**Water-in-water-in-water emulsions formed by cooling mixtures of guar,
amylopectin and gelatin**

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14 **Abstract**

15 Water-in-water emulsions were formed by mixing aqueous solutions of the neutral
16 polysaccharides guar and amylopectin. The effect of adding gelatin B on emulsions, both of a dispersed
17 guar rich phase in a continuous amylopectin phase and the inverse, were investigated using confocal
18 scanning laser microscopy as a function of the gelatin concentration between 0.25 and 1.5 wt% and
19 the pH between pH 4 and pH 6. Gelatin was found to phase separate upon cooling below about 25°C
20 in a narrow pH range around pH 5 both in the pure guar phase and in the pure amylopectin phase
21 forming distinct spherical domains. The phase diagram was established as a function of the gelatin
22 concentration and the pH. In the emulsions, the gelatin phase separated first at the interface between
23 the two phases forming a continuous layer at the droplet surface. In some conditions the layer
24 persisted, but in others it broke up into distinct domains. Excess gelatin phase separated in the bulk
25 amylopectin phase, particularly when it was the continuous phase. The gelatin redispersed into the
26 bulk phases when the emulsions were heated to 30°C.

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28 **Keywords:** aqueous multiphase system; water-in-water emulsion; phase separation; guar;
29 amylopectin; gelatin

30

Introduction

Water-in-water (W/W) emulsions are formed in aqueous solutions that contain two different water soluble incompatible polymers A and B (Frith, 2010). Such emulsions cannot be stabilized by molecular surfactants as can be done for oil-in-water (O/W) emulsions, because the interfacial tension is much smaller. In addition, the interface is formed by semi-dilute solutions of the polymers and therefore expressed on length scales larger than the correlation length, i.e. several nm. However, it has been found that they can in some cases be stabilized by addition of particles (Dickinson, 2018; Esquena, 2016; Nicolai & Murray, 2016) or, more recently, by addition of certain polysaccharides (Tea, Nicolai, & Renou, 2019). Larger particles can span the W/W interfaces and thereby reduce contact between A and B, which leads to adsorption of the particles at the interface if contact between A and B is energetically less favourable than between A or B and the particles (Aveyard, Binks, & Clint, 2003). Self-consistent field calculations showed that depletion effects may cause sufficient difference between the interfacial tension of the particles with phase A and B even if there are no specific interactions between the particles and the polymers (Ettelaie, Murray, & Liu, 2019).

Phase separation within water droplets dispersed in an oil phase (W/W/O) has been studied quite intensively (Cui, Zeng, Wang, & Zhang, 2017; Fransson, Lorén, Altskar, & Hermansson, 2009; Ge, Jin, Li, Wei, & Guo, 2019; Yanagisawa, Nigorikawa, Sakaue, Fujiwara, & Tokita, 2014; Yuan, et al., 2017). It often leads to the formation of so-called Janus droplets containing phase A on one side and phase B on the other side, but it has also been observed that one phase wets the oil phase forming a layer around the dispersed water phase droplets (Yanagisawa, et al., 2014). More complex structures have been observed in aqueous droplets dispersed in an oil phase that contained three incompatible polymers (Cui, et al., 2017).

Phase separation within water droplets dispersed in a continuous water phase leading to water-in-water-in-water (W/W/W) emulsions has been much less investigated. Song et al. (Song, Sauret, & Cheung Shum, 2013; Song & Shum, 2012) injected droplets of a dextran/poly(ethylene glycol)(PEG) mixture into a more concentrated PEG solution. Due to the osmotic pressure water migrated from the droplets to the continuous phase, which caused phase separation between dextran and PEG in the droplets and formation of A/B/A emulsions. Phase separation led to the formation of a continuous layer of the dextran phase that surrounded a central PEG phase. The high viscosity of the dextran layer inhibited coalescence of the PEG phase within the core with the continuous phase, but coalescence of colliding droplets could not be avoided.

A different method to produce A/B/A emulsions, not involving microfluidics, was used by Beldegrun et al. (Beldengrün, et al., 2020) who exploited the temperature dependence of the solubility

of maltodextrin in gelatin. They prepared W/W emulsions by mixing gelatin and maltodextrin at high temperatures. Upon decreasing the temperature, the maltodextrin within the gelatin rich phase that formed the droplets phase separated forming small droplets within the gelatin droplets. These W/W/W emulsions were highly unstable, but the stability could be improved by adding mucin particles that adsorbed at the gelatin droplet interface.

The objective of the present study was to investigate the effect of adding a third incompatible polymer on the morphology of W/W emulsions formed by mixing two incompatible polymers. Our hypothesis is that adding a third incompatible polymer component can be a novel method to stabilize W/W emulsions. We have investigated W/W/W emulsions formed in mixtures of guar, amylopectin and gelatin. W/W emulsions are formed when aqueous solutions of guar and amylopectin from waxy corn are mixed leading either to droplets of the guar phase dispersed in a continuous amylopectin phase (G/A) or the inverse (A/G) (Chen, et al., 2018). Above 30°C, gelatin was miscible with both the guar and amylopectin phases at the concentrations used in this study. However, we found that phase separation occurred when the mixtures were cooled below a temperature that depended on the concentration and the pH. We have exploited this property to induce phase separation of gelatin both in G/A and A/G emulsions by cooling. The gelatin phase was observed to form at the interface as well as within the amylopectin phase. Here we focus on the morphology as a function of concentration, pH and temperature. Elsewhere, we will show that this method can indeed be developed to stabilize the W/W emulsion.

Materials and methods

The amylopectin derived from waxy corn was obtained from Qinhuangdao Lihua Starch Co. Ltd. (Hebei, China). Guar gum was purchased from Shanghai Toong Yeuan Food Technology Ltd. (Shanghai, China). Gelatin from bovine skin (type B) and the fluorescent dyes including fluorescein 5-isothiocyanate (FITC) and rhodamine B isothiocyanate (RITC) were purchased from Sigma-Aldrich. All other chemicals were of analytical grade and used without further purification.

Part of the gelatin and amylopectin was covalently labeled with a fluorescent dye to allow observation by confocal laser scanning microscopy (CLSM). 5.0 g of gelatin powder was added to 100 mL of deionized water and heated at 60°C while stirring for 1 h. Then, 250 µL of RITC (1 wt % dissolved in dimethyl sulfoxide, DMSO) was added and stirred for another 1.5 h in the dark. The labeled gelatin solution was dialyzed for 48 h at 60°C to remove excess dye. The labeled gelatin was lyophilized and stored at 4°C in the dark before use. Amylopectin was labeled with FITC according to De Belder (De Belder & Granath, 1973) with some modifications. 5.0 g of amylopectin powder was dissolved in 100

ml dimethyl sulfoxide with continuous stirring. Several drops of pyridine were added, followed by 250 μ l of 1 wt % FITC dissolved in dimethyl sulfoxide and 0.1 g of dibutyltin dilaurate, and heated to 95°C for 2 h while stirring. The amylopectin was recovered by precipitation with 95% ethanol under centrifugation at 8.10^3 g for 20 min. It was subsequently dissolved with 100 ml deionized water while stirring at 95°C for 1 h. After dialysis for 48 h to completely remove the free dye, the labeled amylopectin was lyophilized and stored at 4°C in the dark before use.

Sample preparation

An amylopectin stock solution (10.0 wt %) was prepared by dispersing the powder in deionized water and incubating at 95°C in a water bath for 1 h while stirring. A guar gum stock solution (1.25 wt %) was made by dispersing the powder in deionized water and stirring for 12 h at room temperature. All the stock solutions were centrifugated at 5.10^4 g for 30 min at 20°C before use in order to remove a small amount of insoluble material. A gelatin stock solution (3 wt %) was prepared by dispersing the powder in deionized water and heating at 60°C for 1 h while stirring. 1 N NaOH and HCl were used to adjust the solutions to the required pH.

The emulsions were prepared by mixing the stock solutions in the required amounts at 60°C using a vortex stirrer. The morphology and behaviour of the emulsions did not depend on the order of mixing nor on the duration and speed of stirring. In fact, strong handshaking was sufficient to produce emulsions with the same microstructure. Elsewhere we showed that even linear shear at relatively low shear rates is sufficient to deform the droplets into thin strands that after cessation of shear break-up into tiny droplets (Tea, Nicolai, Benyahia, & Renou, 2020).

Confocal laser scanning microscopy

CLSM Images were obtained with a Zeiss LSM800 microscope (Carl Zeiss Microscopy GmbH, Germany) with the objective: EC Epiplan 50 $\times$ /0.75 H M27 (NA = 0.75). The samples were inserted into a RheOptiCAD[®] device (CAD Instruments, Les Essarts-le-Roi, France) consisting of two parallel plates that allows visualization with the microscope while controlling the temperature. For a detailed description of the device see Boitte et al. (2012). Part of the amylopectin and the gelatin were labeled with RITC and FITC, respectively. FITC was excited at 488 nm, whereas rhodamine B was excited at 561 nm. Here we show only the signal corresponding to the gelatin. However, one can still distinguish between the two phases, because the gelatin was not distributed equally between the two phases. In addition, the wavelength range over which the RITC signal was detected (590 - 700 nm) contained a small contribution of the signal from FITC corresponding to the amylopectin.

Rheology

The viscosity of guar and amylopectin solutions at the concentration in the phases was measured as a function of the shear rate (0.01 s^{-1} - 10^3 s^{-1}) with a stress-controlled rheometer (MCR 301, Anton Paar) with a couette geometry (No. CC10-SN67089). The temperature was controlled to within 0.1°C using a Peltier plate. The Newtonian viscosity could be determined at low shear rates.

Results and Discussion

Emulsions of droplets of the guar phase in the continuous amylopectin phase (G/A) and vice versa (A/G) were prepared by mixing 0.1 wt% guar with 2.75 wt% amylopectin and 0.54 wt% guar with 0.51 wt% amylopectin, respectively. The phase diagram for mixtures of the amylopectin and guar used in this study was reported by Chen et al. (2018) and shows that at these compositions the mixtures are well within the two phase regime. The compositions were chosen such that the volume fraction of the dispersed phase was 15%, which was convenient for microscopic visualization. The two systems were situated on the same tie-line, which means that the concentration of guar (0.64 wt%) and amylopectin (3.26 wt%) in the respective phases was the same and therefore the interfacial tension was the same (Frith, 2010). The fraction of amylopectin in the guar phase and that of guar in the amylopectin phase was negligible. We found that the phase volumes changed by less than 5% with temperature at least between 10°C and 60°C or with the pH at least between 4 and 7.

Binary mixtures of gelatin with guar or amylopectin

Before discussing the behaviour of the ternary systems, we briefly show the behaviour of binary mixtures of gelatin with guar and amylopectin at the concentrations of the guar and amylopectin phases in the emulsions. At elevated temperatures ($T > 30^\circ\text{C}$), gelatin remained homogeneously dispersed at all pH (4-6) and concentrations ($C \leq 1.5\%$) investigated here. However, when the mixtures were cooled, phase separation could be observed at a rate that depended on the temperature and the gelatin concentration (C). Phase separation was manifested in CLSM observations by the formation of microdomains of a gelatin rich phase. Fig. 1 shows the microstructure of mixtures of different amounts of gelatin in 3.26 wt% amylopectin at pH 5 after standing for 1 h at 10°C . Video 1 of the supplementary information shows an example of the evolution of the system after cooling to 10°C . Results obtained at other pH values and in guar were similar. Larger gelatin microdomains were formed at higher C , but they did not coalesce into a macroscopic phase. Instead, the microdomains stuck together into larger flocs that slowly sedimented over a period of days. It is well known that the

gelatin chains form intermolecular helices below a critical temperature, which leads to aggregation and, at higher concentrations, to gelation (Baziwane & He, 2003; Djabourov, 1988; Ledward, 1986; Ross-Murphy, 1997). The temperature below which phase separation was observed correlated well with the temperature below which gels were formed at higher gelatin concentrations.

It appears therefore that phase separation was induced by aggregation of the gelatin chains. This behaviour is similar to that reported for mixtures of gelatin B and locust bean gum (LBG) (Alves, Antonov, & Gonçalves, 1999). LBG a polysaccharide from the galactomannan family. Alves et al. compared phase separation between gelatin and LBG as a function of the pH between molecularly dispersed gelatin chains and small gelatin aggregates. Phase separation was observed only in a narrow pH range around pH 5 and the solubility of gelatin in the LBG solution was much lower when the gelatin was in the form of aggregates. Here we found that aggregation of gelatin within the microdomains led to the formation of microgels that could not coalesce, but could stick to each other.

Fig.1 CLSM images of mixtures of 3.26 wt% amylopectin and different concentrations of gelatin: 0.25, 0.5, 1 and 1.5 wt%, from left to right. The fluorescence signal of the labeled gelatin is shown as white.

Phase separation initiated when the gelatin aggregates reached a critical size that depended on the temperature, concentration and pH. The rate at which gelatin aggregated also depended on these same parameters. Therefore, phase separation appeared more or less slowly depending on the conditions. Fig. 2 shows state diagrams as a function of the gelatin concentration and the pH at 10°C and at 20°C, where the systems were considered phase separated if the gelatin rich domains appeared within one hour. The behaviour in guar and amylopectin was similar, but the critical concentrations were slightly lower in guar than in amylopectin. The critical concentration was lowest close to pH 5 and increased both at higher and lower pH. This can be explained by the fact that the net charge is close to zero at pH 5 for the type of gelatin used here, because reducing electrostatic interactions favors both aggregation and phase separation. The critical concentration increased with increasing temperature. When phase separated systems were subsequently heated the microdomains rapidly disappeared above a critical melting temperature and the gelatin redispersed homogeneously. The

melting temperature was somewhat higher than the phase separation temperature, because at intermediate temperatures aggregation was too slow to induce phase separation within an hour. All systems studied here rapidly became homogeneous above 30°C after being cooled at 10°C.

Figure 2 State diagram of gelatin in 3.26% amylopectin solution (A) and 0.64% guar solution (B) after cooling at 10°C for 1 h. Open and filled circles or triangles indicate homogeneous and phase separated systems, respectively. Fig. 2A also shows the phase diagram of gelatin in amylopectin at 20°C indicated by open and closed triangles.

Mixtures of gelatin and G/A emulsions

Gelatin B was added to the G/A emulsions containing 0.1 wt% guar and 2.75 wt% amylopectin at 60°C while maintaining the same concentrations of guar and amylopectin. We found that gelatin was distributed homogeneously in the emulsions, but partitioned between the two phases showing a weak preference for the guar phase, see Fig. S1 of the supplementary information. As a consequence, addition of gelatin led to migration of water from the guar phase to the amylopectin phase leading to a small decrease of the volume fraction of the guar phase with increasing gelatin concentration. It

should be stressed though that this was no longer the case at lower temperatures when gelatin aggregated. The results discussed below show that phase separation was accompanied by migration of gelatin towards the amylopectin phase. The latter can be explained by the observation that gelatin aggregates were slightly more compatible with amylopectin than with guar as can be seen by comparing the binary phase diagrams shown in fig. 2.

Fig. 3 shows the microstructure of G/A emulsions at pH 5.0 containing 0.75% gelatin, at different times after rapidly lowering the temperature from 60°C to 10°C. A video showing the evolution over a larger area is provided as supplementary information (video 2). After approximately 4 min at 10°C, a thin layer of gelatin was formed at the droplet surface, without visible phase separation within the phases. However, soon after gelatin rich domains were formed in the continuous amylopectin phase and the layer around the droplets was broken up into distinct domains still adsorbed at the interface. The density of the domains increased with time until after about 10 min the system reached a meta-stable state and not much change was seen for at least an hour. The gelatin domains at the interface protruded almost completely towards the amylopectin phase so that the surface area in contact with the guar phase was minimal. This shows that the interfacial tension between the amylopectin and aggregated gelatin phases was lower than between the aggregated gelatin and guar phases.

Fig. 3 CLSM images of a G/A emulsion (0.1 wt% guar, 2.75 wt% amylopectin) at pH 5. containing 0.75% gelatin at different times after setting the temperature to 10°C: 0 (a), 4.5 (b), 5.5 (c), 9.5 (d), 24.5 min (e). The fluorescence signal of the labeled gelatin is shown as white.

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232 A thin continuous layer of gelatin was seen before microphase separation occurred within the
233 amylopectin phase (Fig. 2b). When the gelatin is positioned between guar and amylopectin, it reduces
234 the interfacial tension between the latter at the cost of creating interfaces with gelatin. The free energy
235 of the system will be reduced in this manner if the interfacial tension between guar and amylopectin
236 is larger than between each of these phases and gelatin, which is the case here. As the gelatin
237 aggregates continued to grow with time, their solubility decreased and phase separation occurred also
238 in the continuous amylopectin phase. Phase separation of aggregated gelatin was not visible in the
239 guar phase during cooling even though it was less compatible with guar than with amylopectin,
240 because the gelatin concentration in the guar phase was low. Phase separation at the interface led to
241 formation of regularly distributed domains at the interface, which can be clearly seen in fig. 3e both in
242 the ring of domains around droplets focussed at their centre and the domains focussed the top of the
243 droplets. At rare occurrences, coalescence of droplets was observed, see video 2 of the supplementary
244 information. Emulsions containing gelatin domains were much more stable than at higher
245 temperatures when the gelatin was homogeneously distributed, which will be discussed in detail
246 elsewhere. When the temperature was increased from 10°C to 20°C, the gelatin concentration in the
247 domains decreased, but the microstructure of emulsion remained qualitatively the same, see video 3
248 of the supplementary information. However, when the temperature was further increased to 30°C the
249 domains melted quickly, see video 4 of the supplementary information.

250 Interestingly, the drive to phase separation did not in all cases lead to break up the continuous
251 layer at the interface. This is shown in fig. 4, where the evolution at 10°C is shown for the same system
252 at pH 5.25. At this pH, the critical gelatin concentration in the amylopectin phase was close to 0.75%
253 implying that the driving force for phase separation was much smaller. A continuous layer was formed
254 at the interface before microphase separation became visible within the amylopectin phase. The layer
255 appeared after approximate 20 min, i.e. much later than at pH 5, because aggregation of gelatin at pH
256 5.25 was much slower. Rapidly after the appearance of the layer, microdomains appeared within the
257 continuous phase that became denser with time until a meta-stable state was reached similarly to the
258 system at pH 5. However, in this case no phase separation occurred in the layer around the droplets.
259 Here also we noted rare cases of coalescence between droplets fully covered with the gelatin layer,
260 which means that even full coverage was not always be sufficient to inhibit coalescence.

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Fig. 4 CLSM images of an G/A emulsion (0.1 wt% guar, 2.75 wt% amylopectin) at pH 5.25 containing 0.75% gelatin at different times after setting the temperature to 10°C: 0 (a), 24 (b), 32 (c), 52 min (d). For clarity only the signal of the labeled gelatin is shown as white.

The effect of the gelatin concentration on the structure of the emulsions at pH 5 after 1 h at 10°C is shown in fig. 5. At 0.25% no gelatin domains were observed. Fewer gelatin domains were formed at 0.5 % than at 0.75%, but the size of the domains was similar. At 1% the domains were larger and it appears that they were formed by partial coalescence of smaller domains before the connectivity between the gelatin in the domains was sufficient to inhibit this.

Fig.5 CLSM images of G/A emulsions (0.1 wt% guar, 2.75 wt% amylopectin) at pH 5.0 containing different amounts of gelatin: 0.25, 0.5, 0.75 and 1.0% from left to right, after standing 1 h at 10°C. For clarity only the signal of the labeled gelatin is shown as white.

The effect of the pH at C = 0.75% is shown in fig. 6. Phase separation of gelatin was observed at pH 4.75, 5.0 and 5.25, but not at pH 5.5 or higher and pH 4.5 or lower. Comparison with the state diagram of gelatin in amylopectin shows that the microstructure of the emulsions is determined by the solubility of gelatin in the amylopectin. At pH 4.75 the gelatin layer at the interface broke up into distinct domains as at pH 5.0 and the gelatin domains within the amylopectin phase were connected

to each other into larger clusters, but without significant coalescence. It is clear that the morphology of the emulsions is sensitive to small changes of the pH. When we repeated these measurements with a newly prepared gelatin we observed the same phenomena, but with a small shift in the pH. With the new gelatin the ring was observed at pH 5.0 and the distinct domains at the interface were observed at pH 4.5 and pH 4.75. We speculate that labeling to different extents slightly changes the net charge of the gelatin and thereby the pH at which the gelatin chains have a particular net charge density.

Fig.6 CLSM images of G/A emulsions (0.1 wt% guar, 2.75 wt% amylopectin) containing 0.75% gelatin at different pH: pH4.75 (a), pH 5.0 (b) and pH5.25 (c), after standing 1 h at 10°C. For clarity only the signal of the labeled gelatin is shown as white.

Mixtures of gelatin and A/G emulsions

In A/G emulsions the gelatin migrates to the amylopectin droplets during cooling. As a consequence, phase separation occurs within the droplets. This is illustrated in fig.7 for an A/G emulsion at pH 5.0 containing 1% gelatin. A video is shown as supplementary information (video 5). After reducing the temperature to 10°C one first observes the migration of gelatin to the amylopectin phase. Then after about 3.5 min phase separation starts at the interface and initially the whole interface is covered with a continuous gelatin layer. Progressively, the gelatin layer transforms into more compact domains within the amylopectin droplet thereby reducing its interface with the guar and amylopectin domains. In a few cases, gelatin particles were formed in the middle of some large droplets.

Fig. 7 CLSM images of an A/G emulsion (0.54 wt% guar and 0.51 wt% amylopectin) at pH 5.0 containing 1.0 wt% gelatin at different times after setting the temperature to 10°C: 0 (a), 2 (b), 3.5 (c), 7 (d) and 60 min (e). For clarity only the signal of the labeled gelatin is shown as bright.

When the temperature was increased to 25°C, the gelatin domains in the middle of the droplets melted and dispersed back into the amylopectin phase, but the continuous gelatin layer at the droplet surface became thinner but remained visible, see Fig. S2 of the supplementary information. Further increasing the temperature led to complete dispersion of the gelatin and partial migration of gelatin back to the guar phase. Decreasing the gelatin concentration led, as might be expected, to a decrease of the gelatin domain size in the droplets and slower phase separation, see fig. 8. At 0.75% gelatin domains no longer formed within the droplets showing again that gelatin preferred to phase separate at the interface. At $C = 0.5\%$, only small hardly visible gelatin domains were observed stuck to the interface and at $C = 0.25\%$, gelatin did not visibly phase separate (results not shown).

Fig. 8 CLSM images of an A/G emulsion at pH 5.0 containing 0.5 % (a), 0.75 % (b) or 1.0% (c) gelatin after 1 h at 10°C. For clarity only the signal of the labeled gelatin is shown as white.

Finally, the effect of the pH at C = 0.75% is shown in fig. 9. At pH 4.5 and 4.75 distinct gelatin domains were formed at the interface. At pH 5.0 the gelatin phase formed a continuous layer at the interface and in some cases a separate domain within the droplets and at pH 5.25 the gelatin phase formed a continuous layer at the interface in some droplets, but in others the layer only covered part of the interface. At higher and lower pH no phase separation was observed after 1 h at 10°C. It is clear that also for A/G emulsions the gelatin has a tendency to form distinct domains at the interface at lower pH and a continuous layer at higher pH similarly to what was found for G/A emulsions. Apparently, the tendency for the gelatin layer at the interface to break-up into distinct domain is favored by a small increase of the number of positive charges on the gelatin.

Fig. 9. CLSM images of an A/G emulsion at 0.75 % gelatin at pH 4.5 (a), 4.75 (b), 5.0 (c) and 5.25 (d) after 1 h at 10°C. For clarity only the signal of the labeled gelatin is shown as white.

The amylopectin droplets in the continuous guar phase were smaller (5-10 μm) and more monodisperse in size than guar droplets in the continuous amylopectin phase (5-50 μm) even though the volume fraction of the dispersed phase was practically the same for both emulsions. This was also found for fresh emulsions in the absence of gelatin. It is probably a consequence of the higher viscosity of the guar phase than the amylopectin phase: 0.17 Pa.s and 0.03 Pa.s, respectively, at 60°C at which the emulsions were prepared. In all cases phases separation of gelatin aggregates is initiated at the interface, which suggests that the gelatin accumulates at the interface even before it phase separates. As was mentioned in the introduction, accumulation of polymers at the interface in W/W emulsions has already been reported (Tea, et al., 2019). Such a layer would be thin and not very dense so it would not necessarily be visible with CLSM. Only later does phase separation occur also in the bulk

amylopectin phase, which is clearly visible when the amylopectin phase is the continuous phase. When amylopectin is the dispersed phase, most of the gelatin remained situated at the interface and only in some cases did gelatin form distinct domains within larger droplets.

As we mentioned above, gelatin continues to aggregate below a critical temperature at a temperature dependent rate. Therefore, the emulsions are expected to evolve with time. We have initiated an investigation of the effect of ageing on the emulsions and preliminary measurements show that indeed the range of pH where phase separation is observed increases with time. We also found that the addition of gelatin stabilizes the emulsions depending on their concentration and pH. These results will be presented elsewhere.

Phase separation of gelatin in W/W emulsions is particular in the sense that it is induced by aggregation of the gelatin which in turn can be induced in-situ by cooling. However, it is likely that similar phenomena can be found in W/W emulsions formed by other combinations of 3 incompatible water soluble macromolecules.

Conclusion

Gelatin B phase separates in guar or amylopectin solutions when the gelatin forms aggregates and it can be induced by cooling below 25°C in a narrow pH range around pH 5. In W/W emulsions formed by mixing guar and amylopectin the gelatin aggregates phase separate preferentially at the interface between the two phases. This leads to formation of a continuous layer of gelatin aggregates at the surface of the dispersed droplets. In some conditions the layer persists, but in others it breaks up into distinct domains. Excess gelatin forms microdomains in the bulk amylopectin phase when it is the continuous phase. When the amylopectin phase forms the dispersed phase most of the gelatin phase remains attached to the interface and only in rare cases are distinct gelatin domains formed within the droplets. Phase separation of a third polymer within a W/W emulsion formed by mixing two kinds of incompatible polymers, can be a novel method to stabilize W/W emulsions.

Supplementary information

Partition of gelatin in amylopectin and guar phases; CLSM images of an A/G emulsion at 25°C; **Video 1.** Evolution of a mixture of 0.75% gelatin and 3.26 wt% amylopectin at pH 5 after cooling to 10°C; **Video 2.** Evolution of a G/A emulsion at pH 5.0 containing 0.75% gelatin after cooling from 60°C to 10°C; **Video 3.** Evolution of a G/A emulsions at pH 5.0 containing 0.5% gelatin at 20°C that after previously kept to 10°C for 1 h; **Video 4.** Evolution of a G/A emulsions at pH 5.0 containing 0.5% gelatin at 30°C that was

previously kept at 10°C for 1 h and thereafter at 20°C for 20 min; **Video 5**. Evolution of an A/G emulsions at pH 5.0 containing 1.0% gelatin after cooling from 60°C to 10°C

Acknowledgement

Jiafeng Chen thanks the China Scholarship Council (CSC) research program for providing funding.

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Supporting Information

Water-in-water-in-water emulsions formed by cooling mixtures of guar, amylopectin and gelatin

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Fig. S1 Phase diagram of guar and amylopectin mixtures. The solid line indicates the binodal. The two compositions studied here are indicated by stars and lie on the tie-line indicated by the dashed line.

Partition of gelatin in amylopectin and guar phases

Fig. S1 concentration ratio of gelatin in guar to amylopectin when A/G or G/A emulsion mixed with different concentration (0.25-1.0 wt%) of gelatin (A) and 0.75 wt% gelatin at pH 4.0-6.0 (B).

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472 Fig. S2 CLSM images of an A/G emulsion (0.54 wt% guar and 0.51 wt% amylopectin) at pH 5.0
473 containing 1.0 wt% gelatin at 25°C after having been cooled to 10°C during one hour.

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