Short communication: Stable water-in-oil emulsions formulated with polyglycerol polyricinoleate and glucono- δ -lactone-induced casein gels

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1. Introduction

Water-in-oil (W/O) emulsions can be employed as encapsulation and delivery matrices for water-soluble nutrients or drugs, such as peptides, proteins, vitamins and flavors (Nadin, Rousseau, & Ghosh, 2014). However, destabilization of W/O emulsions often occurs, which limits their application (Frasch-Melnik, Norton, & Spyropoulos, 2010; Márquez, Medrano, Panizzolo, & Wagner, 2010). Therefore, the development of relatively stable W/O emulsions to obtain W/O emulsions-based products of consistent quality during transport or storage is an important issue.

Due to the high mobility of water droplets, W/O emulsions are mainly destabilized by coalescence and sedimentation (Márquez et al., 2010). These destabilization processes can be partially inhibited by high concentrations of emulsifiers. W/O emulsions are often stabilized by synthetic low molecular weight or polymeric emulsifiers, such as polyglycerol polyricinoleate (PGPR) and sorbitan monostearate (Span 60). It is generally accepted that the use of synthetic emulsifiers in food or drug systems is strictly regulated (Dickinson, 2011). Moreover, the destabilization behavior (such as water transportation) of W/O emulsions-based products stabilized by high concentration of emulsifiers can not be efficiently avoided (Garti, 1997). Hence, many investigators continue to identify alternative methods to deal with the requirement for a high concentration of synthetic emulsifiers, while efficiently stabilizing the W/O emulsions (Andrés, Alejandra, Luis, & Jorge, 2010).

The incorporation of gelling biopolymers into the aqueous phase has been suggested as an effective way of improving the long-term stability of W/O emulsions, especially when the biopolymers added to the aqueous phase can form a network throughout the water droplet (Oppermann, Renssen, Schuch, Stieger, & Scholten, 2015). The mechanism(s) involved in this action has not been fully determined. It has been suggested the biopolymer gels in the inner aqueous phase show soft solid-like properties, making the inner water droplets more resistant to destabilization

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factors (Dickinson, 2011; Massel, Alexander, & Corredig, 2015; Surh, Vladislavljević, Mun, & McClements, 2007; Weiss, Scherze, & Muschiolik, 2005).

However, the preparation of gelled biopolymer droplets in the oil phase is difficult due to rupture of the biopolymer gels into small droplets in the oil phase (Iancu, Chevalie, Popa, & Hamaide, 2009). Therefore, the preparation of these droplets is often carried out by inducing the gelation of biopolymers in the inner aqueous phase after the preparation of W/O emulsions. Some authors have proposed that gelation of the inner aqueous phase in W/O emulsions can be carried out by gelatin or heat denaturation of whey proteins (Oppermann, Renssen, Schuch, Stieger, & Scholten, 2015; Surh, Vladislavljević, Mun, & McClements, 2007).

Herein, we propose a new method of preparing W/O emulsions containing gelled water droplets without a high temperature process. The preparation of these emulsions is shown in the Graphical Abstract. Briefly, the oil (containing the lipophilic emulsifier PGPR) and casein dispersions (containing a certain amount of GDL) were homogenized to form W/O emulsions. Different to other acid reagents such as hydrochloric acid and sulfuric acid which totally dissociate the moment they are added to water, GDL is slowly hydrolyzed to gluconic acid and results in a gradual reduction in the pH of the inner aqueous phase within several hours. When the pH of the inner aqueous phase reaches the isoelectric point ($pI = 4.6$) of casein after ~ 3 h, gelation of the inner aqueous phase occurs (Supporting Materials SFig. 1). Thus, the W/O emulsions containing gelled water droplets are prepared. The objective of the present study was to examine this method, with special reference to the stability of W/O emulsions.

2. Materials and Methods

2.1 Materials

The PGPR (Admul Wol 1403k) was purchased from Kerry Bio-Science (Norwich, New York, NY, USA). Caseinate sodium and GDL were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Soybean oil (Fulinmen Co., Ltd., Tianjin, China) was purchased from a local supermarket and used without further purification. Deionized ($18.2M\Omega$) water was prepared using purification equipment (Sartorius Comfort I, Göttingen, Germany). All other chemical reagents used were of analytical purity.

2.2 Preparation of W/O emulsions

Caseinate sodium (3.0%, w/v) was fully hydrated in water using a magnetic stirrer for 12 h at $4^{\circ}C$. Sodium azide (0.02%, w/v) was added to the casein dispersions to inhibit the microorganisms. Soybean oil and PGPR were mixed to obtain a content of 2.0 and 6.0 g PGPR in 100 g of the total oil phase, followed by heating at $45^{\circ}C$ for 2 min in a water bath to allow the PGPR to fully disperse in the oil phase. Then GDL (0.9%, w/v) was directly added into the

casein dispersion (~25 °C). The dispersion was then magnetically stirred for 30s to totally dissolve the GDL. The casein dispersion containing GDL should be used within 3 h of preparation.

To prepare the W/O emulsions, 90 ml casein dispersion (with 0.9% GDL or without GDL) or water (containing 0.9% GDL as the control) was slowly added to 270 ml oil over approximately 5 seconds, while mixing with an Ultra Turrax homogenization apparatus (IKA T 25, Staufen, Germany) with a dispersing head operating at 17500 rpm for 1 min. The samples were then homogenized using a homogenizer at 60 MPa (NS1001 L-Panda 2K, Niro Soavi S.p.A., Parma, Italy). The prepared emulsions were stored at ambient temperature (~25°C) for 24 h for further analysis.

2.2 Type and stability characterization of the W/O emulsions

The emulsions type was judged by observing the effects of several drops of the emulsions in both oil and water (Destribats, et al., 2014). The W/O (O/W) emulsions can be fully dispersed in oil (water), but remains as drops in water (oil).

The stability of the emulsions was evaluated by three methods. The first was to monitor stability using the optical analyzer Turbiscan (Formulation, L'Union, France). The Turbiscan is capable of detecting the destabilization behavior of a dispersed system much earlier and quicker than by the naked eye, especially for opaque systems (Araújo, Nikolic, Egea, Souto, & Garcia, 2011). A pulsed near-infrared light source and two synchronous detectors are positioned in the detection head, which moves up and down along a cylindrical cell to obtain data from transmission and backscattering. The measurements were performed every 30 min for 19 h at 25 °C. The curves delta backscattering was obtained by subtracting the backscattering profile at $t = 0$ from the profile at t . Global stability of the samples was evaluated by the Turbiscan stability index (TSI), which was calculated using the following formula:

$$TSI = \sqrt{\frac{\sum_{i=1}^n (x_i - x_{BS})^2}{n-1}} \quad (1)$$

where x_i is the backscattering for each measurement, x_{BS} is the mean of x_i , and n is the scan number.

The second method used to assess stability was size changes with time at ambient temperature. The first method in measuring the particle diameter was dynamic light scattering measurements (Horiba SZ-100, Kyoto, Japan). Highly concentrated samples were used to analyze the Z-average diameters with this device. All measurements were performed at 25 °C. The refractive index and viscosity introduced into the test condition were 1.473 and 52.4 mPa·s, respectively. The refractive index and viscosity were measured by a refractometer (Anton Paar Abbemat 500, Ostfildern, Germany) and rheometer (TA Instruments Inc., New Castle, DE, USA), respectively. The second method used in measuring the droplet diameter was Turbiscan (Formulation, L'Union, France), as used by some researchers

(Abisma1, Canselier, Wilhelm, et al., 2000). Three parameters were necessary when calculate the mean size of water droplets: dispersed phase refractive index, continuous phase refractive index and volume fraction. The refractive index of casein dispersions (without GDL) and pure water were 1.338 and 1.332, respectively. The refractive index of oil phase was 1.473. The volume fraction introduced to the calculating condition was 25%.

The third method was visual inspection of the appearance of phase separation following 90 days' storage after preparation of the emulsions. 10 ml emulsions was added to cylindrical bottles, sealed with rubber caps and stored at ambient temperature. The stability was evaluated by the sedimentation index (S), which was calculated using the following formula (Ushikubo & Cunha, 2014):

$$S (\%) = (H/H_0)*100 \quad (2)$$

Where H is the oil phase height at the top of the cylindrical bottles, H_0 is the initial height of the emulsions.

2.3 Statistical analysis

Experiments were independently performed three times. Statistical analyses of the data were carried out using IBM SPSS 21 for Windows 7.0. Duncan's multiple range tests for differences were applied.

3. Results and discussion

The test for emulsions type suggested that all types of emulsions prepared in the present study were W/O emulsions. This indicated that caseinate sodium did not change the type of PGPR-stabilized W/O emulsions.

The TSI values are negatively related to the global stability of emulsions (Álvarez Cerimedo, Iriart, Candal, & Herrera, 2010; Kaombe, Lenes, Toven, & Glomm, 2013). The variation in TSI values of W/O emulsions are usually due to changes in the mean size (coalescence / flocculation) or droplet migration at every position (Liu, et al., 2011). A comparison of the TSI values of the W/O emulsions within 19 h is shown in Fig. 1. As expected, the TSI values of the W/O emulsions prepared with PGPR increased with time, which demonstrated that the emulsions became less stable during storage. At a given PGPR concentration, the TSI values decreased significantly when the casein gels were introduced into the inner aqueous phase. Interestingly, the TSI values of the emulsions containing the gelled aqueous phase and 2% PGPR were even lower than the emulsions with 6% PGPR in the absence of the gelled aqueous phase. This indicated that the destabilization behavior (coalescence and sedimentation, in Supporting Materials SFig. 2) of W/O emulsions formulated with the gelled inner aqueous phase was efficiently inhibited.

The size distribution of the emulsions after 1 or 90 days' storage is shown in Table 2. It can be seen that the size of the gelled water droplets varied little during storage, indicating that no significant coalescence occurred. However, the size of the emulsions containing pure water droplets or casein dispersions without GDL showed a sharp increase

after 90 days' storage, suggesting the coalescence behavior happened easier than emulsions containing gelled water droplets. This could also be verified by the microscopy analysis (Supporting Materials SFig. 3). It is worth noting that the addition of caseinate sodium (in the absence of GDL) to the aqueous phase in W/O emulsions after 90 days' storage led to even larger size than emulsions with pure water droplets. This consistent with previous reports which explained that the high concentrations of unfolded protein (gelatin) hindered the formation of a uniform PGPR layer at the water droplets (not a quite regular circle shape can be seen from the microscopy image in our study) (Scherze, Knoth, & Muschiolik, 2006).

The destabilization process of W/O emulsions is mainly ascribed to coalescence and sedimentation. These two processes are generally known as simultaneous destabilization processes, which can be quantitatively analyzed by the *S* value of the emulsions (Ushikubo, et al., 2014). The *S* value of the W/O emulsions containing 6% PGPR and pure water droplets, 6% PGPR and casein dispersions without GDL, 6% PGPR and casein gels, 2% PGPR and pure water droplets, 2% PGPR and casein dispersions without GDL, 2% PGPR and casein gels after 90 days' storage was 18.4 ± 1.2 , 19.1 ± 0.6 , 5.7 ± 0.3 , 31.5 ± 0.7 , 38.6 ± 1.4 and 10.7 ± 0.6 (mean $\pm$ SD, n=6), respectively. These findings suggested that the phase separation of the W/O emulsions containing casein gelled water droplets can be inhibited. Interestingly, the introduction of casein gels into the inner aqueous phase allowed a significant reduction in the PGPR concentration from 6% to 2%, with no significant changes in the stability of W/O emulsions. This indicated that the incorporation of casein gels into the W/O emulsions can significantly inhibit the destabilization process.

4. Conclusions

The proposed preparation method for W/O emulsions containing gelled water droplets in the inner aqueous phase was found to be simple and effective. No harsh treatments (such as high temperature) are necessary, and thus the functionality of the materials (water soluble drugs, vitamins, proteins, peptide and so on) encapsulated in the inner aqueous phase can be largely preserved. The W/O emulsions containing GDL-induced casein gels in the inner phase showed higher resistance to destabilization caused by coalescence and sedimentation, than the equivalent non-gelled W/O emulsions; thus the long-term stability of W/O emulsions-based products can be significantly improved. To our knowledge, this is the first report on the preparation methods of W/O emulsions containing GDL-induced gelled water droplets.

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5. References

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Figure captions

Fig.1. The stability (Turbiscan stability index values, TSI) of W/O emulsions containing different components. Data shown are expressed as the mean $\pm$ SE of triplicate samples.

Table lists

Table 1 The water droplets diameters in emulsions.

Fig.1. The stability (Turbiscan stability index values, TSI) of W/O emulsions containing different components. Data shown are expressed as the mean $\pm$ SE of triplicate samples.

Table 1 The water droplets diameters in emulsions.

Dia meter (nm)	W			W		
	/O -	W(CN)/O-2	G/O-2	/O -	W(CN)/O-6	G/O-6
	ay	2%PGP	%PGPR	6%PGP	%PGPR	%PGPR
	R			R		
DLS		56		51		
		$8 \pm 28c$	$583 \pm 13c$	$3 \pm$	$525 \pm 16b$	$492 \pm$
			18c	19ab		11a
		18		11		
0		$85 \pm$	$3826 \pm$	$649 \pm$	$1836 \pm$	$521 \pm$
		118d	271e	19b	133d	19a
				34c		
Turbiscan		67		58		
		$2 \pm 14b$	$713 \pm 28c$	-	$659 \pm 17b$	-
		22		17		
		$17 \pm$	$4274 \pm$	-	$2542 \pm$	-
0		94b	316d	79a	161c	

(1) Mean values $\pm$ SD, n=6;

(2) W/O, W(CN)/O and G/O represents pure water in oil emulsions, casein dispersions (without GDL) in oil emulsions and gelled water droplets in oil emulsions;

(3) Mean values with different letters within a row indicate significantly different ($p < 0.05$);

(4) “-” indicates no accurate data were available, since the refractive index of the dispersed phase (casein gels) which is necessary for the Turbiscan analysis could not be measured.

Graphic Abstract