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Food-Grade Covalent Complexes and Their Application as Nutraceutical Delivery Systems: A Review

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Abstract: Food proteins, polysaccharides, and polyphenols are 3 major food constituents with distinctly different functional attributes. Many proteins and polysaccharides are capable of stabilizing emulsions and foams, thickening solutions, and forming gels, although they differ considerably in their abilities to provide these functional attributes. Many plant polyphenols exhibit beneficial physiological functions, such as antitumor, antioxidant, antibacterial, and antiviral properties. Proteins, polysaccharides, and polyphenols can form complexes with each other, which leads to changes in the functional and nutritional properties of the combined systems. Recently, there has been considerable interest in understanding and utilizing covalent interactions between polyphenols and biopolymers (proteins and polysaccharides). The binary or tertiary conjugates formed may be designed to have physicochemical properties and functional attributes that cannot be achieved using the individual components. This article provides a review of the formation, characterization, and utilization of conjugates prepared using proteins, polysaccharides, and polyphenols. It also discusses the relationship between the structural properties and functionality of the conjugates, and it highlights the bioavailability of bioactive compounds loaded in conjugate-based delivery systems. In addition, it highlights the main challenges to be considered when preparing and analyzing conjugates. This article provides an improved understanding of the chemical reactions that occur between major food ingredients and how they can be utilized to develop biopolymer-based delivery systems with enhanced functional attributes.

Keywords: protein–polyphenol–polysaccharide conjugates, colloid delivery systems, nutraceuticals, bioavailability

Introduction

The structural organization and interactions of the constituents in foods influence their physicochemical, sensory, and nutritional properties. Food structure and properties can be controlled by addition of specialized functional ingredients, such as emulsifiers, thickeners, and gelling agents (Buchert and others 2010; Zeeb and others 2014). Many of these functional ingredients are biopolymers, such as proteins or polysaccharides. Biopolymers can be utilized in their natural form, or they can be physically, chemically, or enzymatically modified to extend their functionality (Oliver and others 2006; Jakobek 2015). In this review, we focus on the formation of covalent complexes (conjugates) involving proteins, polysaccharides, and polyphenols. An improved understanding of the chemical interactions between these molecules would facilitate the rational design of food ingredients with improved functional attributes. For example, covalent bonding of polysaccharides to proteins has been shown to improve the solubility, emulsifying, and textural properties of proteins (Oliver and others 2006). Similarly, noncovalent bonding of polysaccharides to polyphenols has been shown to reduce their interactions with salivary proteins under simulated oral conditions, thereby reducing their tendency to cause astringency in some food and beverage products (Carvalho and others 2006; Soares and others 2012).

The interactions between proteins, polysaccharides, and polyphenols may be either reversible or irreversible. Noncovalent bonds (such as hydrogen, π-, hydrophobic, and ionic bonding) are usually involved in reversible (physical) interactions (Ozdal and others 2013; Le Bourvellec and Renard 2012), whereas covalent bonds are involved in irreversible (chemical) interactions (Oliveira and others 2016; Rohn 2014). For certain applications the formation of covalent bonds is more desirable since it leads to stronger, more permanent interactions (Curcio and others 2012).

Numerous chemical and enzymatic methods are available to form covalent conjugates between food-grade molecules. Conjugates can be formed using the Maillard reaction between proteins and polysaccharides (Liu and others 2012a), using free radical grafting between polysaccharides and polyphenols (Curcio and others 2009; Spizzirri and others 2010), and using

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enzyme-catalyzed reactions (such as laccase or tyrosinase) between proteins and polyphenols (Buchert and others 2010). The resultant conjugates can maintain the advantages of both substrates in a single entity, for instance, the antioxidant properties of the polyphenols and the emulsifying properties of the biopolymers.

In this article, we begin by reviewing the various approaches available to fabricate conjugates from food-grade biopolymers, we then discuss methods to characterize conjugate properties, and finally highlight the impact of conjugation on the techno-functional properties and gastrointestinal fate of biopolymers.

Conjugation Mechanisms

Food-grade covalent complexes with different molecular and physicochemical characteristics can be fabricated by controlling the components and preparation conditions used. In general, the formation of food-grade conjugates can be separated into nonenzymatic and enzymatic methods. Nonenzymatic approaches include the Maillard reaction (Oliveira and others 2016), free radical grafting (Curcio and others 2009), alkaline treatment (Kroll and others 2003), and the carbodiimide-mediated coupling reaction (Pasanphan and Chirachanchai 2008). Enzymatic approaches are based on the utilization of specific cross-linking enzymes, such as transglutaminase, laccase, tyrosinase, lipoxygenase, polyphenol oxidase, and peroxidase (Payne and others 2013; Mariniello and others 2014; Zeeb and others 2014). Table 1 provides an overview of the most commonly utilized methods for forming food-grade conjugates.

Substrates: Proteins, polysaccharides, and polyphenols

Initially, a brief description of the properties of the individual components used as substrates to form conjugates is given. Proteins are widely used as structuring ingredients in foods because of their specific functional attributes and high nutritional value (Chen and others 2006). Amino acids are the building blocks of proteins and their type, number, and sequence determine the molecular characteristics of proteins, such as molecular weight, conformation (globular, random coil, helix), electrical properties (charge versus pH), flexibilities (stiff versus flexible), hydrophobicity, thermal stability, and chemical reactivity (Jones and McClements 2010; Whitford 2013). Generally, proteins exhibit a variety of different functional attributes depending on their molecular structure, including emulsification, gelation, foaming, adsorbing to interfaces, catalyzing enzyme reactions, and binding-specific molecules (McClements and others 2009). Proteins from various biological sources can be conjugated with polysaccharides or polyphenols, including animal proteins, such as gelatin (Diftis and others 2005; Spizzirri and others 2009), whey protein (Di Pierro and others 2006; Akhtar and Dickinson 2007), caseinate (Al-Hakkak and Kavale 2002; Flanagan and Singh 2006), bovine serum albumin (BSA) (Rawel and others 2002a; Kim and Shin 2015), and ovotransferrin (You and others 2014), and plant proteins, such as soy protein (Rawel and others 2002b; Yang and others 2015), peanut protein (Liu and others 2012b, Li and others 2015), and corn protein (Takahashi and others 2002). Conjugation is usually achieved using free radical grafting, phenolic oxidation, Maillard reactions, or enzyme-catalyzed reactions.

Polysaccharides are the other major type of biopolymer that are widely used as structuring ingredients in foods (Basu and others 2015). Polysaccharides are polymer chains that consist of monosaccharides linked together by glycosidic bonds; however, the monomer composition of a polysaccharide is typically more uniform than that of a protein (Rinaudo 2008; Li and others 2016). Polysaccharides vary in their molecular weights, conformations, branching, electrical characteristics, flexibility, and hydrophobicities depending on their biological origin and processing conditions (Jones and McClements 2010), which results in differences in their physicochemical and functional properties, such as hydration, gelling, thickening, and surface activity properties (Rinaudo 2008; Li and others 2015). Polysaccharides are usually obtained by biosynthesis in plants (e.g., pectin, alginate), animals (chitosan), and microorganisms (hyaluronan, gellan, or xanthan). Studies have shown that chemical modification of polysaccharides with polyphenols can significantly increase their water solubility and bioactivity (Li and others 2016). In recent years, the utilization of chitosan and alginate has received considerable attention because they contain reactive groups that can easily be modified, such as amino groups on chitosan and carboxylic acid groups on alginate (Alves and Mano 2008; Yang and others 2011; Ryu and others 2015). There are three major methods currently used to conjugate polysaccharides (free radical grafting, Maillard reactions, and enzyme-catalyzed reactions), which are discussed below.

Polyphenols are secondary metabolites produced by many plants. They form an important source of nutraceuticals in our diet, particularly from plant-based foods, such as fruits, vegetables, seeds, and leaves (Petti and Scully 2009). Polyphenols have been reported to reduce the level of oxidative damage in proteins, lipids, carbohydrates, and DNA in living cells and tissues, which may be partly responsible for their reported ability to help prevent chronic diseases such as cancer, neurodegenerative diseases, diabetes, and osteoporosis (Scalbert and others 2005; Fernández-Panchón and others 2008; Cirillo and others 2016). The fact that polyphenols contain multiple phenolic groups makes them unstable to light, elevated temperatures, and alkaline conditions, and reduces their bioavailability (Oliver and others 2016). A number of studies have shown that the conjugation of polyphenols to biopolymers may increase their physical stability, antioxidant activity, and bioavailability (Fang and Bhandari 2010). These conjugates may therefore be used to extend the application range of polyphenols as functional ingredients in nutritional and biomedical applications (Cirillo and others 2016). The main mechanisms for generating polyphenol–biopolymer conjugates involve oxidation of the phenolic groups to form *o*-quinones or *o*-semiquinones using alkaline treatment, carbodiimide-mediated coupling, or enzyme-catalyzed reactions (Le Bourvellec and Renard 2012).

Protein–polysaccharide conjugates

The functional properties of proteins can be improved by conjugating them to polysaccharides using physical, chemical, or enzymatic treatments (Ye 2008; Dickinson 2008). However, chemical modifications are not commonly applied in the food industry because of potential labeling and regulation concerns (Oliveira and others 2016; Kato 2002). A convenient way to prepare protein–polysaccharide conjugates is through the Maillard reaction carried out under conditions of controlled time, temperature, pH, and moisture (Dickinson 2008; Oliveira and others 2016). The Maillard reaction can be carried out using dry-heating conditions, wet-heating conditions (Guo and Xiong 2013), or by applying pulsed electric fields (Guan and others 2010). A simplified schematic representation of the reaction is shown in Figure 1. The electrophilic carbonyl groups on a polysaccharide molecule react with the neutrophilic amino groups on a protein molecule to form a reversible Schiff base with the release of water. Rearrangement of this Schiff base leads to the formation of a more stable ketoamine (Amadori product), and thus to protein–polysaccharide conjugation.

Table 1–Preparation conditions and forming principles of food-grade covalent complexes.

Formation methods	The reaction substrates	Preparation conditions	Forming principles	Representative references
Maillard reaction	Protein—polysaccharide	*Dry heating or heating in solution method*: A mixture of protein and polysaccharide is incubated at controlled heating conditions. *Pulsed electric field method*: A mixture of protein and polysaccharide is subjected to an electric field.	A group of a reducing polysaccharide and an amino group of a protein form a covalent bond.	Guan and others (2010), Liu and others (2014a), Oliveira and others (2016)
Free radical grafting	Protein—polyphenol; polysaccharide—polyphenol	The active species existing on proteins (polysaccharides) react with polyphenol using a redox pair (such as hydrogen peroxide/ascorbic acid) as an initiator system.	Redox pair components generate the hydroxyl radicals, which attack the sensible residues of a polymer backbone (protein or polysaccharide), producing radical species, and then react with the polyphenols, inducing formation of covalent bonds.	Curcio and others (2009), Spizzirri and others (2009)
Enzyme-catalyzed reactions	Protein—polyphenol; polysaccharide—polyphenol; protein—polysaccharide	Reaction between the components is catalyzed by transglutaminase and various oxidative enzymes (tyrosinase, laccase, and peroxidase).	Polyphenol (or aromatic amino acids in protein) are able to be oxidized to quinone in the presence of enzyme and O_2. Which can further cross-link with functional groups present in a protein or polysaccharide.	Di Pierro and others (2006), Flanagan and Singh (2006), Sakai and others (2010), Božič and others (2012a), Božič and others (2012b), Kim and Cavaco-Paulo (2012)
Alkaline treatment	Protein—polysaccharide; Protein—polyphenol	The pH of protein–polyphenol mixture is adjusted to alkaline conditions. After some time under continuous stirring with free exposure to air, the samples are dialyzed against water and finally lyophilized.	Polyphenols may be oxidized with ease in an alkaline solution to its corresponding quinone, which can readily undergo attack by nucleophiles such as lysine, cysteine, and tryptophan moieties in a protein or polysaccharide chain.	Rawel and others (2000), Kroll and Rawel (2001), Rawel and others (2001), Diftis and others (2005)
Carbodiimide-mediated coupling reaction	Polysaccharide—polyphenol	First reaction can occur between polyphenol and EDC, then the reaction solution is gradually added into the polysaccharide dispersion solution. The reaction was carried out heterogeneously in ambient temperature and atmosphere for 24 h.	EDC reacts with carboxylic acid groups of polyphenols to form an active *O*-acylisourea intermediate that is easily displaced by nucleophilic attack from primary amino groups in the reaction mixture.	Pasanphan and Chirachanchai (2008), Xie and others (2014)

However, if the reaction is allowed to proceed further, then these intermediate products degrade leading to the formation of brown-colored pigments (melanoidins) and other advanced glycation end products (Liu and others 2012a; Evans and others 2013). Under carefully controlled reaction conditions, protein–polysaccharide conjugates can be formed with enhanced functional attributes, such as good emulsifying properties, heat stability, antioxidant activity, and antimicrobial activity.

Some examples of protein–polysaccharide conjugates that have been fabricated are listed in Table 2. The conjugates are typically formed from proteins (such as milk, egg, meat, or plant proteins) and polysaccharides (such as dextran, chitosan, alginate, and pectin). In addition, mild alkaline treatment and enzyme-catalyzed reactions are also promising methods to prepare protein–polysaccharide conjugates.

Protein–polyphenol conjugates

Owing to their chemical structure, polyphenols are highly reactive because they can be easily oxidized either nonenzymatically or enzymatically (Rohn 2014). A commonly used nonenzymatic reaction involves the conjugation of proteins with polyphenols in an alkaline solution in the presence of O_2 (Kroll and others 2003). Under these conditions, polyphenols are first oxidized to their corresponding quinones, which then react with nucleophiles such as free amino, lysine, cysteine, and tryptophan groups in proteins (Kroll and others 2003; Rohn 2014). The reaction mechanism

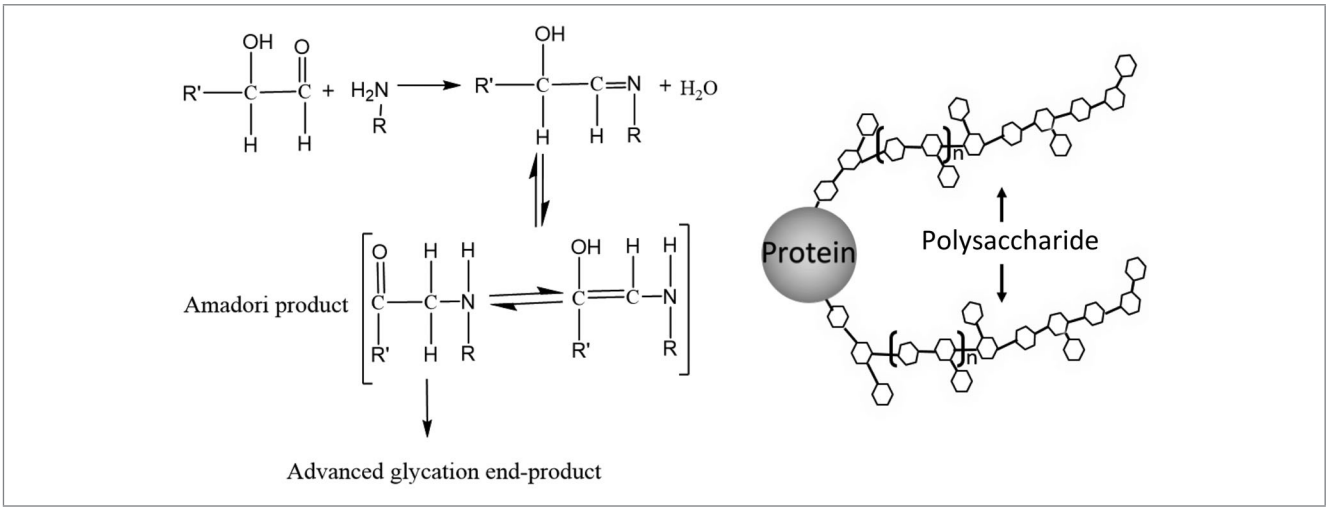

Figure 1–Schematic representation of the Maillard reaction between polysaccharide and protein, leading to protein–polysaccharide conjugate formation.

for the covalent interactions between proteins and phenolics is summarized in Figure 2. The diphenol moiety of a polyphenol readily oxidizes to an orthoquinone, which may then form a dimer (or polymer) in a side reaction, or which reacts with amino or sulfhydryl groups on a protein to form covalent C–N or C–S bonds with the phenolic ring (Jongberg and others 2011; Cao and Xiong 2015), leading to the regeneration of a hydroquinone. Upon further oxidation of this addition product to form its quinone, a second addition may occur, which leads to formation of cross-linked protein polymers (Kroll and others 2003; Strauss and Gibson 2004).

Enzymatic oxidation of polyphenols can be carried out using polyphenoloxidases such as catecholoxidases and laccases (Le Bourvellec and Renard 2012; Budryn and Rachwal-Rosiak 2013). In the presence of oxygen, a catechol group with 2 aromatic hydroxyl groups in the ortho position can form *o*-quinones that can participate in a nucleophilic addition reaction with proteins. Covalent reactions of proteins with polyphenols influence their physicochemical properties (such as solubility, thermal stability, surface activity, gelation properties, and antioxidant activity) as well as their nutritional properties (such as digestibility, bioavailability, and essential amino acid content).

Examples of studies of protein–polyphenol conjugates are shown in Table 3. It is clear from these studies that the nature of the phenolic compounds used to form the conjugates has a major impact on their functional attributes.

Polysaccharide–polyphenol conjugates

Numerous studies have shown that polysaccharides can be conjugated to polyphenols to obtain ingredients with novel or improved functionality. Free radical grafting is one of the most commonly used methods for forming polysaccharide–polyphenol conjugates (Figure 3). This method involves a rapid and ecofriendly procedure that allows covalent attachment of polyphenols onto polysaccharides without the use of organic solvents or toxic radical initiators (Curcio and others 2012). Hydroxyl radicals generated by the interaction between redox pair components (e.g., ascorbic acid/hydrogen peroxide) attack susceptible residues in the polysaccharide molecules, such as H-atoms in the R-methylene ($-CH_2$) or hydroxyl groups ($-OH$) of the hydroxymethylene group of chitosan, producing radical species on the polysaccharides. These radicals then react with the polyphenols promoting the formation of polysaccharide–polyphenol covalent bonds (Curcio and others 2009; Spizzirri and others 2009).

Owing to their advantageous properties, the polysaccharide–polyphenol conjugates have great potential for application in the food industry as additives, to preserve food quality during processing and storage, or as dietary supplements, because they can affect the activity of several enzyme systems involved in the pathogenesis of several diseases (Curcio and Picci 2015). A number of studies that have shown the potential application of enzymes in enhancing the therapeutic effects of polyphenols are shown in Table 4 (Basu and others 2015).

Polysaccharide–polyphenol conjugates can also be obtained by carbodiimide-mediated coupling (Table 4), which involves the formation of amide linkages between amine-containing molecules and carboxylate-containing molecules (Schanté and others 2011; Yang and others 2011). Chitosan–polyphenol conjugates have been successfully formed using this method, and the resultant conjugates were reported to have improved physicochemical and biological properties, such as water solubility, antioxidant capacity, and cell adhesion.

Polysaccharide–polyphenol conjugates can also be formed using various types of enzymes, which often have advantages in terms of being more environmentally friendly. The enzymatic functionalization of chitosan has been performed using oxidative enzymes, such as polyphenol oxidases (tyrosinases and laccases) and peroxidases, as well as by other enzymes such as lipases, phosphorylases, and transglutaminases (Aljawish and others 2015). For instance, in tyrosinase- or laccase-catalyzed reactions, the catechol groups in polyphenol are oxidized to electrophilic *o*-quinones, which are subject to nucleophilic attack from the primary amines present in chitosan backbones (Ryu and others 2015). As shown in Figure 4, reactions between chitosan and quinones can undergo 2 possibilities, producing either Schiff bases or Michael-type adducts (Alves and Mano 2008). Therefore, the products may be a complex mixture of both conjugates. Enzyme-catalyzed conjugation of chitosan and polyphenols are particularly attractive approaches due to the fact that the chitosan derivatives exhibit unique pH-sensitive water solubility and adhesive properties (Yamada and others 2000; Kim and others 2013).

Table 2–Formation, characterization, and functionality of representative protein–polysaccharide conjugates.

Protein–polysaccharide conjugates	Formation methods	Characterization techniques	Changes in functionality or application	References
WPI–dextran; ovalbumin–dextran; PPI-dextran; BSA–dextran; caseinate–dextran; rice protein–dextran; SPI–dextran; soy β-conglycinin–dextran	Maillard reaction	HPSEC–MALLS; SDS–PAGE; fluorescence spectroscopy; circular dichroism; SAX, DLS; circular dichroism; analysis of free amino groups	The thermal stability and solubility of protein at pH 4.5 to 6.0 can be improved by conjugation to dextran; conjugation of dextran could further enhance emulsifying and foaming properties of protein. The stability or bioaccessibility of the bioactive compounds, such as lutein, EGCG, resveratrol, and ibuprofen, could be enhanced when they are encapsulated in protein–dextran conjugates-stabilized nanoparticles.	Choi and others (2005), Diftis and Kiosseoglou (2006), Jung and others (2006), Li and Yao (2009), Liu and others (2012), Zhang and others (2012), Li and others (2013), Li and Gu (2014), Dai and others (2015), Davidov-Pardo and others (2015), Xia and others (2015)
WPI–pectin; sodium caseinate–pectin; egg white–pectin	Maillard reaction	SDS–PAGE; analysis of free amino groups	The conjugates show higher emulsion viscosity, better emulsifying properties and stability than the raw materials.	Neirynck and others (2004), Einhorn-Stoll and others (2005), Al-hakkak and Al-hakkak (2010)
WPI–maltodextrin; sodium caseinate–maltodextrin	Maillard reaction	SDS–PAGE; analysis of free amino groups; HPGPC	These complexes have excellent solubility, exceptional emulsifying and foaming properties under acidic conditions. In addition, the conjugates-stabilized O/W emulsions show better storage stability, freeze–thaw stability, and thermal stability than the raw materials.	Akhtar and Dickinson (2007), Regan and Mulvihill (2009, 2010), Olivas and Sepulveda (2014)
BSA–fucoidan	Maillard reaction	SDS–PAGE; SEC; AFM; DLS; fluorescence spectroscopy; circular dichroism spectroscopy	Fucoidan attachment enhance the solubility, thermal stability, and emulsifying properties of the protein molecules	Kim and Shin (2015, 2016)
SPI–SSPS	Maillard reaction	SDS–PAGE; FTIR spectroscopy; HPSEC	The citral emulsions stabilized by SPI–SSPS conjugate exhibit superior physical stability than those stabilized by SPI or their mixture during prolonged storage, after thermal treatment or under simulated gastrointestinal conditions.	Yang and others (2015)
Gelatin–pectin	Mild alkaline treatment	SDS–PAGE	The emulsions stabilized by the conjugates are very stable against oil droplet coalescence and creaming.	Diftis and others (2005)
Gelatin–chitosan	Enzyme-catalyzed reactions (tyrosinase and transglutaminase)	Rheological properties	Creating gels and it may offer interesting opportunities for in situ applications; the modified gelatin–chitosan conjugate shows better *in vitro* antibacterial activity than unmodified gelatin.	Chen and others (2002, 2003), Wang and others (2015)
WPI–pectin	Enzyme-catalyzed reactions (laccase)	FTIR	The transition of conjugate particles Form glassy to rubbery state at lower temperatures than their parent biopolymers.	Gazme and Madadlou (2014)
Alginate derivative–proteins (gelatin and albumin) derivatives	Enzyme-catalyzed reactions (horseradish peroxidase)	Gelation time; mechanical properties; cell adhesiveness	Scaffolds for tissue engineering and carriers for drug delivery system.	Sakai and others (2010)

Protein–polyphenol–polysaccharide conjugates

Additional functional attributes may be engineered into food ingredients using ternary conjugates consisting of protein, polysaccharide, and polyphenol. Depending on the type of the reactions involved, and the sequence in which they are carried out, it is possible to create conjugates with different properties. As shown in Figure 5, three major strategies have been developed. The first method (a) involves the synthesis of protein–polysaccharide conjugates with exposed reactive groups that are then modified by polyphenols. For example, when protein–polysaccharide conjugates are formed by the Maillard reaction, there are some accessible amino, cysteine, and tyrosine groups in proteins and

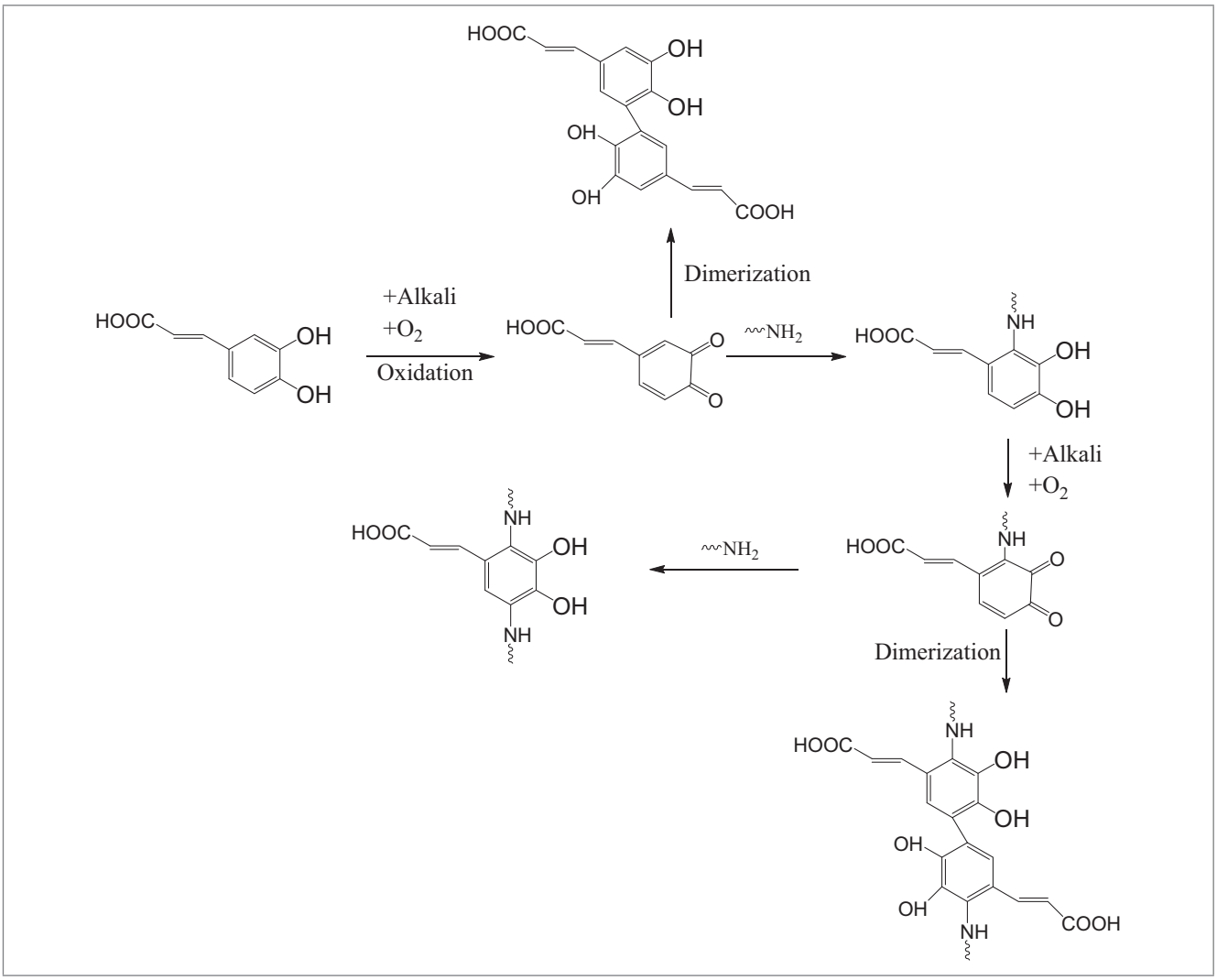

Figure 2–Proposed mechanism for the formation of protein–polyphenol conjugates by alkaline treatment (This figure is from Strauss and Gibson Food Hydrocolloids, 2004, 18, 81-89 with permission).

amine and carboxylic acid groups in polysaccharides available to react with polyphenols. The drawback associated with this method is that the reaction conditions have to be selected so that they do not affect the activity of the protein. The second method (b) involves the formation or protein–polyphenol conjugates that have exposed reactive groups which can form conjugates with polysaccharides. The third method (c) involves the formation of polysaccharide–polyphenol conjugates, which then react with proteins to form ternary conjugates. The last 2 methods have an advantage in terms of purification of the products, because the small molecules (polyphenols), unreacted monomer, and catalyst are easier to remove. Based on these strategies, a diverse range of chemical functionalities can be accomplished. Some examples of the use of the different conjugation strategies are given in Table 5.

Enzymatic approaches for preparing protein–polyphenol–polysaccharide conjugates have also been investigated, for example, using polyphenol oxidases (tyrosinases and laccases). As shown in Figure 6, in the presence of molecular oxygen (O_2), tyrosinase can catalyze the oxidation of polyphenols into reactive *o*-quinones; laccases can catalyze the oxidation of polyphenols by a free radical reaction leading to the formation of the corresponding quinones (Pourcel and others 2007), and then these quinones can react with proteins and polysaccharides to bind them together. Protein–polyphenol–chitosan conjugates have been successfully synthesized using tyrosinase as a catalyst (Chen and others 2001). In this study, various proteins were coupled onto a chitosan film using a tyrosinase-mediated reaction in the presence of a phenolic coupling precursor. In another study, laccase was used to covalently cross-link chitosan, gelatin, and polyphenolic compounds from *Hamamelis virginiana* (Rocasalbas and others 2013). The authors used FTIR analysis to confirm that the phenolic compounds were covalently incorporated into the chitosan/gelatin network and that the cross-linking probably occurred through a Michael addition mechanism. The resultant chitosan–gelatin–phenolic hydrogels were shown to have good antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

In our recent studies, we have prepared protein–polyphenol–polysaccharide conjugates as multifunctional surface-active ingredients (Liu and others 2015a, 2016a). Ternary conjugates composed of lactoferrin, polydextrose, and chlorogenic acid were prepared via alkaline treatment and the Maillard reaction (Figure 7). It was hypothesized that the protein would provide surface activity, the polysaccharide would provide physical

Table 3–Formation, characterization, and functionality of representative protein–polyphenol conjugates.

Protein–polyphenol conjugates	Formation methods	Characterization techniques	Changes in functionality or application	References
Gelatin–gallic acid; gelatin –catechin	Free radical grafting	UV-vis spectroscopy; fluorescence; DSC	Antioxidant; chemoprevention of Alzheimer's disease; inhibition of carbohydrate breakdown and control of glycemic index of food products; anticancer	Spizzirri and others (2009), Cirillo and others (2010)
Ovotransferrin–catechin; β-LG–catechin; α-lactalbumin–catechin	Free radical grafting	SDS–PAGE; ESI-MS; fluorescence; UPLC; MALDI-TOF-MS	Antioxidant; as a novel emulsifier in improving the chemical stability of β-carotene in emulsions	You and others (2014), Yi and others (2015, 2016)
Lactoferrin–EGCG; lactoferrin–chlorogenic acid; lactoferrin–gallic acid	Free radical grafting *or* alkaline treatment	SDS–PAGE; MALDI-TOF-MS; fluorescence	Antioxidant; thermal stability	Liu and others (2015a, 2016a)
Whey proteins–quercetin; whey proteins–rutin; BSA–quercetin; SPI–quercetin; SPI –chlorogenic acid; soy or whey proteins with isoflavones (genistein, daidzein, formononetin, prunetin, biochanin A, and two synthetic isoflavones)	alkaline treatment	UV-Vis spectroscopy; SDS–PAGE; IEF; circular dichroism; RP-HPLC; SELDI-TOF-MS; intrinsic fluorescence; analyses of free amino and thiol groups as well as tryptophan	Antioxidant; true nitrogen digestibility, biological value, and net protein utilization are adversely affected; the pH-dependent solubility of the conjugates is decreased and hydrophilic character is increased.	Rawel and others (2003, 2004), Rohn and others (2004, 2006)
BSA–chlorogenic acid; lysozyme–chlorogenic acid; WPI–chlorogenic acid or –ferulic, –caffeic, gallic acids; myoglobin–chlorogenic, or –caffeic, –quinic acids or *p*–quinone	Alkaline treatment	SDS–PAGE; MALDI-TOF-MS; IEF; DSC; RP-HPLC; analysis of free amino groups; fluorescence	Solubility; denaturation temperature and enthalpy; hydrophobicity; the *in vitro* digestibility of the modified proteins is adversely effected.	Rawel and others (2000, 2001, 2002), Kroll and Rawel (2001)
Lactalbumin–, lysozyme– or BSA–chlorogenic acid	Alkaline treatment or enzyme-catalyzed reactions	SDS–PAGE; MALDI-TOFMS; analysis of free amino groups; DSC	Solubility	Prigent and others (2007)
Gelatin-catechin	Laccase-catalyzed oxidation of catechin	UV-Vis spectroscopy	Amplified activity to inhibit oxidation of low-density lipoprotein.	Chung and others (2003)

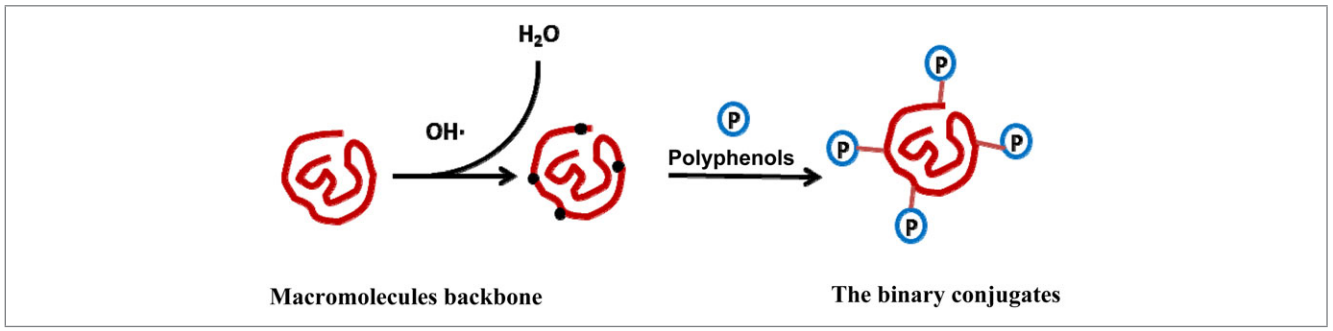

Figure 3–Schematic representation of the covalent attachment of polyphenols to biopolymer molecules using free radical-induced grafting reactions.

stability through steric repulsion, and the chlorogenic acid would provide chemical stability by acting as an antioxidant. In addition, some of the constituents may play multiple roles: The lactoferrin may also act as an antioxidant, and the chlorogenic acid may also help anchor the proteins to the interface. It was shown that the ternary conjugates were good emulsifiers that could effectively inhibit the chemical degradation of encapsulated β-carotene. These types of ternary complexes may therefore be useful for stabilizing chemically labile nutraceuticals in functional foods and beverages.

Complexity of covalent interactions and the potential toxicity of the resulting conjugates

It is important to highlight that the chemical reactions used to conjugate proteins, polysaccharides, and polyphenols are highly complex and may lead to the formation of undesirable side products. For example, melanoidins can be formed during the advanced stages of the Maillard reaction, which are nitrogenous polymers and copolymers of brown coloration (Zhang and Zhang 2007). In addition, a range of potentially toxic reaction products may be formed during the latter states of the Maillard reaction (Zhang

Table 4–Formation, characterization, and functionality of representative polysaccharide–polyphenol conjugates.

Polysaccharide–polyphenol conjugates	Formation methods	Characterization techniques	Changes in functionality or application	References
Chitosan–gallic acid; chitosan–catechin; chitosan–protocatechuic acid or –caffeic acid, –*p*-hydroxybenzoic acid, –vanillic acid; chitosan–caffeic acid, or –ferulic acid, –sinapic acid; chitosan–EGCG	Free radical grafting	UV-vis spectroscopy; FTIR; DSC; ^{1}H NMR; TLC analysis; X-ray diffraction	Antioxidant activity; water–solubility; enhancing α-glucosidase and α–amylase inhibitory activities, antimicrobial activity; enhancing adsorption properties for Fe(II); acetylcholinesterase inhibition; tyrosinase activity inhibition; foodborne pathogens inhibition; preventing oxidative stress in Chang liver cells; as an efficient emulsifier to improve the physical stability of β-carotene emulsion and inhibit the deterioration of β-carotene in emulsions	Curcio and others (2009), Cho and others (2011a, 2011b), Senevirathne and others (2012), Lee and Je (2013), Liu and others (2013, 2015c), Woo and Je (2013), Lee and others (2014), Lei and others (2014a, 2014b)
Alginate–catechin and inulin– catechin; dextran–catechin	Free radical grafting	FTIR; DSC; UV-Vis spectroscopy; ^{1}H NMR; fluorescence; GPC; FTIR	Antioxidant activity; increasing thermal stability and crystallinity; α-amylase inhibitory activity; anticancer activity	Spizzirri and others (2010, 2011), Vittorio and others (2012, 2014), Liu and others (2014b)
Starch–quercetin	Free radical grafting	FTIR, DSC; fluorescence; Folin-Ciocalteu assay	Improving UV stability and retaining the antioxidant properties of free quercetin; potential health functionality in the treatment of Alzheimer disease, diabetes and as skin-whitening agent	Cirillo and others (2012)
Chitosan–gallic acid	Carbodiimide-mediated coupling reaction	FTIR; ^{1}H NMR; XRD	The conjugate clearly shows a synergistic antioxidant activity on hydroxyl radical scavenging; used as a radical and pH-responsive drug carrier	Pasanphan and Chirachanchai (2008), Yu and others (2011)
Carboxymethyl chitosan–quercetin	Carbodiimide-mediated coupling reaction	FTIR; ^{1}H NMR	Antioxidant activity, oral delivery of water-insoluble anticancer drug paclitaxel	Wang and others (2014b)
Hyaluronic acid–EGCG	Carbodiimide-mediated coupling reaction	^{1}H NMR; UV-Vis spectroscopy	Resistance to hyaluronidase–mediated degradation, inhibition of cell growth and scavenging of radicals; as a nanogel, can delivery intracellular protein; targeted gene delivery	Lee and others (2015, Liang and others (2016a, 2016b)
Chitosan–caffeic; Chitosan–gallic acid; Chitosan–quercetin; Chitosan–tannic acid	Enzyme-catalyzed reactions	FTIR and ^{1}H NMR	Antioxidant activity; antimicrobial activity	Božič and others (2012a, 2012b, 2013)

and Zhang 2007). Thus, there may be some safety concerns associated with the utilization of conjugates formed using this approach. Consequently, reaction conditions should be carefully controlled to avoid the formation of these undesirable reaction products (Oliveira and others 2016). In addition, the potential toxicity of any newly formed conjugates should be established before widespread utilization within the food industry.

Conjugates Characterization

A variety of analytical methods is required to characterize the properties of biopolymer conjugates, such as molecular weight, composition, conformation, structure, and bond type (Le Bourvellec and Renard 2012; Bandyopadhyay and others 2012). These methods include electrophoresis, mass spectroscopy, size-exclusion chromatography, light scattering, nuclear magnetic resonance (NMR) spectroscopy, UV–visible spectroscopy, Fourier transform infrared (FTIR) spectroscopy, small-angle X-ray scattering (SAX), and differential scanning calorimetry (DSC). The combination of these techniques with chemical methods, such as amino acid and phenolic acid analysis (Rohn 2014), will lead to a better understanding of the properties of conjugates formed from proteins, polyphenols, and polysaccharides.

Electrophoresis

Electrophoresis involves separating charged molecules in an applied electric field based on differences in their molecular weight, dimensions, conformation, or isoelectric point (Reddy and Raju 2012; Oliveira and others 2016). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli 1970) is a convenient, fast, and inexpensive method to confirm the conjugation of proteins and polysaccharides or proteins and polyphenols. Complex mixtures can be separated to a high resolution using this technique, and the electrophoretic patterns obtained can be used to distinguish pure protein from protein–polysaccharide or

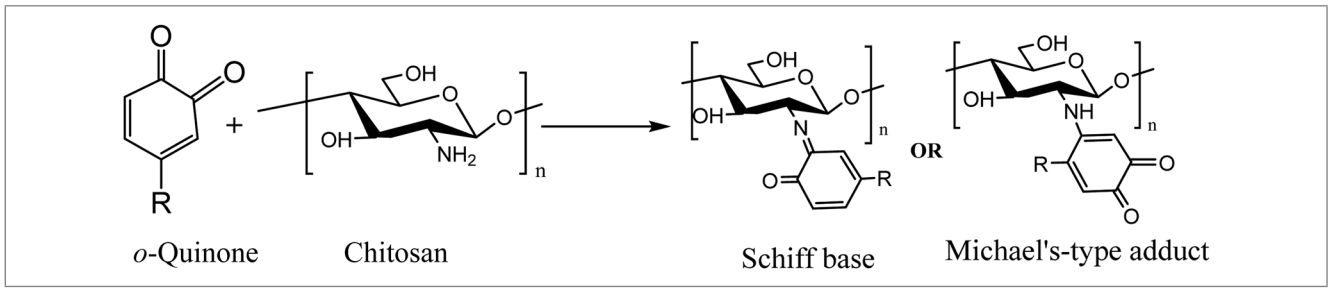

Figure 4–Reaction between chitosan and quinone to form chitosan–polyphenol conjugates.

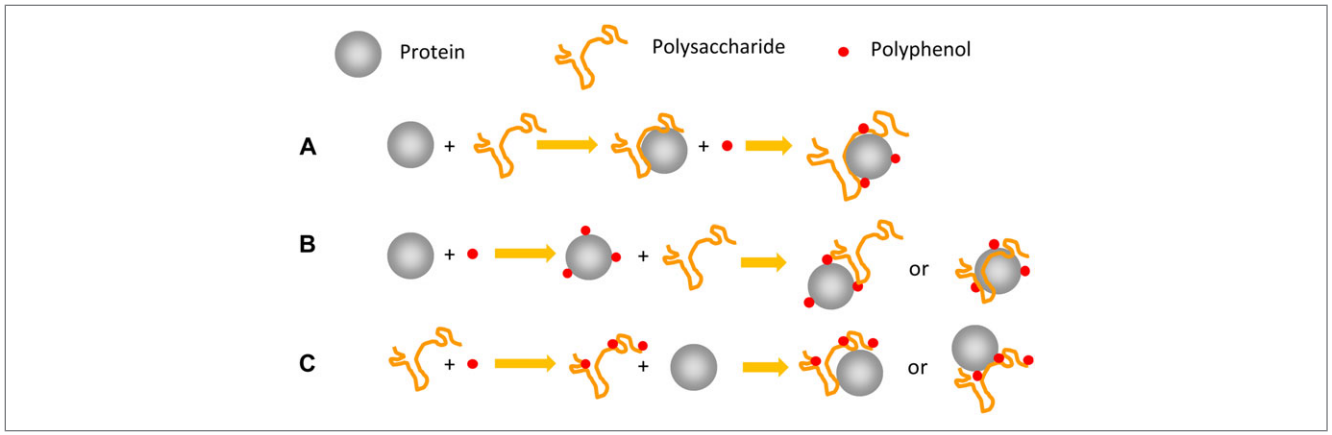

Figure 5–Schematic representation of three synthetic approaches to prepare protein–polysaccharide–polyphenol conjugates.

Table 5–Formation, characterization, and functionality of representative protein—polyphenol—polysaccharide conjugates.

Protein—polyphenol—polysaccharide conjugates	Formation methods	Characterization techniques	Changes in functionality or application	References
Biologically active proteins (cytochrome *c*, OPH, and His-CAT) — methyl gallate or — chlorogenic acid—chitosan	Enzyme-catalyzed reactions (using tyrosinase)	Organophosphorus hydrolase activity assay	Used as a biomaterial (film)	Vachoud and others (2001)
Chitosan—gelatin—plant polyphenols (a mixture of small proanthocyanidins and hydrolysable tannins)	Enzyme-catalyzed reactions (using laccase)	Rheological characterization; FTIR	Antibacterial activity and inhibitory effect on myeloperoxidase and collagenase	Rocasalbas and others (2013)
Chlorogenic acid—lactoferrin—polydextrose	Alkaline treatment and Maillard reaction	MALDI-TOF-MS; fluorescence	Antioxidant activity, thermal stability, and solubility as well as emulsifying properties of protein	Liu and others (2015a, 2016a)

protein–polyphenol conjugates (Kroll and others 2003; Oliveira and others 2016). SDS–PAGE has been employed to characterize the conjugation of gelatin and dextran using protein and carbohydrate stains (Zhou and others 2012). SDS–PAGE has also been applied to investigate the conjugation of ovotransferrin and catechin using the free radical and alkaline methods (You and others 2014).

Capillary electrophoresis (CE) is another powerful analytical tool for characterizing biopolymers and their conjugates. CE methods have been used to distinguish free protein from protein–polyphenol conjugates (Trombley and others 2011). Coupled with chromatographic methods, CE can also be used to characterize conjugates in polyphenol-rich foods and beverages.

Mass spectrometry

Mass spectrometry (MS) can be used to determine the mass-to-charge ratio of molecules, which enables determination of the stoichiometry and structure of conjugates (Le Bourvellec and Renard 2012; Zhu and Fang 2013). In recent studies, matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) and electrospray ionization mass spectrometry (ESI-MS) have played a primary role in the structural characterization of protein-based conjugates. MALDI-MS is a soft ionization technique that can provide quantitative information about the average molecular mass and molecular mass distribution of biopolymers (Rizzarelli and Carroccio 2014). ESI-MS can be readily interfaced with solution-based separation techniques such as HPLC, which makes

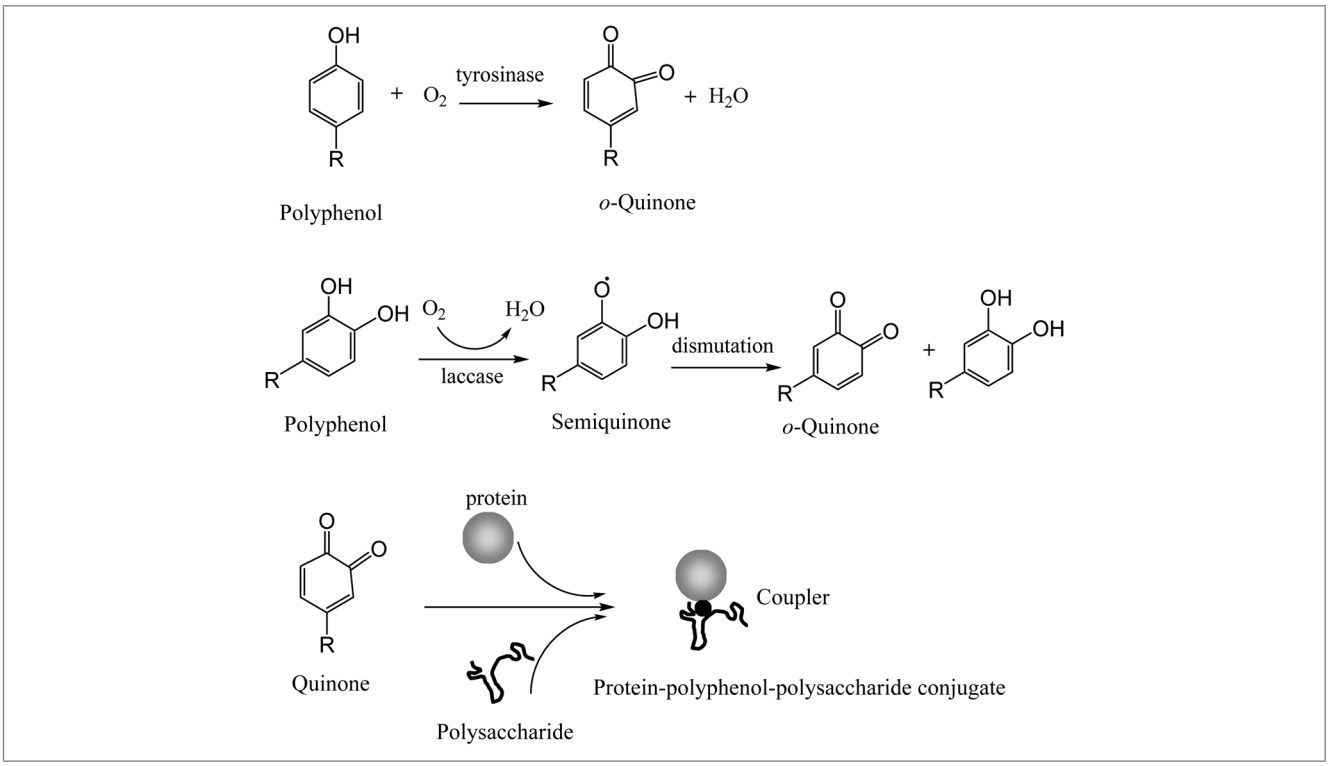

Figure 6–Enzymatic formation of protein–polyphenol–polysaccharide conjugates using tyrosinase or laccase.

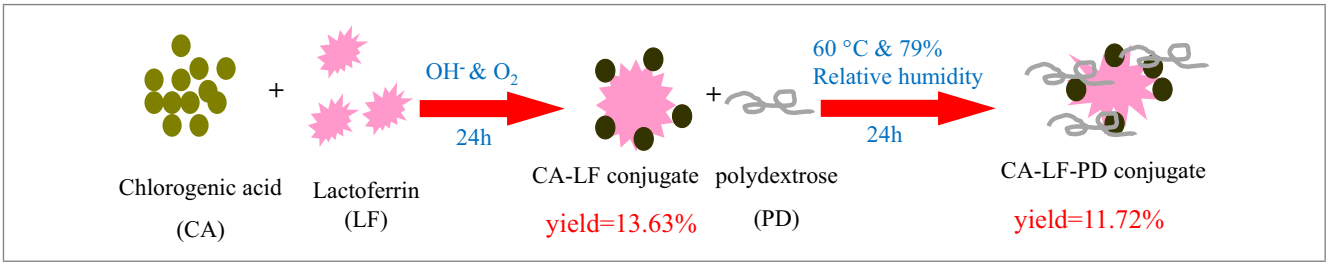

Figure 7–Schematic diagram of the formation of chlorogenic acid–lactoferrin–polydextrose conjugates.

it a powerful tool for characterizing the structural and compositional properties of biopolymers (Rizzarelli and Carroccio 2014). The glycation of dairy proteins has been characterized using MS. For example, Oliver and co-workers systematically summarized the ability of MALDI-MS and ESI-MS to reveal structural information and physicochemical properties about glycoconjugates (Oliver 2011). MS can identify and locate glycated adducts on food proteins, and it is therefore beneficial for understanding the structure–function relationships of glycoconjugates. MALDI-MS and ESI-MS have also been used to characterize conjugates formed using the Maillard reaction between dairy proteins and various carbohydrates (French and others 2002; Luz Sanz and others 2007). Conversely, MS is currently less useful for providing information about the properties of large polysaccharide molecules and their conjugates (Hsu and others 2007).

MS has also been used to confirm the conjugation of polyphenols and proteins (Kroll and others 2003). For example, molecular mass analysis indicated that there were 3 chlorogenic acids attached to one myoglobin molecule. Coupled with a digestion protocol, MS can also be used to identify the reaction sites on a protein by comparison of modified and unmodified peptides (Rawel and others 2005).

Size-exclusion chromatography

Size-exclusion chromatography (SEC) is an analytical procedure used to obtain information about molar mass averages and molar mass distributions of biopolymers based on separating them according to their molecular sizes (Striegel and others 2009). Biopolymers and their conjugates can be resolved by optimizing operating parameters such as mobile phase properties (type and flow rate) and column parameters (length and pore size) (Hong and others 2012). Various detectors may be used to characterize the biopolymers separated by SEC, including refractive index (RI), ultraviolet (UV), multiangle laser-light scattering (MALLS), intrinsic viscometry (IV), and mass spectrometry (MS), which enables detailed characterization of biopolymer samples (Fekete and others 2014). For example, SEC–MALLS can provide information on the size, shape, concentration, and molecular mass of a sample. For example, SEC–MALLS has been used to estimate the conformation of protein–polysaccharide conjugates formed using the

Maillard reaction (Choi and others 2005). This study demonstrated that ovalbumin–dextran conjugates had weight-average molar mass values ranging from 84.4 to 105.1 kDa, and root mean square radius values ranging from 19.6 to 26.4 nm.

The molecular properties of corn fiber gum (CFG)–whey protein conjugates have been characterized by SEC coupled with MALLS and IV detection (Yadav and others 2012). The analysis revealed that CFG–protein conjugates had higher molar mass, polydispersity, and radius of gyration than the unconjugated CFG, but that the intrinsic viscosity (η) was unchanged. In our recent study, the molecular weights of lactoferrin, chlorogenic acid–lactoferrin conjugates, and chlorogenic acid–lactoferrin–glucose/polydextrose conjugates were determined by SEC–MALLS (Liu and others 2016a).

Light scattering

There are 3 fundamental light scattering techniques that are used to characterize biopolymers and their complexes: static (SLS), dynamic (DLS), and electrophoretic (ELS) light scattering (Sapsford and others 2011). SLS is typically used to determine the absolute molar mass of biopolymers by measuring the angular light scattering pattern of a biopolymer solution. DLS is typically used to determine the hydrodynamic radius of biopolymers in solution by measuring their diffusion coefficient from scattered light intensity versus time fluctuations. ELS is typically used to determine the ζ-potential of biopolymers in solution by using light scattering to measure their electrophoretic mobility in an applied electrical field. These techniques can be used in combination to provide information about the molecular weight, hydrodynamic radius, conformation, and charge of conjugates in solution. Moreover, detailed information about different fractions within a conjugate sample can be obtained by combining light scattering methods with SEC. Initially, the samples are passed through a size-exclusion column to separate different fractions based on their molecular dimensions, and then each fraction is analyzed by DLS, SLS, and/or ELS to provide information about their molecular weight, conformation, and charge. This type of information can facilitate the rational development of conjugates as nutraceutical delivery systems (McClements 2007). For example, DLS and ELS have been used to characterize the stability of lutein emulsions prepared using either casein or casein–dextran conjugates as emulsifiers (Gumus and others 2016). This study demonstrated that casein-coated oil droplets were highly unstable to flocculation near their isoelectric point (4 to 5) due to a reduction in electrostatic repulsion. However, casein–dextran-coated droplets were stable from pH 3 to 7 due to a strong steric repulsion associated with the dextran molecules at the droplet surfaces. In another study, DLS and ELS were used to study the pH stability of β-carotene emulsions stabilized by lactoferrin–polyphenol conjugates and polysaccharides (Liu and others 2016b). It was reported that electrostatic deposition of anionic polysaccharides on the oil droplet surfaces improved their pH stability due to strong electrostatic and steric repulsion.

Other possible techniques

As mentioned earlier, there are many other techniques that can be used for conjugation characterization. NMR spectroscopy can be used to elucidate and identify the chemical structures of conjugates (Le Bourvellec and Renard 2012). Zhang and others (2010) studied the cross-linking chemistry of ^{13}C-enriched caffeic acid (LCA) with gelatin using a high-resolution NMR technique in both solution and solid states. Direct evidence was found confirming that chemical reactions occurred between phenolic groups on LCA and amino groups on gelatin leading to the formation of C−N covalent cross-links. SAX can be used to monitor time-dependent conformational changes of proteins during conjugation reactions (Xia and others 2015). The radius of gyration (R_g) of pure BSA increased from 3.8 nm (native) to 5.3 nm (8 h), 7.6 nm (19 h), and 8.9 nm (26 h) as the reaction time increased. However, the R_g values of conjugates just increased from 3.8 nm (native BSA) to 5.6 nm (8 h), 6.8 nm (19 h), and 6.8 nm (26 h). These differences may result from the competition between glycation and aggregation of the protein molecules and suggest that conjugation of BSA with dextran inhibited protein aggregation. Moreover, UV–visible spectroscopy can be used to calculate the amount of polyphenols bound per protein–polyphenol conjugate (Rawel and others 2002b). FTIR spectroscopy methods can be used to provide information about the structure and interactions of both polysaccharides and proteins (Su and others 2010). DSC can be used to determine changes in the thermal stability of proteins or polysaccharides when they are conjugated with polyphenols (Curcio and others 2009; Spizzirri and others 2009).

Potential Advantages of Conjugates

Proteins, polyphenols, and polysaccharides each have their own specific functional attributes. The formation of conjugates from these individual constituents may therefore lead to new ingredients with novel functional properties. At present, the most studied functional attributes of food-grade conjugates are their solubility, antioxidant, thermal, emulsifying, foaming, and gelling properties, and so we will focus on these attributes in this section.

Solubility

Good water solubility is a key requisite for the application of many functional ingredients, for example, ingredients used in products such as fortified waters, sports drinks, and nutritional beverages. The solubility of a biopolymer depends on the balance between solvent–solvent, biopolymer–biopolymer, and biopolymer–solvent interactions, which depends on biopolymer structure, charge, and hydrophobicity (Schmitt and others 1998). Generally, high charge density and low average hydrophobicity result in good biopolymer solubility. Research has shown that conjugation of proteins with polysaccharides or polyphenols affects their water-solubility. Water solubility is usually improved when a protein is conjugated with a polysaccharide because the hydrophilic polysaccharide generates a strong steric repulsion that prevents the proteins from coming close together. For instance, covalent attachment of maltodextrin to caseinate greatly improved the pH stability of the protein (Shepherd and others 2000). Similarly, covalent attachment of dextran to β-lactoglobulin, α-lactalbumin, and BSA molecules improved protein pH stability (Jimenez-Castano and others 2007).

The conjugation of a protein with a polyphenol may either increase or decrease the water solubility of the protein depending on the nature of the system. Covalent attachment of charged polyphenols will alter the electrical characteristics of proteins, particularly the isoelectric point, which will change their pH-solubility profiles (Kroll and others 2003; Budryn and Rachwal-Rosiak 2013). Attachment of nonpolar polyphenols will increase the surface hydrophobicity of proteins, which may not only increase their surface activity, but also reduce their water solubility (Bandyopadhyay and others 2012). The reaction between polyphenols and proteins may promote cross-linking of the proteins, which may also change their water solubility (Ozdal and others 2013). In summary, all these

interactions could affect the solubility of the derivatives. Our recent research demonstrated that conjugation of lactoferrin with chlorogenic acid and (−)-epigallocatechin-3-gallate (EGCG) appreciably altered its solubility: The solubility of the conjugates was relatively high from pH 7 to 11 and from pH 2 to 3, but relatively low at pH 4 and 5 (Liu and others 2016c).

Conjugation may also alter the solubility characteristics of polysaccharides. Many polysaccharides naturally have good water solubility because of their high surface polarity and/or surface charge (Basu and others 2015). Nevertheless, some polysaccharides have limited solubility under certain conditions due to hydrogen-bond formation between helical regions or because of weak electrostatic repulsion due to a low electrical charge (Rinaudo 2008). For instance, the solubility of some polysaccharides is strongly pH dependent because of changes in the ionization of the charged groups around their pK_a values, for example, chitosan loses solubility in neutral and basic conditions due to loss of a proton ($-NH_3^+ \rightarrow -NH_2$), whereas hyaluronic acid loses solubility in acid conditions due to gain of a proton ($-CO_2^- \rightarrow -CO_2H$). The covalent attachment of polyphenols to polysaccharides may be able to improve their water-solubility characteristics. For example, conjugation of chitosan with polyphenols has been shown to improve its solubility at neutral pH (Kumar and others 1999; Kim and others 2013). This enhanced solubility may be ascribed to the good water solubility of the polyphenols used (pyrocatechol and chlorogenic acid) and the increased pK_a value of the amine groups due to conjugation with polyphenols (Ryu and others 2015).

Antioxidant activity

Many polyphenols have strong antioxidant activity, namely, the ability to protect biological molecules against oxidation, scavenge free radicals, and retard reactive oxygen species generation. Therefore, it is one of the most important properties evaluated after conjugation of polyphenols with proteins or polysaccharides. Protein–EGCG conjugates have been reported to exhibit better antioxidant activity than unmodified proteins (Almajano and others 2007). These authors deduced that free phenolic hydroxyl groups with antioxidant properties were still available after the EGCG was conjugated with the protein. Wang and co-workers prepared α-La–EGCG complexes at pH 8.0 and evaluated their antioxidant properties by DPPH radical and ABTS radical scavenging activities assays, compared with the control of α-La (Wang and others 2014a). The α-La–EGCG conjugates had significantly increased antioxidant activity, which was attributed to the fact that EGCG attached to the protein could scavenge radicals and form more stable reaction products, thereby terminating the radical chain reaction.

Reactions between polyphenols and polysaccharides can also produce antioxidant conjugates (Spizzirri and others 2010; Lee and others 2014). In a recent study, chitosan–hydroxycinnamic acid conjugates were synthesized by conjugation of the polysaccharide with caffeic acid, ferulic acid, or sinapic acid (Lee and others 2014). The results demonstrated that all the conjugates had stronger antioxidant activities than unmodified chitosan, confirming that the antioxidant ability of chitosan was increased after conjugation. Finally, the conjugation of proteins and polysaccharides has also been shown to significantly improve the antioxidant capacity compared to the protein or physical protein–polysaccharide mixtures (Nakamura and others 1992; Jing and others 2011; Huang and others 2012).

Thermal stability

Thermal processing is an important step in the production of many food products. However, heating may promote the thermal denaturation and aggregation of globular proteins, which can lead to problems with food quality (Liu and Zhong 2012). Studies have shown that glycation of globular proteins with polysaccharides can inhibit the thermal aggregation of proteins after heating (Liu and Zhong 2012, 2013). For example, it was reported that glycation of whey protein with maltodextrin led to transparent dispersions after heating at 88 °C for 2 min. This improvement in thermal stability was attributed to a number of factors: (i) glycation lowered the isoelectric point of the protein, (ii) glycation increased the denaturation temperature of the proteins, and (iii) glycation increased the steric repulsion between the proteins. Other studies have shown that the thermal stability of globular proteins can be increased by forming covalent conjugates with polysaccharides using the Maillard reaction (Wang and Zhong 2014).

An improvement in thermal stability of globular proteins can also be achieved by conjugation with polyphenols. In our recent study, we showed that conjugation of lactoferrin with polyphenols (EGCG and chlorogenic acid) inhibited its thermal aggregation at neutral pH (Liu and others 2016d). This effect was attributed to an increase in the thermal stability of the protein, as well as to an increase in the steric and electrostatic repulsion between the protein molecules.

Emulsifying properties

Conjugation has been widely used to improve the emulsifying properties of food biopolymers. This can be achieved by increasing the tendency for the biopolymers to adsorb to oil–water interfaces, or by improving the stability of biopolymer-coated droplets to aggregation (Kato 2002). For example, the formation of protein–carbohydrate conjugates using the Maillard reaction can enhance emulsion formation and stability depending on the nature of the constituents used to assemble the conjugates (Evans and others 2013). For instance, the emulsion-stabilizing properties of soy proteins have been improved by conjugation with sodium carboxymethyl cellulose (Diftis and Kiosseoglou 2003), which was attributed to an increase in steric repulsion between the oil droplets due to the presence of the polysaccharide chains.

Development of protein–polyphenol conjugates as novel emulsifiers has also drawn a great deal of interest in recent years. Lactoferrin–polyphenol conjugates prepared using the free radical grafting approach were shown to have better emulsifying activity and emulsion-stabilizing properties than lactoferrin alone (Liu and others 2015b). The improved emulsifying activity was attributed to the increased surface hydrophobicity of the modified protein, whereas the improved emulsion stability was attributed to a stronger repulsion between the conjugate-coated droplets. Wei and co-workers showed that β-carotene emulsions formed using protein–EGCG conjugates were more physically and chemically stable than those formed from protein alone (Wei and others 2015). The improved physical stability was attributed to the formation of smaller droplets with stronger repulsive interactions using the conjugates, whereas the improved chemical stability was attributed to the presence of antioxidant polyphenols at the droplet surfaces.

The presence of polyphenols also affects the emulsifying properties of polysaccharides. Studies have shown that both sugar beet pectin (SBP) and chitosan can be modified by polyphenols to improve emulsion stability. SBP naturally contains ferulic acid and some proteins, which contribute to its good amphiphilic properties (Jung and Wicker 2012). Laccase can be used to catalyze

intermolecular cross-links between the ferulic acid groups on SBP. Conjugated SBP has been shown to produce smaller and more negatively charged droplets than nontreated SBP. In our recent study, conjugates formed by covalent coupling of chitosan to EGCG using the hydroxyl-free radical grafting approach were used to stabilize β-carotene emulsions (Lei and others 2014a). We found that emulsions stabilized by chitosan–EGCG conjugates had smaller mean droplet sizes and better cream layer stability than those formed by chitosan alone.

Foaming properties

The foaming properties exhibited by certain food ingredients are important in determining the quality of numerous food products, including milk, ice cream, whipped creams, cakes, and breads (Oliveira and others 2016). Many proteins are good foaming agents because they can strongly adsorb to the gas–water interface and provide good steric and electrostatic stabilizations (Murray 2007). Many polysaccharides are so hydrophilic that they have a low affinity for the air–water interface; however, they can enhance the stability of protein foams by acting as thickening or gelling agents (Dickinson 2003). The good interfacial properties of proteins and stabilization properties of polysaccharides can be combined to form protein–polysaccharide complexes with enhanced foaming properties (Schmitt and others 1998). Studies have shown that milk proteins conjugated with either pectin or dextran, using the Maillard reaction, are better foaming agents than the original untreated proteins (Hiller and Lorenzen 2010). Similarly, the foaming properties of peanut proteins have been improved by conjugation with dextran using the Maillard reaction (Liu and others 2012b). The improved foaming characteristics of protein–carbohydrate conjugates have been attributed to the fact that their higher water solubility leads to their transfer to the air–water interface faster than the original proteins (Wooster and Augustin 2007). In addition, protein–carbohydrate conjugates form thick viscoelastic layers at the air–water interface, which can provide good protection against air bubble aggregation and Ostwald ripening (Dunlap and Côté 2005).

Gelling properties

A gel is a continuous three-dimensional network composed of molecules linked by covalent and/or noncovalent bonds. Gel networks can be formed using a variety of different approaches, including addition of enzymes (such as transglutaminase or laccase), of mineral counterions (such as potassium, calcium, or tripolyphosphate), pH adjustment (by adding acid or base), or alteration of environmental conditions (such as temperature) (McClements 2014). For example, mixtures of polysaccharides (chitosan) and proteins (gelatin) have been cross-linked by adding an enzyme (tyrosinase) to form a gel (Chen and others 2003). Laccase has also been used to covalently cross-link chitosan and gelatin and form gels using polyphenols as coupling agents (Rocasalbas and others 2013). Gelatin gels have also been formed using polyphenols (gallic acid and rutin) as cross-linking agents, with gel strength depending on polyphenol type and concentration (Yan and others 2011).

A similar tendency was also observed for polysaccharide–polyphenol conjugates. In the presence of carbodiimide and hydroxybenzotriazole, gallic acid could graft onto chitosan in aqueous solution (Xie and others 2014). The resulting chitosan–polyphenol conjugates had a higher viscosity than chitosan alone, which was attributed to the formation of covalent cross-links by oxidation of gallic acid groups. In addition, the high viscosity of the conjugates could be adjusted by modifying the amount of grafted gallic acid. Antioxidant thermoresponsive hydrogels have been synthesized by radical grafting of catechin onto inulin, which may be useful for certain food applications (Spizzirri and others 2011).

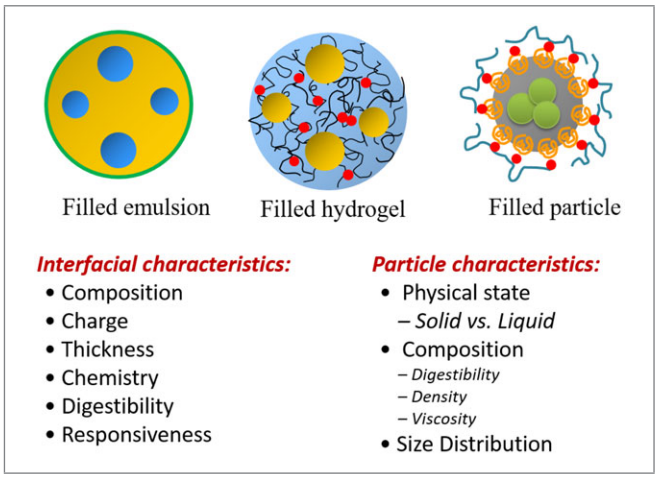

Figure 8–Examples of different kinds of delivery systems stabilized by food-grade conjugates.

Formation and Properties of Conjugates-Based Delivery Systems

Many active food compounds, such as colors, flavors, nutraceuticals, vitamins, and preservatives, benefit from being encapsulated in food-grade delivery systems because this improves their handling, dispersibility, stability, or activity (McClements and others 2009). These delivery systems should not adversely impact the properties of the food product in which they are incorporated, and they should be able to deliver the active compounds to the required site-of-action (Lesmes and McClements 2009). There are currently 3 major types of colloidal delivery systems that utilize food-grade conjugates: (i) emulsions and nanoemulsions, (ii) hydrogels, and (iii) biopolymer nanoparticles and microparticles. As shown in Figure 8, the physicochemical properties and stability of a particular delivery system depend on the composition and physical state of components and the method used to create it. Each system has its own specific advantages and disadvantages for encapsulation, protection, and delivery of functional components.

Emulsions and nanoemulsions

Oil-in-water (O/W) emulsions ($d > 100$ nm) and nanoemulsions ($d < 100$ nm) are widely used for the encapsulation of hydrophobic bioactive compounds that need to be dispersed into aqueous-based foods (McClements 2010). These 2 systems consist of emulsifier-coated oil droplets dispersed in water, but only the smaller droplets in nanoemulsions can lead to more desirable functional attributes, such as improved stability to aggregation and creaming, and higher optical clarity (Tadros and others 2004). There has recently been considerable interest in the application of conjugate-based emulsifiers to create emulsion-based delivery systems to encapsulate bioactive agents such as carotenoids, curcumin, and lutein (Gumus and others 2016; Liu and others 2016c; Wang and others 2016). Some potential advantages and limitations

of using emulsions and nanoemulsions stabilized by conjugates are summarized here:

Potential advantages

- Proteins can provide surface activity, polysaccharides can provide steric stability, and polyphenols can provide antioxidant properties in a single emulsifier.
- Emulsifier conjugates often lead to emulsions with better stability to environmental stresses, such as pH, ionic strength, temperature, and enzymes.
- Emulsifier conjugates often lead to emulsions with improved oxidative stability due to the increased concentration of antioxidants at the oil–water interface.

Potential limitations

- The formation of emulsifier conjugates requires the utilization of physical, chemical, or enzymatic treatments that increase ingredient costs.
- The chemical modification of food ingredients often requires regulatory approval before they can be utilized in food products.
- There are currently only a limited number of food-grade conjugates available to stabilize emulsions.

Despite these limitations, the considerable improvement in physical and chemical stability of emulsions that can be achieved using conjugation may stimulate the food industry to develop new functional ingredients based on this concept.

Hydrogels

Food-grade hydrogels typically consist of three-dimensional networks of aggregated proteins and/or polysaccharides. Conjugation approaches can be used to produce hydrogels with novel or improved functional attributes. Lysozyme-dextran conjugates, formed by the Maillard reaction, can form hydrogel particles, using a heat-gelation process, which are then relatively stable to changes in pH and ionic strength (Li and others 2008). These hydrogel particles were shown to be capable of encapsulating and delivering a drug (ibuprofen).

Polysaccharide–polyphenol conjugates have been shown to form gel-like networks that contain hydrophobic pockets capable of encapsulating bioactive components. Hyaluronic acid–EGCG hydrogel particles have been formed in aqueous solution that are capable of encapsulating bioactive agents (Liang and others 2016a).

Some potential advantages and limitations of using hydrogels as delivery systems for a bioactive compound stabilized by conjugates are summarized here:

Potential advantages

- Hydrogel particles can be prepared from food-grade ingredients (such as proteins, polysaccharides, and polyphenols).
- Hydrogel particles can be prepared using processing procedures that are already commonly used by the food industry, such as mixing, temperature control, and pH adjustment (McClements 2010).
- The composition and properties of hydrogel particles can be designed to protect encapsulated components during storage, and then release them in response to specific environmental triggers, such as pH, ionic strength, temperature, or enzyme activity.

Potential limitations

- Many food-grade hydrogel particles are difficult to thermally process because they dissociate at higher temperatures.
- The preparation of hydrogel particles involves additional ingredients and processing operations that can increase costs.

Biopolymer nanoparticles and microparticles

Food-grade nanoparticles or microparticles can be fabricated from proteins and/or polysaccharides (Jones and McClements 2010; Yao and others 2015). These colloidal particles can be used to encapsulate, protect, and deliver active food ingredients (Velikov and Pelan 2008). The utilization of conjugates to form biopolymer nanoparticles and microparticles is now being actively investigated for their potential application in functional foods and pharmaceuticals.

B-Carotene has been encapsulated in biopolymer nanoparticles fabricated from β-lactoglobulin−dextran conjugates (Yi and others 2014). Conjugation was shown to improve the stability of these nanoparticles under simulated gastrointestinal conditions. In a pharmaceutical study, the delivery of a hydrophobic drug (doxorubicin) was enhanced when it was encapsulated in nanoparticles formed from chitosan conjugated with carbohydrates and fatty acids (Guo and others 2013).

Some potential advantages and limitations of particles stabilized by conjugates are summarized here:

Potential advantages

- Food-grade conjugates provide a new type of building block useful to produce biopolymer nanoparticles or microparticles with structures and functional properties that cannot be achieved with other ingredients.
- Biopolymer particles with improved physicochemical stability and tailored gastrointestinal protection can be designed using conjugates

Potential limitations

- Additional ingredients and processing operations are required to fabricate conjugates, which would increase production costs.
- There may be labeling and regulatory issues associated with using conjugates compared to all-natural ingredients that are already in use.

Bioavailability Characteristics of Conjugate-Based Delivery Systems

The human gastrointestinal tract (GIT) is a highly complex organ consisting of the mouth, esophagus, stomach, small intestine, and large intestine (Figure 9). Knowledge of the physicochemical and physiological conditions within different regions of the GIT is important when designing conjugate-based delivery systems, anticipating control of retention and eventual release of a nutraceutical (Mackie 2012). After ingestion, a series of physical and chemical changes may occur to a delivery system as it moves from the mouth to the large intestine, including changes in pH, ionic composition, surface activity, enzyme activity, agitation, and temperature (McClements and Li 2010). The properties of a delivery system may change due to a variety of processes, including dissociation, disintegration, or digestion (of one or more constituent); changes in interfacial composition due to adsorption/desorption; mass transport mechanisms; and alterations in the ionization state of charged groups (Figure 9).

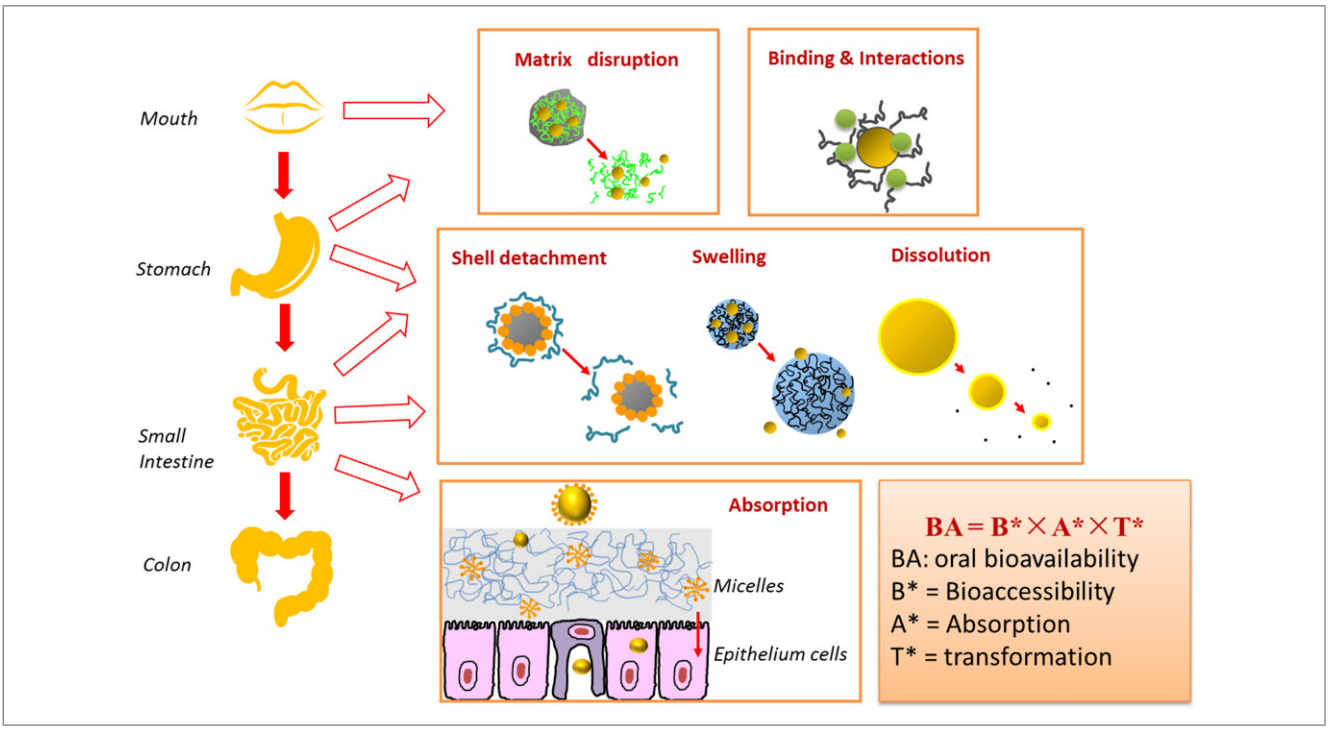

Figure 9–Designing delivery systems to control bioavailability: controlling body–particle interactions.

Oral bioavailability can be defined as the fraction of a specific ingested substance that eventually reaches an individual's circulatory system in an active form (Parada and Aguilera 2007). The overall bioavailability (BA) is determined by 3 main factors (McClements and others 2015):

$$BA = B^* \times A^* \times T^*$$

Here, B^* is the bioaccessibility of the substance within the intestinal fluids obviously change during passage of the GIT. For hydrophilic substances, this may simply be the fraction that is released from the food matrix and dissolved in the intestinal fluids. For hydrophobic substances, this is the fraction of the substance that is solubilized within mixed micelles in the small intestinal fluids. A^* is the absorption of the substance from the gastrointestinal fluids into the body, that is, the fraction that passes through the mucus layer, across the epithelium cells, and into the systemic circulation. T^* is the fraction of the absorbed substance that reaches the site of action in an active state.

The composition and structure of conjugate-based delivery systems can be designed to improve the bioavailability of substances incorporated into it by altering B^*, A^*, and T^*. Curcumin, a bioactive polyphenol derived from the plant *Curcuma longa*, is used here as an example to highlight this approach. Curcumin has been shown to exhibit a range of beneficial biological and pharmacological activities, including antioxidant, anti-inflammatory, and antiseptic activities, but its application as a nutraceutical or drug is currently limited because of its low water solubility, rapid hydrolytic degradation, and poor bioavailability (Dey and Sreenivasan 2014). Curcumin–polysaccharide conjugates have been developed to enhance bioaccessibility and stability. Appreciable enhancements in the water solubility of curcumin were observed upon conjugation with both alginate (Dey and Sreenivasan 2014) and gum arabic (Sarika and others 2015). Indeed, the solubility of a gum arabic–curcumin conjugate was 900-fold higher than that of free curcumin. Moreover, the gum arabic–curcumin conjugate exhibited enhanced accumulation and reduced toxicity in HepG2 cells due to the targeting efficiency of the galactose groups present in the gum arabic.

The bioavailability of a substance can also be improved by increasing the fraction that is solubilized within the mixed micelle phase. For example, resveratrol is a polyphenolic compound that has potential benefits for human health. Research has shown that the bioaccessibility of resveratrol within the mixed micelles was enhanced when it was encapsulated in biopolymer nanoparticles fabricated using caseinate–dextran conjugates (Davidov-Pardo and others 2015). The bioavailability of a substance can also be improved by increasing the amount that reaches the intended site-of-action in an active form. Wang and others synthesized an amphiphilic carboxymethyl chitosan–quercetin conjugate (CQ) for encapsulating and delivering a hydrophobic drug (paclitaxel) (Wang and others 2014b). *In situ* intestinal absorption experiments showed that the polymeric micelles formed by CQ significantly improved the effective permeability of the drug, resulting in strong antitumor efficacy.

In summary, food-grade conjugate-based delivery systems can be deigned to enhance the bioavailability of substances depending on the properties of the starting materials and fabrication methods used. To provide guiding principles for designing more effective delivery systems suitable for application in the food and pharmaceutical industries, more mechanistic investigations are needed to establish the relationship between the structure of the conjugates and the activity of substances within the human GIT.

Concluding Remarks and Future Perspectives

In summary, food-grade conjugates can be formed from proteins, polysaccharides, and polyphenols using specific cross-linking

reactions, including enzymatic methods, the Maillard reaction, free radical grafting, alkaline treatment, and the carbodiimide-mediated coupling reaction. The conjugates produced can be designed to have physicochemical attributes and functional properties that are different from those of the natural starting materials, such as improved physical and chemical stabilities, or controlled release properties. Consequently, these conjugates may be used to develop innovative functional food products specifically designed to enhance human health and performance. On the basis of our review of the literature, we recommend that the future development of studies on conjugates of proteins, polysaccharides, and polyphenols must focus on the following aspects: (i) development of novel food-grade reactions that can be used to produce conjugates with new functional properties; (ii) careful separation and purification of conjugates from the starting materials used so as to establish their specific functional attributes; (iii) the development of new methods to identify the binding sites and bond types on proteins, polyphenols, and polysaccharides; (iv) and utilization of conjugates to design novel delivery systems with improved functional performances.

Abbreviations

ABTS	2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
AFM	atomic force microscopy
BSA	bovine serum albumin
CD	circular dichroism spectroscopy
DLS	dynamic light scattering
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DSC	differential scanning calorimetry
EDC	1-ethyl-3-(30-dimethylaminopropyl) carbodiimide
EGCG	(−)-epigallocatechin-3-gallate
FTIR	Fourier transform infrared spectroscopy
GIT	gastrointestinal tract
GPC–MALLS	gel permeation chromatography–multiple-angle laser light scattering
HPGPC	high−performance gel permeation chromatography
HPSEC	high−performance size−exclusion chromatography
HPSEC–MALLS	high-performance size−exclusion chromatography with multiangle laser light scattering detection
IEF	isoelectric focusing
MALDI	matrix-assisted laser desorption
MALDI-TOF−MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MALLS	multiangle laser light scatter
MRP	Maillard reaction product(s)
MS	mass spectrometry
NMR	nuclear magnetic resonance
pI	isoelectric point
PPI	peanut protein isolate
SDS–PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size-exclusion chromatography
SEM	scanning electron microscopy
SPI	soy-protein isolate
SSPS	soy soluble polysaccharide
TLC	thin-layer chromatography
TOF	time-of-flight
UPLC	ultraperformance liquid chromatography
UV	ultraviolet
WPI	whey protein isolate
XRD	X-ray diffraction
β-LG	β-lactoglobulin

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