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Abstract: The development of food packaging films based on proteins or polysaccharides has been motivated by their biodegradable and renewable characteristics. On the other hand, protein and polysaccharide films have usually poor physico-mechanical performance when compared to their petroleum-derived counterparts. One of the main drawbacks of those films is their water sensitivity, which usually results in their immediate dissolution when in contact with water, limiting their applications for food packaging purposes. One of the main techniques for polymer modification is crosslinking, which consists of linking polymer chains by covalent or noncovalent bonds, forming tridimensional networks with reduced mobility and usually enhanced water resistance as well as mechanical and barrier properties. The main crosslinking agents and mechanisms which have been used for protein and polysaccharide films are reviewed, as well as some possible applications for food packaging.

Dr. Rickey Yada, Editor
Trends in Food Science and Technology

Dear Dr. Yada:

Crosslinking is a promising technique to improve the performance and applicability of biopolymers films as food packaging materials, especially concerning their high water sensitivity which hinders many of their potential applications for food packaging purposes. Crosslinking reduces the mobility of the polymer structure and usually enhances its mechanical and barrier properties as well as its water resistance.

The enclosed manuscript, “Crosslinking in polysaccharide and protein films – a review”, is a summary of published data on the main crosslinking agents and systems which have been used for protein and polysaccharide films, focused on their mechanisms, applications, effects, and limitations. Please consider it for publication in Trends of Food Science and Technology.

Sincerely,

Henriette Azeredo

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CROSSLINKING IN POLYSACCHARIDE AND PROTEIN FILMS - A REVIEW

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Abstract

The development of food packaging films based on proteins or polysaccharides has been motivated by their biodegradable and renewable characteristics. On the other hand, protein and polysaccharide films have usually poor physico-mechanical performance when compared to their petroleum-derived counterparts. One of the main drawbacks of those films is their water sensitivity, which usually results in their immediate dissolution when in contact with water, limiting their applications for food packaging purposes. One of the main techniques for polymer modification is crosslinking, which consists of linking polymer chains by covalent or noncovalent bonds, forming tridimensional networks with reduced mobility and usually enhanced water resistance as well as mechanical and barrier properties. The main crosslinking agents and mechanisms which have been used for protein and polysaccharide films are reviewed, as well as some possible applications for food packaging.

Keywords: biopolymers; water solubility; tensile properties; chemical crosslinking; physical crosslinking.

Introduction

Crosslinking is the process of linking polymer chains by covalent or noncovalent bonds, forming tridimensional networks (Balaguer, Gómez-Estaca, Gavara, &

Hernández-Muñoz, 2011). Chemical crosslinking results from covalent bonding between polymer chains accomplished by irradiation, sulfur vulcanization, or chemical reactions (Bhattacharya & Ray, 2009), while physical crosslinking involves noncovalent bonds, such as ionic interactions, hydrogen bonds or hydrophobic interactions (Hennink & van Nostrum, 2012; Ostrowska-Czubenko & Gierszewska-Druzynska, 2009). Crosslinking may be intra- or intermolecular (Figure 1), and it is one of the prime techniques for polymer modification, apart from grafting, blending, and composite formation (Bhattacharya & Ray, 2009).

Crosslinking is especially useful for biopolymer materials, such as those derived from proteins or polysaccharides. Those kinds of materials have been widely studied because of their biodegradable and renewable characteristics. Their eco-friendly appeal makes them interesting for short-lived applications such as food packaging. On the other hand, biopolymer films have usually poor physico-mechanical performance when compared to their petroleum-derived counterparts, limiting their practical applicability. One of the main drawbacks of biopolymer films is their high water sensitivity, making them to disintegrate when in contact with water, or to have their mechanical and barrier properties impaired because of water absorption and swelling (Sebti, Delves-Broughton, & Coma, 2003). Crosslinking reduces the mobility of the polymer structure and usually enhances its mechanical and barrier properties as well as its water resistance (Balaguer et al., 2011), reducing both its water solubility (Carvalho & Grosso, 2004; Menzel et al., 2013) and swelling (Boanini, Rubini, Panzavolta, & Bigi, 2010). Moreover, it makes the structure more resistant to heat and light, improves dimensional stability, and chemical and solvent resistance. Crosslinking also leads to a decrease in free volume, which results in an increase of glass transition temperature (Bhattacharya & Ray, 2009). Crosslinking reactions may also retard the biodegradation of materials, as reported by some authors (Martucci & Rusecaite, 2009; Rimdusit, Jingjid, Damrongsakkul, Tiptipakorn, & Takeichi, 2008).

The most common crosslinking agents are symmetrical bifunctional compounds with reactive groups with specificity for functional groups present on the matrix macromolecules (Balaguer et al., 2011). Chemical cross-linking is frequently used to improve the performance and resistance of biopolymer films. Nevertheless, many cross-linking agents may leave toxic residues. Since biopolymers are commonly used for applications which require low toxicity (such as food packaging, sometimes including edible films and coatings), many studies have been focused on non-toxic crosslinking agents.

Crosslinking reactions are more commonly applied to proteins than to polysaccharides, since proteins have more functional groups. Gelatin has been especially studied as a matrix biopolymer to be subjected to crosslinking, because of the large number of its functional side groups (Boanini et al., 2010). Moreover, chemical crosslinking of gelatin avoids the physical crosslinks occurring during renaturation and formation of triple helices during gel formation (Carvalho & Grosso, 2004).

This review is a summary of the main crosslinking agents and mechanisms which have been used for protein and polysaccharide films, and which may be applied to food packaging systems.

Crosslinking agents and mechanisms

Several crosslinking agents have been used for proteins and polysaccharides, differing in mechanisms, specificities, and effects. Aldehydes are probably the most commonly used crosslinkers for protein films. However, there have been concerns about the toxicity of aldehyde residues remaining in the films, which could be a problem in case of their migration for food products (Chiou et al., 2006). So, other nontoxic or less toxic crosslinking agents have been studied, which are better alternatives for food packaging applications.

Aldehyde crosslinking agents

Aldehydes, particularly formaldehyde and glutaraldehyde, are widely used crosslinkers since they are relatively inexpensive and react quickly with amine groups in proteins. Formaldehyde (HCHO) is the simplest crosslinking agent and has the broadest reaction specificity. Although it contains just one functional group, it can crosslink with proteins by a two-step process (Figure 2), the first step corresponding to the formation of the methylol compound, and the second one corresponding to formation of methylene bridges between protein chains

(Audic & Chaufer, 2005). Glutaraldehyde is more specific, reacting with lysine, cysteine, histidine and tyrosine (Hernández-Muñoz, Villalobos, & Chiralt, 2004). Protein crosslinking by glyoxal involves lysine and arginine side chain groups (Marquié, 2001) at alkaline pH.

Farris, Song, & Huang (2010) investigated the functional groups involved in gelatin crosslinking with glutaraldehyde. At high pH (close to lysine pK_a), the reaction was reported to be mainly governed by the Schiff's base formation with ε-amine groups of protein side chains, whereas at low pH (when amine groups of lysine are protonated), the reaction may also involve the formation of hemiacetals with hydroxyl groups of hydroxyproline and hydroxylysine.

Cinnamaldehyde has been presented by Balaguer et al. (2011) as a naturally occurring, less toxic aldehyde to be used as a crosslinker. The crosslinking reaction between cinnamaldehyde and gliadins was found to have an optimum pH of 2. The cinnamaldehyde action was ascribed to the formation of intermolecular covalent bonds between "monomeric" gliadins, producing a polymerized network. The authors suggested the participation of noncovalent and disulfide bonds to stabilize the structure of the crosslinked films.

Effects usually reported from crosslinking protein films with aldehydes include enhanced water resistance (Audic & Chaufer, 2005), which has been ascribed by Zárate-Ramírez et al. (2014) to a reduced molecular space available for water molecules to penetrate the matrix; improved barrier properties (Hernández-

Muñoz, Kanavouras, Lagaron, &Gavara, 2005; Balaguer, Cerisuelo, Gavara, & Hernández-Muñoz, 2013); and positive changes in tensile properties (Audic & Chaufer, 2005; Sun, Song, & Zheng, 2007).

Crosslinking reactions between amine groups of proteins (or chitosan) and aldehydes have been evidenced by FTIR, by the formation of a peak associated with imine (C=N) stretching vibration (Singh, Narvi, Dutta, & Pandey, 2006; Yue, Cui, Shuttleworth, & Clark, 2012).

Disulfide crosslinking of proteins

Disulfide bonds (Figure 3) are a common type of naturally occurring crosslinks in proteins. They can be induced via enzymatic catalysis, mechanical mixing, or heat treatment, and consist of oxidation of sulfhydryl groups of two adjacent cysteine residues in a protein, forming inter- and intermolecular bonds which lead to the formation of a protein network, positively affecting gelling properties and heat stability (Buchert et al., 2010).

Hernández-Muñoz, Kanavouras, Villalobos, & Chiralt (2004) added cysteine to gliadin film forming solutions to promote interchain disulfide bonds. Cysteine-treated gliadin films presented improved water resistance and a more rigid structure when compared to a control gliadin film. The polymerization of gliadins via the formation of intermolecular disulfide bonds was evidenced by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE), by the formation of protein aggregates at the top of the separation gel.

Zhao et al. (2012) described another crosslinking reaction which is not based on the formation of disulfide bonds, but also contains disulfide bonds. Alginate was crosslinked with cysteine dimethyl ester or cystamine with di-terminated amine groups by a condensation reaction of carboxyl and amine groups (Figure 4), in the presence of 1-methyl-3 (3-dimethylaminopropyl) carbodiimide (EDC), which activated the alginate carboxyl groups. The FTIR spectra presented evidence for the amidation of carboxylic groups, by the formation of sharp peaks at 1637 and 1568 cm^{-1}, corresponding to the amide I band, C=O stretching, and amide II band, N-H bending, respectively. Also ^1H NMR corroborated the formation of crosslinks by presenting resonance peaks at 2.8 ppm and 3.25 ppm, ascribed to methylene protons of cystamine.

Oxidized polysaccharides

Periodate oxidation specifically cleaves vicinal glycols in polysaccharides, forming their dialdehyde derivatives (Dawlee, Sugandhi, Balakrishnan, Labarre, & Jayakrishnan, 2005), which have received attention as protein crosslinkers. The aldehyde groups can form crosslinks by C=N linkages (Schiff’s base formation) with ε-amine groups of protein side chains (Balakrishnan, Mohanty, Umashankar, & Jayakrishnan, 2005; Mu, Guo, Li, Lin, & Li, 2012), as shown in Figure 5. The degree of oxidation has been reported to affect the number of C=N groups and the consequent benefits on physical properties (Guo, Ge, Li, Mu, & Li, 2014). The extent of crosslinking can be determined by quantifying uncrosslinked ε-amine groups of the protein by UV spectrometry before and after the reaction (Boanini et al., 2010).

Several dialdehyde polysaccharides have been proven effective crosslinkers on gelatin, such as alginate dialdehyde (Balakrishnan et al., 2005; Boanini et al., 2010), dialdehyde starch (Martucci & Ruseckaite, 2009), dialdehyde carboxymethylcellulose (Mu et al., 2012), oxidized xanthan gum (Guo et al., 2014). The crosslinked structures presented reduced water solubility (Martucci & Ruseckaite, 2009) and water uptake (Martucci & Ruseckaite, 2009; Mu et al., 2012), improved barrier (Guo et al., 2014; Martucci & Ruseckaite, 2009) and tensile properties (Guo et al., 2014; Mu et al., 2012) as well as thermal stability (Guo et al., 2014). Guo et al. (2014) observed that the oxidation of xanthan gum improved the film barrier against UV light while having little effect on visible light transmission, keeping film transparency.

However, Guo et al. (2014) reported degradation of xanthan gum backbone resulting from the progressive periodate oxidation, indicating that highly oxidized xanthan gum might behave somehow as short chain aldehydes. This degradation ultimately resulted in impaired tensile properties of films, probably because it retarded interactions and entanglement between polymer chains.

The presence of amine groups in chitosan make it also suitable for crosslinking with oxidized poly- or oligosaccharides, as reported by Gómez-Mascaraque, Méndez, Fernández-Gutiérrez, Vázquez, & San Román (2014) and Zhang, Qadeer, & Chen (2011) using oxidized dextrins and dextrans, respectively. Gómez-Mascaraque et al. (2014) developed hydrogel networks based on agarose and chitosan using oxidized dextrins as crosslinking agents for chitosan. The stiffness of the materials was strongly affected by the oxidation degree of the crosslinker.

Phenolic acids

Several natural phenolic compounds derived from plants have been used as crosslinkers to modify protein films, proline-rich proteins such as gelatin being especially susceptible (Ozdal, Capanoglu, & Altay, 2013). Several potential interactions may be involved, including covalent as well as noncovalent bondings (Zhang et al., 2010).

The postulated main chemical pathway (Figure 6) involves oxidization of diphenol moieties of phenolic acids or other polyphenol, under alkaline conditions, producing quinone intermediates which react with nucleophiles (mainly amine or sulfhydryl side chains of proteins) to form covalent C-N or C-S bonds with the phenolic ring (Ou, Wang, Tang, Huang, & Jackson, 2005; Strauss & Gibson, 2004). The regenerated hydroquinone can be reoxidized and bind another polypeptide chain, resulting in a crosslink. Alternatively, two quinones, each carrying one polypeptide chain, can dimerize to produce a crosslink (Strauss & Gibson, 2004). Zhang et al. (2010) found evidence to crosslinking by high-resolution NMR via investigation on small molecular model systems or using ^{13}C enriched caffeic acid (LCA) in the reaction with gelatin, confirming reactions between the phenolic groups of the phenolic acids and the amino groups in gelatin to form C - N covalent crosslinks.

Aewsiri, Benjakul, Visessanguan, Wierenga, & Gruppen (2010) reported two types of crosslinking between gelatin and tannic acid. Unoxidized tannic acid (TA) formed non-covalent interactions with gelatin at pH 7, whereas oxidized tannic acid (OTA) formed covalent bonds with gelatin at pH 9. Different non-covalent interactions may have occurred, such as hydrogen bonds between hydroxyl groups of TA and polar carbonyl groups of gelatin, and hydrophobic interactions between aromatic rings of TA and the hydrophobic side chain of gelatin (Aewsiri et al., 2010; Ozdal et al., 2013). The covalent bonding probably occurred between electrophilic quinone (oxidized form of tannic acid) and nucleophilic amine groups of lysine. Non-covalent interaction produced a higher degree of tannic acid incorporation into gelatin than covalent bonding, as evidenced by a lower free amine group content, and higher contents of phenolic, hydroxyl and aromatic groups by FTIR, as well as higher antioxidant activity.

Ou et al. (2005) added different concentrations of ferulic acid (FA) to soy protein isolate film forming solutions. An optimal FA concentration (1 wt% on a dry basis) improved tensile properties of films. The water vapor permeability of films was decreased by oxidized ferulic acid (OFA), but it was unaffected by the unoxidized FA. The crosslinking was evidenced by a much longer absorbance wavelength for the peak of ferulic acid-protein than for the components alone.

Moreover, FA is also a natural crosslinker for polysaccharides. In wheat, FA esterifies the C-5 hydroxyl group of some arabinose residues of arabinoxylans (Barberousse et al., 2009). FA is sometimes oxidatively coupled at 5-5 of the aromatic rings to form dehydrodiferulates, and this dimerization of polysaccharide-bound ferulates (Figure 7) is a known crosslinking mechanism (Buranov & Mazza, 2008). However, no reports have been found relating this mechanism in polysaccharide films, except by films including chitosan (Mathew & Abraham, 2008), which, thanks to its amine groups, is crosslinked by the same mechanism described to proteins.

The addition of 5 wt% OFA to starch-chitosan films reduced their water vapor permeability and oxygen permeability in about 19% and 75%, respectively, and increased their tensile strength in 50%. At higher OFA concentrations, however, films exhibited phase separation, which impaired some physical properties. FTIR studies indicated interactions between chitosan amine groups and hydroxyl groups of starch and ferulic acid.

Some studies have described phenolic acids acting rather as film plasticizers instead of crosslinking agents. Arcan & Yemenicioglu (2011) reported that gallic and ferulic acids remarkably decreased the tensile strength and increased the elongation of zein films. Similarly, Hager, Vallons, & Arendt (2012) observed that gallic acid had a plasticizing effect rather than the expected crosslinking effect on gluten films.

Besides acting as crosslinkers, phenolic acids are also antioxidant agents. Actually, their antioxidant properties, combined to their oxygen barrier enhancing capacity by effect of crosslinking, may act together to help reduce oxidation reactions in foods (Fabra, Hambleton, Talens, Debeaufort, & Chiralt, 2011).

Enzymatic crosslinking

Enzymatic crosslinking of biopolymers is an attractive option because of the specificity of enzymes and mild reaction conditions needed. The crosslinking can be a result of direct enzymatic catalysis of crosslink formation or can occur indirectly by enzymatic production of a crosslinking agent, such as H_2O_2, which in turn is able to oxidize reactive structures with subsequent crosslink formation (Buchert et al., 2010).

Microbial transglutaminase (TGase) catalyzes acyl transfer reactions between γ-carboxyamide groups of glutamine residues (acyl donor) and ε-amine groups of lysine residues (acyl receptor), forming ε-(γ-glutamyl) lysine intra- and intermolecular crosslinkings into food proteins (Bae et al., 2009), as shown in Figure 8. The degree of crosslinking can be measured by reaction of 2,4,6-trinitrobenzenesulfonic acid (TNBS) with primary amino groups of the protein to detect the absorbance of noncrosslinked groups by UV spectroscopy (Carvalho & Grosso, 2004; Yi, Kim, Bae, Whiteside, & Park, 2006).

TGase was reported by Carvalho & Grosso (2004) to significantly reduce the water vapor permeability (WVP) and the water solubility of gelatin films. Yi et al. (2006) presented TGase crosslinking as a practical way to improve the applicability of fish gelatin films (whose overall performance is usually poorer than those of mammalian gelatins), increasing gelatin solution viscosity, and improving tensile strength, oxygen permeability, and thermal stability of films.

Jin & Zhong (2013) incorporated soy protein-coated montmorillonite (MMT) as intercalated nanofillers to a soy protein matrix biopolymer, and used TGase to crosslink proteins. There was a drastic increase in storage modulus (G’) in treatments including MMT and TGase, suggesting the occurrence of crosslinking between proteins coating MMT and those in the continuous phase.

Laccase is an oxidoreductase which catalyzes one-electron oxidations in aromatic amines and phenols, resulting in radicals that may undergo polymerization. It is a useful enzyme for polymerizing and crosslinking phenolic structures, requiring only oxygen as a co-substrate and displaying both ligninolytic and polymerizing activities on lignin (Oinonen, Areskogh, & Henriksson, 2013). A process involving laccase-catalyzed crosslinking of lignin bound to high molecular weight hemicelluloses (galactoglucomannans) was presented by Oinonen et al. (2013), which resulted in improved tensile properties of galactoglucomannan films.

Alginic acid

Alginic acid is a major structural polysaccharide of some brown seaweeds, consisting of a linear anionic copolymer of 1,4 linked β-D-mannuronic acid (M) and 1,4 linked α-D-guluronic acid (G) arranged as homopolymeric (GG or MM) or heteropolymeric (GM) blocks (Mitra et al., 2011b). Alginic acid was demonstrated by Mitra et al. (2011b) to crosslink with collagen by multiple intermolecular hydrogen bonding, and by covalent amide bonds between carboxyl groups of alginic acid amine groups of collagen (Figure 9). The formation of –CONH (amide) bonds was evidenced by a number of changes on FTIR spectra.

Polycarboxylic acids

Compounds containing at least two carboxyl groups are able to interact ionically with amine groups of two different chains. Oxalic acid has been reported to crosslink to amine groups of chitosan (Melina, Heuzey, & Begin, 2006) and collagen (Mitra, Sailakshmi, Gnanamami, & Mandal, 2011a). Figure 10 presents a scheme of the crosslinking between a dicarboxylic acid and a protein.

Moreover, di- and polycarboxilic acids have been reported to form hydrogels with polysaccharides. Citric acid (CA) has been used in several studies (Bonilla, Talón, Atarés, Vargas, & Chiralt, 2013; Coma, Sebti, Pardon, Pichavant, & Deschamps, 2003; Olsson, Hedenqvist, Johansson, & Järnström, 2013a) as a crosslinking agent for polysaccharide films. One of the advantages of using CA as a crosslinker is that any unreacted CA is not only nutritionally acceptable but it can also act as a plasticizer, as reported by Shi et al. (2008). The proposed crosslinking mechanism (Figure 11) is attributed to covalent intermolecular di-ester formation between hydroxyl groups of the polysaccharide and two carboxyl groups of the crosslinker (Hashem, Sharaf, El-Hady, & Hebeish, 2013; Olsson et al., 2013a). The presence of an ester bond can be revealed by FTIR by the formation of a carbonyl peak around 1725-1738 cm^{-1} (Coma et al., 2003; Dastidar & Netravali, 2012; Demitri et al., 2008), attributed to carbonyl stretching related to anhydride formation, which is an intermediary reaction for the crosslinking (Demitri et al., 2008).

Some studies have reported the use of a catalyst such as sodium hypophosphite (Reddy & Yang, 2010), which supposedly weaken the hydrogen bonding between carboxylic acid groups, increasing the speed of formation of the cyclic anhydride intermediate (Sauperl & Stana-Kleinschek, 2010). However, Coma et al. (2003) did not observe significant effects of the catalyst on the reaction.

The crosslinking reaction has been reported to be favored by a high temperature curing process on the dried films (Coma et al., 2003; Dastidar & Netravali, 2012; Olsson et al., 2013a, 2013b). Dastidar & Netravali (2012) observed that starch films crosslinked with malonic acid presented more ester bonds and less unreacted malonic acid after being cured at 120˚C.

Wang, Ren, Li, Sun, & Liu (2014) observed two different (sometimes apparently inconsistent) effects from CA in polyvinyl alcohol/xylan films. When added at concentrations of up to 10%, it acted as a crosslinker, but further increases in CA concentrations from 10% to 50% resulted in decreased tensile strength and sharply increased elongation, characterizing a plasticizing effect of CA. On the other hand, the water vapor permeability of the films kept decreasing with increasing of CA contents, which is consistent with a crosslinking effect.

A series of studies has been conducted on the effects of with different CA concentrations at different temperatures on barrier and solubility properties of starch films (Menzel et al., 2013; Olsson et al., 2013a, 2013b). Olsson et al. (2013a) observed that the water vapor permeability of starch-based films decreased with increasing CA contents (up to 30% CA), and that a curing treatment (at 150˚C for 10 min) on the dried crosslinked films improved the CA effect on barrier properties. Menzel et al. (2013) reported that increasing concentrations (up to 30%) of CA as a crosslinker increased the degree of crosslinking and decreased the water solubility of starch films, and that a significant crosslinking of starch films was possible even at a relatively low temperature (70˚C), although the degree of crosslinking had increased at higher temperatures. Olsson et al. (2013b) focused on the two concurrent reactions promoted by CA affecting starch molecular weight and consequent starch film properties – hydrolysis and crosslinking. They demonstrated that the hydrolysis reaction was more strongly affected by pH changes than the crosslinking, and that starch-coated papers presented minimum water vapor permeability when the starch coating solution had pH 4, which corresponds to a point where hydrolysis was hindered while a significant degree of crosslinking still occurred.

Genipin

Genipin, a naturally occurring low-toxicity crosslinker, is an aglycone derivative from an irinoid glycoside (geniposide) present in fruits of *Gardenia jasminoides* Ellis, a traditional Chinese herbal medicine (Ma, Tang, Yin, Yang, & Qi, 2013; Nickerson, Patel, Heyd, Rousseau, & Paulson, 2006). It is obtained by enzymatic hydrolysis with β-glucosidase (Nickerson et al., 2006). Genipin covalently binds ε-amine groups thorough a nucleophilic substitution reaction (Nickerson et al., 2006) which produces a dark-blue coloration (Pujana, Pérez-Álvarez, Iturbe, & Katime, 2013). The proposed mechanism for the crosslinking reaction is summarized in Figure 12.

The appearance and intensity of a peak at 605 nm in UV-vis spectra (blue pigment) may be considered as an evidence for the crosslinking reaction (Pujana et al., 2013). The degree of crosslinking can also be estimated as the difference between the number of free ε-amine groups before and after reaction (Cui, Jia, Guo, Liu, & Zhu, 2014). FTIR spectra can provide evidence for the crosslinking reaction as well; an increased intensity of C-N band at 1078 cm^{-1} at the expense of the C-O band at 1026 cm^{-1} was considered by Cui et al. (2014) as evidence for the formation of heterocyclic compounds of genipin crosslinked to polymers.

Genipin crosslinking was used by Ma et al. (2013) to retard the release of lysozyme from gelatin films, suggesting its usefulness for a controlled release of active compounds from crosslinked matrices.

Ionic crosslinking between polyuronates and divalent cations

Pectin and alginate (both polyuronates) are characteristic examples of ionic polysaccharides forming crosslinking upon addition of divalent cations such as Ca^{2+} (Fang et al., 2008). Alginates, extracted from brown algae, are block copolymers of guluronic (G) and mannuronic (M) acids, which are present in the linear macromolecule in homopolymeric M-blocks and G-blocks, as well as blocks of alternating sequence (Fang et al., 2008; Russo, Malinconico, & Santagata, 2007). Pectins, a major structural component of cell walls composed of galacturonic acid residues, are classified as high methoxyl pectins (HMP) or low methoxyl pectins (LMP), depending on more or less than 50% of their carboxyl groups being methyl esterified, respectively (Silva, Bierhalz, & Kieckbusch, 2009). Similarly to alginates, LMP form gel with divalent cations (Fang et al., 2008; Silva et al., 2009).

Calcium induced gelation results from interactions between calcium ions and uronate blocks (guluronate and galacturonate in alginate and pectin, respectively). The resulting chain-chain associations constitute junction zones, which have been represented by the so called “egg-box” model (Figure 13). However, the random distribution pattern of galacturonate groups in pectin generates some “defects” during the formation of egg-box structures when compared to the blockwise distribution pattern of alginate (Fang et al., 2008). This may be the reason why Galus & Lenart (2013) observed that crosslinked LMP film networks were less organized than crosslinked alginate films, as evidenced by microscopic images.

Other authors have described the formation of crosslinked films from divalent cation treatment of alginate (Olivas & Barbosa-Cánovas, 2008; Rhim, 2004; Russo et al., 2007), LMP (Kang, Jo, Lee, Kwon, & Byun, 2005) or alginate and LMP composites (Silva et al., 2009).

Polyelectrolyte complexes

Polyelectrolyte complexes (PECs) may be formed between cationic and anionic polyelectrolytes. A well-known example is the PEC formed by ionic bonds between carboxylate groups of the anionic alginate and ammonium groups of the cationic chitosan (Ostrowska-Czubenko & Gierszewska-Druzynska, 2009), according to the scheme presented in Figure 14. The PEC formation was confirmed by water solubility tests, which revealed that, while pure alginate and pure chitosan films were highly soluble, the PEC film presented low water solubility (Ostrowska-Czubenko & Gierszewska-Druzynska, 2009). Moreover, those authors reported some changes in FTIR spectra providing additional evidences for the PEC formation, namely, a slight downshift of the symmetric stretching vibration of carboxylate group (1415 cm^{-1}) band, and a new band at 1635 cm^{-1} (antisymmetric N-H deformation) indicating the formation of $–NH_3^+$ ions. On the other hand, Lawrie et al. (2007) reported no significant changes in band position of the carbonyl vibrations from alginate upon interaction with chitosan. Two new bands have been described by Lawrie et al. (2007) to have resulted from the chitosan-alginate interactions: one at 1710 cm^{-1} ascribed to protonation of carboxylate groups of alginate, and another at 1530 cm^{-1} due to partial protonation of amine groups of chitosan.

Crosslinking of film-reinforcing cellulose whiskers

Cellulose nanocrystals (CNC), also known as cellulose whiskers (CW), have been increasingly used as a reinforcing phase in polymer nanocomposite films. Goetz, Mathew, Oksman, Gatenholm, & Ragauskas (2009) described the development of nanocomposite films wherein CW were crosslinked to poly(methyl vinyl ether-co-maleic-acid) (PMVEMA) and polyethylene glycol (PEG). The crosslinking of CW with PMVEMA and PEG occurred via an esterification reaction between the hydroxyl groups on the cellulose, terminal hydroxyl groups of PEG and the carboxylic acid groups on the PMVEMA. The films, formed by curing the three components at high temperature, were reported to be capable of absorbing up to about 900% water without losing its structural integrity, forming a stable hydrogel which could be used for food packaging applications. The crosslinking formation was evidenced by an ester peak at 1734 cm^{-1} on FTIR spectra of samples previously treated with NaOH (to remove the influence of unreacted carboxylic acid groups).

Final considerations

Crosslinking is a promising technique to improve the performance and applicability of protein and polysaccharide films as food packaging materials, especially concerning their usual water sensitivity which hinders many of their potential applications for food packaging purposes. Many types of crosslinking agents and mechanisms have been used, with different applications, effectivities, and limitations. Some aldehydes are especially effective, but their use in food packaging systems has been avoided because of concerns about migration of aldehyde residues to food products. Less toxic compounds have thus been studied as alternative crosslinking agents for food packaging applications. However, effort is still needed to scale up biopolymer processing and crosslinking techniques from the experimental to industrial level and to assure their practical applications.

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Figure 1. Schematic diagram of intermolecular (A) and intramolecular (B) crosslinking.

Figure 2. Crosslinking between formaldehyde and ε-amine group of a protein. (Modified from Audic & Chaufer, 2005).

Figure 3. Scheme of a disulfide bond.

Figure 4. Crosslinking of alginate with cystine or cystamine. (Modified from Zhao et al., 2012).

Figure 5. Scheme of the periodate oxidation of a polysaccharide and the Schiff's reaction between a protein and dialdehyde polysaccharide. (Modified from Mu et al., 2012).

Figure 6. Reactions between a phenolic acid and amine side chains of proteins. (Modified from Strauss & Gibson, 2004).

Figure 7. (A) Structure of ferulic acid. (B) Covalent diferulate cross-link between polysaccharides. (Modified from Buranov & Mazza, 2008).

Figure 8. Cross-linking reaction catalyzed by TGase. (Modified from Rachel & Pelletier, 2013).

Figure 9. Scheme of covalent bonding between alginic acid and a protein. (Modified from Mitra et al., 2011b).

Figure 10. Ionic crosslinking between a dicarboxylic acid and a protein. (Modified from Mitra et al., 2011a).

Figure 11. Proposed mechanism for covalent crosslinking between a dicarboxylic acid and a polysaccharide. (Modified from Hashem et al., 2013).

Figure 12. Summarized proposed mechanism for the crosslinking between genipin and a protein. (Modified from Yoo et al., 2011).

Figure 13. Ionotropic gelation by interaction between anionic COO^- groups on alginate and divalent metal ions (Ca^{2+}). (Modified from Sun et al., 2012).

Figure 14. The PEC formed between chitosan and alginate. (Source: Ostrowska-Czubenko & Gierszewska-Druzynska, 2009).

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Submission checklist

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Figure 1

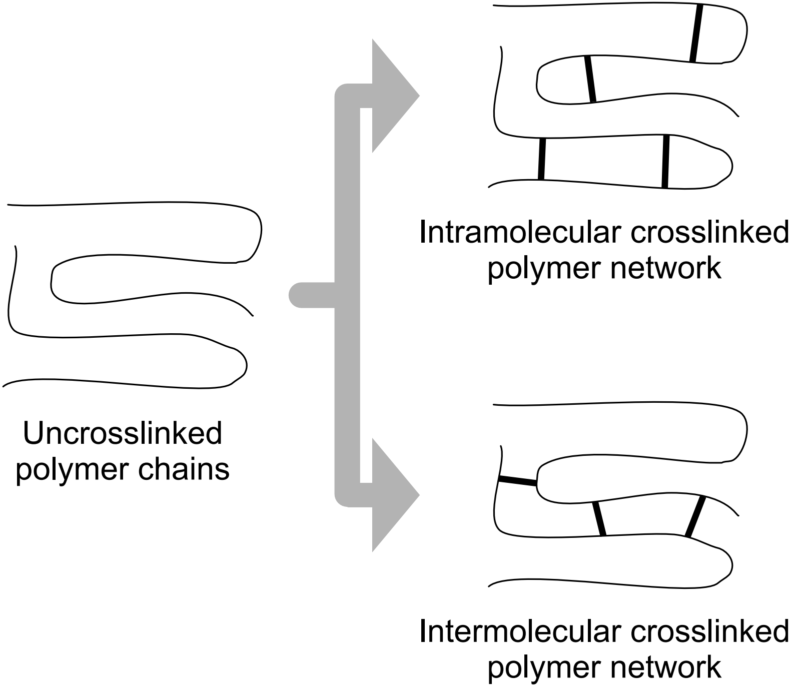
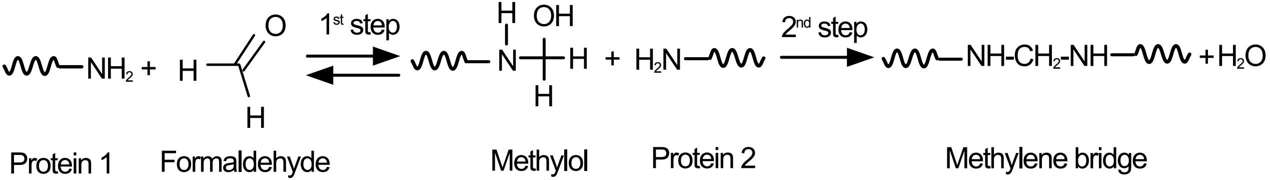

Uncrosslinked polymer chains
Intramolecular crosslinked polymer network
Intermolecular crosslinked polymer network

Figure 2



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2nd step
NH$_2$
+
H
H
N
H
H
+
H$_2$N
NH-CH$_2$-NH
+H$_2$O
Protein 1
Formaldehyde
Methylol
Protein 2
Methylene bridge

Figure 3

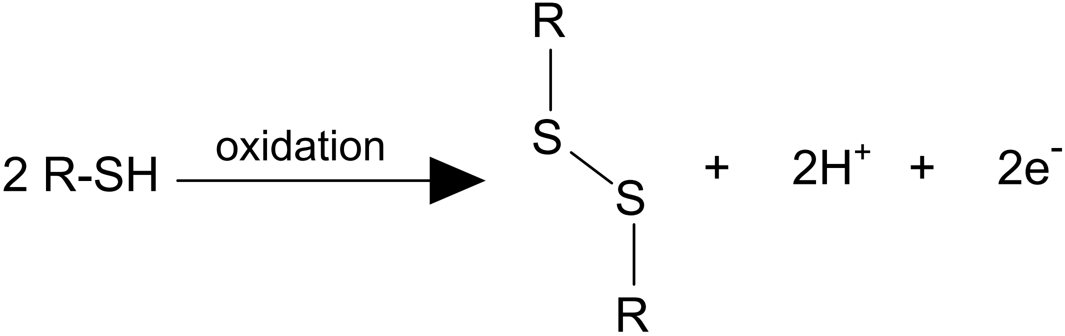
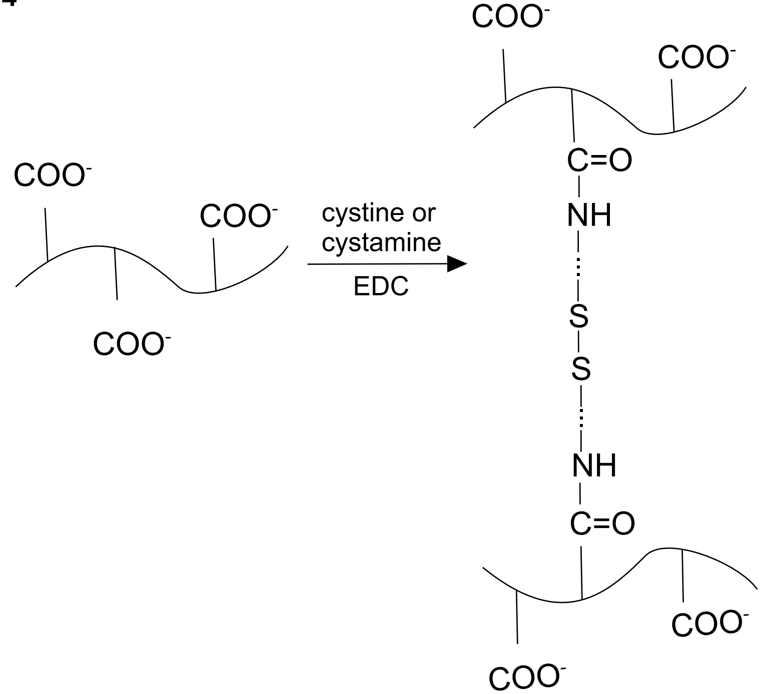


Figure 4



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C=O
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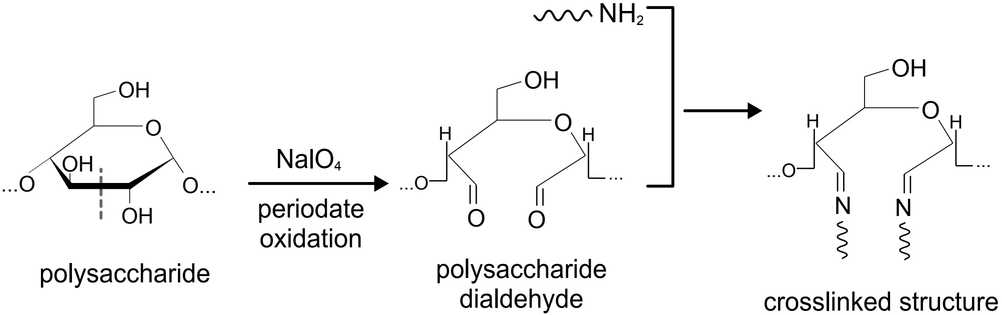
COO-

Figure 5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polysaccharide
dialdehyde
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N
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crosslinked structure

Figure 6

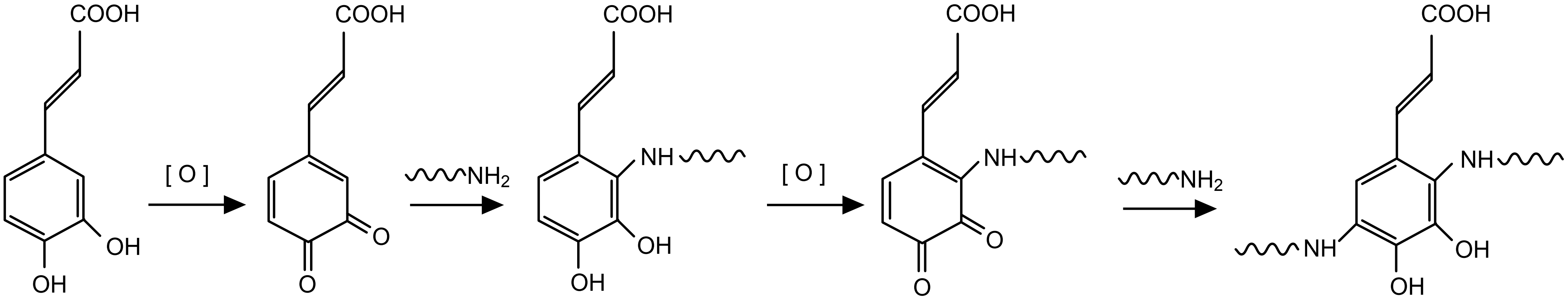
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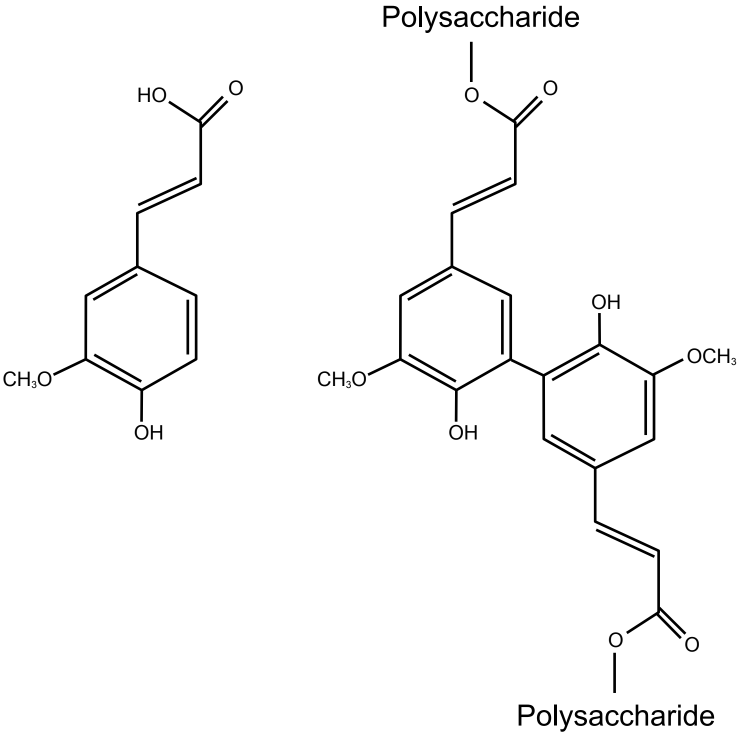
Figure 7



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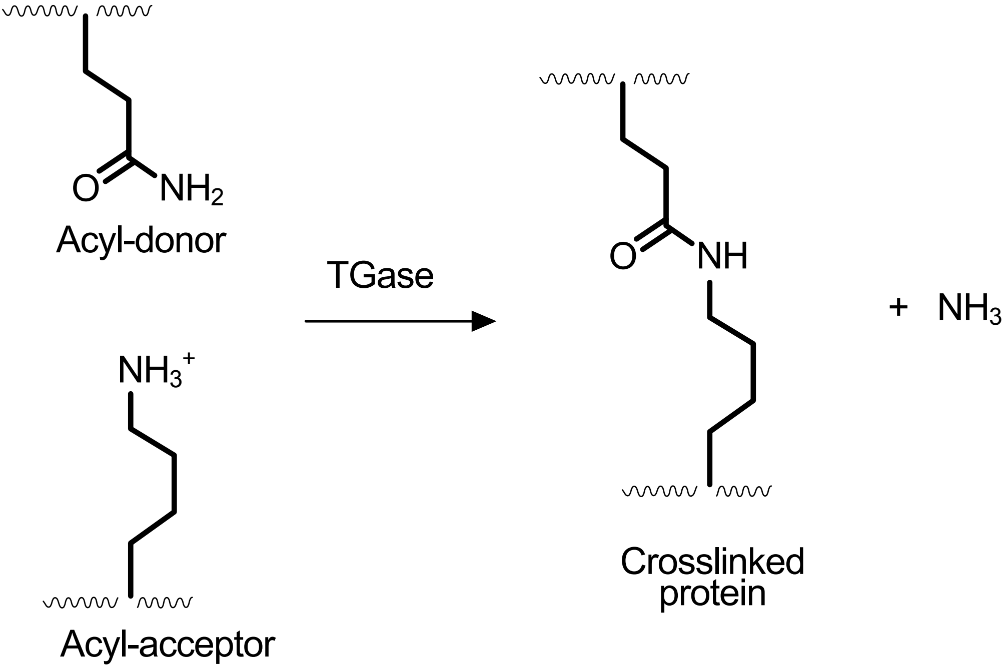
Polysaccharide

Figure 8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NH_2
Acyl-donor
NH_3^+
Acyl-acceptor
TGase
O
NH
+ NH_3
Crosslinked protein

Figure 9

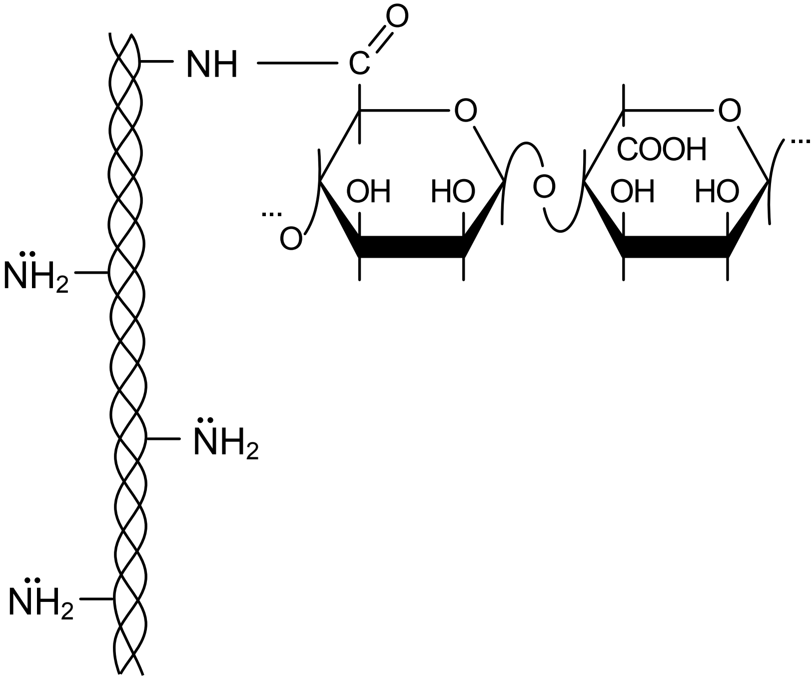


Figure 10

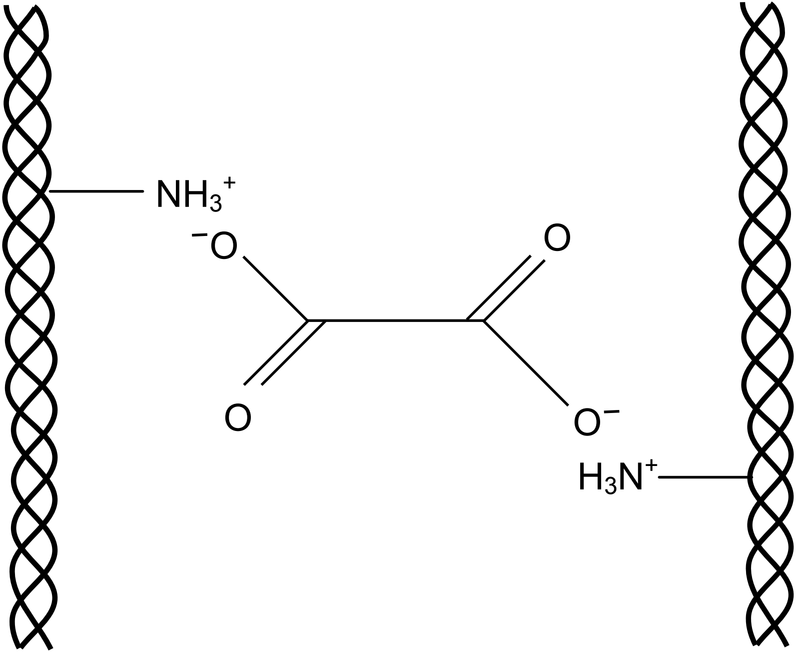
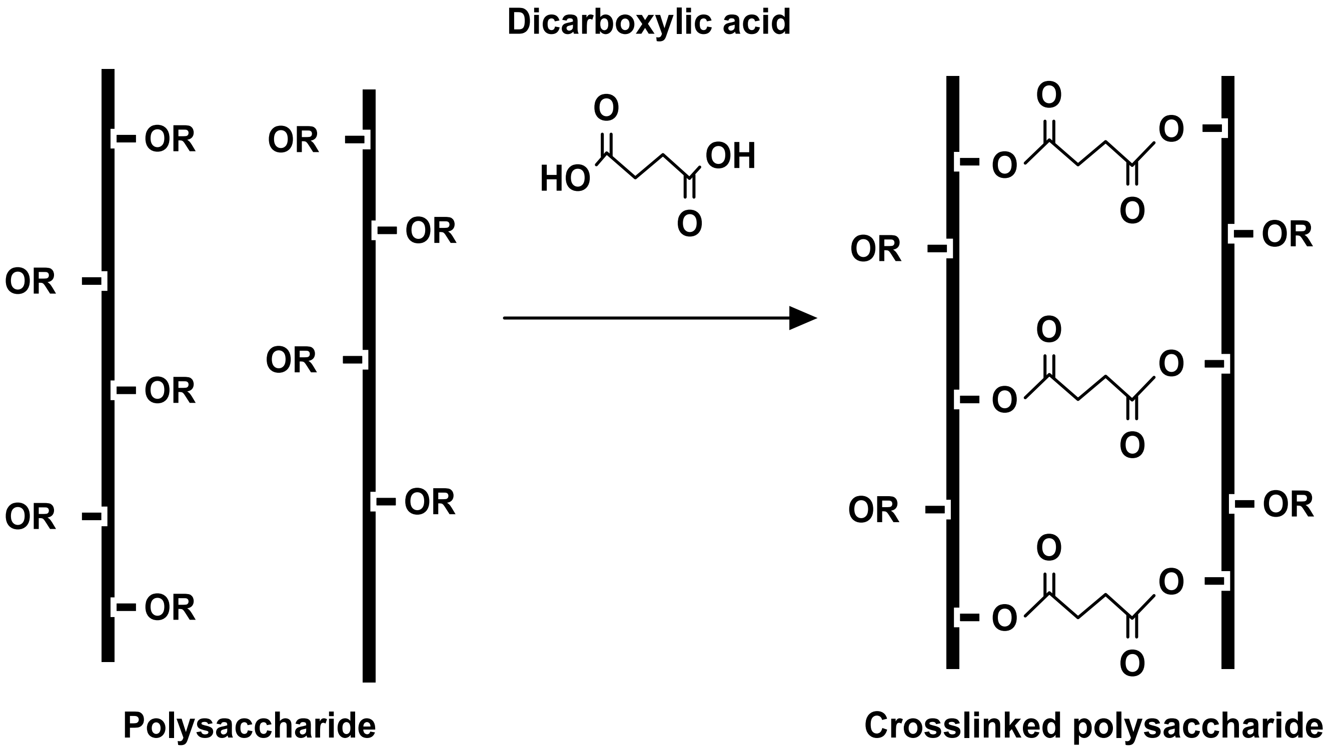


Figure 11


Dicarboxylic acid
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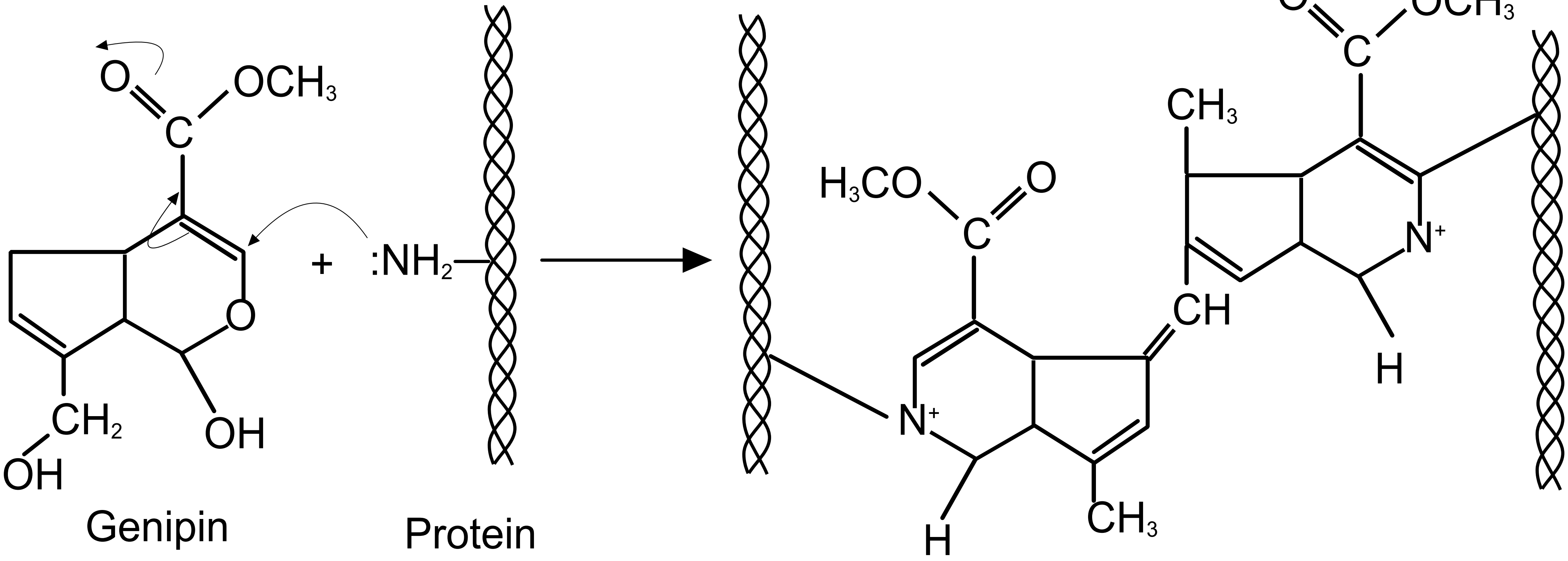
Polysaccharide
Crosslinked polysaccharide

Figure 12


O
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Genipin
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Figure 13

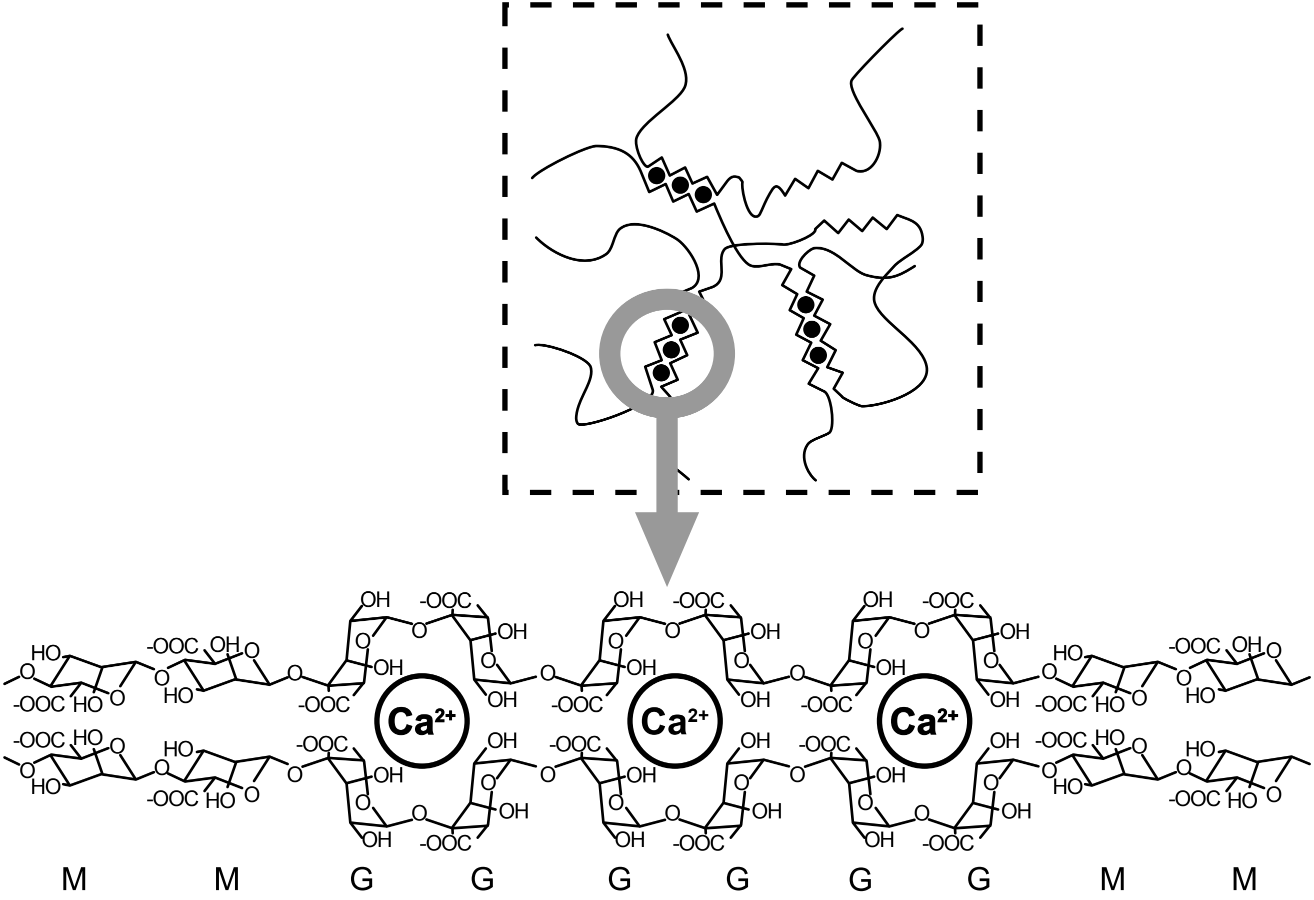
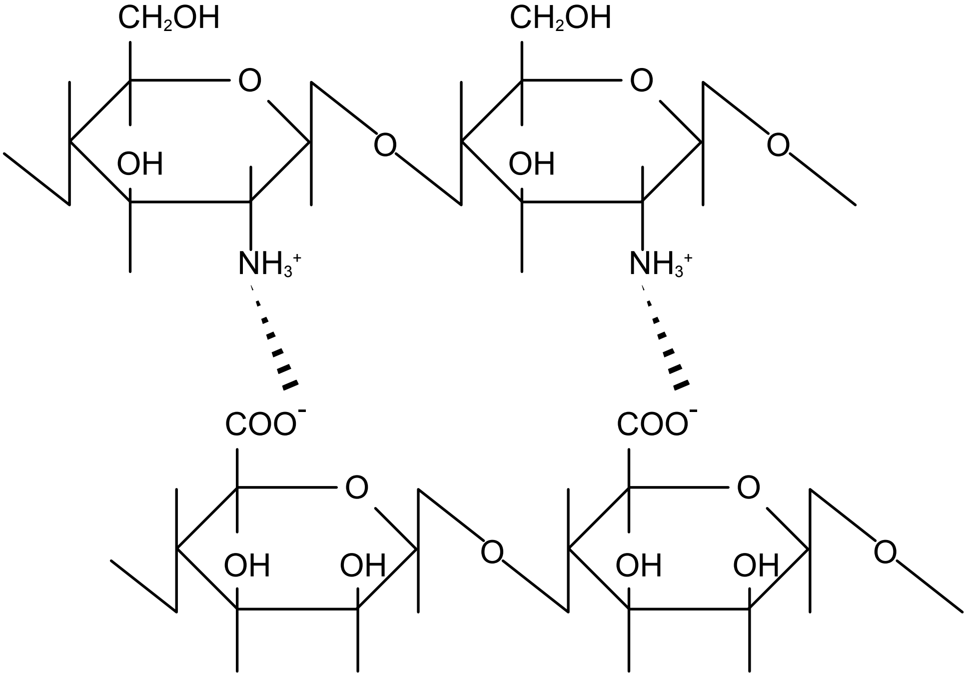


Figure 14



***Highlights (for review)**

Polysaccharide and protein films have been studied because of their biodegradability. > However, they have poor properties when compared to petroleum-derived polymers. > Crosslinking enhances film performance, notably water resistance. > Crosslinking mechanisms used for protein and polysaccharide films are reviewed. > The effects, applications and drawbacks of crosslinking agents are summarized.