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

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23
24 **Highlights**

- 25 • Syntheses of casein-based conjugates and graft copolymers.
26 • Maillard reactions of mono and polysaccharides on casein.
27 • Polymerization of a monomer initiated by the casein backbone.
28 • Properties of casein-based graft copolymers: mechanical properties, water solubility,
29 hydrophilicity/hydrophobicity, degradability, micellization.

30
31 **Keywords** Casein, conjugates, graft copolymers, Maillard reaction, polysaccharides

32 **Declarations of interest:** none

35 **Abstract**

36

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

48

49

50 **1. Introduction**

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

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

63 From an industrial and economic point of view, according to data from Future Market Insights (FMI), the
64 global casein market in 2023 is \$2.7 billion and expected to reach a value of \$4.9 billion by 2033, with a
65 compound annual growth rate (CAGR) of 6.3% between 2023 and 2033 (Future Markets Insights, 2023).
66 This growth is mainly due to increasing demand for high-quality, functional dairy products, as well as
67 growing demand for casein in the pharmaceutical and cosmetics industries (Observatory of Economic
68 Complexity 2021). The main current exporters are New Zealand, Ireland, the Netherlands and France.
69 Casein's market share is currently estimated at around 35% for the food industry, 20% for paints and
70 coatings and 20% for pharmaceuticals.

71 From a structural point of view, casein comes in four different structures: α S1 (36%), β (37%), κ (10.5%),
72 α S2 (12%), and γ (4.7%) (Bonfatti et al., 2011). Each casein structure (Yang & Reddy, 2012) has a specific
73 amino-acid sequence, resulting in different properties, especially in terms of
74 hydrophobicity/hydrophilicity. Casein chain contains numerous amino acids, some of which are
75 responsible for casein's chemical reactivity. Thus, a casein chain contains approximately 14 lysine (NH₂
76 groups), 30 serine/threonine/tyrosine (OH groups) and 30 aspartic/glutamic acids (COOH groups). As the
77 carboxylic acid/amine ratio of the casein backbone is greater than 1, an aqueous casein solution is slightly
78 acidic, with an isoelectric point around pH=4.6. Casein is thus soluble at basic pH (as caseinate) and
79 becomes almost insoluble at slightly acidic pH around 4.5-5. Sodium caseinate is a water-soluble form of
80 casein obtained by solubilizing casein in a sodium hydroxide medium. In the following, we'll use the
81 terms casein or caseinate indifferently, considering that we're referring to the same compound in two pH-
82 dependent forms. The main functional properties of casein are water solubility, viscosity, gelation,
83 emulsifying and foaming properties. Casein has a major application as an additive in food industry
84 (Wusigale et al., 2020), but increasingly in other areas such as emulsifiers (Liao et al., 2022; X. Ma &
85 Chatterton, 2021), paint (Nevin et al., 2008), adhesive (Tundisi et al., 2021), packaging (Khan et al.,
86 2021), incorporation and delivery of active drugs (Głab & Boratyński, 2017; Nascimento et al., 2020;
87 Sadiq et al., 2021), textile manufacturing (Belkhir et al., 2021) etc....The industrial production,
88 specifications and regulatory aspects of casein are summarized in a previous review (O'Regan &
89 Mulvihill, 2011).

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

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

117 More generally, despite its many qualities, casein has shortcomings such as excessive hydrophilicity,
118 processing complexity, high degradation rate, poor machinability, poor mechanical properties and low
119 environmental resistance (particularly to water), which considerably limit the possibilities for industrial
120 applications. For example, casein-based films are quite brittle and it is difficult to get films on which
121 tensile tests can be performed properly (Chevalier et al., 2018). These films are not stretchable, have poor
122 water resistance, and are easily susceptible to mold and crazing (N. Wang et al., 2004). Therefore, in
123 many film formulations, a plasticizer (usually glycerol) should be added that generates a free volume to
124 improve film flexibility (Chevalier et al., 2018). In the food industry glycerol is also known as the additive
125 E422 that preserves food from desiccation. But the additive eventually migrates out of the films. Tannins
126 are occasionally added to improve some properties of casein (increase in antioxidant and antimicrobial
127 activities and decrease in water solubility, vapor permeability and stretchability) (Cano et al., 2020).
128 To improve the properties of casein and to confer new ones, it is necessary to make structural
129 modifications to the protein. These modifications can be i) of physicochemical nature where only
130 secondary bonds are involved or ii) of chemical nature involving new covalent bonds. In both cases, polar
131 groups of casein (amines, alcohols, and carboxylates), which are also reactive functions, are involved.
132 Physicochemical modifications are generally obtained by mixing, with or without heating, where the
133 primary structure of the casein is generally preserved. Complexes are also formed by changes in pH or
134 pressure, by the addition of multivalent ions of chelating agents or ligands (Broyard & Gaucheron, 2015).
135 The addition of folic acid (vitamin B9) to increase the stability of casein (Bourassa & Tajmir-Riahi, 2012),
136 or the complexation with curcumin to give nano-formulations are also noted (Sahu et al., 2008). But in
137 most cases, the physicochemical modifications are relatively unstable because they only involve a low
138 stabilization energy and therefore are unsuitable for most industrial applications.
139 Chemical modifications are therefore preferred to obtain medium and long-term changes. Chemically, the
140 main reactive functions in casein are the primary amine (from lysine), alcohol/phenol (from tyrosine,
141 serine, and threonine) and carboxylic acid groups (from aspartic and glutamic acids) (Broyard &
142 Gaucheron, 2015).

143

144 **2. Chemical reactions on the amine functions of casein**

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

148

149 **2.1. Maillard reactions**

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

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

160

161 **2.1.1. Reactions of sugars and polysaccharides on casein**

162 The reactions of sugars with casein are well documented in the literature. Most casein-sugar conjugates
163 are prepared by a dry-heating method where caseinate and sugar are dissolved in phosphate buffer at
164 pH=7, freeze-dried, and the lyophilized mixture is typically heated to 50-60°C (Markman & Livney,
165 2012). The dry state is known as the conventional method but it has the disadvantage of the formation of
166 browning products, determined with a scanner and analyzed using Image J software, the proportion of
167 which increases with reaction time (Tavasoli et al., 2022). The brown color was caused by the formation
168 of (co)polymers containing nitrogen, such as melanoidins, which are produced through condensations in
169 the last stage of the Maillard reaction. Dry heating leads to a decrease in solubility and a loss of
170 digestibility due to the formation of hydrophobic bonds and uncontrolled cross-linking reactions (Zenker
171 et al., 2020). The percentage of dissolved milk protein powders in the simulated milk ultrafiltrate (SMUF)

172 depends on the reaction temperature: 75% for untreated casein, 30% when treated at low temperature
173 (60°C) and 20% when treated at high temperature (130°C).

174 Casein-sugar conjugates are also prepared by wet heating, generally in phosphate buffer at 60-100°C for
175 a few hours in a sealed vessel to hinder water evaporation. In this process, the conjugate is often recovered
176 after dialysis against redistilled water, and then dried under vacuum. (Cardoso et al., 2011; Consoli et al.,
177 2018). Grafting glucose or beta-cyclodextrin onto casein can also be achieved using a microwave-assisted
178 wet heating approach, which has reduced reaction time to a few minutes and yielded compounds with
179 improved gelation properties (Bi et al., 2015).

180 Glycation of casein with glucose, fructose and lactose was described long ago for food applications (H.
181 S. Lee et al., 1979). In the presence of 1000 M excess of sugar, the degree of modification was 80% with
182 glucose, 62% with fructose, and 17% with lactose. Compared with native casein, sugar casein conjugates
183 have been shown to have slightly lower in vitro digestibility by α -chymotrypsin and lower nutritive values
184 in rat feeding experiments. Significantly lower growth rates were observed in rats fed diets based on
185 casein grafted with glucose, fructose or lactose. Food intake and feed efficiency were also reduced in the
186 groups receiving these casein derivatives. In addition, serious growth depression was observed in rats
187 feeding glucose-derived casein. Despite this, there is currently growing interest in the reaction of casein
188 with sugars and polysaccharides for other applications.

189 Generally, reactions with sugars are carried out at neutral or basic pH on sodium caseinate, which is more
190 soluble than casein, due to the formation of carboxylate groups. It is noted that the molar mass, structure,
191 and charge of the reagents are important in determining their reactivity in the Maillard reaction
192 (O'Mahony et al., 2017). For example, the reactivity of sugars/polysaccharides decreases with increasing
193 molecular weight, due to the contribution of steric hindrance as molecular weight increases: Galactose
194 (180Da) > lactose (342Da) >> dextran (10.000Da). The percentage of modification of free amine
195 functions in a casein-dextran conjugate is only 25.6% compared to 76.1% in galactosylated casein
196 (Courthaudon et al., 1989). More generally, monosaccharides are more reactive with casein than
197 disaccharides or oligosaccharides under conditions that favor conjugation (Corzo-Martínez et al., 2010).
198 The main consequence is that glycation with dextran does not produce any substantial improvement in
199 caseinate properties, particularly rheological properties, due to a limited extent of the reaction. While

200 degrees of modification close to 80% are obtained by coupling glucose or galactose, they are only 20%
201 with fructose (a ketose instead of an aldose), demonstrating the greater reactivity of aldose compared with
202 ketose (Courthaudon et al., 1989).

203 The success of the grafting reactions is confirmed by the increase in molar mass from 23,400Da to 28.700
204 Da, analyzed by SDS-PAGE, in the case of maltodextrin grafting (Markman & Livney, 2012). Titrations
205 of the remaining lysine residues with O-phthalaldehyde (Markman & Livney, 2012), trinitrobenzene
206 sulfonate (O'Regan & Mulvihill, 2009) or by enzymatic galactose assay (X.-P. Wang & Zhao, 2017) are
207 used to measure the degree of glycation of lactose in casein and soybean proteins.

208 In a general way, the increase in solubility is a key feature of the reaction of casein with sugars. Glycation
209 of casein with glucose, fructose, galactose, mannose or lactose resulted in conjugates with improved water
210 solubility, especially near the isoelectric point ($\text{pH} \approx 4.5$) where casein is initially poorly soluble, as a result
211 of the grafting of highly water-soluble sugars at any pH (Courthaudon et al., 1989). The solubility of 76%
212 galactosylated casein at $\text{pH} = 4.5$ is 8 times greater than that of unmodified casein at the same pH.

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

222 To improve the variations in the properties of casein-sugar conjugates, sugars may be replaced by
223 polysaccharides to give casein-g-polysaccharide copolymers in which the Maillard reaction introduces
224 covalent bonds between proteins and carbohydrates, both of which are food constituents and sustainable
225 compounds. As a result, the casein-g-polysaccharide copolymers obtained are both biodegradable and
226 sustainable compounds. Moreover, they are all formed from compounds used in the food industry.
227 Extensive research has been carried out to utilize this reaction to improve the functionality of proteins.

228 For the same level of substitution, it increases the proportion of sugar units. However, from a chemical
229 point of view, as mentioned above, the reactivity of polysaccharides with proteins is much lower than that
230 of simple sugars, leading to lower grafting degrees due to i) steric hindrance and ii) the presence of a
231 single reactive terminal carbonyl group at the chain end (Kato, 2002; Kato et al., 1992). Dry-heating
232 method is preferred to prepare protein-polysaccharide conjugates due to ease of handling reagents and
233 control of the reaction conditions. Some examples of casein-g-polysaccharide are discussed below.

234 Reactions of maltodextrin and dextran with casein have resulted in new types of casein-g-polysaccharide
235 copolymers, as summarized in a recent review (Abd El-Salam & El-Shibiny, 2020). As for sugars, water
236 solubility is the main effect of grafting dextran onto casein: β -casein is nearly insoluble ($\cong 0.1\text{mg/mL}$) in
237 water at its isoelectric point ($\text{pH}=4.6$), whereas dextran is soluble in water at any pH. However, the
238 solubility of a mixture of β -casein and dextran at acidic pH is the same as that of β -casein alone, implying
239 that the simple addition of dextran has no effect on the solubility of the protein at acidic pH (M. Li, Wen,
240 Wang, Liu, et al., 2022). In contrast, the solubility of casein-g-polysaccharide copolymers is much higher
241 than that of casein alone (T. Wu et al., 2022). It is a reasonable hypothesis that the increased solubility of
242 casein-g-polysaccharide is due to the formation of micelles in this pH range, where the highly hydrophilic
243 dextran is located on the micelle surface and protects the β -casein from further aggregation (Mu et al.,
244 2006). Reaction time, casein/dextran molar ratio and dextran molecular weight play an important role in
245 the water solubility of casein-g-dextran: the copolymer becomes soluble at any pH when the degree of
246 grafting and the molecular weight of dextran are sufficiently high (Pan et al., 2006). With 10.000Da
247 dextran, precipitation at pH 4.6 (isoelectric point of casein) can be prevented if dextran/casein ratio
248 (mol/mol) in the copolymer is $\geq 1/2$. For 1.5kDa and 6 kDa dextrans, precipitation occurs at $\text{pH}=4.6$ no
249 matter what the molar ratio is. Similarly, solubilities of casein-g-maltodextrin (35.7% w/v), casein-g-
250 pectin (33.5% w/v) and casein-g-gum arabic (25.6% w/v) copolymers are significantly higher than that
251 of casein (1.6% w/v) at pH 4.5 (Kumar et al., 2021).

252 Protein-g-polysaccharide compounds are almost always better emulsifiers than the original protein: the
253 emulsifying activity of casein-g-dextran and casein-g-galactomannan is 1.5 times higher than that of
254 casein (Kato et al., 1992). Casein-g-dextran shows also special emulsification properties, in terms of
255 emulsification activity index (EAI) and emulsification stability index (ESI) compared to casein (Y. Li et

256 al., 2023) : EAI of Casein-Dextran10, Casein-Dextran40 and Casein-Dextran70 (10, 40, 70 are the
257 grafting percentages of 70kDa dextran onto casein) increased by 12.8, 28.6 and 30.85 %, respectively as
258 compared with genuine casein. The stability of the casein-*g*-polysaccharide emulsions -estimated by
259 measuring the half-time of the turbidity immediately after the emulsion had formed- is 10 times higher
260 than that of casein (Kato et al., 1992). Another advantage with casein-*g*-polysaccharide conjugates is their
261 emulsion stability in acidic and high-salt concentrations. Casein-*g*-dextran nanoparticles are more stable
262 to aggregation than casein nanoparticles, even under simulated gastrointestinal conditions, probably due
263 to greater steric repulsion as a result of the presence of dextran chains (Davidov-Pardo et al., 2015).
264 Maillard conjugates of casein also lead to improved physicochemical properties (zeta potential, viscosity
265 at constant shear, microstructure...) as in the case of the casein-sugar beet pectin conjugate (J. Zhang &
266 Wolf, 2019). Surface hydrophobicity, emulsifying capacity, stabilizing capacity are dependent on the
267 molar mass of the grafted dextran: casein-*g*-dextran formed from intermediate molecular weight dextrans
268 (MM=40.000 and MM=70.000) showed enhanced emulsification activity and stability as well as
269 improved stability during in vitro digestion (Liu et al., 2019). Casein-*g*-dextran is used as a stabilizer for
270 fish oil emulsions (sources of polyunsaturated fatty acids) and the presence of casein-*g*-dextran in the
271 gastrointestinal tract influences zeta potential and mean particle diameter, resulting in a significant delay
272 in fatty acid release (Liu et al., 2019; Wei et al., 2018). In addition to their stability to acidic conditions,
273 casein-*g*-dextran emulsions are also resistant to enzymatic digestion by pepsin, thus avoiding problems
274 of premature release of encapsulated lutein (a natural colorant or nutraceutical present in many foods) in
275 the stomach (Gumus et al., 2016). The hydrodynamic diameter of micelles is around 100 nm, compared
276 with 10-15 nm for the casein alone, with a critical micellar concentration (CMC) around 10 mg/L, a
277 particularly low value (Mu et al., 2006). As expected, the hydrodynamic diameter of the micelles increases
278 with the molar ratio and the molecular weight of the grafted dextran.

279 Casein-*g*-dextran micelles can incorporate insoluble β -carotene into a micellar core-shell structure, to be
280 further released by hydrolysis in the presence of pepsin or trypsin (Pan et al., 2007). The nanoparticles
281 are formed with casein and β -carotene core and dextran shell. They have a spherical shape and are stable
282 in aqueous solution against dilution, pH change, ionic strength change, oxidation, and storage. Self-

283 assembled casein-g-dextran micelles act as protective carriers for curcumin and are superior to casein
284 micelles for curcumin radical scavenging activity (Y. Wu & Wang, 2017).
285 Synthesis and emulsifying properties (Semenova et al., 2016; Shepherd, 2000), structural and
286 thermodynamic characteristics (Grigorovich et al., 2012) of casein-g-maltodextrin are described. These
287 copolymers form core-shell co-assemblies for the nanoencapsulation of hydrophobic nutraceuticals,
288 including vitamin D, in beverages (Markman & Livney, 2012). They also provide enhanced protection
289 against oxidation of omega-3 and omega-6 fatty acids (Semenova et al., 2016). They enhance the stability,
290 in vitro release, anti-oxidant properties and cell viability of eugenol-olive oil nano emulsions (Nagaraju
291 et al., 2021).
292 Similarly, casein-carrageenan conjugates have been used to improve the stability of oil-in-water
293 emulsions (Seo & Yoo, 2022), to microencapsulate the red pigment of paprika and to confer an
294 exceptional protective effect of the microcapsules on this red pigment (J. Qiu et al., 2018).
295 Grafting of chitosan onto casein has been showed to improve the proliferation and osteogenic
296 differentiation of human mesenchymal stem cells (HMSCs) (Y. Li et al., 2016). It also improves the
297 stability of emulsions, the antioxidant activity, the inhibition of lipid oxidation (Y. Wu & Wang, 2017)
298 and facilitates the incorporation and release of curcumin (Meng et al., 2021).
299 The reaction of casein phosphopeptides (CPP) (a type of protein containing multiple surfactant groups)
300 on soluble dietary fibers (polysaccharide that contain large amounts of active surface groups) is used to
301 bind calcium ions: an increase in calcium binding of up to 46.8% over the original CPP is observed (Gao
302 et al., 2018). New calcium binding and delivery systems based on casein-g-(oligochitosan) copolymers
303 have recently been proposed, prepared by Maillard reaction with or without transglutaminase linkages
304 (Zhu et al., 2020). They prolong the release of calcium from delivery vehicles in digestive system. These
305 calcium delivery systems could promote bone synthesis to prevent calcium deficiency, but also promote
306 intestinal health.
307 Some delivery systems from casein-g-polysaccharide are listed in Table 1.

308

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	Y. Wu & Wang, 2017
casein-g-maltodextrin	hydrophobic nutraceuticals	Markman & 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	Zhu et al., 2020; Gao et al., 2018

309

310 Rheological properties are also enhanced after glycation of sodium caseinate (Corzo-Martínez et al., 2010;
311 O'Mahony et al., 2017). An increased viscosity with a non-Newtonian behavior was observed after
312 reaction of casein with fructose or fructo-oligosaccharides (Oliver et al., 2006). Gelation temperature of
313 sodium caseinate/maltodextrin dispersions increased by 7 °C after conjugation (L. Zhang et al., 2020).
314 In conclusion, most Maillard reactions of casein with sugars or polysaccharides are aimed at forming new
315 water-soluble stable self-assemblies in aqueous media for various food, environmental, or biomedical
316 applications (Y.-Y. Lee et al., 2017). It should be noted, however, that studies on the degradation products
317 of compounds obtained with Maillard reactions and their possible toxicity are still under discussion. (Wei
318 et al., 2018).

319

320

2.1.2 Crosslinking reactions on casein

321 Maillard reactions can be applied to a dialdehyde instead of a monoaldehyde, resulting in casein cross-
322 linking reactions. As a rule, the properties of casein-based compounds are quite radically different before
323 and after crosslinking, in terms of solubility, mechanical properties, degradation, etc.... Applications after
324 crosslinking are mainly related to drastically reduced water solubility, improvement of mechanical
325 properties, and obtaining hydrogels and solid drug delivery devices. A clear advantage of these systems
326 is that they give biocompatible, biodegradable, and inexpensive materials. Furthermore, in vivo studies
327 have shown that rats fed with cross-linked casein had a normal growth rate compared to rats fed with

328 native casein (Seguro et al., 1996). The main chemical crosslinking agents are glutaraldehyde, glyoxal
329 and formaldehyde (a monoaldehyde) (Nascimento et al., 2020). Their structures are shown in Figure 1.

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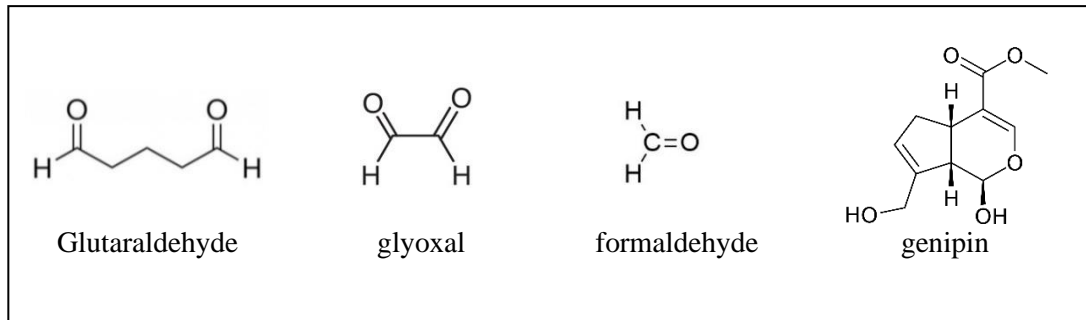
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Figure 1 : Main crosslinking agents of casein-based compounds

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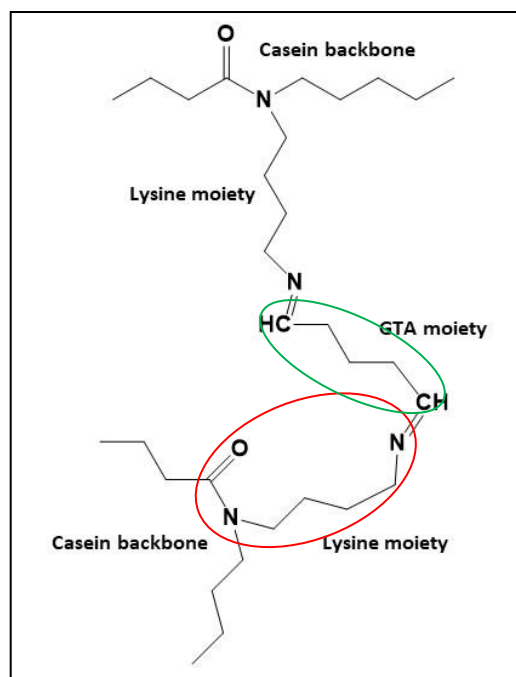
2.1.2.1. Crosslinking with glutaraldehyde

341 The reaction of sodium caseinate with glutaraldehyde (GTA) resulted in a crosslinked material (

342 Figure 2) with a significant decrease in solubility, a shrinkage and improved mechanical, thermal,

343 degradation and barrier properties (Muthulakshmi et al., 2022; Pereda et al., 2010).

344



345

346

Figure 2 :Scheme of the chemical crosslinking of Sodium caseinate with GTA

348 Depending on the dry or wet method used to prepare cross-linked casein, total soluble matter decreases

349 with the percentage of GTA added, due to the formation of a denser network (Table 2). Mechanical

350 properties (Table 2), film shrinkage and yellowish coloration generated by the Schiff base increased with
351 GTA content (Pereda et al., 2010).

352
353 *Table 2: Percentages of Total Soluble Matter and Mechanical properties of casein based GTA-*
354 *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

355
356 GTA-crosslinked casein formulations are used as bioprotheses (vascular grafts, ligament substitutes,
357 pericardial grafts...) (Jayakrishnan & Jameela, 1996). Tissue valves made from GTA-crosslinked casein
358 have the advantage of being biocompatible, non-thrombogenic and not requiring anticoagulant therapy
359 after implantation.

360 Applications of the GTA-crosslinked casein in Drug Delivery Systems in the forms of films, composites,
361 hydrogels, beads, and micro- and nanoparticles are described (Elzoghby et al., 2011; Nascimento et al.,
362 2020), including for the delivery of progesterone (Latha et al., 2000), theophylline (Latha et al., 1995),
363 curcumin (T. Wang et al., 2016), methotrexate (Vino et al., 2011), diethylstilboestrol (Bayomi & El-
364 Sayed, 1994). As expected, in all cases a reduction in drug release rate was observed.

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

372 However the toxicity of residual glutaraldehyde is still the subject of debate, even though this compound
373 is widely used (Takigawa & Endo, 2006; Xu et al., 2016). Some inflammatory reactions, cytotoxicity, or
374 calcification have been attributed to residual glutaraldehyde, but this mainly depends on the amount used

375 for crosslinking (McPherson et al., 1986). Residual glutaraldehyde in bioprostheses has been involved in
376 calcification as well as in local cytotoxicity inducing endothelial cell death (Moritz et al., 1991). This
377 appears to be a feature of glutaraldehyde cross-linking, which requires excellent purification of the
378 resulting products, especially for biological applications.

379
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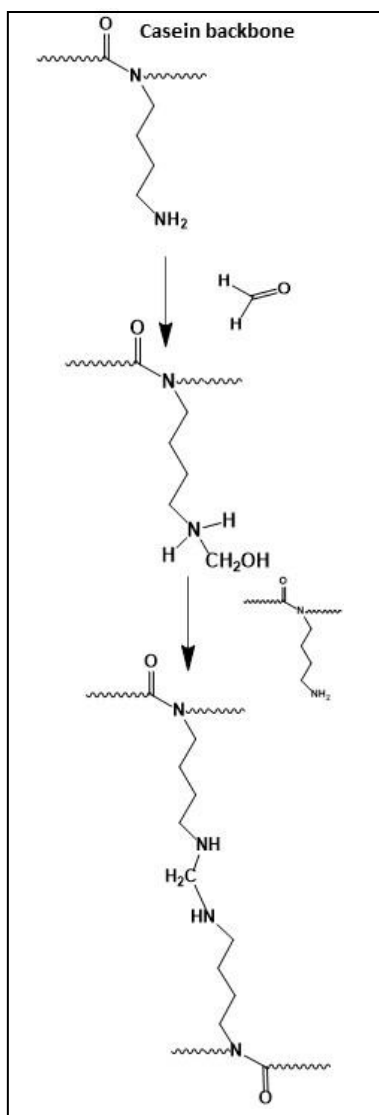
2.1.2.2. Crosslinking with glyoxal

381 As for glutaraldehyde, crosslinking of casein with glyoxal (at 80°C or under UV irradiation) leads to
382 improved mechanical (improved E-modulus, yield strength and strain at break, reduced ductility) and
383 degradation properties, giving potential applications in the biomedical field when long-term treatment is
384 required (G. A. Silva et al., 2003; Vaz et al., 2003). The cross-linking effect of glyoxal on casein is shown
385 by the prolonged release of lysozyme with sustained antimicrobial effects as compared to physically
386 cross-linked films obtained by pH variation (Mendes de Souza et al., 2010). Moreover, the amount of
387 glyoxal required is very low: for a glyoxal content of 0.05%, a small but already remarkable effect on
388 lysozyme release was observed. For glyoxal contents of 0.5% and 1% more radical effects are observed,
389 with a more obvious prolongation of lysozyme release. On the other hand, a slight toxicity is also observed
390 on the product after cross-linking, probably due to the persistence of traces of glyoxal (G. A. Silva et al.,
391 2003).

392
393

2.1.2.3. Crosslinking with formaldehyde

394 As formaldehyde is a mono aldehyde, crosslinking is carried out in 2 steps i) formation of a α -amino
395 alcohol by reaction of formaldehyde on an amine function of casein and ii) reaction of this α -amino
396 alcohol on another casein amine function giving a methylene bridge (Figure 3) (Audic & Chaufer, 2005).



397
398 *Figure 3 : Reaction scheme of casein crosslinking with formaldehyde.*

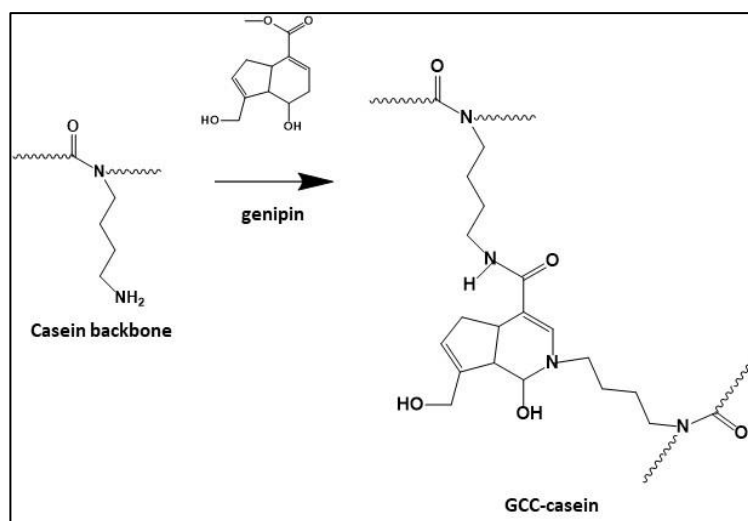
399 Formaldehyde-crosslinked casein is typically prepared from a caseinate/formaldehyde dispersion in water
400 that is left to react with gentle stirring at room temperature at pH= 8.6 for 6h. This method resulted in
401 biodegradable films with drastic decrease in water solubility, improved water resistance (Audic &
402 Chaufer, 2010), and thermal stability (Somanathan et al., 1997). However, crosslinking by formaldehyde
403 cannot prevent the exudation of triethylamine (TEA), a hydrophilic plasticizer, when a crosslinked film
404 is placed in an aqueous medium: after 10 min immersion in distilled water, around 80% of the TEA has
405 migrated out of the film, regardless of formaldehyde content (Audic & Chaufer, 2010). Here again, the
406 potential toxicity of residual formaldehyde that could be released from the films should also be
407 highlighted.

408 *2.1.2.4. Crosslinking with other crosslinkers*

409 In general, the main drawback of the inexpensive chemical aldehydes as crosslinking agents is their
410 potential cytotoxicity or that of their byproducts, although most of the prepared materials are considered
411 non-toxic. Given these uncertainties about potential toxicity, citric acid, regarded as non-toxic, may be
412 used as a crosslinking agent. Fibers crosslinked with 1% citric acid show nearly twice the strength, about
413 10 times improvement in elongation, and approximately 30% increase in modulus compared to the
414 undrawn and non-crosslinked fibers. However, the resulting films were not favorable to attachment and
415 growth of mouse fibroblasts and were therefore considered as cytotoxic (Yang & Reddy, 2012). The use
416 of a low-cost plant-derived phenolic compound, tannic acid (TA), as crosslinking agent for producing
417 plasticized casein films is also described (Picchio et al., 2018). Like most cross-linked casein derivatives,
418 films formulated by TA retard water vapor transmission, improve water resistance without degrading
419 mechanical strength, have a higher modulus of elasticity and excellent UV-blocking properties.

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

432



433

434 *Figure 4: A possible crosslinking mechanism for the reaction of casein with genipin in water*

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

440 Casein nanomicelles crosslinked with different amounts of genipin yield microgels particularly resistant
 441 to dissociating agents such as citrate or urea, and with gradual changes in their colloidal properties (N. F.
 442 N. Silva et al., 2014). The cross-linking reaction is intramicellar, as no aggregated particles are observed
 443 by DLS. These crosslinked nanomicelles can also be used for prolonged release of Alfuzosin
 444 hydrochloride (Elzoghby et al., 2013). In the same way, a β -Casein-g-Dextran copolymer crosslinked by
 445 genipin gives stable nano micelles with diameters in the range 28.4 to 53.7 nm that can be loaded with
 446 naringenin (M. Li, Wen, Wang, Wang, et al., 2022). The controlled drug release properties of naringenin
 447 and the resistance to attack by pepsin and trypsin were enhanced. A casein/chitosan mixture crosslinked
 448 with genipin yields microparticles for oral delivery of curcumin (Razi et al., 2018), or nattokinase, a
 449 thrombolytic enzyme used for the prevention and cure of thrombosis-related cardiovascular diseases (X.
 450 Zhang et al., 2020). Genipin-crosslinked caseino-phosphopeptide (CPP)-chitosan (CS) nanoparticles
 451 show great potential for oral delivery of polyphenols (B. Hu et al., 2014). It has been shown that the
 452 anticancer activity of polyphenols is maintained in loaded nanoparticles.

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

455 However, enzymatic crosslinking of casein with low toxicity transglutaminase (TCC) is also widely
456 mentioned in the literature for food and biomedical applications (Chambi & Grosso, 2006; Raak et al.,
457 2020). Generally speaking, protein modification by TG can be exploited in a number of food industry
458 applications, as it improves rheological properties, gel formation, protein solubility, foaming properties
459 and water retention properties. (Jaros et al., 2006; Matheis & Whitaker, 1987; Nonaka et al., 1992).

460 Historically, the use of transglutaminase in the food industry started with the manufacturing of surimi
461 (fish paste) products in Japan or more generally in seafood (Kuraishi et al., 2001). The production of
462 restructured meat using transglutaminase and sodium caseinate is more convenient. Cross-linking has
463 great potential for the development of high-quality restructured foods. There is no loss of taste and flavor,
464 and the strong binding is preserved even during cutting, freezing and cooking. (Kuraishi et al., 1997).

465 However, although its acute toxicity appears to be very low, there are questions about its responsibility in
466 the transfer of acyl groups between lysine and glutamine, and about the possibility of mutagenicity
467 (Bernard et al., 1998).

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

476 Hydrogels are also obtained (Kruif et al., 2015; Nascimento et al., 2020) by TCC, which can incorporate
477 hydrophobic (aspirin) or hydrophilic (vitamin B12) drugs and allow their sustained release (X. Wang et
478 al., 2021). It should also be noted that TCC can be microwave-assisted to yield compounds with high
479 molecular weight (>130 kDa) (Chen & Hsieh, 2016; Huppertz et al., 2007). The use of microwaves
480 resulted in a 3-fold increase in the polymerization rate of milk proteins in the presence of microbial

481 transglutaminase, compared with a sample containing microbial transglutaminase but not treated with
482 microwaves.

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

485

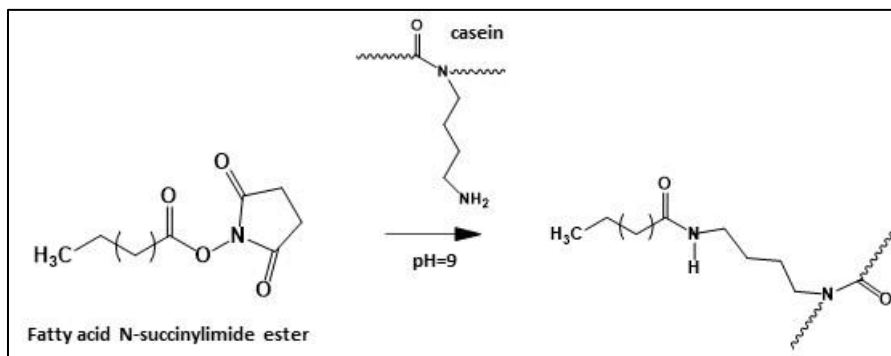
486 **2.2. N- Acylation of casein**

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

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

507 the solubility of succinylated casein is maintained, unlike unsubstituted casein, and Zn⁺⁺ uptake is
508 significantly higher.

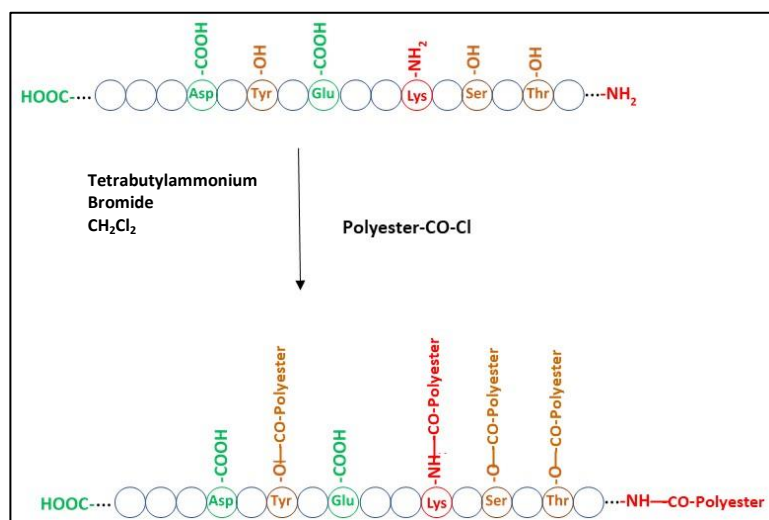
509 In the field of packaging, it is now accepted that the use of non-degradable, non-recyclable packaging
510 derived from non-renewable resources must be avoided. In this field casein and its derivatives, which
511 meet biodegradability and renewable resource criteria as mentioned above, have a promising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



523 *Figure 5 : General scheme for the formation of fatty acid-alkylated casein*

524 According to Zhang's procedure, the degree of substitution of fatty acids on casein varies between 72.9%
525 (C8: octanoic acid) and 11.8% (C16: palmitic acid), showing the importance of steric hindrance on
526 grafting. The surface hydrophobicity increases, but this increase is interestingly inversely proportional to
527 the length of the alkyl chain for similar grafting degrees. The authors explained this amazing trend by the
528 fact that a longer chain is more easily buried in the center of the protein. To overcome this difficulty, our
529

530 team recently reported a new mild synthetic method for grafting oleic acid, a C16 carboxylic acid, in a
 531 biphasic medium (water/CH₂Cl₂) by Phase Transfer Catalysis at room temperature (Tichané et al., 2023).
 532 In this method, oleyl chloride, diluted in the organic phase (CH₂Cl₂), reacts with sodium caseinate in the
 533 aqueous phase, in the presence of tetrabutylammonium bromide, under stirring at 8,000 rpm with an Ultra-
 534 Turrax®. This method allows oleyl chloride to react with both amine and alcohol functions of casein, and
 535 not only with amine functions, thus increasing the proportion of oleic acid incorporated into each casein
 536 chain. In addition, a substitution degree of 56% is obtained instead of 11.8% for the grafting of C16
 537 palmitic acid using Zhang's method. This results in a weight percentage of grafted oleic acid of 24%. In
 538 the same way, new casein-g-poly(ε-caprolactone) (Cas-g-PCL) and casein-g-poly(lactic acid) (Cas-g-
 539 PLA) are described, prepared by reacting acid chlorides of chain end of PCL and PLA with caseinate,
 540 according to the biphasic method (Viora et al., 2023) (Figure 6). Unlike most copolymers grafted by the
 541 "from" method described below, these graft copolymers are fully biodegradable. The effective grafting
 542 and the absence of homopolymers has been unambiguously demonstrated by DOSY NMR. It is shown
 543 by Scanning Electron Microscopy (SEM) that these copolymers are excellent compatibilizers for
 544 casein/PCL and casein/PLA blends.
 545



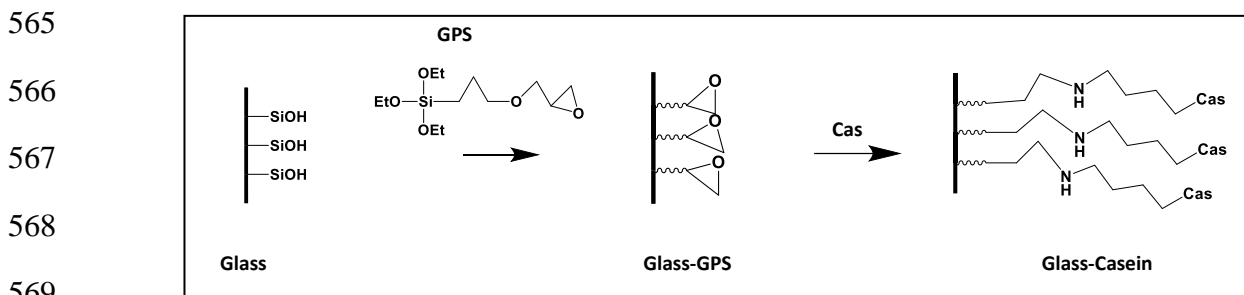
546
 547 *Figure 6 : Synthesis scheme of casein-g-PLA and casein-g-PCL copolymers*

548 A direct grafting of stearic acid (a C18 fatty acid) has been achieved in water at 60° and at pH=10 by
 549 reacting casein with stearic acid in the presence of glycerol (Sophia A & Malar Retna, 2018). The

550 mechanical properties of the graft copolymer (with 30% w/w of stearic acid) show increased tensile
551 strength (from 10.35 MPa to 31.4 MPa) and Young's modulus (from 18.5 MPa to 82.9 MPa) and a
552 decreased elongation at break (from 55.85% to 37.84%). However, the analyses carried out do not allow
553 us to affirm that all the stearic acid is effectively grafted onto casein.

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

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



570 *Figure 7 : Schematic chemical activation of casein surface with GPS*

571
572

3. Other grafting reactions on casein.

573

3.1. Graft copolymers by the “grafting from” method.

574 Maillard reactions of caseinate with polysaccharides are examples of the formation of caseinate-based
575 graft copolymers by a so-called “grafting onto” method, where pre-formed polymers are grafted onto the
576 caseinate chain. The “grafting from” method involves the polymerization of a monomer initiated by some
577 reactive functions in the caseinate chain, notably amine and alcohol groups. Most of the monomers,

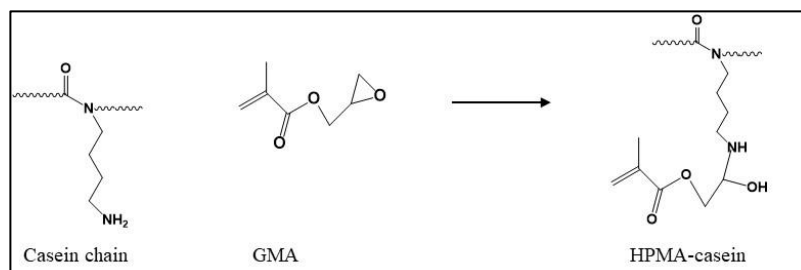
578 usually acrylate or methacrylate derivatives, are derived from petroleum, unlike sugars or
579 polysaccharides. Accordingly, the obtained copolymers are not totally bio-sourced and are only partially
580 biodegradable. The main changes in the properties compared with casein are increased hydrophobicity
581 and improved mechanical properties of the films formed, in particular flexibility, due to the reduction in
582 the number of hydrogen bonds between the C=O and NH functions (Allasia et al., 2019). This is
583 particularly interesting as films formed with casein alone are extremely fragile. These casein-based graft
584 copolymers are described below, together with their main characteristics and applications.

585

586 **3.1.1. Casein-g-poly(meth)acrylate copolymers.**

587 Most casein-g-poly(meth)acrylate copolymers are prepared from the casein-glycidyl methacrylate
588 conjugate to minimize the potential formation of Poly Methyl Methacrylate (PMMA) homopolymer,
589 although methods for polymerizing Methyl Methacrylate (MMA) directly from casein are also used (P.
590 Li et al., 2002). In this case, the homopolymer PMMA is extracted with chloroform for 48 hours using a
591 Soxhlet.

592 Glycidyl methacrylate (GMA) is grafted onto casein by reaction of the oxirane group with the amine
593 functions of casein. Typically, GMA is added to a casein solution containing Na_2CO_3 ($\text{pH} > 10$ to ensure
594 mixture solubility) and the mixture is reacted at 50°C for 4 hours to give the conjugate (2-hydroxypropyl
595 methacrylate)-casein (HPMA-casein) (Figure 8). The control of the grafting degree of GMA is achieved
596 by varying the GMA/casein ratio. A quantitative grafting degree is obtained for GMA/casein molar ratio
597 of 2 and the maximum functionality reached is 32 vinyl double bonds per molecule of casein (Picchio et
598 al., 2016).



600

Figure 8 : HPMA formation reaction scheme.

601 The (meth)acrylate monomers are then polymerized from the HPMA-casein conjugate via emulsion
 602 polymerization in an aqueous medium. Typically, HPMA-casein is dissolved in water and mixed with
 603 methyl methacrylate (MMA) under a nitrogen atmosphere, tertiary butyl hydroperoxide is then added and
 604 the mixture is heated to 80°C for 2 hours. The grafting efficiency was estimated at 40% (Picchio et al.,
 605 2015). Latexes are obtained, giving cast films whose hydrophobicity and mechanical properties could be
 606 modulated with the nature and proportion of the methacrylate part in the copolymer. However, a high
 607 degree of HPMA substitution leads to a decrease in the polymerization rate of (meth)acrylate monomers
 608 and in the chain length of grafted segments (Picchio et al., 2016). This behavior can be explained by the
 609 interaction of hydroperoxide molecules with the remaining amino groups in casein. (P. Li et al., 2002).
 610 Other similar copolymers, such as casein-*g*-poly(*n*-butyl acrylate) are promising candidates for packaging
 611 films, taking account of their water resistance, mechanical behavior and thermal stability. In a general
 612 way, their mechanical properties are highly dependent on the proportion of casein in the copolymer
 613 (Picchio et al., 2018), as shown in table 3.

614 *Table 3: Variations of mechanical properties of casein-g-poly(n-butyl acrylate) as a function of the*
 615 *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

616
 617 Thermal stability, water resistance, mechanical strength, anti UV properties and coating performance of
 618 poly(meth)acrylate-modified casein are improved over those of casein (Fan et al., 2015). The
 619 simultaneous incorporation of butyl acrylate and methyl methacrylate in the casein chain improves anti-
 620 blocking properties, film hardness, resistance to organic solvents and biodegradability in soil (Picchio et
 621 al., 2016). Casein-*g*-poly(meth)acrylic copolymers are good compatibilizers for (meth)acrylic/casein
 622 blends (Picchio et al., 2015). Similarly, a (meth)acrylate/casein nanocomposite made from a
 623 casein/poly(butyl acrylate)/poly(octyl acrylate)/Poly(methyl methacrylate) copolymer showed increased
 624 hydrophobicity after incorporation of a low fraction of zein, a water-repellent protein (Allasia et al., 2019).

625 A strong compatibilizing effect of the copolymer for casein/zein blends is highlighted, considering that a
626 simple mixing of the components leads to phase separation. The plant-derived tannic acid can be added
627 to give a crosslinking reaction between the residual amine groups of casein-*g*-PMMA and the phenolic
628 groups of tannic acid (Cencha et al., 2021).

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

635

636 **3.1.2. Casein-*g*-polyacrylonitrile copolymers (casein-*g*-PAN)**

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

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

661

662 *Table 4: Variations of mechanical properties of 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

663

664

665 A less common PAN-g-casein copolymeric structure (with a PAN backbone and casein grafts), obtained
 666 by grafting casein onto poly(acrylonitrile) via an "onto" process, is also reported. This structure was
 667 obtained by hydrolysis of PAN followed by chlorination of the carboxylic acids of the hydrolyzed PAN

668 and reaction with casein (Jia & Du, 2006). Although this structure has not been clearly evidenced, it is
669 probably a mixture of casein-*g*-PAN and PAN-*g*-casein structures.

670

671 **3.1.3. Other Casein-based graft copolymers**

672 Casein-*g*-polyacrylamide (casein-*g*-PAAm) was prepared by microwave-initiated graft polymerization of
673 acrylamide onto casein backbone in the presence of ceric ammonium nitrate (Sinha, 2013). Poly
674 acrylamide (PAAm) is widely used as a flocculant in water and mineral treatment and an additive in the
675 paper industry. Casein-*g*-PAAm is also a flocculant for the treatment of coal washing effluents with a
676 flocculation profile dependent on the grafting percentage: the grade with highest percentage grafting
677 showed maximum flocculation efficacy, due to the high level of PAAm. The improvement in the
678 flocculation characteristics of these copolymers is due to the increase in hydrodynamic volume. Grafting
679 of polyacrylamide onto casein also improved water solubility, because PAAm is infinitely soluble in
680 water. Accordingly, the higher the grafting percentage of PAAm on casein, the greater its solubility
681 (Sinha, 2018). In the result, casein-*g*-PAAm can be used as an efficient flocculant for coal washing
682 effluent and wastewater.

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

693 Recently, casein-*g*-PNIPAAm unimers have been prepared and applied to cancer therapy (Cuggino et al.,
694 2020). They self-assemble into micelles capable of incorporating doxorubicin and transporting the drug
695 in physiological medium before being released in acidic medium (pH=5). The role of pH and the presence

696 of trypsin, interesting because it is overexpressed in several cancer tumors, on the release rate has been
697 demonstrated. Trypsin allows faster and more complete release (60% of doxorubicin is released in 50h,
698 compared with 30% in the absence of trypsin). Release is also faster at acid pH than at physiological pH
699 due to the doxorubicin's greater solubility at acid pH. However, a release plateau between 20 and 60% is
700 observed in all cases, with no indication of the fate of the remaining doxorubicin.

701 The synthesis of casein-g-polystyrene is also reported using potassium diperiodatonickel-casein as
702 initiator for styrene polymerization. High grafting efficiency (>98%) and a weight proportion of grafted
703 polystyrene chains/casein backbone >300 % are obtained (Weiqi Zhou et al., 2006). The effect of reaction
704 parameters, such as monomer/casein weight ratio, initiator concentration, pH, time, and temperature is
705 investigated. Optimized conditions are monomer/casein weight ratio =3.06 mol/L, time =3 h, temperature
706 =55°C, and pH =12.83. The proof of grafting was obtained from FTIR spectroscopy, X-ray diffraction,
707 and SEM tests. However, the grafting sites on casein and the possibility of obtaining polystyrene
708 homopolymer are not discussed and no properties of this copolymer are indicated.

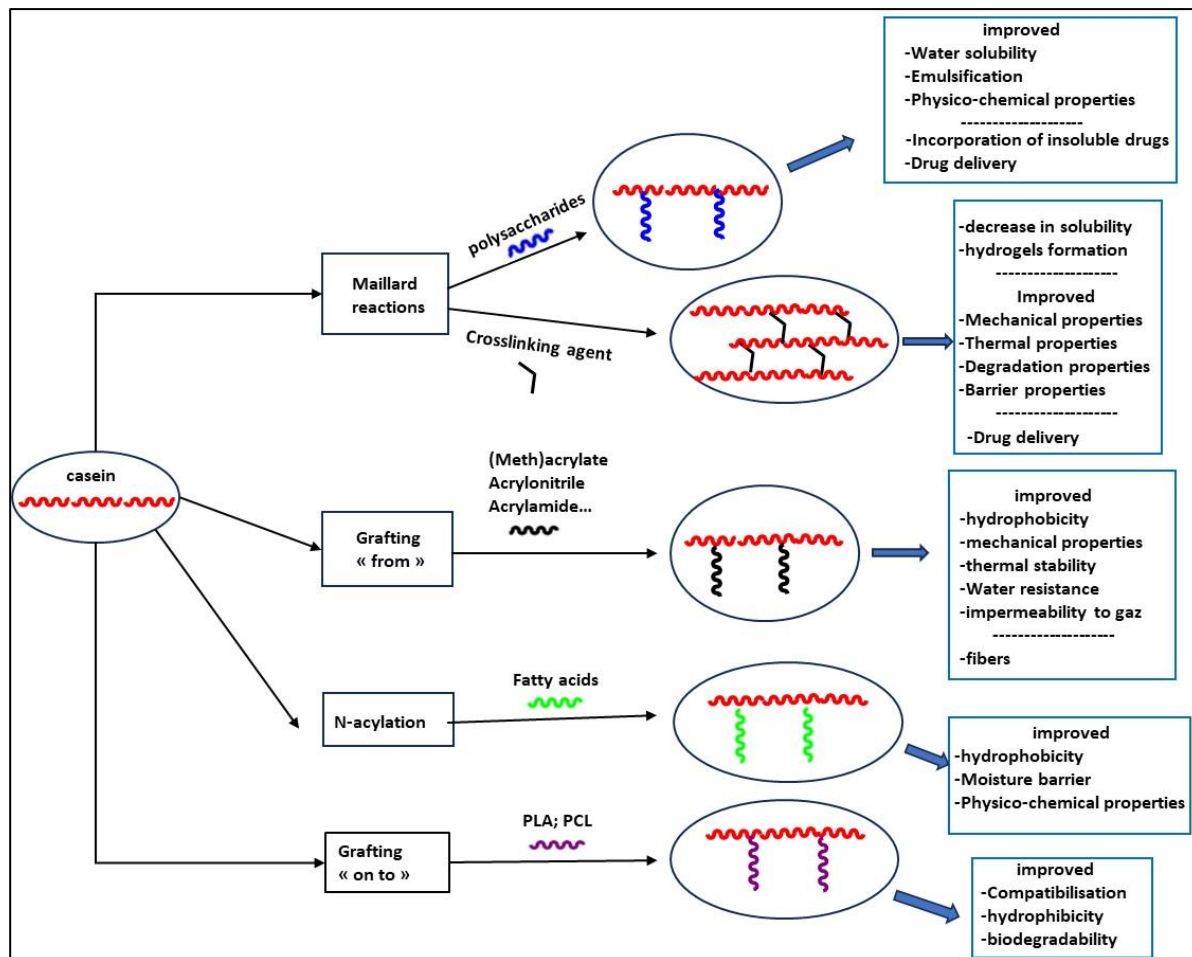
709

710 **3.2. Esterification of carboxylic acid functions of casein.**

711 While reactions on the free amine functions of casein are numerous, reactions on the free carboxylic acid
712 functions (\cong 30 per casein chain) are less frequent. In most cases, they involve esterification with various
713 alcohols (typically ethanol and propanol) in the presence of concentrated hydrochloric acid at 4°C for a
714 few hours (Sitohy et al., 2002). Lin *et al.* described the reaction of the carboxylic acid functions of casein
715 with the alcohol function of triethanolamine followed by reaction of alcohol groups of the grafted
716 triethanolamine on fatty acids to prepare new surfactants (L.-H. Lin et al., 2009). Some properties of
717 esterified and native casein, such as isoelectric point, water solubility, emulsifying activity (Chobert et
718 al., 1990), sensitivity to trypsinolysis (Sitohy et al., 2001) were compared. A decrease in water solubility
719 and a variation in the isoelectric point (from 5.3 for casein to 5.75-9.75 for esterified casein, depending
720 on the degree of esterification) are observed. Potential inhibition of DNA replication by esterified β -casein
721 and esterified α - or β -lactalbumin was investigated and showed that no inhibitory effect was observed
722 with esterified β -casein, unlike esterified α - or β -lactalbumin (Sitohy, 2001). Finally, to our knowledge,

723 there is no description in the literature of a polymeric chain grafted onto casein by reaction with the
724 carboxylic acid functions of casein.

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



727
728 *Figure 9 : Reactions leading to casein graft copolymers and their main properties*

729
730 **4. Conclusion**

731 This review gives an overview of current chemical modifications of the edible casein chain aimed at
732 synthesizing conjugates and/or graft copolymers, as well as some related applications, mainly in the
733 environmental (food chemistry, film formation and packaging) and biomedical (biomaterials, drug
734 encapsulation and delivery) fields. Various types of reactions are described, most of them involving the
735 free amine functions of casein. Maillard reactions are the most frequent, particularly with sugars and
736 polysaccharides, leading to new amphiphilic compounds whose main feature is an increased solubility in

737 water, especially at acid pH. When applied to dialdehydes or other cross-linking agents, these reactions
738 give three-dimensional polymers that are insoluble in water or form chemical hydrogels. The main
739 properties changes concern the formation of stable micelles and hydrogels, and improvements in the
740 rheological and mechanical properties of the resulting biomaterials. Graft copolymers are also obtained
741 by polymerization of various monomers, often (meth)acrylate derivatives, initiated by the amine functions
742 of casein. In contrast to the reaction products obtained with polysaccharides, these copolymers show
743 increased hydrophobicity and resistance to hydrolysis. All these reactions offer a wide range of properties,
744 and therefore potential applications, for casein-based graft copolymers, even though relatively few
745 industrial applications for these products have been developed to date, particularly in the food sector.
746 However, apart from casein's free amine functions, other reactive functions, notably alcohols and
747 carboxylic acids, give rise to only a few chemical reactions. Research in this direction is likely to lead to
748 new structures that will improve the properties of casein-based copolymers and thus open the way to new
749 applications. In any case casein-based graft copolymers are natural or "semi-natural" compounds of
750 animal origin, for the most part nontoxic and partially or fully biodegradable, and therefore promising in
751 food, packaging, environmental and biological fields.

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