement in colloidal stabilising power of the protein. In a few cases where the attachments are seen to be beneficial, the predicted improvement is still not sufficient to prevent aggregation and possible coalescence of emulsion droplets at pH ~ pI of protein. This conclusion is clearly borne out and supports the experimental observations in previous sections.

4. Conclusions

Protein-polysaccharide conjugates prepared via simple dry-heating treatment have excellent emulsifying and stabilising properties under unfavourable environmental conditions such as pH values close to pI of protein. Sugar impurity, present during synthesis, can compete with polysaccharide for reaction with protein. This can hinder the attachment of the two biopolymers. Large disparity between the molecular weight of small sugar molecules and large polysaccharide chains will mean that even a small amount of contamination may be problematic in achieving good efficiency in producing these complexes. In large scale industrial production of conjugates for use in food industry, such impurities are unavoidable. We have studied the impact of the presence of sugar contaminant on interfacial behaviour of the conjugates. We show that the Whey Protein Isolate + Maltadextran system (WPI-MD19 (1:2 w/w)) is not overtly sensitive to the existence of lactose during the Maillard reaction, even at a relatively high molar ratio (1:10 MD19 : lactose). The protein-polysaccharide covalent complexes, formed in presence of lactose, remain effective emulsifiers and stabilisers of emulsion droplets, as demonstrated by the study of the stability of 20 vol% oil in water emulsion systems here.

438 The usual argument for the improved colloidal stabilising power of conjugates is attributed to
439 the presence of large polysaccharide chains. It is assumed that through their linkage with protein, the
440 otherwise hydrophilic polysaccharide chains are enticed to adsorb on the surface of droplets. This
441 provides thick interfacial layers, with good provision of repulsive steric force that keeps the droplets
442 apart. However, one may also attribute some of this improvement to the higher solubility of the
443 covalent complex, especially at pH values close to pI of the protein. It is known that sufficient levels of
444 solubility remain an important parameter in determining the suitability of dispersants in colloidal
445 formulations in general (Dickinson, 1992). To examine this proposition, we have also studies both
446 theoretically and experimentally the emulsifying/stabilising properties of WPI-lactose complexes. We
447 have found the results to be inferior to that of WPI-MD19 systems. Theoretical calculations show that
448 slight improvement in emulsion stabilising properties of protein may be possible, but this is only
449 achievable by careful and selected choice of the position of sugar attachments on the protein backbone.
450 Therefore, from a practical point of view, we conclude that covalent complexes of small sugars with
451 proteins are not particularly advantageous compare to protein itself.

452 The relatively small influence of lactose on the preparation of WPI-MD19 conjugates indicates
453 that the Maillard reactions are biased towards the larger MD19 chains. This is somewhat surprising, as
454 the smaller sugar moieties are expected to have a higher degree of mobility, particularly in the relatively
455 dry environment required by Maillard reactions. One possible reason for this may be that the sugar
456 molecules undergo caramelisation which leads to much larger polymer like structure. Even a degree of
457 polymerisation of say 10 is enough to reduce the molar ratio of lactose : MD19 from 10:1 down to 1:1.
458 This then gives the dextran chains a reasonable chance of reacting with protein. Further experiments
459 are needed to verify this possibility. In meantime, the insensitivity of protein + polysaccharide
460 conjugates to the presence of sugar at the time of their synthesis, implies a less stringent level of purity
461 of whey protein, thus reducing the possible cost of making these superior food dispersants.

462

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Figure captions

Figure 1. An illustration of WPI, MD19 and lactose mixture before (**A**) and after (**B**) heating treatment; (1) MD19 : lactose (molar) = 1:1; (2) MD19 : lactose (molar) = 1:2; (3) MD19 : lactose (molar) = 1:4; (4) MD19 : lactose (molar) = 1:6; (5) MD19 : lactose (molar) = 1:10

Figure 2. Photograph of O/W emulsions stabilised at pH4.6 by different complexes, after 28 days of storage, kept at 30 °C. In each case the emulsifier was synthesised as follows: (1) WPI-MD19 (1:2 w/w) with no sugar contamination; (2) WPI-MD19 with lactose impurity present at a molar ratio of 1:10 (MD19 : lactose); (3) WPI-lactose (2:1 w/w) with no maltodextrin present; (4) a mixture of WPI and MD19 (1:2 w/w) without any heat treatment. In Fig. 2 (3), the position of cream layer is indicated by an arrow.

Figure 3. Degree of linkage for protein-polysaccharide/sugar conjugates, with lactose at various molar ratios to MD19. Sample in column 1 is a mixture of WPI and lactose solutions that has not undergone any heat treatment.

Figure 4. The change of average droplet size (ADS) of emulsions (20 vol% oil, pH 4.6) stabilised by different conjugates during 28 days of storage, kept at 30 ^{0}C.

Figure 5. The comparison between droplet-size distribution (DSD) of emulsions (20 vol% oil, pH 4.6) freshly prepared and after 28 days.

Figure 6. The flow behaviours, as characterised by *k* and *n* index for a power law fluid, for various emulsions stabilised by three different conjugates: WPI-MD19 (1:2 w/w), WPI-MD19 (1:2 w/w) with lactose impurity present during synthesis at a molar ratio of 1:10 (MD19 : lactose) and WPI-lactose (2:1 w/w), respectively. All results are for emulsions kept at 30 ^{0}C for 4 weeks.

Figure 7. The relationship between shear rates and apparent viscosity of emulsions stabilised by WPI-MD19 (1:2 w/w), WPI-MD19 (1:2 w/w) with lactose present at a mole ratio of 1:10 (MD19 : lactose) and WPI-lactose (2:1 w/w) without maltodextrin, after a storage period of 28 days.

Figure 8. The CLSM images of emulsions stabilised by WPI-MD19 (1:2 w/w), WPI-MD19 (1:2 w/w) with lactose impurity at the molar ratio 1:10 (MD19 : lactose) and without maltodextrin at WPI-lactose (2:1 w/w), after emulsion preparation and after four weeks of storage.

Figure 9. Colloidal interaction potential between a pair of 1 μm droplets, covered by various protein + lactose covalently bonded complexes, adsorbed on their surface. There is only a

single sugar moiety bonded to a proline residue of α_{s1}-casein in each case, but the position of the linkage is different for each graph, as follows: pro-2 (dash-dotted line), pro-12 (long dashed line), pro-73 (dotted line), pro-102 (solid line) and the dashed line represents casein without modification.

Figure 10. As in Fig. 9, but now with some of the conjugates involving multiple bonds with several lactose molecules, as follows: Lys-7 + Pro 12 (dotted lone), Pro-5 + Pro-12 (dash-dotted line), Pro-5 + Lys-7 + Pro-12 (solid line). The graphs for unmodified casein (dashed line) and that with best single modification, at Pro-12 (long dashed line) are also included for comparison.

Figure 1

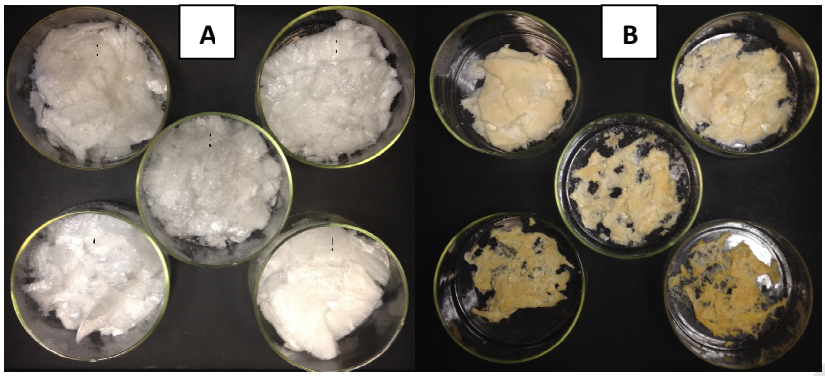


Figure 2

Figure 3

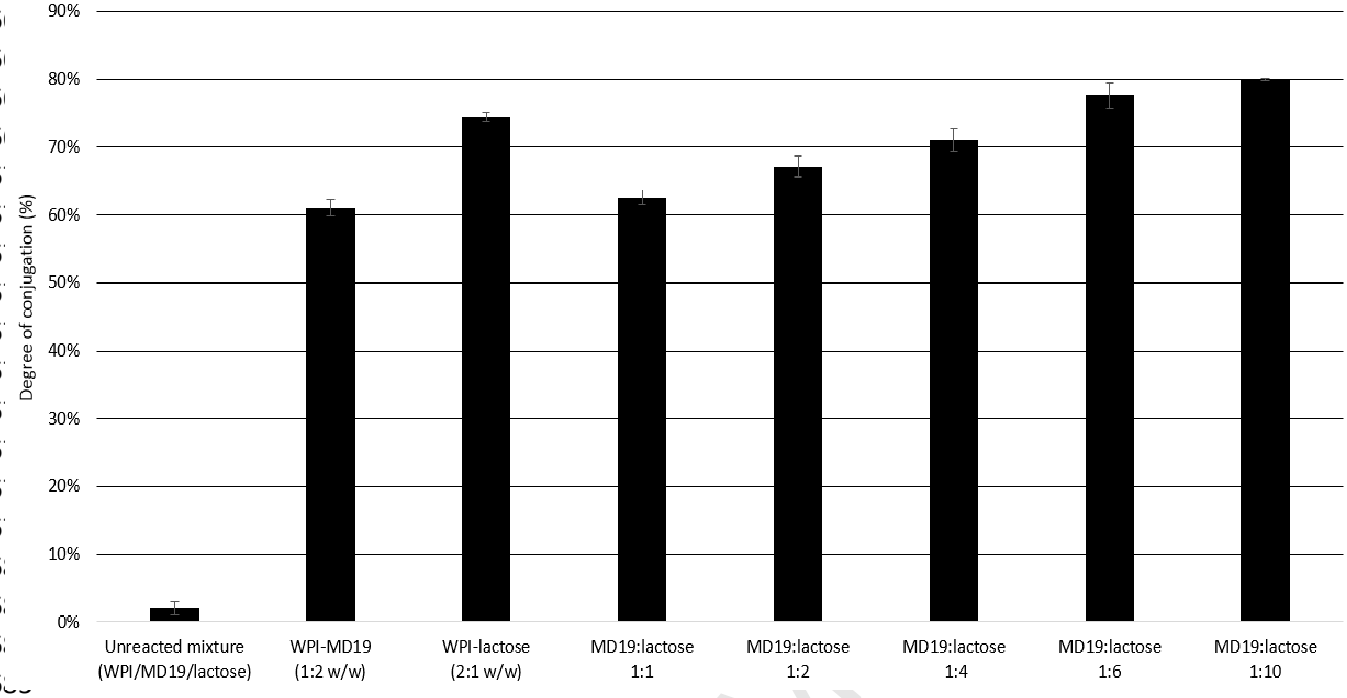


Figure 4

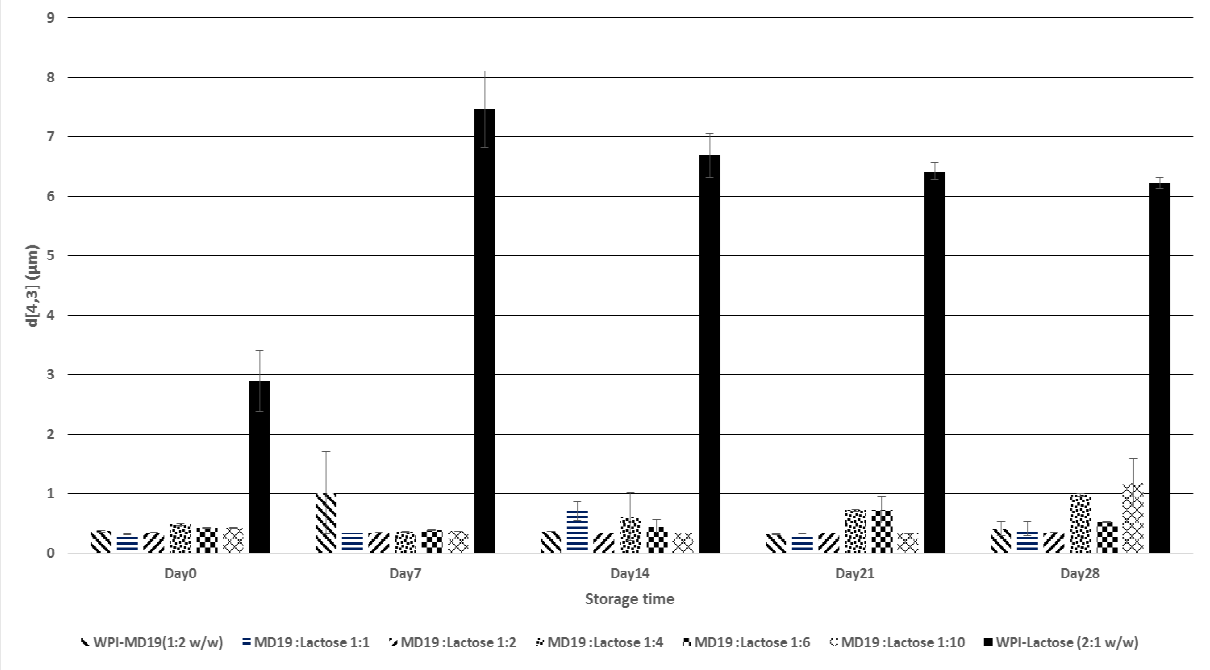


Figure 5

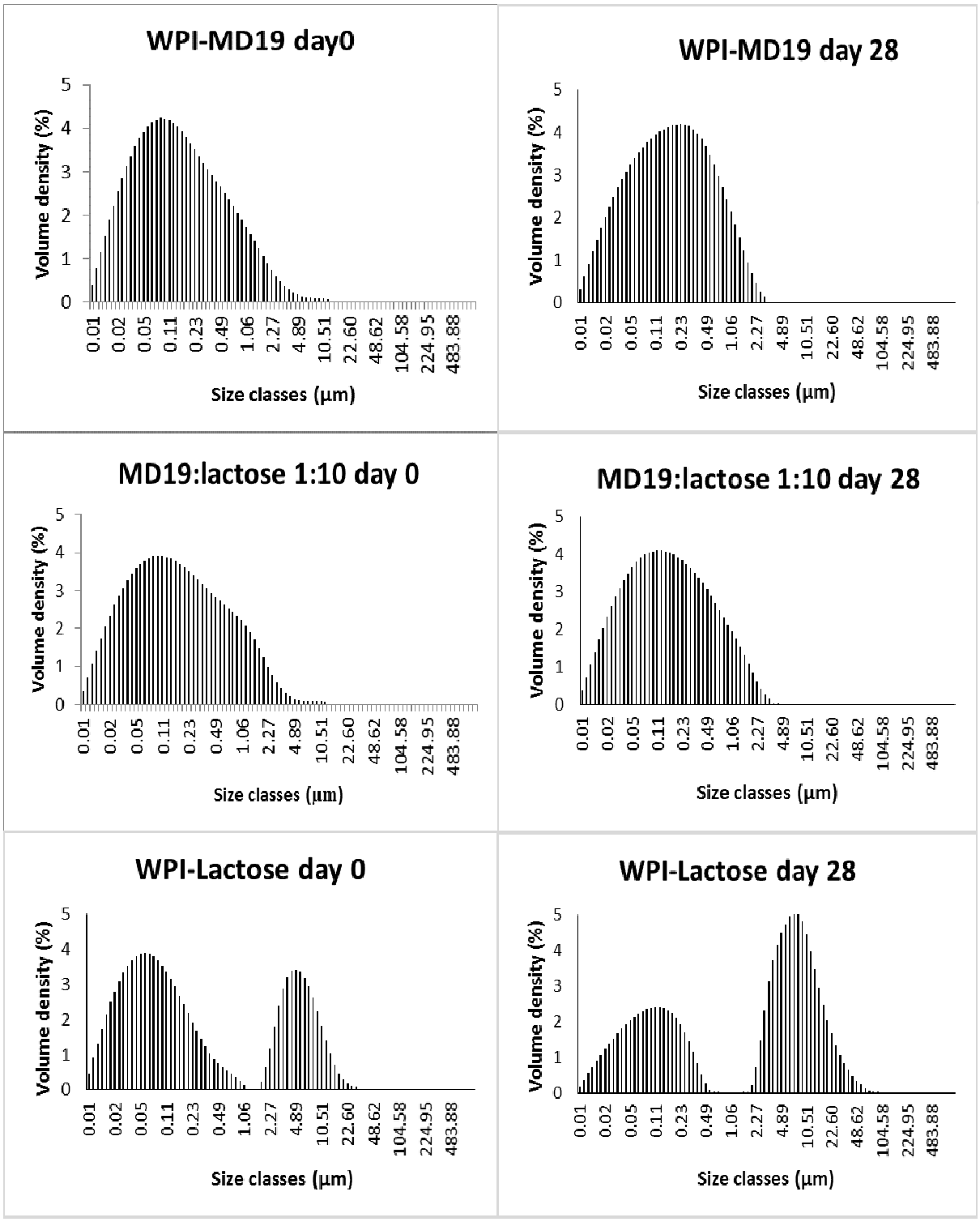


Figure 6

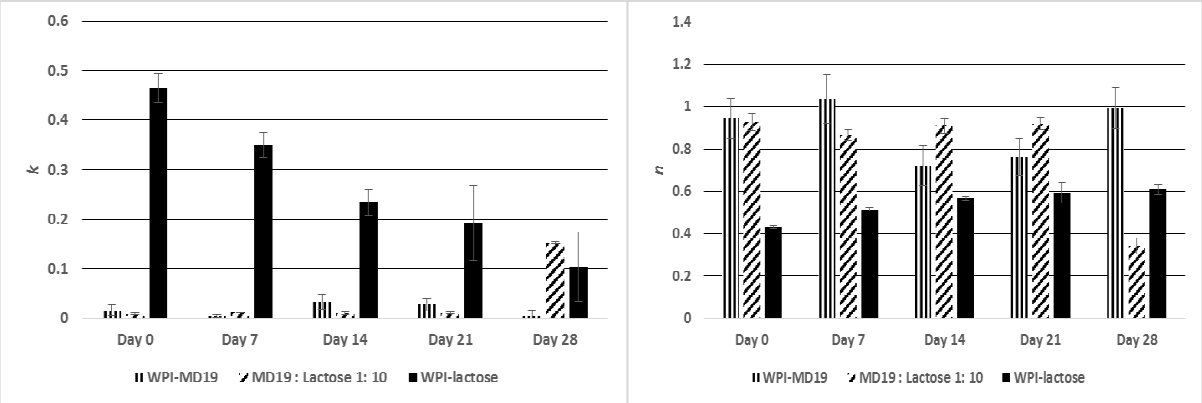


Figure 7

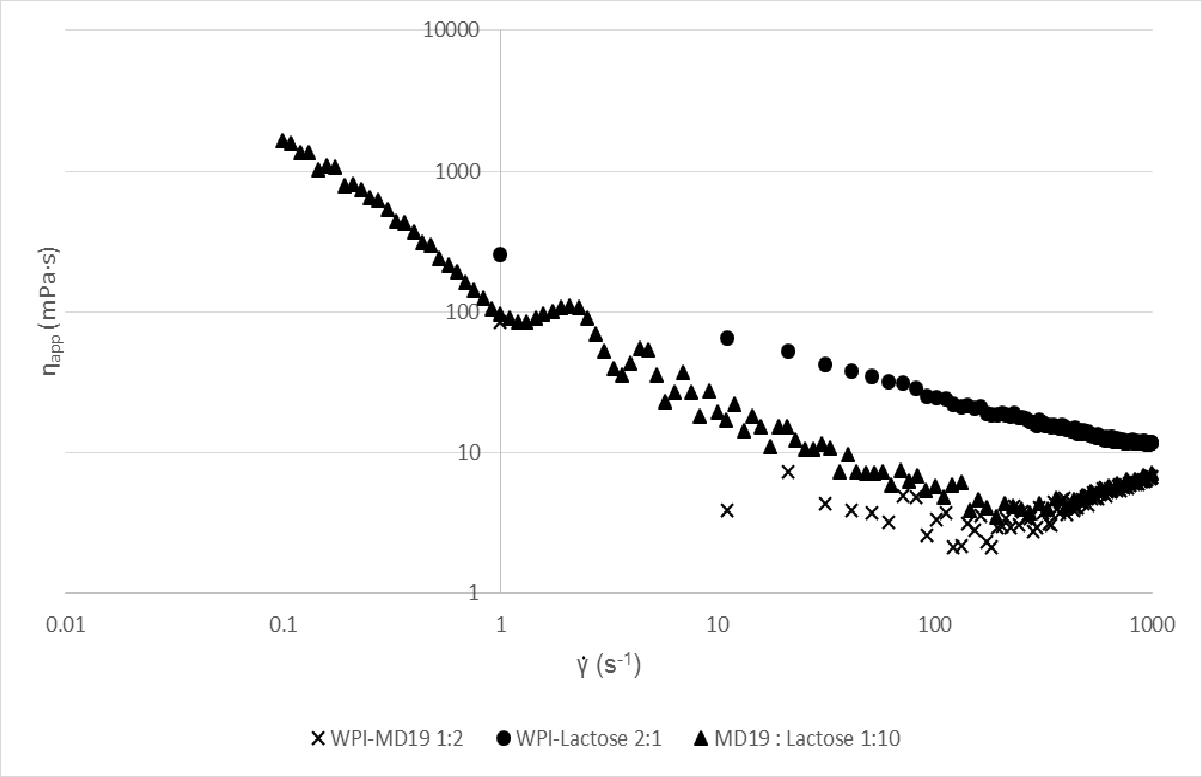
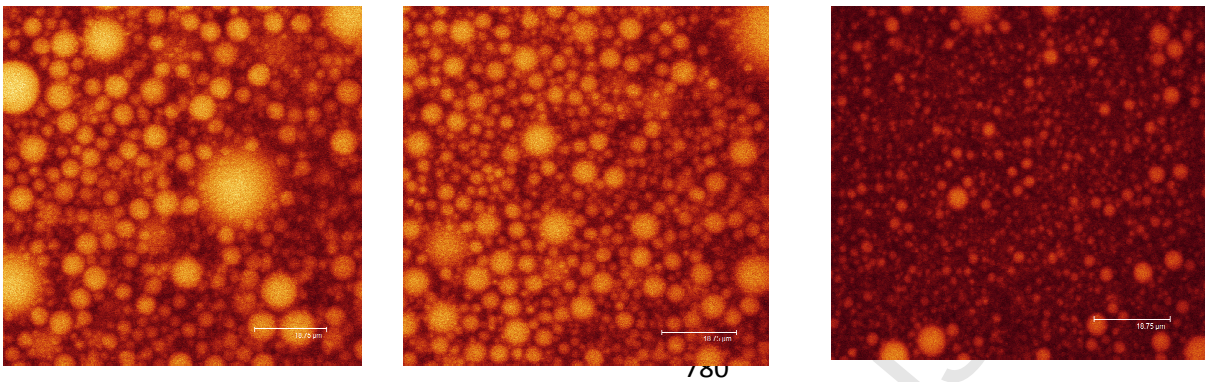


Figure 8

Day 0

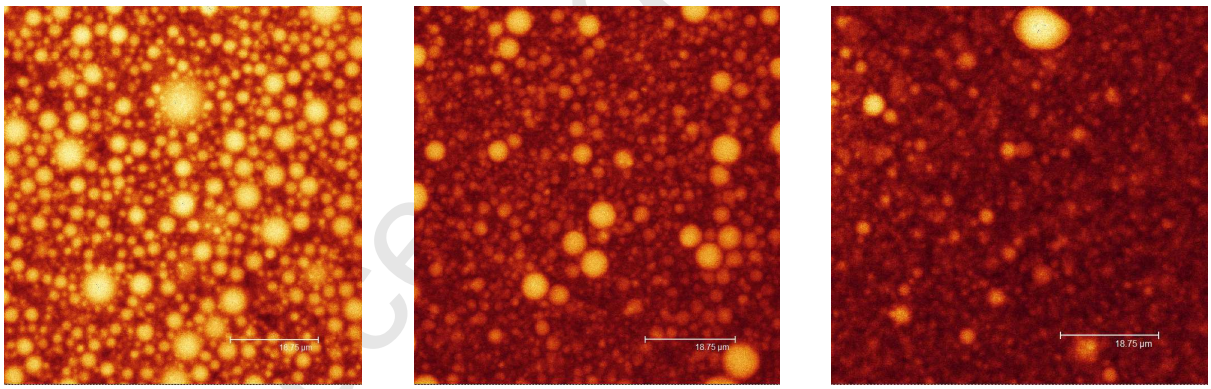


Stabiliser: WPI-MD19

Stabiliser: MD19: lactose 1:10

Stabiliser: WPI-lactose

Day 28



Stabiliser: WPI-MD19

Stabiliser: MD19: lactose 1:10

Stabiliser: WPI-lactose

796 **Figure 9**

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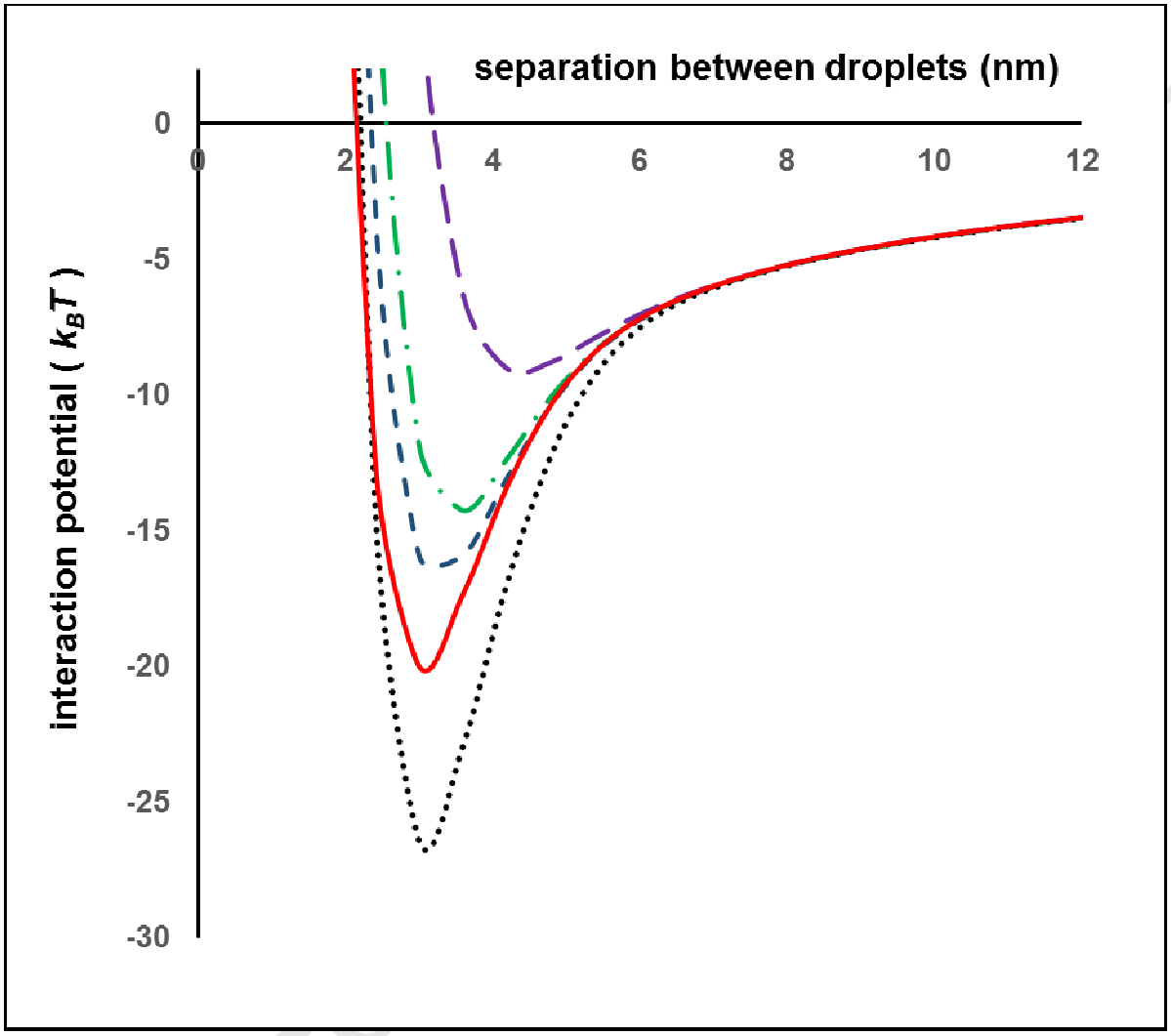

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Figure 10

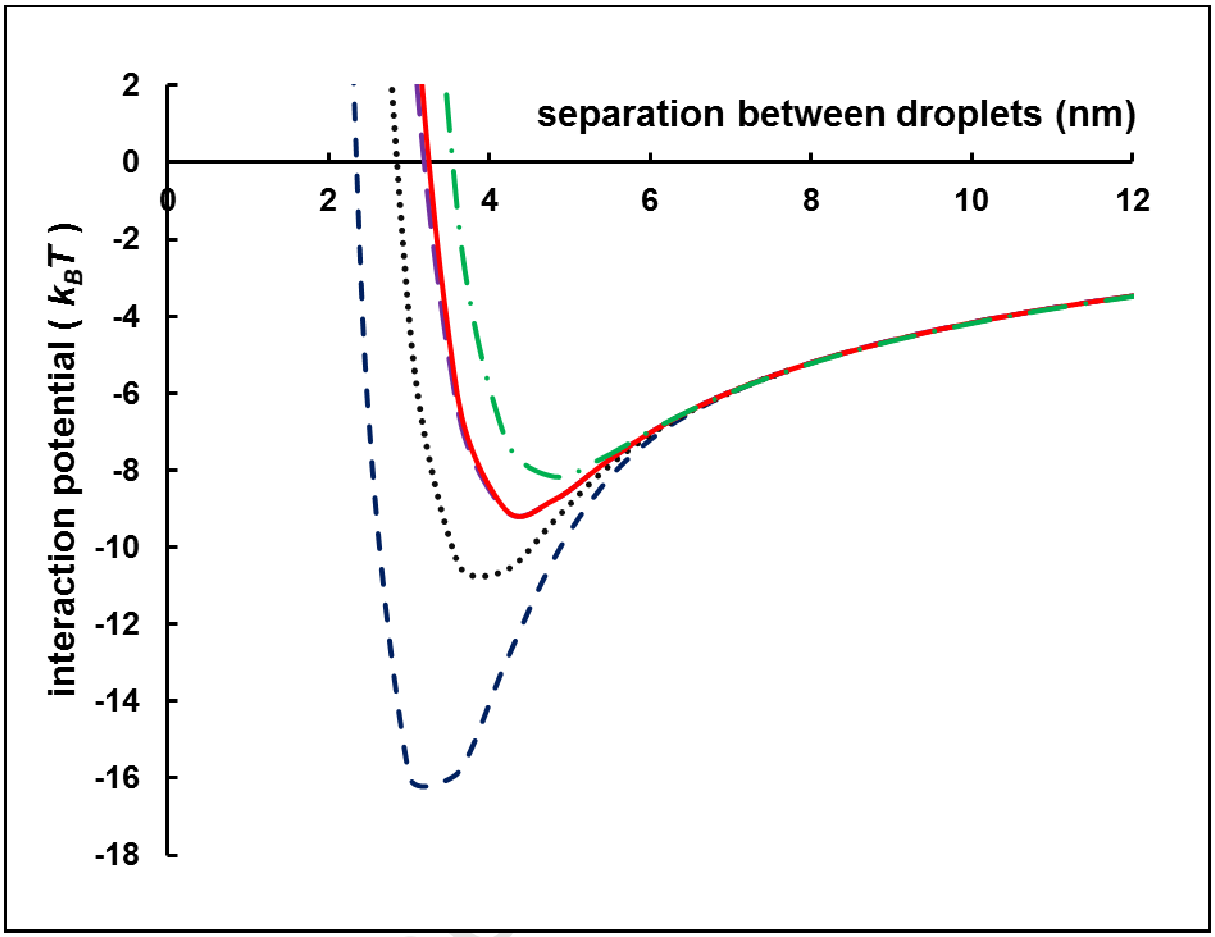

Table 1. Recipe of WPI-MD19 conjugate with various molar ratios of lactose before the Maillard reaction

WPI (g)	MD19 (g)	Lactose (g)	MD19 : Lactose (molar) *

1	2	0.0787	1:1
1	2	0.1574	1:2
1	2	0.3135	1:4
1	2	0.4703	1:6
1	2	0.7839	1:10

816 * MD19 (Mw = 8.7 kDa); Lactose (Mw = 342.3 g/mol)

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818