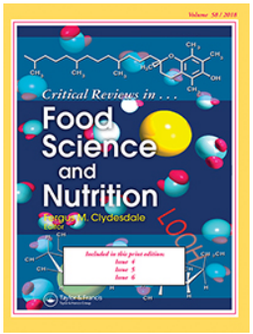

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Multiple layers and conjugate materials for food emulsion stabilization

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ABSTRACT

A single emulsifier material is seldom sufficient to cater to the requirements of complex emulsion-based food formulations that have to function over a wide range of pH, ionic strength, storage time, and temperature. Food emulsions have to be designed to satisfy several requirements for use which could be achieved by combining the beneficial properties of multiple emulsifiers. The present article reviews novel biological materials that are used to design oil-in-water (o/w) emulsions. More specifically, the major focus is to discuss (o/w) interfaces stabilized by multiple biopolymers. Prevalent ways by which two or more emulsifiers could be combined is by (i) forming multi-layered emulsions and (ii) conjugation of two compounds of beneficial traits. Multilayer emulsions make use of a combination of protein, phospholipids, and carbohydrates to stabilize (o/w) interfaces. On the other hand, covalent bonding between a protein and polysaccharide is induced to form a single entity known as conjugate that is superior to the individual biopolymers in terms of emulsion stability. Therefore, properties required to maintain emulsion stability such as surface activity, solubility, steric and electrostatic repulsion, and antioxidant effects from two different hydrocolloids could be integrated.

KEYWORDS

Protein–polysaccharide; Maillard reaction; layer-by-layer electrostatic deposition; secondary emulsion; tertiary emulsion; conjugates

1. Introduction

Many foods have emulsion structure (Vincent, 2008). Some of the existing food including milk, cream, butter, beverages, and mayonnaise are emulsions. Emulsions essentially have three components: the dispersed phase, the interface, and the continuous phase. The dispersed phase (oil or water) is present as small droplets suspended in a continuous phase (water or oil) separated by an interface. The interface is made up of surfactant molecules that reduce the surface tension and thus, prevents the oil and water phases from separating (McClements, 2015). The emulsions are thermodynamically unstable systems. There is a breakdown of the emulsion system due to their ability to expand interfacial area after emulsification (Choi et al., 2011). Therefore, food emulsions have an innate nature to separate into oil and water phases. The complexity in maintaining an emulsion stable could be visualized by understanding the diverse instability mechanisms such as flocculation and creaming or sedimentation. In addition, other instability mechanisms include coalescence and Oswald ripening. Coalescence occurs in emulsions owing to thinning of the interfacial membrane. Meanwhile, the chemical potential gradient between the materials inside large and tiny droplets results in Ostwald ripening (Vianna-Filho et al., 2013).

Food emulsions are divided into different classes based on the distribution of phases. The most common type of food emulsions are conventional emulsions. They are classified into two types such as water-in-oil and oil-in-water emulsions. In addition to conventional emulsions, formation of several sophisticated emulsions have been attempted (McClements, 2015). Pickering emulsion, multilayer emulsions, multiple emulsions, solid lipid particles and filled hydrogel particles are other systems that could be created based on emulsion technology (McClements et al., 2007). Multiple emulsions have a continuous phase with suspended droplets which in turn have droplets within (Chu et al., 2007). Emulsions with particle stabilized interfaces are referred to as Pickering emulsions (Mwangi et al., 2016). Multilayer emulsions have more than one interfacial layers (Fioramonti et al., 2015a).

Multilayer emulsions make use of more than one biopolymer interacting electro-statistically to stabilize the interface of an emulsion system. The primary colloidal lipid dispersion is formed by homogenizing an aqueous phase, oil phase, and a primary emulsifier of suitable proportion. Multilayer emulsions are colloidal systems that have a secondary layer consisting of biopolymer adsorbing onto the surface of a primary emulsion droplet (Chen et al., 2011a). The lipid droplets enclosed by membranes composed of two layers of opposite charge are referred to as secondary emulsions or bilayer emulsions. Emulsions with three-layer interfacial membrane are known as triple layer emulsions or tertiary emulsions (McClements, 2015).

In addition to multilayer emulsions, two biopolymers can interact at emulsion interface in the form of a protein-polysaccharide conjugate. Protein-polysaccharide conjugates have a protein and carbohydrate component linked together by a covalent bond (Kasran et al., 2013a). Covalent bonding may be induced by promoting natural Maillard type reaction

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(Tamnak et al., 2016) or by using enzymes (Liu et al., 2015). In the past few years, there is an improved interest in creating emulsions with a composite interface that consists of many emulsifiers (Xu et al., 2013; Drapala et al., 2016; Liu et al., 2016e). These are attempts to improve emulsion stability to stress factors like variation in pH, temperature, ionic strength, and freeze–thaw treatment.

In general, both proteins and polysaccharides are functional biopolymers and can act as good emulsifiers (Najafi et al.). For proteins, when the pH of solution approaches isoelectric point, the net charge on the hydrocolloid becomes zero and they cease to remain in solution. Because proteins are vulnerable to destabilization near their isoelectric point (Xiang et al., 2015), polysaccharides could be considered as alternative emulsifiers. Polysaccharides provide emulsion stability over wide pH ranges and ionic strengths by steric stabilization mechanism (Dickinson, 2008). However, the hydrophobic residues in polysaccharides are meager and require the use of high concentration of emulsifier to achieve efficient coating of droplet interface (Gharsallaoui et al., 2010b; Lim and Roos, 2015).

Therefore, the merits of polysaccharides and proteins could be combined by complexation process (Bengoechea et al., 2011). The benefit of protein stabilized emulsions is that tiny emulsion droplets are produced. Meanwhile, the benefit of carbohydrate stabilized emulsion is that it can be used to stabilize emulsions over a wide range of environmental conditions (Calero et al., 2013). Rather than the use of protein or polysaccharide as a monolayer emulsifier, more than one biopolymer is often complexed by electrostatic deposition of emulsifiers and polyelectrolytes (Choi et al., 2011; Fioramonti et al., 2015b; Xiang et al., 2016) or by forming covalent linkages (Kasran et al., 2013b; Liu et al., 2016e; Tamnak et al., 2016). The complex so formed is believed to have improved physical and chemical properties than any of the biopolymers used as a single emulsifier (Liu et al., 2015).

Multilayer and conjugate stabilized emulsions can perform various functions in food systems. For instance, accumulation of several layers at the interface improves the stability of multilayered and conjugate stabilized emulsions to environmental stresses compared to monolayer emulsions. In addition, the dispersed phase could be functionalized to carry active ingredients such as antioxidants (Esfanjani et al., 2015; Wei and Gao, 2016), ω-3 fatty acids (Jo et al., 2015; Kaushik et al., 2015), and antimicrobials (Niu et al., 2016); thus, acting as delivery tools in food environment. Triggered release of active ingredients can be achieved by carefully and systematically manipulating the properties and composition of polyelectrolytes that build the interface (McClements, 2006).

In this work, we have reviewed the various emulsifiers that help in emulsion stabilization with or without a covalent linking. We emphasize different biopolymer combinations that have been studied for preparing multilayer and conjugate-stabilized emulsions. In this manuscript, X/Y and X/Y/Z refers to bilayer and triple-layered interfaces, respectively where X, Y, and Z are individual biopolymers. However, A-B indicates a conjugate where both A and B can be either proteins, polysaccharides, or polyphenols.

2. Emulsions stabilized by multiple layers

Droplets of multilayer emulsions possess a complex interface. The formation of multiple layers on interface is dictated by the charge on the droplet and emulsifying conditions (Đorđević et al., 2015). Almost all primary emulsions are characterized by a certain amount of surface charge due to the biopolymers interacting with oil droplets. Biopolymers acting as surface active compounds could be non-ionic, anionic or cationic in nature. Ionic groups present in the biopolymers decide the overall charge on the emulsion droplet. Even in case of non-ionic emulsifiers, there is a small net negative charge on the emulsion droplet contributed by the lipid components such as fatty acids (Güzey and McClements, 2006). The surface charge of biopolymers is emphasized due to its role in electrostatic deposition of multiple coatings.

When polyelectrolytes with opposite charge to the electric potential possessed by primary emulsion come in contact, they interact constructively and form a second layer above the first layer, with charge reversal of the bilayer droplet. The procedure may be repeated with hydrocolloids of opposite charge to form more layers, depending upon the requirement and feasibility (Wei and Gao, 2016). This method is referred to as layer-by-layer (l-b-l) electrostatic deposition (Liu et al., 2016d; Xiang et al., 2016) and is illustrated in Figure 1. However, when the biopolymers used for preparing multiple layers amounts to neutral charge or do not possess sufficient charge, electrostatic or steric repulsion may prevail (Gharsallaoui et al., 2010b). In this way, more layers with appropriate charge can interact constructively or when interactions are not favorable, respond destructively. Emulsion produced by l-b-l approach enhance stability against changes in ionic strength, pH, thermal processing, ageing, drying, lipid oxidation, and freeze–thaw cycling (Lim and Roos, 2015). Table 1 enlists the multitude of materials that could be used to construct multilayer interfaces.

2.1. Secondary emulsions

Secondary emulsions are constructed from two individual layers of biopolymers. Secondary emulsions when compared to primary emulsions have more stability to freeze–thaw cycling and fluctuations in ionic strength, pH, and temperature (Zhao et al., 2015). The change of ζ-potential of the secondary emulsion after addition of polyelectrolytes to the primary emulsion indicates adsorption of a second layer on top of the interfacial layer of the primary emulsion (Kaltsa et al., 2014; Zhao et al., 2015; Xu et al., 2016b). These systems enable the protection of lipophilic food components that are retained in the oil droplets using a variety of proteins, phospholipids, and polysaccharides (Dima et al., 2015).

2.1.1. Protein stabilized interfaces

Considerable research has demonstrated that food proteins can be used successfully as interfacial-stabilizing components in secondary emulsions. Studies have attempted the use of lactoferrin and β-lactoglobulin (β-lg) having isoelectric points of 8.5 and 5.0, respectively, to form laminated coatings on emulsion droplets in order to confer specific functional characteristics. The coatings formed from mixed interfacial systems were

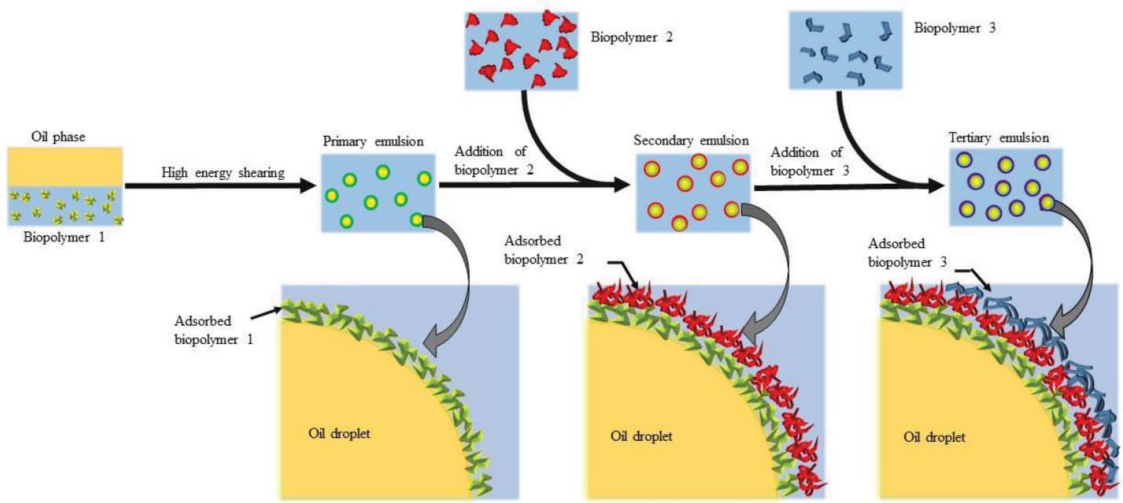

Figure 1. The general steps involved in the formation of multilayer emulsions are represented in the figure. The oil phase and the aqueous phase along with emulsifier is taken. Shearing causes breakdown of the separate oil phase into tiny droplets that are dispersed throughout the aqueous phase. Addition of a second biopolymer to the primary emulsion, forms secondary emulsion.

found to be stable in the pH range 3.0–7.0. In addition, these emulsions demonstrated stability under thermal treatment for 20 min at 21 to 90°C and ionic strength 0–500 mM NaCl as well as 0–60 mM $CaCl_2$ (Schmelz et al., 2011). The experiments of Mao et al. (2013) observed that interfaces laminated with β-lg/lactoferrin and lactoferrin/β-lg were more resistant to thermal treatment up to 90°C than mixed systems of the proteins.

Delivery of active ingredients through foods could benefit from the bilayer emulsion system that can protect sensitive lipophilic compounds. Emulsions of ω-3 oils encapsulated in a double-layer protein coating of caseinate and lactoferrin was physically stable and the oil phase demonstrated improved oxidative stability in terms of TBARS and peroxide value compared to primary caseinate emulsions (Lesmes et al., 2010). Chaprenet et al. (2014) demonstrated that surface charge of

Table 1. An overview of different components of multilayered emulsion.

	Layers					
Oil phase	Primary	Secondary	Tertiary	Active ingredient	Stress factors studied	References
Corn oil	Lactoferrin β-Lactoglobulin	β-Lactoglobulin Lactoferrin	—	—	pH, ionic strength, thermal treatment	Schmelz et al. (2011)
Menhaden oil	Caseinate	Lactoferrin	—	—	pH, ionic strength,	Lesmes et al. (2010)
Soybean oil	Protein of nonfat dry milk	ι-Carrageenan, high-, low-methoxyl pectin, or gelatin	—	—	pH	Tippetts et al. (2013)
Red chili oleoresin	Chitosan–calcium phosphate complex	Mesquite gum	—	Carotenoid	—	García-Márquez et al. (2015)
Sunflower oil	OSA-starch	Xanthan gum	—	—	—	Krstonošić et al. (2015)
Oleoresin capsicum	Tween 80	Alginate	Chitosan	Capsaicin	—	Choi et al. (2011)
Medium-chain triglyceride (MCT) oil	Soybean soluble polysaccharides	Chitosan	—	β-Carotene	—	Hou et al. (2010)
Fish oil PUFA rich oil	Soy β-conglycinin Pea protein isolate	High methoxyl pectin Pectin	—	—	—	Xiang et al. (2016) Gharsallaoui et al. (2010b); Aberkane et al. (2014)
Orange oil	Lactoferrin	Beet pectin or Soybean soluble polysaccharides	—	Limonene decanal, octanal and geranial	—	Zhao et al. (2015)
Sunflower oil	Soy protein isolate	OSA-starch	Chitosan	—	pH, thermal treatment, ionic strength and freeze–thaw cycling	Noshad et al. (2016); Noshad et al. (2015)
Medium chain triacylglycerol	Whey protein isolate (WPI)	Flaxseed gum	Chitosan	Lutein	—	Xu et al. (2016a)
Fish oil	Tween 20	Chitosan	Low methoxyl pectin	*Trans*-cinnamaldehyde	—	Jo et al. (2015)

Note: The table lists multilayered emulsion systems that have been studied for their physical and chemical stability. Several proteins, polysaccharides, and phospholipids have been employed as emulsifiers under various combinations

multilayered emulsions consisting of primary sodium caseinate and secondary lactoferrin layers had to be sufficiently high for the emulsions to be stable. However, it was observed that the surface charge had little role to play in the protection of vulnerable active compounds in the lipid phase. Hu et al. (2003) also emphasized that apart from the magnitude of droplet charge, underlying factors such as free radical scavenging of amino acids, interfacial film thickness and chelating property of proteins could affect oxidative stability of lipids.

Bilayer emulsions coated with inner anhydrous milk protein and outer gelatin was found to be consistent at pH 7.0 and unstable at pH 3.0 and 5.0. The emulsion behavior was studied using scanning electron microscopy (Tippetts et al., 2013). Layer-by-layer (l-b-l) deposition of fish gelatin and whey protein isolate (WPI) was used to stabilize and prevent oxidative breakdown of fish oil-in-water emulsions for incorporation into milk and citrus beverage (Taherian et al., 2011). Such multiple layer coatings could be considered apt for human consumption as they are made from food-grade ingredients and are digested easily. Oil droplets covered by lactoferrin/β-lg or β-lg/lactoferrin nanolaminates were digested easily in simulated gastrointestinal conditions (Schmelz et al., 2011).

2.1.2. Carbohydrate stabilized interfaces

Natural polysaccharides occur with varied properties and structure. Two polysaccharides are combined in this approach in order to achieve enhanced chemical and physical functionality (Ramírez-Santiago et al., 2012). Bilayer emulsion of internal cationic calcium phosphate–chitosan composites and outer anionic mesquite gum were fabricated by l-b-l approach to protect chili oleoresin. Stability against degradation of carotenoids and aggregation of the prepared oleoresin-in-water emulsion significantly improved when the mass ratio of calcium phosphate–chitosan complex and mesquite gum was 1:10 (García-Márquez et al., 2015).

A primary conventional emulsion stabilized with 10% (w/w) octenyl succinic anhydride starch (OSA-starch) was studied for its interaction with xanthan gum. Improvement in emulsion stability was associated with thickening droplet interface and increase in viscosity of aqueous phase (Krstonošić et al., 2015). Oleoresin capsicum was formulated as double-layer nanoemulsion by self-assembly using alginate and chitosan interfacial layers to increase stability. The study claimed the size of the nanoemulsion formed to be less than 20 nm (Choi et al., 2011).

Hou et al. (2010) demonstrated that the concentration of secondary layer in multilayer emulsion played an important role in stabilizing β-carotene consisting of soybean soluble polysaccharide as primary emulsifier. Below a critical concentration of 0.33% (w/w) of secondary emulsifier chitosan, droplet size had reached elevated levels indicating aggregation; above 0.33% (w/w), the globule size dropped. The most stable as well as least β-carotene degradable emulsion was obtained when the concentration of chitosan was 0.5% (w/w) for an oil-in-water emulsion with 10% (w/w) oil phase. Layer over layer deposition of emulsifiers was concluded from the charge reversal of emulsions from −34 to 58.2 mV, as positively charged chitosan adsorbed on to the soybean soluble polysaccharide stabilized anionic droplets (Hou et al., 2010). Molecular weight of chitosan was also found to influence the stability of carotenoid emulsions (Hou et al., 2012).

2.1.3. Protein and carbohydrate combined interfaces

Bilayer emulsions prepared using protein and carbohydrate biopolymers are most prevalent as l-b-l deposition technique requires the two layers to have opposite charges. Proteins and polysaccharides are charged entities in solution. Multilayer interface is contributed by monolayers of different interacting biopolymers. It is observed that proteins and carbohydrates form a primary monolayer coating and sufficient biopolymer is required to completely cover the surface before the adherence of subsequent layers (Đorđević et al., 2015).

2.1.3.1. Emulsion physical stability of protein and carbohydrate combined interfaces. Using l-b-l deposition technique, bilayer emulsions of thickness 80–170 nm of inner β-conglycinin and outer high methyl pectin layers were formed and its surface morphology was characterized. Positively charged fish oil-in-water primary dispersion using β-conglycinin was prepared by high-shear mixing, followed by pectin deposition assisted by homogenization (Xiang et al., 2016). The thickness of the interface could be measured at the submicron level after freeze fracturing the emulsion droplets using cryo-SEM (Humblet-Hua et al., 2012). However, such bilayer emulsions could not be formed with primary emulsion of WPI, on which bacterial cellulose was added (Paximada et al., 2016). Therefore, it can be inferred that not all positively charged proteins and negatively charged polysaccharides form an electrical bilayer and there are alternative interactions that influence bilayer emulsion formation.

Addition of pectin was associated with the formation of multiple interfacial coatings on pea protein isolate stabilized primary emulsions. Secondary emulsions were stable except for bridging flocculation observed at low pH values and low pectin concentration. The rigidity of interfacial membrane was believed to have increased by pectin addition (Gharsallaoui et al., 2010b). Sufficient beet pectin was required for complete coverage of the surface of primary emulsion, if not, bridging flocculation was prevalent and the uniformity of the emulsion collapsed. Also high concentration of pectin increased the viscosity which substantially enhanced the physical stability of olive oil-in-water emulsions (Kaltsa et al., 2014). The physical stability of bilayer emulsions of sodium caseinate and κ-carrageenan were evaluated at two different pH 3.5 and 7.0. At less than 0.25% (w/v) of κ-carrageenan concentration, bridging flocculation was observed at both pH. However, stable emulsions were obtained at 1% (w/v) polysaccharide coated on a primary emulsion surrounded by 0.5% (w/v) sodium caseinate at the evaluated pH (Perrechil and Cunha, 2013).

The interaction of microcrystalline cellulose (MCC) with soy protein hydrolysate (SPI) stabilized curcumin emulsion could be potentially viewed as multilayer system as the interface became more negatively charged with the interaction with MCC (Xu et al., 2016b). Hydrolysates of SPI were prepared in the test using papain enzyme and was found to be a safe and well accepted method of modification (Chen et al., 2011b).

In multicomponent emulsion preparation, l-b-l approach is preferred over mixed emulsion formation as illustrated by

Azarikia and Abbasi (2015). When mixed systems of whey protein and tragacanth gum were created, lower apparent viscosity was observed which was associated with bridging flocculation on sonication. When emulsions with similar combination of emulsifiers was prepared by l-b-l, stable dispersions were obtained. In addition, l-b-l emulsions demonstrated superior stability to varying oil concentration at pH 5.0 and produced small-sized droplets than mixed systems. Tragacanth gum is obtained from an Asiatic species called *Astragalus*. The hydrocolloid is best known for its suitability for acidic emulsions (Abdolmaleki et al., 2016).

Multilayered emulsions were suitable for delivery of active components through human food. At low pH, flocculated sunflower oil-in-water emulsions deposited with coatings of internal WPI and external gum Arabic was found to be stable suggesting deflocculation into individual oil particles and the system destabilized at higher pH condition. Such variations in pH could be expected in human alimentary canal where the active component is to be protected at the low pH of stomach and enhance the absorption of active component in small intestine (Lim and Roos, 2015).

The digestibility of lipids and bioaccessibility of functional compounds can be tailored by the rational selection of protein and polysaccharide that constitute the bilayer interfaces. Carotenoid incorporated medium-chain triglyceride (MCT) oil-in-water emulsions were prepared with single or l-b-l deposited biopolymers such as soy protein isolate (SPI), SPI/alginate, or SPI/chitosan. Digestibility studies performed by in vitro digestion model revealed reduced oil digestion (Zhang et al., 2015b). However, secondary emulsions stabilized by gliadin/xanthan gum or gliadin/pectin demonstrated enhanced lipid digestion in simulated mouth, gastric, and small intestinal phases. When compared to the monolayer systems, secondary emulsions enhanced digestibility due to reduced aggregation that enabled the exposure of large surface area of lipid droplets to the gut secretions (Qiu et al., 2015). Similarly, double-layer emulsions of gelatin/cross-linked pectin had appreciably high lipid digestibility than gelatin primary dispersions. The authors postulated that either the displacement of bilayer hydrocolloids from the interface or the permeability of the interfacial membrane to digestive secretions is the cause of improved digestion of bilayer emulsions (Zeeb et al., 2015). Pinheiro et al. (2016) studied the digestive behavior via dynamic gastrointestinal model of curcumin loaded nanoemulsions created using lactoferrin monolayer or lactoferrin/alginate bilayers. It was noted that curcumin bioavailability was high for lactoferrin/alginate stabilized emulsions.

Enzymatic cross-linking of the secondary pectin layer formed a coating that was associated with superior physical stability in the presence of deleterious factors. The primary layer surrounding the oil droplets was WPI. Beet pectin was facilitated to adsorb on to the primary emulsion by pH adjustments to form the secondary emulsions. Subsequently, the enzyme horseradish peroxidase (HRP) was added to induce cross-linking between pectin molecules (Li et al., 2012). Enzymatically cross-linked multilayer emulsions have been attributed to various functional properties. For instance, emulsified oils were prepared to reduce the absorption of lipids in the gastrointestinal tract (GIT) as an attempt to combat obesity. The release of fatty acids from the emulsion was studied using a simulated GIT consisting of mouth, gastric, and intestinal phases. The cross-links among adsorbed beet pectin molecules was induced by using laccase enzyme. In addition, the cross-linked gelatin-beet pectin bilayer was found to be slightly stable in upper gut but were digested in simulated small intestine (Zeeb et al., 2015).

Multilayered emulsions have also been explored for the protection of probiotic microorganisms in food systems. Probiotics can improve immune system of humans and food products with a minimum of 10^6 CFU/g is recommended (Chávarri et al., 2010). Because of its numerous health benefits, consumption of live beneficial microbes has gained importance. The main hindrance in incorporating probiotics in food is that it is liable to destruction during processing and the harsh environment of the gut such as peristalsis, pH variation, and changes in enzymes and bile concentrations (Marteau et al., 1997). The organisms have to survive in the large intestine after gastrointestinal transit (Sathyabama and Vijayabharathi, 2014). Multilayered emulsion prepared from sodium caseinate or whey protein and pectin as primary and secondary layers respectively was spray dried. Subsequently, they were used to encapsulate *Lactobacillus salivarius* NRRL B-30514 and were found to improve cell viability (Zhang et al., 2015c).

2.1.3.2. Oxidative stability of protein and carbohydrate combined interfaces. Polyunsaturated fatty acids within emulsions are vulnerable to oxidative degradation due to the presence of double bonds and suitable emulsion interface needs to be designed that will form a protective barrier against pro-oxidants. Conventionally, antioxidants and chelators are used for protection of lipids in emulsion system. The manipulation of interface to form a dense and physically thick layer around the oil globule with an overall positive charge is believed to pose a barrier that separates pro-oxidants and lipid hydroperoxides (McClements and Decker, 2000; Scheffler et al., 2009). The segregation between the metal ions and oxygen in the aqueous phase from lipids or lipid peroxides in the oil phase prevents the progress of further chemical reaction leading to degradation products (Gharibzahedi et al., 2013).

Thick and dense bilayered interfaces have been created for the protection of vulnerable lipophilic compounds. Secondary emulsion of inner pea protein and outer pectin layers were subjected to dry processing and showed that stability was ensured using size, charge, microstructure, and creaming measurements (Gharsallaoui et al., 2010a). Spray-dried secondary emulsions consisting of pea protein isolate and pectin as interfaces showed higher resistance to oxidative breakdown of polyunsaturated lipids (Aberkane et al., 2014). Water-in-oil-in-water double emulsions were fabricated to protect saffron extract by making use of whey protein concentrate and pectin double layers (Esfanjani et al., 2015).

Lactoferrin stabilized orange oil-in-water primary emulsions were made into bilayer systems by coating with two polysaccharides: soybean soluble polysaccharide and beet pectin (one at a time) in an attempt to protect the orange oil from oxidation and the bilayer emulsions were compared. The oil consisted of volatile compounds including octanal, decanal, limonene, and geranial. Bilayers consisting of internal lactoferrin and beet

pectin were found to significantly improve the protection of volatile compounds (Zhao et al., 2015). Xiang et al. (2015) also studied the encapsulation of citral emulsions using milk proteins (lactoferrin, β-lg, or α-lactalbumin) and beet pectin. The bilayer consisting of internal lactoferrin and external beet pectin exhibited enhanced protection of neral and geranial against oxidative degradation during storage at 25°C.

2.2. Tertiary emulsions

Emulsion interface stabilized with three layers are referred to as tertiary emulsions. Several instability mechanisms such as depletion and bridging flocculation becomes prominent when the interface is a stratified layer of several biopolymers. If a biopolymer does not adhere around the oil droplet creating a bare interface, droplet-droplet interaction may cause depletion flocculation. On the contrary, bridging flocculation occurs if a single biopolymer molecule adsorbs onto multiple oil droplets at the same time, creating an interconnected bridge. In this situation, the droplets come close enough to be flocculated (Blijdenstein et al., 2004). The concentration of emulsifiers that form triple-layered emulsion has to be carefully monitored so that destabilization by depletion or bridging flocculation can be prevented and reduce the concentration of unadsorbed emulsifiers. Triple layer emulsions were formed using emulsifiers SPI/OSA-starch/chitosan by l-b-l deposition and compared with secondary emulsions of SPI/OSA-starch layers and primary emulsion prepared from SPI monolayer. The superiority among primary, secondary, and tertiary emulsions were established on the basis of ζ-potential, microstructure analysis, and diameter of droplet in response to varying pH, thermal treatment, and salt concentration. The results indicated that tertiary emulsions had a better steric stabilization and protected the integrity of emulsion from stress factors that are presumed to be a part of the aqueous phase (Noshad et al., 2016). Other multiple layer emulsion membrane produced by electrostatic interaction that protected the oil phase from environmental stresses include sodium dodecyl sulfate/chitosan/pectin membranes (Aoki et al., 2005) and β-lg/ι-carrageenan/gelatin membranes (Gu et al., 2005, 2007). The stability of 0.3 μm diameter tri-layered oil droplets formed by Aoki et al. (2005) was attributed to the resistance of the interface to breach against stresses and increased repulsive interaction between colloidal droplets.

The buffer solutions that are used to maintain pH during emulsion preparation was found to influence the emulsion stability in triple-layered emulsion systems consisting of a combination of WPI, pectin, and fish gelatin. It was noticed that citrate buffer was unsuitable for making up the aqueous phase of multilayer emulsion and the ionic composition played a key role in maintaining the emulsified globules in dispersed form (Zeeb et al., 2013). Noshad et al. (2015) showed that triple-layered emulsions were superior to freeze–thaw treatment than bilayer or monolayer o/w emulsions which was attributed to the thick interface. When the water phase was frozen, primary and secondary emulsions exhibited droplet flocculation whereas triple-layered emulsions were stable.

Lutein emulsions were protected using a stable triple-layered interfacial membrane made up of WPI/flaxseed gum/chitosan which exhibited low lutein degradation than secondary and primary emulsions. Chitosan had to be added at a minimum level of 1% (w/w) for a stable oil-in-water emulsion at pH 3.0 containing 10% (w/w) medium chain triacylglycerol oil into which lutein was incorporated (Xu et al., 2016a). Jo et al. (2015) attempted to produce stable triple layer fish oil-in-water emulsion that reduced the off-flavors due to oxidation of oil. Also *trans*-cinnamaldehyde was included into the oil phase so as to mask the undesirable odor. It was noticed that oxidative stability improved with an increase in interfacial thickness due to additional layers. In addition, fish oil release was prevented with primary 1.25% (w/w) Tween-20, secondary 0.1% (w/w) chitosan, and tertiary 0.2% (w/w) low methoxyl pectin layer combination.

Tertiary emulsions could be employed to tailor the digestibility of dispersed lipid phase. Li et al. (2010) investigated the in vitro digestion of primary (β-lg), secondary (chitosan), and tertiary (pectin or alginate) emulsions. Digestion was delayed for secondary and tertiary emulsions than primary β-lg coated droplets. The authors postulated that the polysaccharide layers prevented the diffusion of lipase enzyme into the emulsified lipid, thus retarding lipolysis of secondary and tertiary emulsions. However, results of Mun et al. (2006) suggested that triple-layered membranes of lecithin/chitosan/pectin did not influence lipase activity probably due to the displacement of the membrane from the emulsion interface.

3. Emulsions stabilized by conjugates

A conjugate is a biomaterial prepared from the covalent linking of a protein and polysaccharide. In nature, similar structures exist in plant-based foods such as gum Arabic, sugar beet pectin, and corn fiber gum. Comparable nature-inspired conjugates have been attempted from food grade polysaccharides and proteins as discussed in Table 2. The conjugation is brought about primarily by Maillard type reaction induced by carefully controlling temperature, humidity, and concentration of ingredients. Such conjugates are distinguished from multilayer emulsions by the presence of covalent linkages between proteins and polysaccharides (Dickinson, 2008).

Gum Arabic is an emulsifier with natural conjugation between protein and polysaccharide. The protein fraction comprises only 1.7% (w/w; Matsumura et al., 2003). Three different fractions of varying polysaccharide and protein content are accepted by researchers to exist in gum Arabic (Gulrez et al., 2011). The first fraction comprises of polysaccharides entirely. The second fraction, gum Arabic glycoprotein (or GAGP) complex is a high molecular weight glycoprotein making about 1% (w/w). The third fraction is also a glycoprotein with 50% (w/w) protein and contributes to 1% (w/w) of the total mass. The major difference between the second and third fraction is their amino-acid sequence (Dror et al., 2006). The functional properties of gum Arabic is primarily due to the adsorption of the high molecular weight protein-polysaccharide complex (GAGP) at the oil–water interface. The structure of protein-polysaccharide complex is explained by the "wattle blossom" model which assumes the polysaccharide to take spheroidal shape and as being attached to the contiguous protein chain (in the form similar to the inflorescence of wattle plant). Charge has an inconspicuous role in emulsion stabilization by gum

Table 2. An overview of different components of conjugate-stabilized emulsion: Various combinations of proteins and carbohydrates that have been linked by covalent linkage leading to conjugate formation are tabulated.

	Conjugate stabilizer					
Oil phase	Protein	Carbohydrate	Control emulsions system/ comparing standards	Active ingredient	Stress factors studied	References
Soybean oil	Soy protein hydrolysates	Dextran, glucose, maltose, or maltodextrin	Soy polypeptides	—	—	Li et al. (2016)
Orange oil	β-Lactoglobulin or whey protein isolate	Corn fiber gum	Whey protein isolate, β-lactoglobulin and corn fiber gum	—	—	Yadav et al. (2010)
Canola oil	Pea protein isolate	Pectin	Pectin	—	—	Tamnak et al. (2016)
Tomato oleoresin	Soy protein isolate-gum acacia	Gum acacia	Gum acacia an soy protein isolate mixtures	Lycopene	—	Li et al. (2015)
Canola oil	Soy whey protein isolate (SWPI)	Fenugreek gum	SWPI/fenugreek gum mixtures and swpi	—	—	Kasran et al. (2013b)
Soybean oil	Oat protein isolate (OPI)	Dextran	Native OPI and heated OPI	—	pH and ionic strength	Zhang et al. (2015a)
Soya oil	Sodium caseinate	Maltodextrin	Sodium caseinate, sodium caseinate–maltodextrin mixtures	—	Freeze-thaw cycling	O'Regan and Mulvihill (2010)
Canola oil	Deamidated wheat protein	Dextran	—	—	—	Wong et al. (2011)
Medium-chain triacylglycerol	Whey protein isolate	Beet pectin	WPI, unheated mixtures of WPI and beet pectin	β-Carotene	—	Xu et al. (2012)
Soybean oil albumin	Bovine serum Albumin and corn fiber gum	Corn fiber gum	Bovine serum	—	—	Liu et al. (2015)
Corn Oil	β-Conglycinin	Dextran	β-Conglycinin, products of enzymatic hydrolysis of β-conglycinin–dextran conjugates		pH, thermal treatment, and ionic strength	Zhang et al. (2012a)

Note: The table also mentions the emulsifiers that were used as standard of comparison to judge and compare conjugated emulsifiers.

Arabic though a small negative potential is detected (−10 to −20 mV; Dror et al., 2006; Dickinson, 2008).

Sugar beet pectins have good emulsification power, which depends on the amount of protein fraction present (Schmidt et al., 2015). Hydrophobic components within beet pectin such as ferulic acid and protein adsorbs to the oil surface. There are lateral chains primarily of galactose and arabinose that link the hydrophobic groups on the surface of oil to the main chain. The main chain mostly consists of galacturonic acid monomers. The lateral and main chain provides steric hindrance forming a film, whereas ferulic acid and protein components anchor to the hydrophobic end of the interface (Chen et al., 2016).

Corn fiber gum (CFG) is a natural protein-polysaccharide complex. It is a by-product from corn milling operations. CFG is obtained via alkaline extraction from fibrous components of corn kernels like pericarp, endosperm cell-wall fractions, and tip cap. The various sugars present in corn fiber gum are D-xylose, galactose, L-arabinose, and glucuronic acid (Yadav et al., 2007). The small amount of protein molecules anchors at the oil–water interface with the carbohydrate fractions extending into the aqueous phase, thus acting as a barrier. However, not all CFG has enough protein fraction to conjugate and artificial conjugation has been attempted to improve the emulsifying capacity (Liu et al., 2015).

In an attempt to mimic the naturally occurring conjugates, initially glycoproteins were formed by Maillard reaction between β-lg and several sugars like glucose, lactose, galactose, ribose, and arabinose and improvement in solubility, emulsifying capacity, foaming, and heat stability were reported (Chevalier et al., 2001). Also the results of Liu et al. (2016e) indicated that the hydrophilicity of lipid globules and steric repulsion was increased by the conjugation of chlorogenic acid-lactoferrin to carbohydrates such as glucose or polydextrose. Meanwhile, improvement in emulsifying properties were attempted by conjugating proteins with glucose and dextran (Wong et al., 2009, 2011). It was observed that when β-lg was conjugated with CFG, emulsifying properties substantially improved compared to sugars (Yadav et al., 2010). Carbohydrates of low molecular weight like sugars were not as efficient in providing steric stabilization as highly branched and large molecular weight polysaccharides like dextran (Wong et al., 2011).

3.1. Emulsions stabilized entirely by conjugate emulsifier

3.1.1. Maillard type conjugates

Maillard reaction is named after Louis Camille Maillard (1912), a French chemist who first observed the formation of brown pigments on heating aqueous solution of glucose and lysine. The mechanism of Maillard browning was proposed by Hodge (1953) and it remains valid till date (Martins et al., 2000; Eskin et al., 2013). Maillard conjugates are multifunctional biopolymers because they have found a vast array of application in multiple fields. Maillard reaction is primarily induced by conjugating a protein with polysaccharide under elevated temperatures. The favorable reaction to form conjugate occurs between a reducing end carbonyl group of polysaccharide and mainly lysine residue of proteins that has an ε-amino group (Al-Hakkak and Al-Hakkak, 2010; Wong et al., 2011; Eskin et al.,

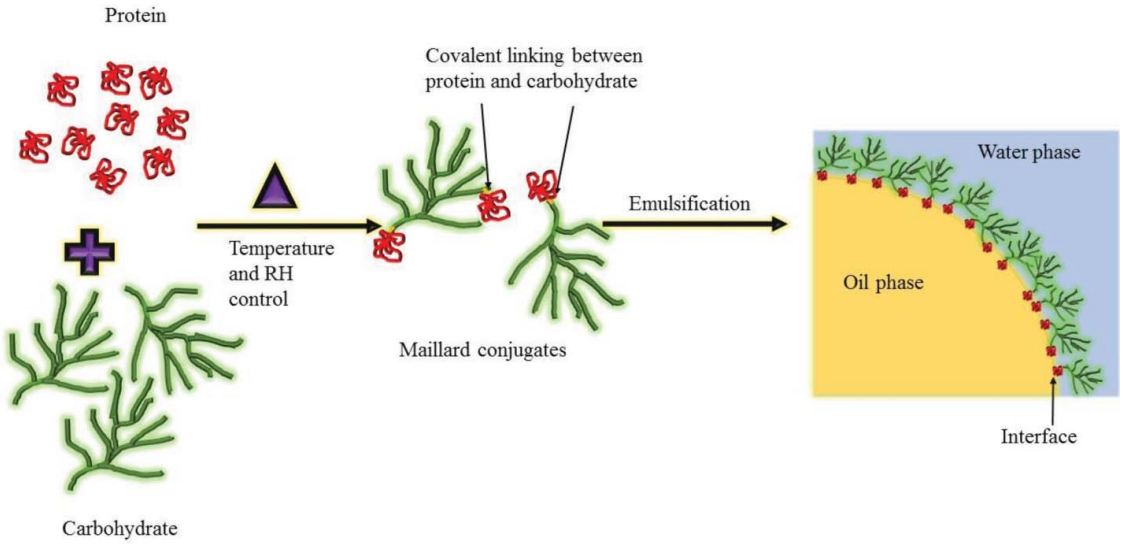

Figure 2. Schematic representation of controlled Maillard reaction between protein and polysaccharide to establish conjugate bonding between the two. The conjugate so formed is used along with an oil phase, water phase to form an emulsion where the conjugate stabilizes the interface by self-assembly. The alignment of the Maillard conjugate on the droplet interface is represented after emulsification.

2013). Figure 2 is a generalized schematic depicting the conjugation of a model protein and polysaccharide. In spite of research over a century on Maillard reaction, the complete mechanism involving amino acids, sugar, and thermal treatment is not entirely understood (Somoza and Fogliano, 2013).

The complex set of reactions contributing Maillard browning are understood by subdividing into initial, intermediate, and final stages. The initial stage of Maillard browning involves sugar-amine condensation and Amadori rearrangement. Carbonylamino reaction is the beginning step that involves the covalent attachment of α- or ε-NH_2 groups present in proteins or free amino acids to carbonyl group of reducing sugars giving rise to an addition compound. It readily loses water to form a Schiff's base which undergoes cyclization to its corresponding N-substituted glycosylamine. This compound is tremendously unstable and undergoes isomerization from an aldose (N-substituted glycosylamine) to the corresponding ketose sugar derivative (fructose-amino acid), a process referred to as Amadori rearrangement. This process generates Amadori products which are stable to a certain extent. Meanwhile, such products are vulnerable to additional degradation processes (Hodge, 1953; Reynolds, 1963; Friedman, 1996; Eskin et al., 2013).

The next two phases primarily involves degradation reactions of the formed Amadori compounds. Such associated reactions are considered detrimental for the preparation of protein-polysaccharide conjugates. The intermediate stage involves sugar dehydration, sugar fragmentation, and Strecker degradation (amino acid break-down). Depending on pH and environmental conditions, sugar dehydration proceeds in two different pathways. At pH less than 7.0, the Amadori products undergo 1, 2 enolization by the loss of three molecules of water to generate furfural and hydroxymethylfurfural when pentose and hexose sugars are involved respectively. Under pH greater than 7.0, Amadori compounds principally advances through 2, 3 enolization to develop reductones by the loss of two water molecules. Sugar fragmentation progresses majorly by retroaldolization and oxidative fragmentation to give rise to a variety of fission products including glycolaldehyde, formaldehyde dihydroxyacetone, glyceraldehyde, 2-oxopropanal, acetol, ethanal, acetic acid, acetoin, aldol, butanedione, pyruvic acid, propanal, formic acid, lactic acid, saccharinic acid, and levulinic acid (Martins et al., 2000; de Oliveira et al., 2016). Strecker degradation refers to the oxidative breakdown of α-amino acids to its corresponding aldehyde in the presence of α-dicarbonyls or similar dicarbonyl compounds from the concomitant reactions of Amadori products (Eskin et al., 2013).

The final stages of Maillard browning involves aldol condensation and aldehyde–amine condensation. The aldehydes formed from the intermediate reactions as well as from lipid oxidation in the food system condense with one another. This reaction known as aldol condensation is effectively catalyzed by the presence of amines and their salt. Aldehydes, specifically α- and β- unsaturated forms undergo condensation with amines at low temperature to give polymeric melanoidins (Namiki, 1988; de Oliveira et al., 2016). To produce protein-polysaccharide conjugates, the breakdown of Amadori compounds to produce melanoidins must to be prevented. Melanoidins are poorly soluble in water and are the colored end products of Maillard browning (Hui, 2006; Yadav et al., 2010).

There are several techniques employed to induce Maillard reaction between the protein and polysaccharide. Different strategies include wet-heating, dry-heating, and molecular crowding (Zhuo et al., 2013). Figure 3 schematically illustrates the general steps involved in the formation of Maillard conjugates by dry-heating and wet-heating methods. The most preferred technique used to induce condensation reaction between amino group of protein and carbonyl group of polysaccharides is dry-heating the raw materials under controlled water activity and temperature (O'Regan and Mulvihill, 2010). In this method, protein and polysaccharide of interest are dispersed in water and freeze-dried to ensure sufficient contact. It is then powdered and heated in an apparatus where time, temperature, and relative humidity (RH) are monitored. For example,

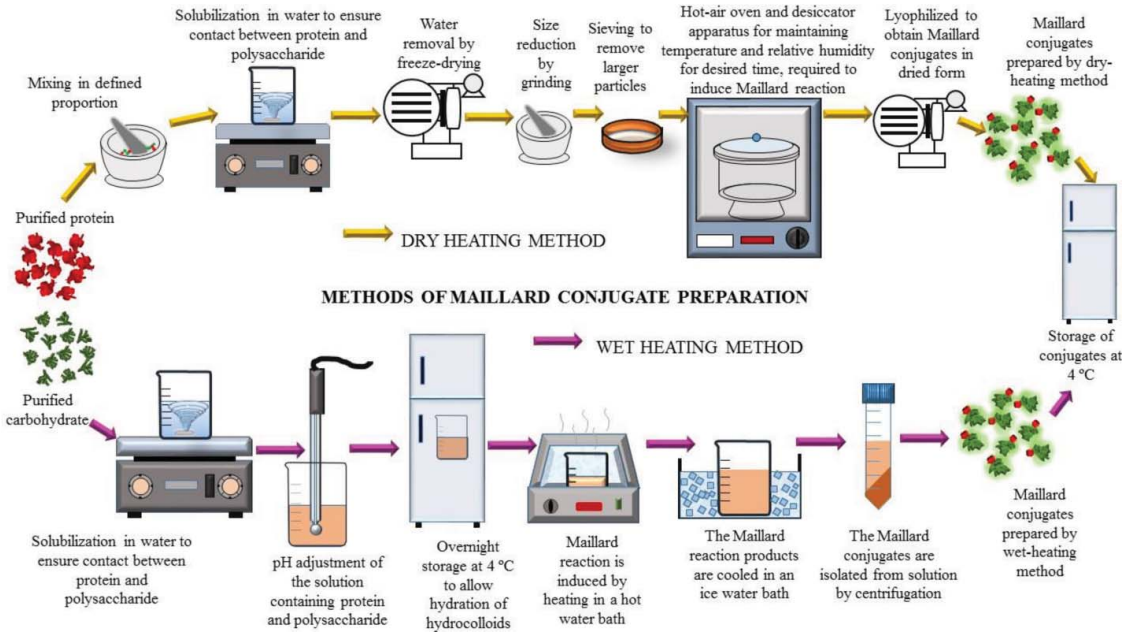

Figure 3. Two main methods of Maillard conjugate preparation are illustrated schematically. The dry raw materials, namely protein and polysaccharide is conjugated by heat either in dry-powdered state (dry-heating method) or in solution (wet-heating method). The dry-heating method involves mixing the raw materials in water to ensure sufficient contact, drying, grinding and sieving to collect fine powder and then subjecting the powder to controlled heat treatment for desired time to obtain Maillard conjugates. The wet-heating method of conjugate preparation involves hydration of hydrocolloids at desired pH, heating the protein and polysaccharide in solution, and cooling. The cooled solution containing Maillard conjugates could be used directly for emulsion preparation or the conjugates could be isolated by centrifugation and stored for later use.

Tamnak et al. (2016) prepared pectin-pea protein isolate conjugate by dry-heating. The protein (4%, w/w) and polysaccharide (4%, w/w) was dissolved individually in phosphate buffer (0.1 M, pH 7.0) and hydrated overnight. They were mixed in desired proportion (1:1, 2:1, 3:1, and 1:2, w/w) prior to lyophilization to obtain uniformly mixed freeze-dried powder. This was followed by milling and sieving to get uniform-size particles. Maillard reaction was induced by incubation at 60°C and 79% RH for varying intervals. The resulting product containing conjugates were freeze-dried and stored. The limiting factor that restricts the progress of the desired Maillard reaction to form conjugate include nonuniform proximity of the reacting species. The resulting mixture could be a protein-polysaccharide conjugate, unbound polysaccharides, rigid or folded proteins in an unreacted state or intermediate or final stage products of the Maillard reaction (Zhuo et al., 2008, 2013). It is necessary for the conjugates to be separated and refined using certain techniques. This renders the dry-heating process unattractive from the industrial perspective (Zhu et al., 2008; Zhang et al., 2012b). To exert some control over the progress of Maillard reaction, wet-heating approach has been attempted (Guan et al., 2006; Zhang et al., 2012b; Li et al., 2016).

Wet-heating is another method of preparing protein-saccharide conjugate and is achieved by heating the ingredients present in buffer solution for specified hours (Zhuo et al., 2013; Perusko et al., 2015). For instance, Li et al. (2016) conjugated soy peptides with varying size dextran by wet-heating method. The raw materials were mixed in a ratio of 1:1, pH was adjusted to 7.0, overnight time was allowed for thorough hydration, and was heated in a water bath at 60°C for a period of 3 days. Similarly, a conjugate of oat protein isolate and dextran was formed by glycating the materials at pH 9.0 and 90°C that enhanced the emulsifying property at oil–water interface by providing steric hindrance. On storage, smaller and uniform oil droplets were observed through confocal laser scanning microscopy. Also resistance to environmental stresses of varying salt concentration and pH was enhanced (Zhang et al., 2015a). In comparison to dry-heating technique, the wet-heating approach relatively shortens the reaction time and demonstrates improved control over the reaction advancement. However, glycosylation levels may be low due to protein aggregation at elevated temperatures. Glycosylation yield may be improved by raising the concentration of reactants but protein aggregation and polymerization also increases simultaneously (Zhu et al., 2008).

Zhu et al. (2008) put forth macromolecular crowding effect as a remedy to overcome the problems in Maillard conjugate preparation. Molecular crowding is a condition in which the Maillard reaction could proceed under less adverse treatments in aqueous solutions. A strategy devised to reduce the extent of destruction of native protein structure during harsh conditions is to bring about the crowding effect in solution (Zhuo et al., 2013; Zhang et al., 2015a). According to excluded volume theory, when reactions involving biomolecules are conducted under conditions of macromolecular crowding (high concentration of macromolecules), the reaction will proceed in a direction that will favor the formation of species with smaller excluded volume. The native proteins would have a more compact structure than the denatured forms. Therefore, the native protein structure will be favored and the degree of polymerization and denaturation would be minimal under harsh conditions required to raise the conjugate yield. As Maillard reaction between a protein and polysaccharide progresses slowly, the reaction could be terminated by rapid reduction in temperature

to approximately 0°C (Zhu et al., 2008). The conjugate formed by the protein adducted to the bulky polysaccharide is adequately stable due to steric factors (Zhu et al., 2008; Eskin et al., 2013) and thus leads to maximum yield. Perusko et al. (2015) attempted to increase the glycation efficiency of whey proteins using ultrasound and macromolecular crowding. The Maillard reaction products generated showed improved thermal stability, pH, and oxidative stability. Zhu et al. (2008) demonstrated WPI-dextran conjugate formation under macromolecular crowding. At temperature 60°C and pH 6.5, the formation of Schiff base was indicated. In addition to being a reactant, dextran restrained protein denaturation and aggregation thus playing the role of a protective reagent.

Several studies have created conjugates by the Maillard reaction for use as surface-active materials for the stabilization of oil–water interfaces. Chemically, CFG is an arabinoxylan having ability to produce solutions with low viscosities (Yadav et al., 2007). As the protein fraction in CFG is limited, conjugation with WPI and β-lg was attempted using nonenzymatic browning reactions performed under 75 °C and 79% relative humidity for a duration of 1–2 days. Longer duration of treatment produced more conjugate, but of inferior quality as the solubility of conjugates reduced. The emulsions formed using β-lg-CFG and WPI-CFG conjugates were stable to high ionic strength and low pH as compared with emulsions where one of CFG, β-lg, or WPI was used as the sole emulsifier (Yadav et al., 2010).

When Maillard reaction was carried for a long duration, strong linkage was found to be established between protein and polysaccharide. Long incubation time of 48 h at 60 °C was necessary to form pea protein isolate (PPI)- pectin conjugate. This conjugate had better emulsifying activity and was stable to physical separation after 1 month of storage. PPI-pectin conjugate had high negative ζ-potential (−49.97 mV) that made the oil droplets stable in aqueous phase (Tamnak et al., 2016). Maillard conjugates obtained after reaction for 3 days at 60°C and 75% relative humidity prepared from soy soluble polysaccharide and SPI was found to exhibit a uniform size even up to 70 days of storage. The recoalescence of oil droplets was prevented by the combined properties of protein and polysaccharide in conjugate form (Yang et al., 2015). Maillard type protein-polysaccharide conjugates were prepared by heating SPI and gum Arabic to a temperature of 60°C, relative humidity 79%, and best results were obtained when the treatment period was 6 days. Emulsion formed from conjugates had superior properties like greater apparent viscosity when compared to an unheated mixture of SPI and gum Arabic. Human colon carcinoma (HT-29) cells were used to study the toxicity of various concentration of SPI-gum Arabic conjugates. This in vitro toxicity study supported the idea that the conjugate possessed better biocompatibility than Tween-80, a small molecule surfactant (Li et al., 2015).

The influence of molecular weight of conjugated carbohydrate was investigated using dextrans and β-lg based emulsions which were flocculated using calcium in order to assess the emulsion stability. It was found by coating latex spheres with β-lg that it formed an interface of 3 nm thickness. Similar methods found that conjugates of β-lg-dextran (M_w of dextran: 18.5 kDa) formed 5 nm and conjugates of β-lg-dextran (M_w of dextran: 440 kDa) formed 20-nm-thick interface and both the conjugated emulsions exhibited equivalent surface activity. Linking of dextran to globular protein by carbodiimide linkage improved emulsion stability but the molecular weight of linked polysaccharide had negligible role (Wooster and Augustin, 2006). However, Wong et al. (2011) showed conjugation of high molecular weight dextran of 41 kDa added an extra 6 nm to the interface thickness than that offered by soluble isolated wheat protein fraction alone, and formed more stable emulsion than the low molecular weight dextran (6.4 kDa). However, Li et al. (2016) found no significant correlation between the varied chain length of saccharides bonded to SPIs with respect to functional properties.

Conjugates of protein and polysaccharide have been established to resist flocculation, creaming and coalescence, and to improve solubility, thus enhancing the overall physical stability of emulsions. This may be achieved by modifying the rheology of continuous phase such as thickening, structuring, and gelling the matrix (Xu et al., 2012). The nature of polysaccharides involved in Maillard reaction influence the properties of conjugate. Pectins are heteropolysaccharides primarily made up of D-galacturonic acids and several other monomers including ferulic acid (Pérez et al., 2003). The degree of esterification in citrus pectin was found to affect emulsion characteristics when pectin was used to form conjugate with WPI. Low methoxyl pectin favored high conjugate yield and formation of small droplets within 100 nm range (Schmidt et al., 2016).

In addition to emulsion stability, antioxidative properties of certain conjugates have also been explored. The Maillard type glycoprotein that has antioxidant potency is explained by the amido-sugar model and sometimes by protein-sugar model. The formation of a physical barrier at interface to prevent oxygen promoting agents may also contribute to their ability to prevent oxidation of the bioactive compounds (Xu et al., 2012). Egg white protein and high methyl esterified pectin were used to form emulsions in two forms: (a) after conjugating and (b) after physical mixing. Emulsions formed with conjugated emulsifier had improved stability and formed viscous emulsions than those formed from physical mixtures (Al-Hakkak and Al-Hakkak, 2010).

Emulsions stabilized by Maillard conjugates have also been prepared for the encapsulation of nutritionally-active compounds such as β-carotene against chemical deterioration. β-carotene is a natural component obtained from vegetables and demonstrates significant health benefits including prevention of cancer, colorectal adenomas and heart disease. It also behaves as an antioxidant and a coloring agent within food systems. The primary hindrance in β-carotene incorporation in food systems is its unstable nature, tendency to degrade due to oxidation and low water solubility (Xu et al., 2013; Lim and Roos, 2016). In addition, carotenes including β-carotene are sensitive to light and heat. Owing to the hindrances faced in the incorporation of β-carotene to complex food environment, oil-in-water emulsions can be a viable option. The interfacial stabilizers have to be cautiously selected as they can significantly influence the stability of β-carotene dispersions (Xu et al., 2012; Lei et al., 2014a). Conventionally, emulsions stabilized by octenyl succinate starch (Mao et al., 2009; Mao et al., 2010; Sweedman et al., 2014), whey protein (Mao et al., 2009;

Hou et al., 2014; Wang et al., 2015), soy glycinin (Liu and Tang, 2016), sodium alginate (Soukoulis et al., 2016), α-phosphatidylcholine (Verrijssen et al., 2015), pectin (Verrijssen et al., 2014) and mixtures of chitosan and soybean polysaccharide (Hou et al., 2012) have been used to protect β-carotene degradation.

Treatment of WPI and beet pectin to temperatures 80, 90, and 100°C of varying weight ratios and relative humidity of 79% produced covalent coupling between protein and polysaccharide. The treatment time was varied from 1 to 9 h. Emulsions were formed using β-carotene incorporated within medium-chain triacylglycerol oil and phosphate buffer (pH 7.0, 10 mM) using emulsifiers including WPI, unheated mixtures of WPI, and beet pectin and conjugates of WPI-beet pectin. As compared with WPI and unheated WPI–beet pectin mixtures, the conjugates of WPI-beet pectin had better physical stability to creaming, uniform size distribution, and reduced degradation of β-carotene. In pectin molecule, the carboxylic acid group of ferulic acid is esterified to oxygen linked to the C-6 of galactose or C-2 of arabinose, thereby, the hydroxyl group of ferulic acid is vulnerable to the action of free radicals and could behave as an antioxidant. Also, a denser and thicker interfacial layer could have conferred protective action to the encapsulated β-carotene (Xu et al., 2012). Even the fraction of emulsifiers that did not adsorb to the interface contributed to the stability of β-carotene present in the oil phase of emulsions. The authors also observed that β-carotene was more stable at higher pH values (Xu et al., 2013).

The digestibility of active ingredients within oil-in-water emulsions was also affected by the use of conjugated emulsifiers. Emulsions stabilized by Maillard conjugates can be used to modulate the digestibility of consumed fats. Food matrices that induce satiety by reducing the digestibility of lipids have been attempted using β-lg-dextran Maillard conjugates in simulated gastrointestinal conditions. Raising the molecular weight of dextran in the conjugate increased steric repulsion and thus retarded lipid digestibility (Lesmes and McClements, 2012). When lipid digestion is restrained by emulsion matrices, more of undigested food reaches the ileum. Therefore, the *illeal* break mechanism which induces hunger and satiety is stimulated (McClements and Li, 2010). Eventually, lower levels of fats are absorbed that leads to improved nutrition. Xu et al. (2014) hypothesized that the protein-polysaccharide conjugate could modulate the permeability of the lipid droplet interface and limit the contact between the emulsion oil phase and digestive enzymes.

Conjugated emulsifiers influenced the release and bioavailability of active components in emulsion systems. The active component in the emulsion matrix needs to be delivered at a suitable location in the GIT. For carotenoids, the appropriate region of absorption is considered as small intestine. Liu et al. (2016a) investigated the bioavailability of β-carotene primary emulsions stabilized by ternary conjugates of (chlorogenic acid-lactoferrin)- dextran in simulated stomach and small intestinal conditions. Bioavailability was determined by quantification of β-carotene fraction in mixed micelle phase obtained at the end of simulated lipid digestion. The ternary conjugate stabilized emulsions had high bioavailability of 47% attributed to small-sized droplets that had large surface area exposed to digestive secretions. The use of active compound citral having selective inhibition of intestinal pathogens in animals is restricted by the lack of suitable carrier during deteriorative gastric transit (Si et al., 2009). In this study, optimized SPI and soy soluble polysaccharide Maillard reaction products substantially improved the stability of citral in simulated gastric and intestinal fluids. These results were favorable for the intestinal lutein release (Yang et al., 2015). However, the lutein emulsions formulated by Gumus et al. (2016) using sodium caseinate-dextran Maillard conjugate had similar bioaccessibility as that of sodium caseinate stabilized emulsions. This result was attributed to the similar charge and size of the tested emulsions after simulated small intestinal digestion.

Studies have also focused on systems where a protein and polysaccharide are intrinsically present as naturally occurring substances and emulsifying property is brought about by inducing Maillard reaction. For example, okra hydrocolloid mucilage has both proteins and characteristic slimy polysaccharides. Heating at 100°C for 6 h formed conjugates that showed better emulsification than the conjugate formed between okra polysaccharides and bovine serum albumin (BSA). Okra raw mucilage did not show significant emulsion stabilizing property at pH 7.0 whereas heat treatment produced conjugates of superior quality at the same pH without any added compounds. This combination of ingredients are natural and the reaction is similar to that occurring during cooking of the fruit. Therefore, the conjugate can be considered as a safe product (Temenouga et al., 2016).

3.1.2. Conjugates produced by enzymatic and nonenzymatic methods

Protein-polysaccharide conjugates can also be produced by enzymatic methods. Maillard reactions tend to take several days or weeks for establishing a covalent bond between the amino group of protein and the reducing-end carbonyl group of polysaccharide. As an alternative to Maillard reaction, enzyme-induced conjugation have been attempted. The enzymatic conjugation has several advantages including the requirement of smaller amount of reactants and rapid conjugate production (Liu et al., 2015).

The HRP was used to cross-link BSA and corn fiber gum. BSA has tyrosine moieties located at the outer surface of compact globular protein, enabling better contact with enzyme and facilitating conjugation. Hetero-conjugation is achieved between the phenolic acids present as part of corn fiber gum and the tyrosine moieties of BSA. The resulting conjugate was found to have better emulsifying potential than either BSA or CFG. The conjugates had a high CFG to BSA ratio as well as good stability over a wide range of pH, freeze–thaw cycling, and high ionic strength (Liu et al., 2015).

β-conglycinin and dextran (67 kDa) was cross-linked by bringing about Maillard reaction. In an attempt to improve the emulsion forming and stabilizing ability, the conjugates were subjected to enzymatic breakdown using protease trypsin at 2.2 and 6.5% degree of hydrolysis (DH; Zhang et al., 2012a). DH is defined as the ratio of peptide bonds cleaved to the total peptide bonds present in a gram of protein, expressed in percentage (Adler-Nissen, 1979). Emulsion prepared using hydrolysates of β-conglycinin-dextran conjugates of 2.2% DH exhibited higher protein adsorption, a significantly reduced saturation surface

load and resilience to change in pH, temperature, and ionic strength for 4 weeks against emulsions prepared with conjugates of 6.5% DH and β-conglycinin (Zhang et al., 2012a).

In addition, polyphenol and protein conjugates can be prepared by noncovalent or covalent interactions, which modifies the properties of proteins. For instance, such interactions can improve thermal stability and alter the solubility of proteins. When covalent bonding prevails, protein-polyphenol conjugates are known to be formed in solution (Liu et al., 2016b). Interactions between protein and polyphenol are affected by pH, temperature, structure, type of phenolic compound, and concentration and type of protein (Ozdal et al., 2013).

Free radical grafting is an alternative method for the preparation of polyphenol conjugates. Chitosan-epigallocatechin-3-gallate (EGCG) conjugates were designed to demonstrate potential emulsifying and antioxidant ability. UV, H-NMR, and FT-IR spectroscopic techniques revealed the formation of conjugates by using a redox pair of H_2O_2 and ascorbic acid. Chitosan-EGCG conjugates were better antioxidants than chitosan alone as studied by 2,2-diphenyl-1-picrylhydrazyl scavenging activity. Also superior emulsion stabilizing property was observed as compared with chitosan (Lei et al., 2014b). The previously mentioned conjugate prepared by free radical grafting method was assessed for its ability to stabilize β-carotene present in MCT oil from degradation. In addition, the physical stability of emulsion was also tested. The active compound β-carotene was quite stable to degradation after emulsification using conjugate. However, the emulsion physical stability as expressed from accelerated creaming studies was almost equivalent to the chitosan control emulsion (Lei et al., 2014a). Ternary conjugates of chlorogenic acid–lactoferrin–glucose and chlorogenic acid–lactoferrin–polydextrose were also formed. The latter combination protected β-carotene in emulsion from degradation under ultraviolet exposure and freeze–thaw cycling (Liu et al., 2016e).

3.2. Emulsions stabilized by conjugate combinations

Some emulsions are made functional by using multilayers of which one or more layer(s) consist of conjugates. For instance, chitosan is a polysaccharide that dissolves in aqueous phase only in acidic pH. Chitosan is rendered soluble in neutral pH by conjugating with EGCG. Because of the presence of hydroxyl groups in the EGCG component of chitosan-EGCG conjugate, the compound is believed to be soluble. This conjugate was coated as the secondary layer on top of a primary β-carotene loaded oil-in-water emulsion stabilized by α-lactalbumin or sodium caseinate (Wei and Gao, 2016).

A multilayer emulsion was formulated in which the primary layer was a conjugate between lactoferrin (LF) and EGCG or chlorogenic acid, whereas the secondary layer was an anionic polysaccharide beet pectin or soybean soluble polysaccharide for the encapsulation of β-carotene. The secondary emulsions showed improved protection of β-carotene and enhanced physical stability was exhibited by LF-EGCG/beet pectin combination (Liu et al., 2016c). However, LF-EGCG stabilized primary emulsions were more effective at shielding β-carotene loaded MCT oil-in-water emulsion from UV light and heat induced degradation than secondary emulsions of LF/LF-polyphenol conjugates and LF-polyphenol conjugates/LF (Liu et al., 2016d).

4. Conclusion

The present paper reviewed the state-of-the-art in the area of biopolymers that were used to form multilayers or conjugates for food emulsion stabilization. Based on our literature search, materials that found important applications in the designing of multilayer emulsions include proteins such as lactoferrin, β-lg, caseinate, and polysaccharides such as carrageenan, mesquite gum, chitosan, alginate, high methoxyl pectin, and starch-OSA. However, conjugates have been prepared from a host of molecules which includes proteins, polysaccharides, and polyphenols. Some of the major proteins are SPIs, β-lg, pea protein isolate, oat protein isolate, sodium caseinate, and β-conglycinin. Meanwhile, polysaccharides included dextran, corn fiber gum, beet pectin, and okra hydrocolloids among a multitude of other compounds. It was also found that both multilayer and conjugate stabilized emulsions could be used as a delivery vehicle for the protection and controlled release of active ingredients within food systems. Future work should focus on (a) finding novel low-cost, edible materials that could be used for emulsifier preparations; (b) reactions between the emulsifier and food components; and (c) application of such systems in real food products.

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