Casein-based conjugates and graft copolymers. Synthesis, properties, and applications

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Casein-based conjugates and graft copolymers. Synthesis, properties, and applications

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Abstract

Biobased natural polymers, including polymers of natural origin such as casein, are growing rapidly in the light of the environmental pollution caused by many mass-produced commercial synthetic polymers. Although casein has interesting intrinsic properties, especially for the food industry, numerous chemical reactions have been carried out to broaden the range of its properties, most of them preserving casein's nontoxicity and biodegradability. New conjugates and graft copolymers have been developed especially by Maillard reaction of the amine functions of the casein backbone with the aldehyde functions of sugars, polysaccharides, or other molecules. Carried out with dialdehydes, these reactions lead to the cross-linking of casein giving three-dimensional polymers. Acylation and polymerization of various monomers initiated by amine functions are also described. Other reactions, far less numerous, involve alcohol and carboxylic acid functions in casein. This review provides an overview of casein-based conjugates and graft copolymers, their properties, and potential applications.

KEYWORDS
casein, conjugates, graft copolymers, Maillard reaction, polysaccharides

1 | INTRODUCTION

For many years, materials based on natural products have been the focus of considerable attention, due to their environmental impact and, in many cases, their absence of toxicity. Among these, materials of animal origin, such as casein, chitosan (CS), or collagen, are becoming more and more important.

Casein is a protein that is the main nitrogen component of milk. Casein is an inexpensive protein found in milk (80% by mass in cow's milk) and dairy products. It is obtained by precipitating milk at an acidic pH (4.6). Casein has important nutritional properties: It is a "complete" protein, that is, it contains all nine essential amino acids our body needs to start building muscle. An important property is that it digests very slowly, which is of great interest in sports nutrition. Casein is used in the food industry, notably in cheeses, and as an additive for its thickening and emulsifying properties. Casein is also the base for nonfood applications (coatings [paint, ink, paper, and packaging], adhesive, plastic, or surfactant) (Audic et al., 2003) and pharmaceuticals. Casein is also used in the textile industry for the manufacture of artificial fibers (Bier et al., 2017; Yang & Reddy, 2012).

From an industrial and economic point of view, according to data from Future Market Insights, the global casein

market in 2023 is $2.7 billion and expected to reach a value of $4.9 billion by 2033, with a compound annual growth rate of 6.3% between 2023 and 2033 (Future Markets Insights, 2023). This growth is mainly due to increasing demand for high-quality, functional dairy products, as well as growing demand for casein in the pharmaceutical and cosmetics industries (Observatory of Economic Complexity, 2021). The main current exporters are New Zealand, Ireland, the Netherlands and France. Casein's market share is currently estimated at around 35% for the food industry, 20% for paints and coatings, and 20% for pharmaceuticals.

From a structural point of view, casein comes in four different structures: αS1 (36%), β (37%), κ (10.5%), αS2 (12%), and γ (4.7%) (Bonfatti et al., 2011). Each casein structure (Yang & Reddy, 2012) has a specific amino-acid sequence, resulting in different properties, especially in terms of hydrophobicity/hydrophilicity. Casein chain contains numerous amino acids, some of which are responsible for casein's chemical reactivity. Thus, a casein chain contains approximately 14 lysine (NH_2 groups), 30 serine/threonine/tyrosine (OH groups), and 30 aspartic/glutamic acids (COOH groups). As the carboxylic acid/amine ratio of the casein backbone is greater than 1, an aqueous casein solution is slightly acidic, with an isoelectric point around pH = 4.6. Casein is thus soluble at basic pH (as caseinate) and becomes almost insoluble at slightly acidic pH around 4.5–5. Sodium caseinate is a water-soluble form of casein obtained by solubilizing casein in a sodium hydroxide medium. In the following, we will use the terms casein or caseinate indifferently, considering that we are referring to the same compound in two pH-dependent forms. The main functional properties of casein are water solubility, viscosity, gelation, emulsifying, and foaming properties. Casein has a major application as an additive in food industry (Wusigale et al., 2020), but increasingly in other areas, such as emulsifiers (Liao et al., 2022; Ma & Chatterton, 2021), paint (Nevin et al., 2008), adhesive (Tundisi et al., 2021), packaging (Khan et al., 2021), incorporation and delivery of active drugs (Głąb & Boratyński, 2017; Nascimento et al., 2020; Sadiq et al., 2021), and textile manufacturing (Belkhir et al., 2021). The industrial production, specifications, and regulatory aspects of casein are summarized in a previous review (O'Regan & Mulvihill, 2011).

Many useful properties of casein, such as gelation (Horne, 2002; Nicolai & Chassenieux, 2021; Vasbinder et al., 2004), viscosity (Dunn et al., 2021; Hayes et al., 1968), emulsification, micelles, and foam formation (Hassan et al., 2023; Kuan et al., 2011; Lam & Nickerson, 2013; Sánchez & Patino, 2005), are due to its amphiphilic structure (Broyard & Gaucheron, 2015). The major structures and uses of casein micelles, especially in the field of drug delivery, are described in a review (Rehan et al., 2019). Casein micelles can be used to incorporate hydrophobic bioactives, food products, low-fat products, essential oils, vegetable oils, and polyphenols. The role of beta casein is particularly important for the formation of nanomicelles, given its rather low critical micellar concentration (CMC) between 0.05% and 0.2% w/v (Kharisov et al., 2016).

Furthermore, casein has some filmogenic properties, which allow the production of film-forming emulsions and edible films (Hiemenz & Rajagopalan, 1997). Casein-based films have good oxygen permeability thanks to the polar groups (amines, alcohols/phenols, and carboxylic acids) in its chain. This results in a material used as edible coatings and films, but the presence of these polar groups results also in a high water vapor permeability and a poor water resistance that reduces applications in packaging (Mihalca et al., 2021). Packaging is a key element in the transport and conservation of foodstuffs, protecting them from various external factors (light, water vapor, oxidation, dust, microbes, etc.). Today's food packaging is based on synthetic polymers, almost all of which are nonbiodegradable and are derived from nonrenewable petroleum-based sources, giving rise to serious and well-known environmental problems. (Bio)degradable packaging is made from (co)polymers derived from both renewable natural products (cellulose derivatives, poly lactic acid (PLA), poly α-hydroxy acids (PHA)), and synthetic products (poly-ε-caprolactone etc.). Degradable edible films are now widely used for various food categories, such as meat products, vegetables, or dairy products. In particular, proteins are proving to be excellent materials for edible and nonedible coatings and films (Mihalca et al., 2021). Casein is undoubtedly considered a sustainable raw material (Indrie et al., 2023; Verrillo et al., 2023). It is used as a sustainable flame retardant for cotton, acrylic coatings, PLA polypropylene, or polyurethane (Cobos et al., 2023). Recently, a company (lactips) manufactured and marketed a casein-based polymer for packaging in granulated form via a twin-screw corotative extruder and a film blowing machine (Belyamani et al., 2014; Chevalier et al., 2018).

More generally, despite its many qualities, casein has shortcomings, such as excessive hydrophilicity, processing complexity, high degradation rate, poor machinability, poor mechanical properties, and low environmental resistance (particularly to water), which considerably limit the possibilities for industrial applications. For example, casein-based films are quite brittle and it is difficult to get films on which tensile tests can be performed properly (Chevalier et al., 2018). These films are not stretchable, have poor water resistance, and are easily susceptible to mold and crazing (Wang et al., 2004). Therefore, in many film formulations, a plasticizer (usually glycerol) should be added that generates a free volume to improve film flexibility (Chevalier et al., 2018). In the food industry glycerol is also known as the additive E422 that preserves food from

desiccation. But the additive eventually migrates out of the films. Tannins are occasionally added to improve some properties of casein (increase in antioxidant and antimicrobial activities and decrease in water solubility, vapor permeability, and stretchability) (Cano et al., 2020).

To improve the properties of casein and to confer new ones, it is necessary to make structural modifications to the protein. These modifications can be (i) of physicochemical nature where only secondary bonds are involved or (ii) of chemical nature involving new covalent bonds. In both cases, polar groups of casein (amines, alcohols, and carboxylates), which are also reactive functions, are involved.

Physicochemical modifications are generally obtained by mixing, with or without heating, where the primary structure of the casein is generally preserved. Complexes are also formed by changes in pH or pressure, by the addition of multivalent ions of chelating agents or ligands (Broyard & Gaucheron, 2015). The addition of folic acid (vitamin B9) to increase the stability of casein (Bourassa & Tajmir-Riahi, 2012) or the complexation with curcumin to give nano-formulations are also noted (Sahu et al., 2008). But in most cases, the physicochemical modifications are relatively unstable because they only involve a low stabilization energy and therefore are unsuitable for most industrial applications.

Chemical modifications are therefore preferred to obtain medium and long-term changes. Chemically, the main reactive functions in casein are the primary amine (from lysine), alcohol/phenol (from tyrosine, serine, and threonine), and carboxylic acid groups (from aspartic and glutamic acids) (Broyard & Gaucheron, 2015).

2 | CHEMICAL REACTIONS ON THE AMINE FUNCTIONS OF CASEIN

Among all the reactive functions of the casein backbone, the lysine-derived amine functions are by far the most widely used to produce new conjugates or graft copolymers with a wide range of properties. They are mainly Maillard reactions and, to a lesser extent, acylation reactions.

2.1 | Maillard reactions

Maillard reactions and their applications in the food industry have been known universally for a very long time. They produce flavors and aromas during cooking process. They are nonenzymatic browning reactions between amino acids and reducing sugars. They involve the free amine functions of casein reacting with the aldehyde or hemiacetal functions of sugars or polysaccharides, leading to a glycosylamine that can evolve into an imine and then a ketosamine via an Amadori rearrangement (O'Mahony et al., 2017).

Unlike other chemical methods, Maillard reactions are sometimes spontaneous and natural and do not require the addition of other chemicals, which may be of interest in the food and biomedical fields. In addition, after coupling with sugars or polysaccharides, the functionality of the conjugate is improved over that of casein, due to the presence of many new alcohol functionalities.

2.1.1 | Reactions of sugars and polysaccharides on casein

The reactions of sugars with casein are well documented in the literature. Most casein–sugar conjugates are prepared by a dry-heating method where caseinate and sugar are dissolved in phosphate buffer at pH = 7, freeze-dried, and the lyophilized mixture is typically heated to 50–60°C (Markman & Livney, 2012). The dry state is known as the conventional method, but it has the disadvantage of the formation of browning products, determined with a scanner and analyzed using ImageJ software, the proportion of which increases with reaction time (Tavasoli et al., 2022). The brown color was caused by the formation of (co)polymers containing nitrogen, such as melanoidins, which are produced through condensations in the last stage of the Maillard reaction. Dry heating leads to a decrease in solubility and a loss of digestibility due to the formation of hydrophobic bonds and uncontrolled cross-linking reactions (Zenker et al., 2020). The percentage of dissolved milk protein powders in the simulated milk ultrafiltrate depends on the reaction temperature: 75% for untreated casein, 30% when treated at low temperature (60°C) and 20% when treated at high temperature (130°C).

Casein–sugar conjugates are also prepared by wet heating, generally in phosphate buffer at 60–100°C for a few hours in a sealed vessel to hinder water evaporation. In this process, the conjugate is often recovered after dialysis against redistilled water and then dried under vacuum (Cardoso et al., 2011; Consoli et al., 2018). Grafting glucose or beta-cyclodextrin onto casein can also be achieved using a microwave-assisted wet heating approach, which has reduced reaction time to a few minutes and yielded compounds with improved gelation properties (Bi et al., 2015).

Glycation of casein with glucose, fructose, and lactose was described long ago for food applications (Lee et al., 1979). In the presence of 1000 M excess of sugar, the degree of modification was 80% with glucose, 62% with fructose, and 17% with lactose. Compared with native casein, sugar casein conjugates have been shown to have slightly lower in vitro digestibility by α-chymotrypsin and lower

nutritive values in rat feeding experiments. Significantly lower growth rates were observed in rats fed diets based on casein grafted with glucose, fructose, or lactose. Food intake and feed efficiency were also reduced in the groups receiving these casein derivatives. In addition, serious growth depression was observed in rats feeding glucose-derived casein. Despite this, there is currently growing interest in the reaction of casein with sugars and polysaccharides for other applications.

Generally, reactions with sugars are carried out at neutral or basic pH on sodium caseinate, which is more soluble than casein, due to the formation of carboxylate groups. It is noted that the molar mass, structure, and charge of the reagents are important in determining their reactivity in the Maillard reaction (O'Mahony et al., 2017). For example, the reactivity of sugars/polysaccharides decreases with increasing molecular weight, due to the contribution of steric hindrance as molecular weight increases: galactose (180 Da) > lactose (342 Da) ≫ dextran (10,000 Da). The percentage of modification of free amine functions in a casein-dextran conjugate is only 25.6% compared to 76.1% in galactosylated casein (Courthaudon et al., 1989). More generally, monosaccharides are more reactive with casein than disaccharides or oligosaccharides under conditions that favor conjugation (Corzo-Martínez et al., 2010). The main consequence is that glycation with dextran does not produce any substantial improvement in caseinate properties, particularly rheological properties, due to a limited extent of the reaction. Although degrees of modification close to 80% are obtained by coupling glucose or galactose, they are only 20% with fructose (a ketose instead of an aldose), demonstrating the greater reactivity of aldose compared with ketose (Courthaudon et al., 1989).

The success of the grafting reactions is confirmed by the increase in molar mass from 23,400 to 28,700 Da, analyzed by SDS–PAGE, in the case of maltodextrin grafting (Markman & Livney, 2012). Titrations of the remaining lysine residues with O-phthalaldehyde (Markman & Livney, 2012), trinitrobenzene sulfonate (O'Regan & Mulvihill, 2009), or by enzymatic galactose assay (Wang & Zhao, 2017) are used to measure the degree of glycation of lactose in casein and soybean proteins.

In a general way, the increase in solubility is a key feature of the reaction of casein with sugars. Glycation of casein with glucose, fructose, galactose, mannose, or lactose resulted in conjugates with improved water solubility, especially near the isoelectric point (pH ≅ 4.5) where casein is initially poorly soluble, as a result of the grafting of highly water-soluble sugars at any pH (Courthaudon et al., 1989). The solubility of 76% galactosylated casein at pH = 4.5 is eight times greater than that of unmodified casein at the same pH.

The biological activity of casein modified with sugars (glucose, fructose, and ribose) was evaluated in Caco-2 cell culture (Jing & Kitts, 2004). Casein–glucose conjugates show ferrous ion chelation and consequently antioxidant activity. A significant protective effect of Caco-2 cells against radical-induced cytotoxicity as well as iron and copper-induced toxicity was observed. Antioxidant and anti-inflammatory activities are higher in the galactose–casein conjugate than in the casein–glucose conjugate, although this remains to be explained (Oh et al., 2017). In the field of drug delivery, casein–glucose conjugate was used to form stable emulsion containing fish oil (1% w/w) that was delivered in simulated gastric (SG) and intestinal (SI) fluids. The amount of oil released is <2% in acidic SG and 36% in alkaline SI (Kosaraju et al., 2009).

To improve the variations in the properties of casein–sugar conjugates, sugars may be replaced by polysaccharides to give casein-*g*-polysaccharide copolymers in which the Maillard reaction introduces covalent bonds between proteins and carbohydrates, both of which are food constituents and sustainable compounds. As a result, the casein-*g*-polysaccharide copolymers obtained are both biodegradable and sustainable compounds. Moreover, they are all formed from compounds used in the food industry. Extensive research has been carried out to utilize this reaction to improve the functionality of proteins. For the same level of substitution, it increases the proportion of sugar units. However, from a chemical point of view, as mentioned above, the reactivity of polysaccharides with proteins is much lower than that of simple sugars, leading to lower grafting degrees due to (i) steric hindrance and (ii) the presence of a single reactive terminal carbonyl group at the chain end (Kato, 2002; Kato et al., 1992). Dry-heating method is preferred to prepare protein–polysaccharide conjugates due to ease of handling reagents and control of the reaction conditions. Some examples of casein-*g*-polysaccharide are discussed below.

Reactions of maltodextrin and dextran with casein have resulted in new types of casein-*g*-polysaccharide copolymers, as summarized in a recent review (Abd El-Salam & El-Shibiny, 2020). As for sugars, water solubility is the main effect of grafting dextran onto casein: *β*-casein is nearly insoluble (≅0.1 mg/mL) in water at its isoelectric point (pH = 4.6), whereas dextran is soluble in water at any pH. However, the solubility of a mixture of *β*-casein and dextran at acidic pH is the same as that of *β*-casein alone, implying that the simple addition of dextran has no effect on the solubility of the protein at acidic pH (Li, Wen, Wang, Liu, et al., 2022). In contrast, the solubility of casein-*g*-polysaccharide copolymers is much higher than that of casein alone (Wu et al., 2022). It is a reasonable hypothesis that the increased solubility of casein-*g*-polysaccharide is due to the formation of micelles in this pH range, where

the highly hydrophilic dextran is located on the micelle surface and protects the β-casein from further aggregation (Mu et al., 2006). Reaction time, casein/dextran molar ratio, and dextran molecular weight play an important role in the water solubility of casein-*g*-dextran: The copolymer becomes soluble at any pH when the degree of grafting and the molecular weight of dextran are sufficiently high (Pan et al., 2006). With 10,000 Da dextran, precipitation at pH 4.6 (isoelectric point of casein) can be prevented if dextran/casein ratio (mol/mol) in the copolymer is $\geq$1/2. For 1.5 and 6 kDa dextrans, precipitation occurs at pH = 4.6 no matter what the molar ratio is. Similarly, solubilities of casein-*g*-maltodextrin (35.7% w/v), casein-*g*-pectin (33.5% w/v), and casein-*g*-gum arabic (25.6% w/v) copolymers are significantly higher than that of casein (1.6% w/v) at pH 4.5 (Kumar et al., 2021).

Protein-*g*-polysaccharide compounds are almost always better emulsifiers than the original protein: The emulsifying activity of casein-*g*-dextran and casein-*g*-galactomannan is 1.5 times higher than that of casein (Kato et al., 1992). Casein-*g*-dextran shows also special emulsification properties, in terms of emulsification activity index (EAI) and emulsification stability index compared to casein (Li et al., 2023): EAI of casein-dextran10, casein-dextran40, and casein-dextran70 (10, 40, and 70 are the grafting percentages of 70 kDa dextran onto casein) increased by 12.8%, 28.6%, and 30.85%, respectively, as compared with genuine casein. The stability of the casein-*g*-polysaccharide emulsions—estimated by measuring the half-time of the turbidity immediately after the emulsion had formed—is 10 times higher than that of casein (Kato et al., 1992). Another advantage with casein-*g*-polysaccharide conjugates is their emulsion stability in acidic and high-salt concentrations. Casein-*g*-dextran nanoparticles are more stable to aggregation than casein nanoparticles, even under simulated gastrointestinal conditions, probably due to greater steric repulsion as a result of the presence of dextran chains (Davidov-Pardo et al., 2015).

Maillard conjugates of casein also lead to improved physicochemical properties (zeta potential, viscosity at constant shear, microstructure, etc.) as in the case of the casein–sugar beet pectin conjugate (Zhang & Wolf, 2019). Surface hydrophobicity, emulsifying capacity, and stabilizing capacity are dependent on the molar mass of the grafted dextran: Casein-*g*-dextran formed from intermediate molecular weight dextrans (MM = 40,000 and MM = 70,000) showed enhanced emulsification activity and stability as well as improved stability during in vitro digestion (Liu et al., 2019). Casein-*g*-dextran is used as a stabilizer for fish oil emulsions (sources of polyunsaturated fatty acids) and the presence of casein-*g*-dextran in the gastrointestinal tract influences zeta potential and mean particle diameter, resulting in a significant delay in fatty acid release (Liu et al., 2019; Wei et al., 2018). In addition to their stability to acidic conditions, casein-*g*-dextran emulsions are also resistant to enzymatic digestion by pepsin, thus avoiding problems of premature release of encapsulated lutein (a natural colorant or nutraceutical present in many foods) in the stomach (Gumus et al., 2016). The hydrodynamic diameter of micelles is around 100 nm, compared with 10–15 nm for the casein alone, with a CMC around 10 mg/L, a particularly low value (Mu et al., 2006). As expected, the hydrodynamic diameter of the micelles increases with the molar ratio and the molecular weight of the grafted dextran.

Casein-*g*-dextran micelles can incorporate insoluble β-carotene into a micellar core–shell structure, to be further released by hydrolysis in the presence of pepsin or trypsin (Pan et al., 2007). The nanoparticles are formed with casein and β-carotene core and dextran shell. They have a spherical shape and are stable in aqueous solution against dilution, pH change, ionic strength change, oxidation, and storage. Self-assembled casein-*g*-dextran micelles act as protective carriers for curcumin and are superior to casein micelles for curcumin radical scavenging activity (Wu & Wang, 2017).

Synthesis and emulsifying properties (Semenova et al., 2016; Shepherd, 2000) and structural and thermodynamic characteristics (Grigorovich et al., 2012) of casein-*g*-maltodextrin are described. These copolymers form core–shell co-assemblies for the nanoencapsulation of hydrophobic nutraceuticals, including vitamin D, in beverages (Markman & Livney, 2012). They also provide enhanced protection against oxidation of omega-3 and omega-6 fatty acids (Semenova et al., 2016). They enhance the stability, in vitro release, antioxidant properties, and cell viability of eugenol-olive oil nano emulsions (Nagaraju et al., 2021).

Similarly, casein–carrageenan conjugates have been used to improve the stability of oil-in-water emulsions (Seo & Yoo, 2022), to microencapsulate the red pigment of paprika, and to confer an exceptional protective effect of the microcapsules on this red pigment (Qiu et al., 2018).

Grafting of CS onto casein has been showed to improve the proliferation and osteogenic differentiation of human mesenchymal stem cells (Li et al., 2016). It also improves the stability of emulsions, the antioxidant activity, the inhibition of lipid oxidation (Wu & Wang, 2017) and facilitates the incorporation and release of curcumin (Meng et al., 2021).

The reaction of casein phosphopeptides (CPP) (a type of protein containing multiple surfactant groups) on soluble dietary fibers (polysaccharides that contain large amounts of active surface groups) is used to bind calcium ions: An increase in calcium binding of up to 46.8% over the original

TABLE 1 Sustained-release systems from casein-*g*-polysaccharides.

Copolymer	Drug	References
Casein-g-dextran	β-carotene	Pan et al. (2007)
Casein-g-dextran	Curcumin	Wu and Wang (2017)
Casein-g-maltodextrin	Hydrophobic nutraceutical	Markman and Livney (2012)
Casein-g-maltodextrin	Fatty acids	Semenova et al. (2016)
Casein-g-maltodextrin	Eugenol	Nagaraju et al. (2021)
Casein-g-carrageenan	Red pigment	Qiu et al. (2018)
Casein-g-chitosan	Curcumin	Meng et al. (2021)
CPP/oligo-chitosan	Calcium ions	Gao et al. (2018) and Zhu et al. (2020)

Abbreviation: CPP, casein phosphopeptides.

CPP is observed (Gao et al., 2018). New calcium binding and delivery systems based on casein-*g*-(oligochitosan) copolymers have recently been proposed, prepared by Maillard reaction with or without transglutaminase linkages (Zhu et al., 2020). They prolong the release of calcium from delivery vehicles in digestive system. These calcium delivery systems could promote bone synthesis to prevent calcium deficiency but also promote SI health.

Some delivery systems from casein-*g*-polysaccharide are listed in Table 1.

Rheological properties are also enhanced after glycation of sodium caseinate (Corzo-Martínez et al., 2010; O'Mahony et al., 2017). An increased viscosity with a non-Newtonian behavior was observed after reaction of casein with fructose or fructo-oligosaccharides (Oliver et al., 2006). Gelation temperature of sodium caseinate/maltodextrin dispersions increased by 7°C after conjugation (Zhang, Versteege, et al., 2020).

In conclusion, most Maillard reactions of casein with sugars or polysaccharides are aimed at forming new water-soluble stable self-assemblies in aqueous media for various food, environmental, or biomedical applications (Lee et al., 2017). It should be noted, however, that studies on the degradation products of compounds obtained with Maillard reactions and their possible toxicity are still under discussion (Wei et al., 2018).

2.1.2 | Crosslinking reactions on casein

Maillard reactions can be applied to a dialdehyde instead of a monoaldehyde, resulting in casein cross-linking reactions. As a rule, the properties of casein-based compounds are quite radically different before and after crosslinking, in terms of solubility, mechanical properties, degradation, and so on. Applications after crosslinking are mainly related to drastically reduced water solubility, improvement of mechanical properties, and obtaining hydrogels and solid drug delivery devices. A clear advantage of these systems is that they give biocompatible, biodegradable, and inexpensive materials. Furthermore, in vivo studies have shown that rats fed with cross-linked casein had a normal growth rate compared to rats fed with native casein (Seguro et al., 1996). The main chemical crosslinking agents are glutaraldehyde (GTA), glyoxal, and formaldehyde (a monoaldehyde) (Nascimento et al., 2020). Their structures are shown in Figure 1.

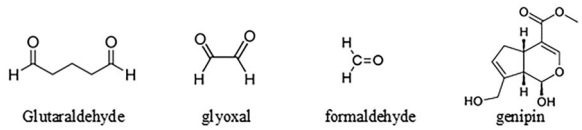

FIGURE 1 Main crosslinking agents of casein-based compounds.

Crosslinking with glutaraldehyde

The reaction of sodium caseinate with GTA resulted in a cross-linked material (Figure 2) with a significant decrease in solubility, a shrinkage and improved mechanical, thermal, degradation, and barrier properties (Muthulakshmi et al., 2022; Pereda et al., 2010).

Depending on the dry or wet method used to prepare cross-linked casein, total soluble matter decreases with the percentage of GTA added, due to the formation of a denser network (Table 2). Mechanical properties (Table 2), film shrinkage, and yellowish coloration generated by the Schiff base increased with GTA content (Pereda et al., 2010).

GTA-cross-linked casein formulations are used as bioprostheses (vascular grafts, ligament substitutes, and pericardial grafts…) (Jayakrishnan & Jameela, 1996). Tissue valves made from GTA-cross-linked casein have the advantage of being biocompatible, non-thrombogenic, and not requiring anticoagulant therapy after implantation.

Applications of the GTA-cross-linked casein in drug delivery systems in the forms of films, composites,

TABLE 2 Percentages of total soluble matter and mechanical properties of casein-based glutaraldehyde (GTA)-crosslinked films.

GTA%	Total soluble matter		Modulus E (MPa)	Stress σ_b (MPa)	Strain ε_b (%)
	Dry method	Wet method			
0	25	100	251	6.3	63
3	18.5	30	132	3.3	58.5
5	15.4	25.4	250	5.8	37
10	13.1	16.7	448	9.1	11.3

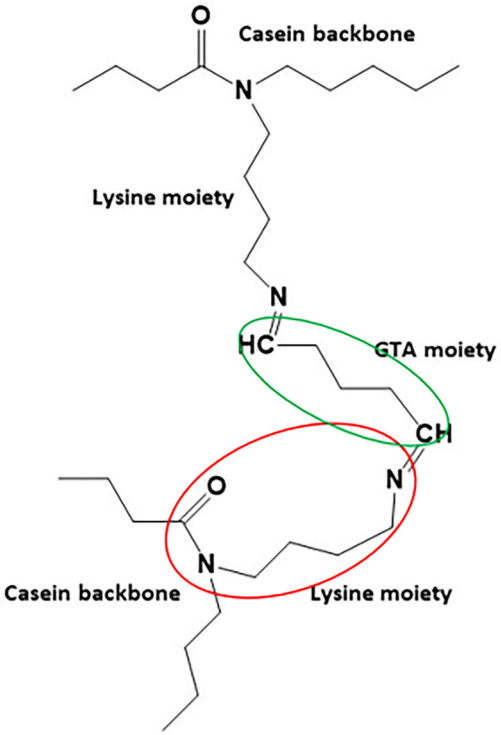

FIGURE 2 Scheme of the chemical crosslinking of sodium caseinate with glutaraldehyde (GTA).

hydrogels, beads, and micro and nanoparticles are described (Elzoghby et al., 2011; Nascimento et al., 2020), including for the delivery of progesterone (Latha et al., 2000), theophylline (Latha et al., 1995), curcumin (Wang et al., 2016), methotrexate (Vino et al., 2011), and diethylstilboestrol (Bayomi & El-Sayed, 1994). As expected, in all cases a reduction in drug release rate was observed.

GTA-cross-linked casein is also used as a matrix to promote regeneration of peripheral nerve in adult rats. A rapid morphological and functional recovery for disrupted rat sciatic nerves repaired with the GTA-cross-linked casein conduits is observed (Wang et al., 2011). A mixture of sodium caseinate and a conjugate stearic acid–CS was cross-linked in the presence of GTA to give polymeric nanoparticles with exceptional gastrointestinal stability as oral delivery vehicles for lipophilic bioactives. Internalization of the nanoparticles by Caco-2 cells showed the non-cytotoxicity of the copolymer. A successful encapsulation and sustained release of curcumin were also demonstrated (Hu et al., 2019).

However, the toxicity of residual GTA is still the subject of debate, even though this compound is widely used (Takigawa & Endo, 2006; Xu et al., 2016). Some inflammatory reactions, cytotoxicity, or calcification have been attributed to residual GTA, but this mainly depends on the amount used for crosslinking (McPherson et al., 1986). Residual GTA in bioprostheses has been involved in calcification as well as in local cytotoxicity inducing endothelial cell death (Moritz et al., 1991). This appears to be a feature of GTA cross-linking, which requires excellent purification of the resulting products, especially for biological applications.

Crosslinking with glyoxal

As for GTA, crosslinking of casein with glyoxal (at 80°C or under UV irradiation) leads to improved mechanical (improved E-modulus, yield strength and strain at break, reduced ductility) and degradation properties, giving potential applications in the biomedical field when long-term treatment is required (Silva et al., 2003; Vaz et al., 2003). The cross-linking effect of glyoxal on casein is shown by the prolonged release of lysozyme with sustained antimicrobial effects as compared to physically cross-linked films obtained by pH variation (Mendes de Souza et al., 2010). Moreover, the amount of glyoxal required is very low: For a glyoxal content of 0.05%, a small but already remarkable effect on lysozyme release was observed. For glyoxal contents of 0.5% and 1% more radical effects are observed, with a more obvious prolongation of lysozyme release. On the other hand, a slight toxicity is also observed on the product after cross-linking, probably due to the persistence of traces of glyoxal (Silva et al., 2003).

Crosslinking with formaldehyde

As formaldehyde is a mono aldehyde, crosslinking is carried out in two steps (i) formation of a α-amino alcohol by reaction of formaldehyde on an amine function of casein and (ii) reaction of this α-amino alcohol on another casein amine function giving a methylene bridge (Figure 3) (Audic & Chaufer, 2005).

Formaldehyde-cross-linked casein is typically prepared from a caseinate/formaldehyde dispersion in water that is left to react with gentle stirring at room temperature at

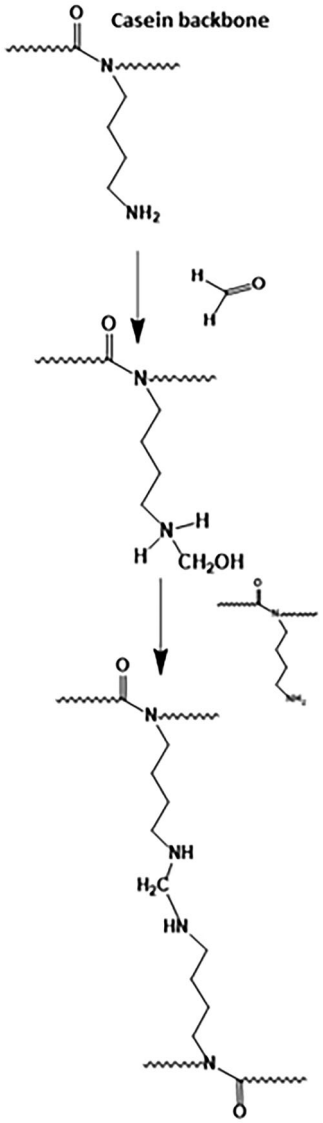

FIGURE 3 Reaction scheme of casein crosslinking with formaldehyde.

pH = 8.6 for 6 h. This method resulted in biodegradable films with drastic decrease in water solubility, improved water resistance (Audic & Chaufer, 2010), and thermal stability (Somanathan et al., 1997). However, crosslinking by formaldehyde cannot prevent the exudation of triethylamine (TEA), a hydrophilic plasticizer, when a cross-linked film is placed in an aqueous medium: After 10 min immersion in distilled water, around 80% of the TEA has migrated out of the film, regardless of formaldehyde content (Audic & Chaufer, 2010). Here again, the potential toxicity of residual formaldehyde that could be released from the films should also be highlighted.

Crosslinking with other crosslinkers

In general, the main drawback of the inexpensive chemical aldehydes as crosslinking agents is their potential cytotoxicity or that of their byproducts, although most of the prepared materials are considered nontoxic. Given these uncertainties about potential toxicity, citric acid, regarded as nontoxic, may be used as a crosslinking agent. Fibers cross-linked with 1% citric acid show nearly twice the strength, about 10 times improvement in elongation, and approximately 30% increase in modulus compared to the undrawn and non-cross-linked fibers. However, the resulting films were not favorable to attachment and growth of mouse fibroblasts and were therefore considered cytotoxic (Yang & Reddy, 2012). The use of a low-cost plant-derived phenolic compound, tannic acid (TA), as crosslinking agent for producing plasticized casein films is also described (Picchio et al., 2018). Like most cross-linked casein derivatives, films formulated by TA retard water vapor transmission, improve water resistance without degrading mechanical strength, have a higher modulus of elasticity and excellent UV-blocking properties.

Other natural compounds such as genipin and transglutaminase, given their lower toxicity compared to synthetic aldehyde-based crosslinking agents, are frequently used for casein crosslinking. Genipin (structure in Figure 1), with its very low toxicity (Hobbs et al., 2018), is preferred for food and medical applications despite its high cost. Genipin is actually 10,000 times less cytotoxic than the commonly used GTA solution (Sung et al., 1999). Genipin-cross-linked casein (GCC) has resulted in edible and biodegradable films with lower water vapor permeability than genuine casein, which can be used as food packaging materials (Lin et al., 2020). In this case, the cross-linked bridge consists of polymerized genipin macromers or oligomers (7–88 monomers units) via ring-opened intermediates (Mi et al., 2005). GCC (genipin was used at 1 mg/g sodium caseinate) gives films with enhanced mechanical and antimicrobial properties when lysozyme is incorporated (Qiu et al., 2020). GCC also gives hydrogels used in controlled drug delivery of bovine serum albumin (Song et al., 2009). The authors propose a possible crosslinking mechanism for GCC (Figure 4).

Depending on the amount of genipin used, the casein hydrogels obtained show various swelling and drug release characteristics under simulated gastrointestinal tract conditions: The release rate and the proportion of BSA released are functions of both pH and the genipin proportion used for cross-linking: the higher the genipin proportion, the lower the release rate. Release rate is also faster at physiologic pH (pH = 7.4) than at acid pH (pH = 1.2).

Casein nanomicelles cross-linked with different amounts of genipin yield microgels particularly resistant to dissociating agents such as citrate or urea and with gradual changes in their colloidal properties (Silva et al., 2014). The cross-linking reaction is intramicellar, as no aggregated particles are observed by DLS. These

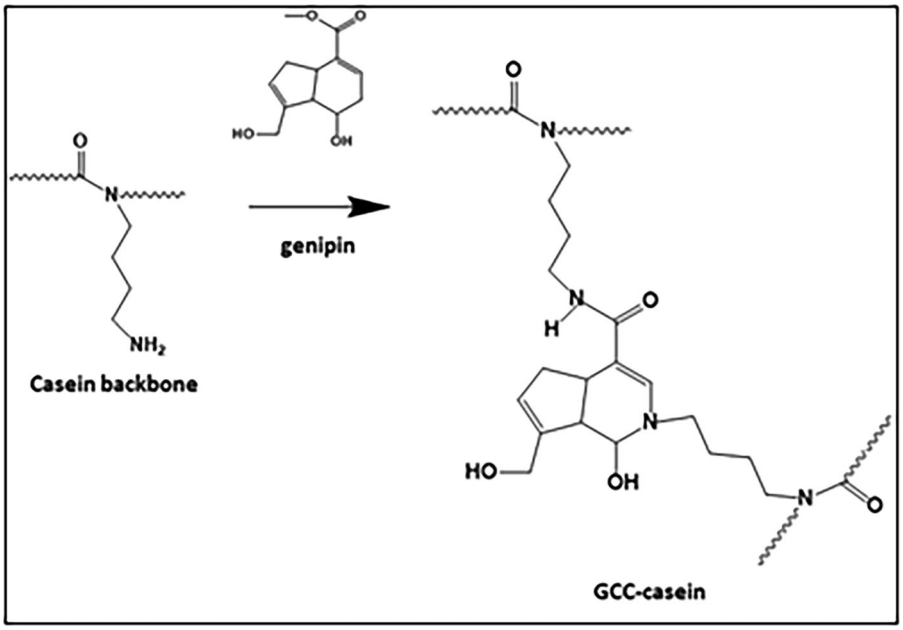

FIGURE 4 A possible crosslinking mechanism for the reaction of casein with genipin in water.

cross-linked nanomicelles can also be used for prolonged release of alfuzosin hydrochloride (Elzoghby et al., 2013). In the same way, a β-casein-*g*-dextran copolymer cross-linked by genipin gives stable nanomicelles with diameters in the range 28.4–53.7 nm that can be loaded with naringenin (Li, Wen, Wang, Wang, et al., 2022). The controlled drug release properties of naringenin and the resistance to attack by pepsin and trypsin were enhanced. A casein/CS mixture cross-linked with genipin yields microparticles for oral delivery of curcumin (Razi et al., 2018) or nattokinase, a thrombolytic enzyme used for the prevention and cure of thrombosis-related cardiovascular diseases (Zhang, Lyu, et al., 2020). Genipin-cross-linked CPP-CS nanoparticles show great potential for oral delivery of polyphenols (Hu et al., 2014). It has been shown that the anticancer activity of polyphenols is maintained in loaded nanoparticles.

As a conclusion, genipin is the most widely used cross-linking agent for all kinds of health and food applications, thanks to its nontoxicity.

However, enzymatic crosslinking of casein with low toxicity transglutaminase (TCC) is also widely mentioned in the literature for food and biomedical applications (Chambi & Grosso, 2006; Raak et al., 2020). Generally speaking, protein modification by TG can be exploited in a number of food industry applications, as it improves rheological properties, gel formation, protein solubility, foaming properties, and water retention properties (Jaros et al., 2006; Matheis & Whitaker, 1987; Nonaka et al., 1992). Historically, the use of transglutaminase in the food industry started with the manufacturing of surimi (fish paste) products in Japan or more generally in seafood (Kuraishi et al., 2001). The production of restructured meat using transglutaminase and sodium caseinate is more convenient. Cross-linking has great potential for the development of high-quality restructured foods. There is no loss of taste and flavor, and the strong binding is preserved even during cutting, freezing, and cooking (Kuraishi et al., 1997). However, although its acute toxicity appears to be very low, there are questions about its responsibility in the transfer of acyl groups between lysine and glutamine, and about the possibility of mutagenicity (Bernard et al., 1998).

The stabilization of emulsions or nano/microparticles is probably the most outstanding characteristic of TCC: TCC is used to stabilize casein-based emulsions or microparticles (Gebhardt et al., 2022; Ma et al., 2012; Zeeb et al., 2013). The cross-linked micelles obtained by TCC are stable to disruption by urea or citrate (Huppertz et al., 2007). β-casein micelles, cross-linked at 35°C with transglutaminase, are less susceptible to monomerization (O'Connell & Fox, 1999). In the presence of transglutaminase, a mixture of casein and octylamine gives materials with an increased surface hydrophobicity, better emulsifying activity, and improved thermal stability, due to the grafting of hydrophobic octyl groups onto casein (Zhang et al., 2014).

Hydrogels are also obtained (Kruif et al., 2015; Nascimento et al., 2020) by TCC, which can incorporate hydrophobic (aspirin) or hydrophilic (vitamin B12) drugs and allow their sustained release (Wang et al., 2021). It should also be noted that TCC can be microwave-assisted to yield compounds with high molecular weight (>130 kDa) (Chen & Hsieh, 2016; Huppertz et al., 2007).

The use of microwaves resulted in a threefold increase in the polymerization rate of milk proteins in the presence of microbial transglutaminase, compared with a sample containing microbial transglutaminase but not treated with microwaves.

Other minor crosslinking biologics, tyrosinase (Xu et al., 2016), laccase (Buchert et al., 2010), and peroxidase (Wen et al., 2014) are also discussed.

2.2 | *N*-Acylation of casein

It is noteworthy that dietary proteins are often naturally *N*-acylated, as covalent attachment of several acyl groups, including fatty acids acyl groups, is present in many plants and animals cells (Ferjancic-Biagini et al., 1998). Free amine functions of casein can react with activated carboxylic acids to give new amide groups. The carboxylic acids are beforehand activated in the form of anhydride and the most frequently used are acetic anhydride and even more succinic anhydride. Generally, acylation is performed at room temperature by adding the anhydride (1–3 equivalents per lysine group) dropwise to the protein solution, at pH ≅ 9 to keep amines in non-protonated form (Gerbanowski et al., 1999).

Succinylation of food proteins is listed in a recent review where the properties of water solubility, emulsification, foaming, thermal stability, rheology, nutrition, encapsulation, and drug release are highlighted (Basak & Singhal, 2022). Succinylation gives a higher degree of acylation than acetylation or acylation in general. Degrees of succinylation close to 100% can be achieved using excess of succinic anhydride. Highest degree of succinylation was achieved at the level of 4 mol of succinic anhydride/mol of lysine in sodium caseinate (Shilpashree, Arora, Chawla, & Tomar, 2015). An increase in water solubility of succinylated casein is observed at basic pH due to the greater number of carboxylate groups (Vidal et al., 2002). More generally, viscosity, foamability capacity and emulsifying activity of succinylated casein increase and zeta potential decreases compared to unsubstituted casein (Shilpashree, Arora, Chawla, Vakkalagadda, et al., 2015). For example, the zeta potential decreases from −14 mV in sodium caseinate to −36 mV in succinylated sodium caseinate with a substitution degree = 96%. The presence of additional carboxylate groups after succinylation also improves the divalent metal (especially Zn) binding efficiency of milk proteins (Shilpashree et al., 2022). In the presence of high amounts of Zn^{2+}, the solubility of succinylated casein is maintained, unlike unsubstituted casein, and Zn^{2+} uptake is significantly higher.

In the field of packaging, it is now accepted that the use of nondegradable, nonrecyclable packaging derived from nonrenewable resources must be avoided. In this field casein and its derivatives, which meet biodegradability and renewable resource criteria as mentioned above, have a promising future. For packaging applications, the fatty acids are probably the most important category of compounds mixed or grafted with casein, as they are hydrophobic, nontoxic, and often edible. Their hydrophobicity allows them to compensate for casein's excessive hydrophilicity. Although caseinate can readily form thermoplastic films, its high hydrophilicity results in poor moisture resistance. In contrast to grafting sugars or polysaccharides onto casein, amidation generally leads to a reduction in water solubility and resistance to aggregation. The addition of fatty acids to caseinate-based films formulations improves the film's moisture barrier (Fabra et al., 2008). However, the ratio of added fatty acid is limited to ≅1% to minimize phase separation prior to film formation (Aliheidari et al., 2013). To avoid this phase separation, fatty acids with C8–C16 alkyl groups were covalently grafted onto the sodium caseinate chains after activation of the carboxylic acid to an *N*-succinimidyl form (Zhang et al., 2018) (Figure 5).

According to Zhang'procedure, the degree of substitution of fatty acids on casein varies between 72.9% (C8: octanoic acid) and 11.8% (C16: palmitic acid), showing the importance of steric hindrance on grafting. The surface hydrophobicity increases, but this increase is interestingly inversely proportional to the length of the alkyl chain for similar grafting degrees. The authors explained this amazing trend by the fact that a longer chain is more easily buried in the center of the protein. To overcome this difficulty, our team recently reported a new mild synthetic method for grafting oleic acid, a C16 carboxylic acid, in a biphasic medium (water/CH_2Cl_2) by phase transfer catalysis at room temperature (Tichané et al., 2023). In this method, oleyl chloride, diluted in the organic phase (CH_2Cl_2), reacts with sodium caseinate in the aqueous phase, in the presence of tetrabutylammonium bromide, under stirring at 8000 rpm with an Ultra-Turrax. This method allows oleyl chloride to react with both amine and alcohol functions of casein and not only with amine functions, thus increasing the proportion of oleic acid incorporated into each casein chain. In addition, a substitution degree of 56% is obtained instead of 11.8% for the grafting of C16 palmitic acid using Zhang's method. This results in a weight percentage of grafted oleic acid of 24%. In the same way, new casein-*g*-poly(ε-caprolactone) and casein-*g*-poly(lactic acid) are described, prepared by reacting acid chlorides of chain end of PCL and PLA with caseinate, according to the biphasic method (Viora et al., 2023) (Figure 6). Unlike most copolymers grafted by the "from" method described below, these graft copolymers are fully biodegradable. The effective grafting and the absence of homopolymers have been unambiguously demonstrated by DOSY NMR. It is shown by scanning electron

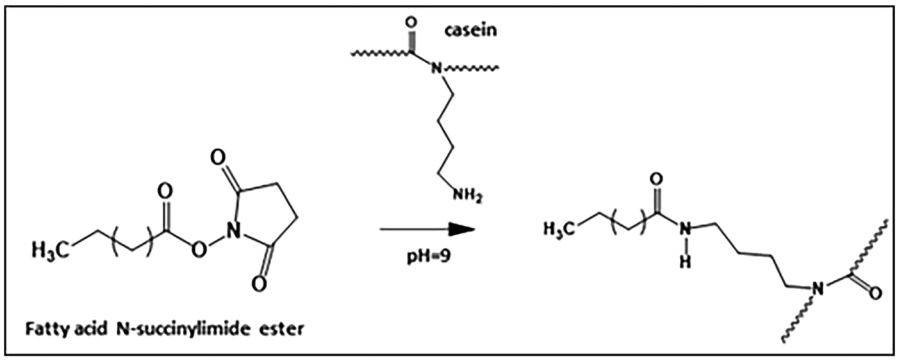

FIGURE 5 General scheme for the formation of fatty acid-alkylated casein.

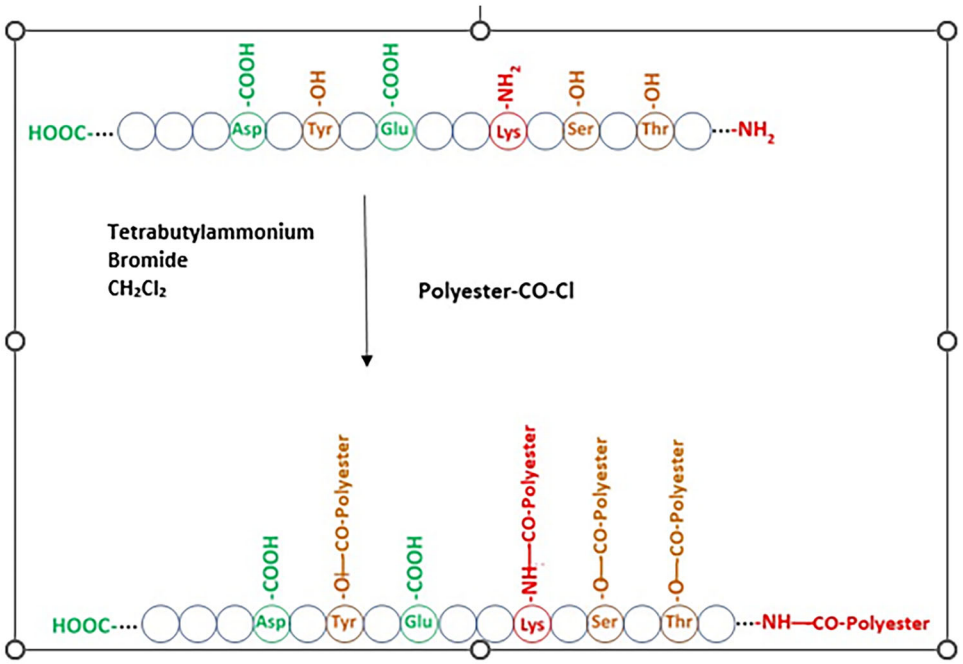

FIGURE 6 Synthesis scheme of casein-*g*-poly(lactic acid) (casein-*g*-PLA) and casein-*g*-poly(ε-caprolactone) (casein-*g*-PCL) copolymers.

microscopy (SEM) that these copolymers are excellent compatibilizers for casein/PCL and casein/PLA blends.

A direct grafting of stearic acid (a C18 fatty acid) has been achieved in water at 60° and at pH = 10 by reacting casein with stearic acid in the presence of glycerol (Sophia & Malar Retna, 2018). The mechanical properties of the graft copolymer (with 30% w/w of stearic acid) show increased tensile strength (from 10.35 to 31.4 MPa) and Young's modulus (from 18.5 to 82.9 MPa) and a decreased elongation at break (from 55.85% to 37.84%). However, the analyses carried out do not allow us to affirm that all the stearic acid is effectively grafted onto casein.

In a book chapter, Feeney and Whitaker described the chemical coupling between casein amine groups and protein carboxylic acids activated by *N*-carboxy anhydrides or *N*-hydroxy succinimide (Feeney & Whitaker, 1982). High substitution levels (80%–90%) were obtained but result in only small changes in relative viscosities. However, this work does not demonstrate an effective grafting rate of 80%–90%.

A few special surface reactions are described, including activation of glass surfaces with 3-glycidyloxypropyl triethoxysilane (GPS) or 3-aminopropyltriethoxysilane. The subsequent reaction with the free amine functions of casein results in grafting casein onto the glass surface (Han et al., 2011) (Figure 7). The density of grafted casein on the surface, the change in contact angle (from 1° on glass surface to 54° on glass-GPS-casein surface), and the adhesion of bacterial spores to the surface were evaluated. The main result is that grafting leads to a decrease of surface density of Geobacillus spores by an order of magnitude in comparison with glass.

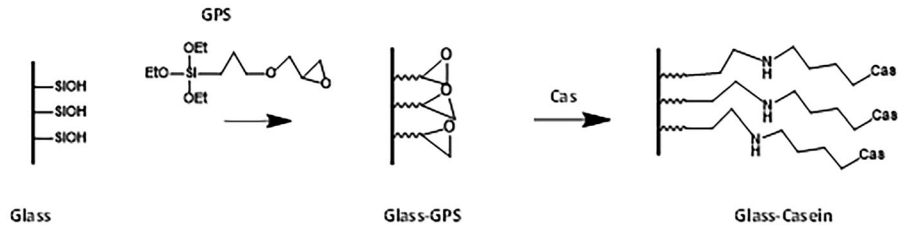

FIGURE 7 Schematic chemical activation of casein surface with 3-glycidyloxypropyl triethoxysilane (GPS).

3 | OTHER GRAFTING REACTIONS ON CASEIN

3.1 | Graft copolymers by the "grafting from" method

Maillard reactions of caseinate with polysaccharides are examples of the formation of caseinate-based graft copolymers by a so-called "grafting onto" method, where preformed polymers are grafted onto the caseinate chain. The "grafting from" method involves the polymerization of a monomer initiated by some reactive functions in the caseinate chain, notably amine and alcohol groups. Most of the monomers, usually acrylate or methacrylate derivatives, are derived from petroleum, unlike sugars or polysaccharides. Accordingly, the obtained copolymers are not totally bio-sourced and are only partially biodegradable. The main changes in the properties compared with casein are increased hydrophobicity and improved mechanical properties of the films formed, in particular flexibility, due to the reduction in the number of hydrogen bonds between the C = O and NH functions (Allasia et al., 2019). This is particularly interesting as films formed with casein alone are extremely fragile. These casein-based graft copolymers are described later, together with their main characteristics and applications.

3.1.1 | Casein-*g*-poly(meth)acrylate copolymers

Most casein-*g*-poly(meth)acrylate copolymers are prepared from the casein-glycidyl methacrylate (GMA) conjugate to minimize the potential formation of polymethyl methacrylate (PMMA) homopolymer, although methods for polymerizing methyl methacrylate (MMA) directly from casein are also used (Li et al., 2002). In this case, the homopolymer PMMA is extracted with chloroform for 48 h using a Soxhlet.

GMA is grafted onto casein by reaction of the oxirane group with the amine functions of casein. Typically, GMA is added to a casein solution containing Na_2CO_3 (pH > 10 to ensure mixture solubility) and the mixture is reacted

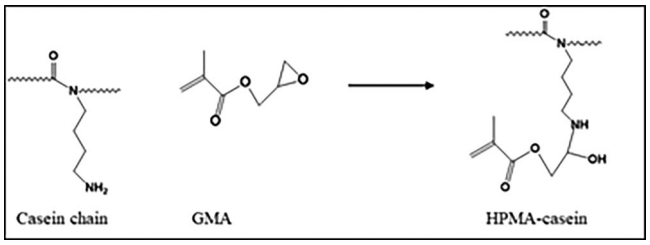

FIGURE 8 HPMA formation reaction scheme.

at 50°C for 4 h to give the conjugate (2-hydroxypropyl methacrylate)-casein (HPMA-casein) (Figure 8). The control of the grafting degree of GMA is achieved by varying the GMA/casein ratio. A quantitative grafting degree is obtained for GMA/casein molar ratio of 2 and the maximum functionality reached is 32 vinyl double bonds per molecule of casein (Picchio et al., 2016).

The (meth)acrylate monomers are then polymerized from the HPMA-casein conjugate via emulsion polymerization in an aqueous medium. Typically, HPMA-casein is dissolved in water and mixed with MMA under a nitrogen atmosphere, tertiary butyl hydroperoxide is then added and the mixture is heated to 80°C for 2 h. The grafting efficiency was estimated at 40% (Picchio et al., 2015). Latexes are obtained, giving cast films whose hydrophobicity and mechanical properties could be modulated with the nature and proportion of the methacrylate part in the copolymer. However, a high degree of HPMA substitution leads to a decrease in the polymerization rate of (meth)acrylate monomers and in the chain length of grafted segments (Picchio et al., 2016). This behavior can be explained by the interaction of hydroperoxide molecules with the remaining amino groups in casein (Li et al., 2002). Other similar copolymers, such as casein-*g*-poly(*n*-butyl acrylate), are promising candidates for packaging films, taking account of their water resistance, mechanical behavior, and thermal stability. In a general way, their mechanical properties are highly dependent on the proportion of casein in the copolymer (Picchio et al., 2018), as shown in Table 3.

Thermal stability, water resistance, mechanical strength, anti UV properties, and coating performance of poly(meth)acrylate-modified casein are improved

TABLE 3 Variations of mechanical properties of casein-*g*-poly(*n*-butyl acrylate) as a function of the proportion of casein in the copolymer.

% Of casein	Young's modulus (MPa)	Elongation at break (%)
3	0.35	1118
6	5.1	693
12	20.9	636
25	93.1	156
50	283.7	64

over those of casein (Fan et al., 2015). The simultaneous incorporation of butyl acrylate and MMA in the casein chain improves anti-blocking properties, film hardness, resistance to organic solvents, and biodegradability in soil (Picchio et al., 2016). Casein-*g*-poly(meth)acrylic copolymers are good compatibilizers for (meth)acrylic/casein blends (Picchio et al., 2015). Similarly, a (meth)acrylate/casein nanocomposite made from a casein/poly(butyl acrylate)/poly(octyl acrylate)/poly(methyl methacrylate) copolymer showed increased hydrophobicity after incorporation of a low fraction of zein, a water-repellent protein (Allasia et al., 2019). A strong compatibilizing effect of the copolymer for casein/zein blends is highlighted, considering that a simple mixing of the components leads to phase separation. The plant-derived TA can be added to give a crosslinking reaction between the residual amine groups of casein-*g*-PMMA and the phenolic groups of TA (Cencha et al., 2021).

To obtain casein emulsions with a modified nanoscale core–shell structure, Ma et al. prepared casein-*g*-poly(caprolactam)/poly(butyl acrylate) copolymer (Ma et al., 2010) or casein-*g*-poly(caprolactam)/poly[butyl acrylate/MMA /vinyl acetate] copolymers (Ma et al., 2015). The result was 40–50 nm particles with a uniform size distribution and a clear core–shell structure. These latexes form films with increased hydrophobicity and better storage thermostability than films made with pure casein. Acrylic casein copolymers give also latexes with adhesive properties (Aguzin et al., 2020).

3.1.2 | Casein-*g*-polyacrylonitrile copolymers (casein-*g*-PAN)

From an industrial point of view, the main advantages of poly acrylonitrile (PAN) materials are their resistance to aromatic hydrocarbons, their impermeability to gases and their good resistance to cold, which are not features of casein. Casein-*g*-polyacrylonitrile (casein-*g*-PAN) copolymers have been synthesized to improve the properties of casein in this way. These copolymers are prepared by graft radical polymerization of acrylonitrile onto casein ("grafting from" method) in concentrated sodium thiocyanate solution with AIBN as initiator (Dong & Gu, 2002a) or in an aqueous solution of triethanolamine at 60°C with potassium persulfate as initiator (Dong & Hsieh, 2000). PAN homopolymer is also formed that is removed by extraction with DMF for 24 h.

Casein-*g*-PAN is mainly used as a textile fiber with a good dyeability, with either acidic or cationic dyes. The mechanical properties, hygroscopy, and dyeability of the copolymer were investigated. The effect of environmental factors, including relative humidity (RH), on the mechanical properties of casein-*g*-PAN was measured (Somanathan, 1996). Casein-*g*-PAN fiber has good mechanical properties, which can meet the requirement of wearing fiber (Dong & Gu, 2002b) and it has been considered a new type of "silk-like" modified fiber. Casein-*g*-PAN shows also better wet rub resistance and reduced growth of microorganisms in the textile industry, as well as new fracture properties (Somanathan & Sanjeevi, 1994). In any case, casein-*g*-PAN absorbs significantly less water than casein, whatever the RH (Somanathan, 1996). However, tensile strength, elongation at break, and modulus of elasticity were drastically altered by changes in RH (Table 4). Stress–strain curves of casein-*g*-PAN are also highly dependent on temperature: The stress at break increased, reached a maximum value at 40°C, and then decreased. For clothing applications alkali resistance is an important property in determining the success of fiber processing as the pretreatment and reactive dyeing processes are generally carried out in an alkaline environment. Unfortunately, degradation of a casein-*g*-PAN fiber containing about 25% casein in an alkaline environment at 60 and 80°C for 120 min showed that the fiber lost up to 10% of its mass, indicating poor alkali resistance (Tang & Mei, 2008).

A less common PAN-*g*-casein copolymeric structure (with a PAN backbone and casein grafts), obtained by grafting casein onto poly(acrylonitrile) via an "onto" process, is also reported. This structure was obtained by hydrolysis of PAN followed by chlorination of the carboxylic acids of the hydrolyzed PAN and reaction with casein (Jia & Du, 2006). Although this structure has not been clearly evidenced, it is probably a mixture of casein-*g*-PAN and PAN-*g*-casein structures.

3.1.3 | Other casein-based graft copolymers

Casein-*g*-polyacrylamide (casein-*g*-PAAm) was prepared by microwave-initiated graft polymerization of acrylamide onto casein backbone in the presence of ceric ammonium

TABLE 4 Variations of mechanical properties of casein-*g*-polyacrylonitrile (casein-*g*-PAN) with relative humidity (RH).

RH (%)	Tensile strength (MPa)	Elongation at break (%)	Elastic modulus (MPa)
17	221	3.5	6900
37	207	19.5	4400
50	200	42.3	2800
67	193	51.4	3000
72	89	91.3	100
95	15.5	194	6

nitrate (Sinha, 2013). Polyacrylamide (PAAm) is widely used as a flocculant in water and mineral treatment and an additive in the paper industry. Casein-*g*-PAAm is also a flocculant for the treatment of coal washing effluents with a flocculation profile dependent on the grafting percentage: The grade with highest percentage grafting showed maximum flocculation efficacy, due to the high level of PAAm. The improvement in the flocculation characteristics of these copolymers is due to the increase in hydrodynamic volume. Grafting of PAAm onto casein also improved water solubility, because PAAm is infinitely soluble in water. Accordingly, the higher the grafting percentage of PAAm on casein, the greater its solubility (Sinha, 2018). In the result, casein-*g*-PAAm can be used as an efficient flocculant for coal washing effluent and wastewater.

Involving the response to stimuli of poly(*N*-isopropylacrylamide) (PNIPAAm), a water-soluble casein-*g*-poly(*N*-isopropylacrylamide) copolymer (casein-*g*-PNIPAAm) sensitive to stimuli, responding to both temperature and pH changes, was synthesized by polymerizing NIPAAm from casein ("grafting from" method) in water in the presence of t-butyl hydroperoxide (Cao et al., 2010, 2012; Cuggino et al., 2020). Casein-*g*-PNIPAAm self-assembles into core–shell particles with collapsed PNIPAAm as core as well as inverse core-hair particles with expanded casein as core, depending on temperature and pH, as shown by transmission electron microscopy (Cao et al., 2012). Transmittance analyses at 25°C showed that, unlike casein, casein-*g*-PNIPAAm did not precipitate at the isoelectric pH of casein for the same concentration, that the minimum transmittance changed with pH and that a temperature transition occurred around 33°C. These results suggest a greater hindrance to casein aggregation.

Recently, casein-*g*-PNIPAAm unimers have been prepared and applied to cancer therapy (Cuggino et al., 2020). They self-assemble into micelles capable of incorporating doxorubicin and transporting the drug in physiological medium before being released in acidic medium (pH = 5). The role of pH and the presence of trypsin, which is interesting because it is overexpressed in several cancer tumors, on the release rate has been demonstrated. Trypsin allows faster and more complete release (60% of doxorubicin is released in 50 h, compared with 30% in the absence of trypsin). Release is also faster at acid pH than at physiological pH due to the doxorubicin's greater solubility at acid pH. However, a release plateau between 20% and 60% is observed in all cases, with no indication of the fate of the remaining doxorubicin.

The synthesis of casein-*g*-polystyrene is also reported using potassium diperiodatonickelate–casein as initiator for styrene polymerization. High grafting efficiency (>98%) and a weight proportion of grafted polystyrene chains/casein backbone >300% are obtained (Weiqi Zhou et al., 2006). The effect of reaction parameters, such as monomer/casein weight ratio, initiator concentration, pH, time, and temperature, is investigated. Optimized conditions are monomer/casein weight ratio = 3.06 mol/L, time = 3 h, temperature = 55°C, and pH = 12.83. The proof of grafting was obtained from FTIR spectroscopy, X-ray diffraction, and SEM tests. However, the grafting sites on casein and the possibility of obtaining polystyrene homopolymer are not discussed and no properties of this copolymer are indicated.

3.2 | Esterification of carboxylic acid functions of casein

Although reactions on the free amine functions of casein are numerous, reactions on the free carboxylic acid functions (≅ 30 per casein chain) are less frequent. In most cases, they involve esterification with various alcohols (typically ethanol and propanol) in the presence of concentrated hydrochloric acid at 4°C for a few hours (Sitohy et al., 2002). Lin et al. described the reaction of the carboxylic acid functions of casein with the alcohol function of triethanolamine followed by reaction of alcohol groups of the grafted triethanolamine on fatty acids to prepare new surfactants (Lin et al., 2009). Some properties of esterified and native casein, such as isoelectric point, water solubility, emulsifying activity (Chobert et al., 1990), sensitivity to trypsinolysis (Sitohy et al., 2001), were compared. A decrease in water solubility and a variation in

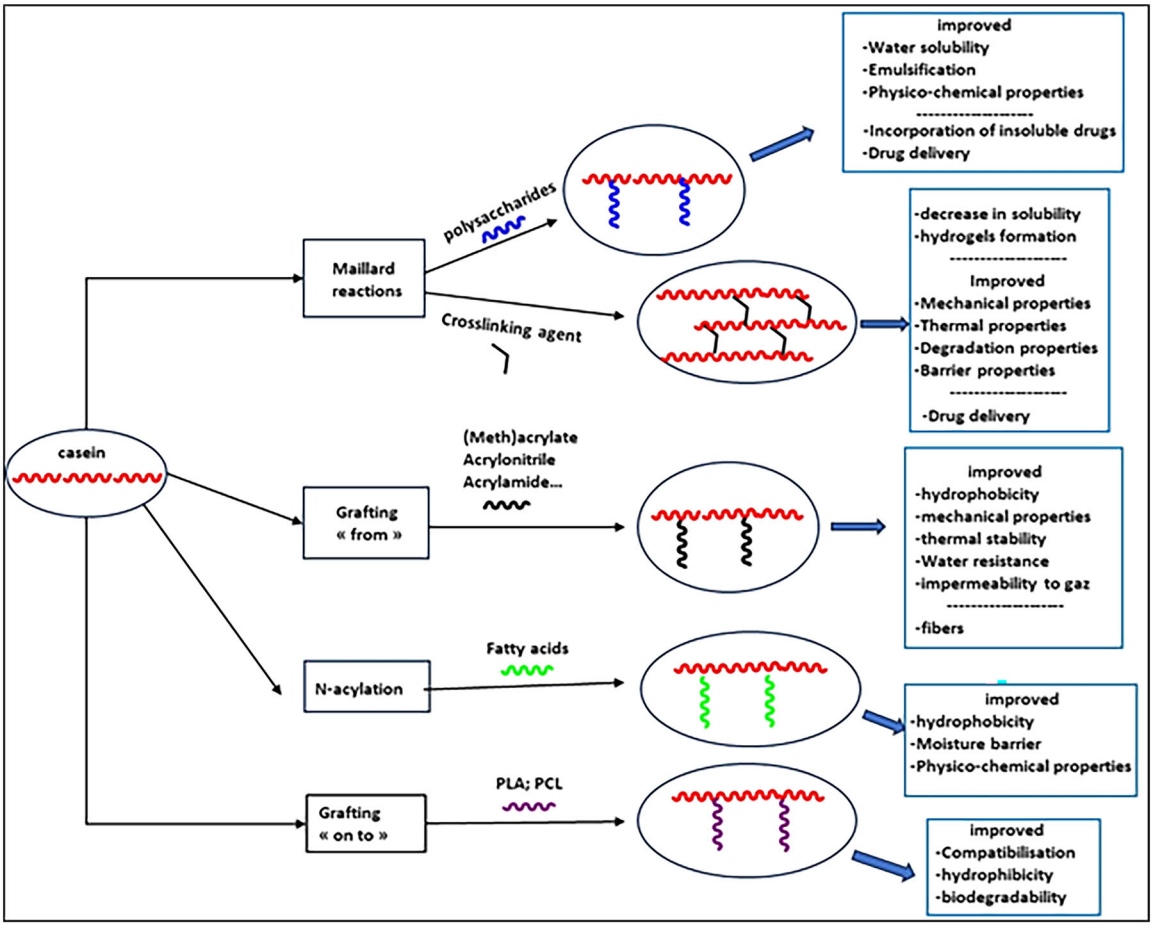

FIGURE 9 Reactions leading to casein graft copolymers and their main properties.

the isoelectric point (from 5.3 for casein to 5.75–9.75 for esterified casein, depending on the degree of esterification) are observed. Potential inhibition of DNA replication by esterified β-casein and esterified α- or β-lactalbumin was investigated and showed that no inhibitory effect was observed with esterified β-casein, unlike esterified α- or β-lactalbumin (Sitohy, 2001). Finally, to our knowledge, there is no description in the literature of a polymeric chain grafted onto casein by reaction with the carboxylic acid functions of casein.

The main reactions leading to casein-based graft copolymers and their principal properties are shown in Figure 9.

4 | CONCLUSION

This review gives an overview of current chemical modifications of the edible casein chain aimed at synthesizing conjugates and/or graft copolymers, as well as some related applications, mainly in the environmental (food chemistry, film formation, and packaging) and biomedical (biomaterials, drug encapsulation, and delivery) fields. Various types of reactions are described, most of them involving the free amine functions of casein. Maillard reactions are the most frequent, particularly with sugars and polysaccharides, leading to new amphiphilic compounds whose main feature is an increased solubility in water, especially at acid pH. When applied to dialdehydes or other crosslinking agents, these reactions give three-dimensional polymers that are insoluble in water or form chemical hydrogels. The main property changes concern the formation of stable micelles and hydrogels and improvements in the rheological and mechanical properties of the resulting biomaterials. Graft copolymers are also obtained by polymerization of various monomers, often (meth)acrylate derivatives, initiated by the amine functions of casein. In contrast to the reaction products obtained with polysaccharides, these copolymers show increased hydrophobicity and resistance to hydrolysis. All these reactions offer a wide range of properties, and therefore potential applications, for casein-based graft copolymers, even though relatively few industrial applications for these products have been developed to date, particularly in the food sector. However, apart from casein's free amine functions, other reactive functions, notably alcohols and carboxylic acids, give rise to only a few chemical reactions. Research

in this direction is likely to lead to new structures that will improve the properties of casein-based copolymers and thus open the way to new applications. In any case, casein-based graft copolymers are natural or "seminatural" compounds of animal origin, for the most part nontoxic and partially or fully biodegradable, and therefore promising in food, packaging, environmental, and biological fields.

AUTHOR CONTRIBUTIONS

Laurianne Viora: Writing—original draft. **Teddy Tichané**: Writing—original draft. **Benjamin Nottelet**: Visualization; writing—review and editing. **Julia Mouton**: Writing—review and editing. **Xavier Garric**: Validation; supervision. **Hélène Van Den Berghe**: Methodology; writing—review and editing; investigation; project administration; funding acquisition. **Jean Coudane**: Conceptualization; methodology; investigation; writing—original draft; funding acquisition; project administration; validation.

CONFLICT OF INTEREST STATEMENT

None.

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