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Modifications of structures and functions of caseins: a scientific and technological challenge

Camille Broyard · Frédéric Gaucheron

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Abstract Casein molecules are used in food industry as ingredients. They can be used as isolated forms and under micellar form consisting in an association of different casein molecules and calcium phosphate. In this review, after a brief reminder of the main characteristics of casein molecules and casein micelles, the modifications of caseins induced by physical, chemical, and enzymatic actions are reported. The resulting new physicochemical properties (mineral and casein compositions, charge, hydrophobicity, aggregation state, and morphology) and techno-functionalities (heat stability, viscosity, gelation, emulsifying, and foaming properties) are described and discussed with a special attention paid to the results obtained in our laboratory since several decades.

Keywords Casein micelles · Physical, chemical, and enzymatic methods · Minerals · Stability · Functionality

1 Introduction

The four casein molecules (α_{S1} -, α_{S2} -, β -, and κ -caseins) are well known in terms of amino acid composition, molecular weight, concentrations in milk, and global physicochemical properties (Table 1). All these casein molecules are more or less phosphorylated. This confers to these proteins a good capacity to bind calcium phosphate. Another important characteristic is the glycosylation of 50% of κ -casein. Due to this glycosylation, its C-terminal part is hydrophilic. This characteristic is important for the micellar structure and stability. On the other hand, casein molecules have high contents in prolyl residues conferring open and flexible conformations. Due to their high flexibility, casein molecules have excellent surface-active and stabilizing properties.

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Table 1 General properties of casein molecules

	α_{s1} -casein (B variant)	α_{s2} -casein (A variant)	β -casein (A ² variant)	κ -casein (B variant)
Molecular weight (Da)	23 614	25 230	23 983	19 023
Concentration (g.L ⁻¹)	10	2.6	9.3	3.3
Amino acids	199	207	209	169
Proline	17	10	35	20
Cysteine	0	2	0	2
Glutamic acid	24	25	18	13
Aspartic acid	8	11	5	1
Phosphoserine	8	11	5	1
Apolar residues (%)	36	40	33	33
Glycosylation	0	0	0	0–5
Charge at pH 6.6	-21	-15	-12	-3

The casein molecules are associated together to form casein micelles. Lot of researches focused on the understanding of the organization of casein micelles via different approaches and methods (from molecular to colloidal scale) and with special attention paid to protein-protein and protein-mineral interactions (Walstra 1990, 2002; Schmidt 1982; Horne 1986; Holt 1992; Holt and Horne 1996; Fox and McSweeney 1998; Swaisgood 1992, 2003; Tuinier and de Kruif 2002; De Kruif and Holt 2003; Dalgleish et al. 2004; Walstra et al. 2006; Qi 2007; Fox and Brodtkorb 2008; Dalgleish 2011; Dalgleish and Corredig 2012; De Kruif et al. 2012; Holt et al. 2013). The casein micelles contain α_{s1} -, α_{s2} -, β -, and κ -caseins in proportions 40, 10, 35, and 15% (w/w), respectively. Casein molecules are associated together by protein-protein interactions (hydrophobic, hydrogen, and electrostatic binding) and also by the presence of calcium and phosphate (6–8% in weight). They have an average diameter close to 150 nm with a huge polydispersity, a zeta potential of about -20 mV, and a high amount of associated water (4 g of water/g of protein). Other micellar physico-chemical characteristics are reported in Table 2. Different models of micellar organization were proposed thanks to the improvement of the methods used to their characterization. The main proposed models are (1) a submicellar model where the micelles correspond to submicelle assemblages linked together by nanoclusters of calcium phosphate (Morr 1967a, b; Schmidt 1982; Ono and Obata 1989; Walstra 1999), (2) a model with an open structure (Holt and Horne 1996), and (3) an open model like sponge (Marchin et al. 2007; Bouchoux et al. 2010; Dalgleish 2011). In this last proposed structure, dense regions composed of caseins in interaction with nanoclusters of calcium phosphate and water channels containing salts and caseins are described. In all of these models, the glycosylated forms of κ -casein are located at the surface of casein micelles, conferring them a negative charge and stability due to electrostatic repulsion and steric hindrance.

The aqueous phase of milk contains calcium, magnesium, sodium, and potassium as main cations and inorganic phosphate, citrate, and chloride as main anions. All these ions are associated together to form salts like calcium phosphate, calcium citrate, and sodium chloride. One part of them is also free under ionic form. Taking into account

Table 2 Main physico-chemical characteristics of casein micelles

Characteristic	Value
Diameter	150 nm (range 50–500 nm)
Surface area	$8 \times 10^{-10} \text{ cm}^2$
Volume	$2.1 \times 10^{-15} \text{ cm}^3$
Density (hydrated)	1.0632 g.cm^{-3}
Mass	$2.2 \times 10^{-15} \text{ g}$
Water content	63%
Hydration	$3.7 \text{ g H}_2\text{O.g}^{-1}$
Voluminosity	$4.4 \text{ cm}^3.\text{g}^{-1}$
Molecular weight (hydrated)	$1.3 \times 10^9 \text{ g.mol}^{-1}$
Molecular weight (dehydrated)	$5 \times 10^8 \text{ g.mol}^{-1}$
Number of peptide chains	5×10^3
Number of particles per milliliter of milk	$10^{14}\text{--}10^{16}$
Surface of micelles per milliliter of milk	$5 \times 10^4 \text{ cm}^2$
Mean free distance	240 nm

these different associations and free forms, the aqueous phase of milk at the native pH of 6.6–6.7 is supersaturated in calcium phosphate and has an ionic strength of about 70 mM (Holt 1997; Gaucheron 2004a, b; 2005). Casein micelles are in dynamic equilibrium with the aqueous phase. According to the physico-chemical conditions, they can exchange casein molecules, calcium, inorganic phosphate, and water with the aqueous phase (Fig. 1). In some cases, ions and casein molecules can be transferred from the micellar phase to the aqueous phase. In other cases, these constituents can be integrated in casein micelles.

The techno-functionalities of caseinates (sodium, potassium, or calcium) are largely used in the food industry. Caseinates can improve viscosity, structure, texture of gel, and emulsifying and foaming properties of different food products (Fox and Mulvihill 1983; Rollema 2003; Dickinson 2006). Conversely, the techno-functionalities of casein micelles are less studied and used in industry. However, understanding the relationship between their structures and functions is of major interest to propose new and innovative dairy products. The relationships between structures and techno-functionalities of purified casein molecules or casein micelles are complex but can be schematically represented as in Fig. 2 with intrinsic (casein) and extrinsic (environment) factors. All modifications of intrinsic or/and extrinsic factors can change the techno-functionalities of caseins. These modifications were either used to manufacture different dairy products or applied as strategy to understand the structure-function relationships of the casein molecules or casein micelles.

Different processes or physico-chemical conditions can alter the structure and the techno-functionalities of proteins (Foegeding and Davis 2011; Chobert 2012). This is especially true for the casein molecules and casein micelles (Gaucheron 2004a, b; Augustin and Udabage 2007; Dalgleish and Corredig 2012). In this review, physico-chemical, chemical, and enzymatic modifications are presented, as summarized in Table 3. The studies described in this review were performed with (1) purified casein

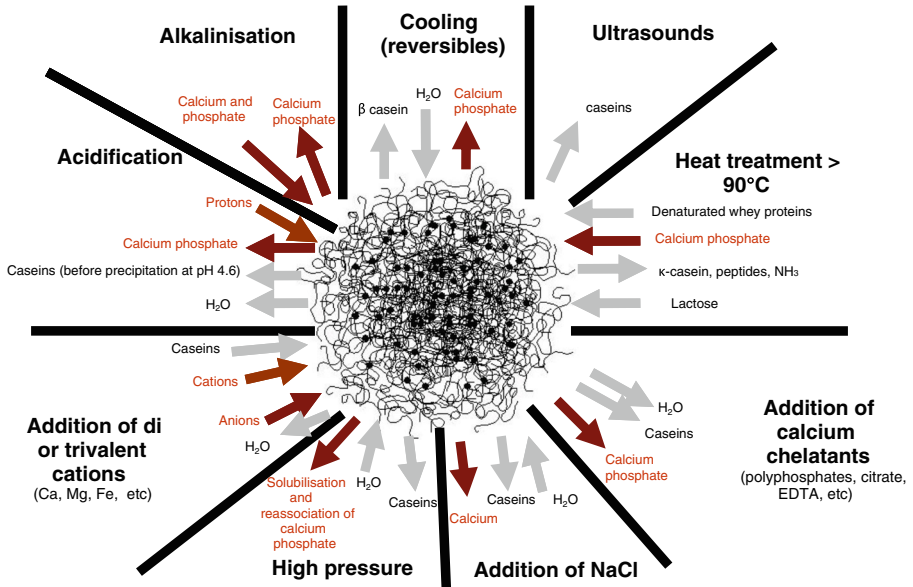


Fig. 1 Schematic exchanges of minerals, water, and casein molecules as a function of different physico-chemical conditions (from Gaucheron 2004b)

molecules or (2) milk or (3) native casein micelles (also named native phosphocaseinate). The casein micelles are prepared from raw skim milk by microfiltration (pore size of 0.1 μm) combined with diafiltration with water to eliminate components of the soluble phase of milk (minerals, whey proteins, peptides, lactose...) (Pierre et al. 1992; Schuck et al. 1994). After their purification by membrane filtration, the micellar suspension is spray-dried. The advantage of this purification is to have the possibility to study the casein micelles alone in controlled conditions. It is indeed easier

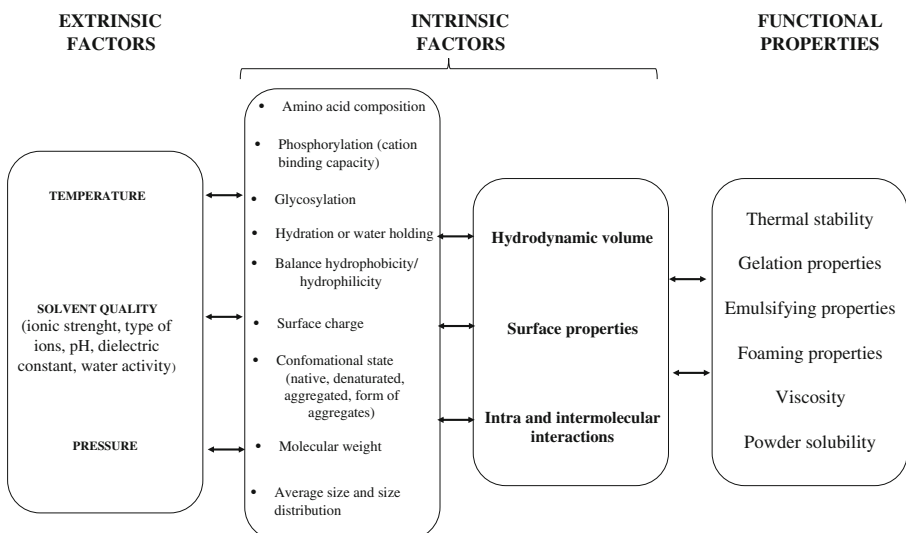


Fig. 2 Schematic relationships between structure and functionality of proteins

to study suspended micellar casein in well-defined solutions. However, it is noteworthy that, after the use of membrane filtration, the casein micelles are not exactly in the same state as in the original milk. Alexander et al. (2011) and more recently Ferrer et al. (2014) reported that during ultrafiltration of milk combined with diafiltration with water, some changes in the physico-chemical properties of casein micelles were observed. Among the changes, they described progressive and irreversible removal of micellar calcium especially after diafiltration.

2 Modification of casein molecules and casein micelles: actions and consequences on their structures and techno-functionalities

2.1 Physico-chemical modifications

2.1.1 Heating

The casein molecules are remarkably heat-stable due to their lack of tertiary structure. However, different modifications depending on the intensity of the heat treatment have been highlighted. Some biochemical modifications are described such as desamidation of asparagine and glutamine residues, proteolysis (Gaucheron et al. 2001; Gaucheron 2001), and reticulation between amino acids, inducing protein polymerization, breakdown of disulfide bridges, and exchange of free thiols present on cysteine residues. The mineral fraction and especially calcium phosphate present in the aqueous phase is less soluble during heat treatment. The newly formed precipitate can interfere with casein micelles (Rose and Tessier 1959; Davies and White 1960) by modifying their surface. The composition, location, and interaction of this newly formed “micellar calcium phosphate” are not well defined in the literature. If the heat treatment is inferior to 95 °C for a few minutes, the modifications of salt equilibria are reversible. In contrast, severe heat treatments (for example 120 °C during 20 min) lead to irreversible changes for casein micelles and salt distribution. At temperatures higher than 110 °C, phosphoserine residues of caseins can be partially hydrolyzed (Van Boekel 1999). For a heat treatment

Table 3 Methods to modify casein molecules or casein micelles

Physical methods	Chemical methods	Enzymatic methods
Heating or cooling	Glycation/lactosylation	Dephosphorylation
High pressure	Reticulation	Deamidation
Ultrasound	Succinylation/acylation	Reticulation
Decrease or increase of pH	Phosphorylation	Deglycosylation Proteolysis
Addition of di- and trivalent cations		
Addition of NaCl		
Removal of diffusible ions		
Addition of calcium chelatants		
Addition of external ligands		

higher than 70 °C for several minutes, covalent binding of whey proteins, mainly β -lactoglobulin, with caseins and aggregation are described (Oldfield et al. 2000; Donato and Guyomarc'h 2009). Increase in the hydrodynamic size of casein micelles and modification of their surface are reported as consequences (Anema and Li 2003; Tran Le et al. 2008). Lactose is also modified during heat treatment. It can be transformed in several compounds like organic acids, lactulose, and Maillard reaction products inducing browning of milk. Lactosylation of protein is also an important reaction induced by heat treatment. This reaction implies NH_2 groups of basic amino acid residues like lysine and lactose (Van Boekel 1998). This reaction is relatively well described on whey proteins but not on casein micelles.

The technological consequences of heat treatment are multiple and described in different reviews, book chapters, and specific articles (Lucey et al. 2001; Vasbinder et al. 2003; Raikos 2010; Kethireddipalli et al. 2010; Dalgleish and Corredig 2012). The most well-known consequence of milk heat treatment is its loss of coagulability by rennet with an increase of the time of gelation and a decrease of gel firmness. By contrast, heating of milk improves firmness of acidic gels.

2.1.2 Cooling at 4 °C

Cooling of milk is a common practice in dairy industry to microbiologically preserve milk before its transformation and consumption. The studies reporting the physico-chemical changes caused by cooling are few and old. Ichilczyk-Leone et al. (1981) showed an increase of about 1% of calcium and inorganic phosphate in the aqueous phase after cooling skim milk. Koutina et al. (2014) confirmed this trend and reported that at 4 °C, the amounts of calcium and phosphorus in the soluble phase were more important than at 40 °C. This is explained by a better solubility of calcium phosphate at low temperature. In the same time, a decreased amount of water associated to casein micelles and a release of β -casein from the micellar structure were described (Downey and Murphy 1970; Creamer et al. 1977; Pierre and Brulé 1981; Pouliot et al. 1994; Holt 1995). Indeed, temperature reduction modifies interactions between proteins, and consequently, this casein can be transferred into the aqueous phase. These changes are reversible and the previous partition may be re-established after milk warming. Nevertheless, the native structure is not perfectly recovered because β -casein do not come back to their previous place. Liu et al. (2013) confirmed that the structure of casein micelle and the mineral system of milk were dependent on temperature in the range 10–40 °C by showing that the amount of soluble casein, hydration, and apparent voluminosity of casein micelles decreased as a function of increasing temperature. Moreover, the dynamic response of the mineral system to changes in temperature appears almost immediate when heating whereas re-equilibration of casein micelles is slower during cooling. Due to these modifications of interaction at low temperature, microfiltration of cold milk was proposed to purify β -casein at industrial level (Pouliot et al. 1994; Van Hekken and Holsinger 2000). Moreover, it might be interesting to use these new casein micelles (less mineralized, depleted in β -casein and more hydrated) to obtain new techno-functionalities. For example, Koutina et al. (2014) showed that the storage modulus G' at pH 4.8, 4.7, and 4.6 (gel formation) was higher for decreasing temperature (4 > 20 > 30 > 40 °C). They concluded that the final texture of dairy products such as cheese could be modulated by the choice of the couple pH and temperature.

2.1.3 High pressure

The structure of casein micelles is also modified by pressure treatments. Depending on the pressure level, the technology is called high-pressure homogenization or isostatic high pressure (up to 150–200 MPa) and ultra-high-pressure homogenization or dynamic high pressure (up to 350–400 MPa).

Concerning the effects of high-pressure treatment, several results obtained on milk with a special attention paid on milk proteins have been published for 25 years (Buccheim and Prokopeck 1992; Johnston et al. 1992; Shibauchi et al. 1992; Desobry-Banon et al. 1994; Gaucheron et al. 1997a; Schrader and Buchheim 1998; Huppertz et al. 2004a, b; Orlien et al. 2006; Huppertz and Kruif 2007; Bravo et al. 2013). The modifications of casein structure by high-pressure treatments are influenced by process parameters (pressure level, holding time, pressure release rate, temperature, and type of equipment) and by the composition of the dairy liquid (pH, ionic strength, solvent composition, and protein concentration). The first visible impact of a high-pressure treatment (higher than 150 MPa) is a decrease of milk whiteness. The physico-chemical analyses of pressurized skim milk revealed a decrease in the hydrodynamic diameter of casein micelles, a decrease in turbidity, an increase in viscosity, an increase in casein micelle hydration, and slight solubilizations of micellar phosphorus and calcium. Partial solubilizations of α_{s1} - and α_{s2} -caseins were also described (Regnault et al. 2006). These modifications are probably due to weakening of interactions between casein molecules themselves and also with colloidal calcium phosphate during the pressure treatment (Menéndez-Aguirre et al. 2014). After a return to atmospheric pressure, molecules of casein reassociate between themselves but not in the same organizational state as before the pressure treatment. The rate and the extent of casein disruption and reassociation were not affected by the presence of major whey proteins (i.e., β -lactoglobulin and α -lactalbumin) (Huppertz and Kruif 2007). Results of Merel-Rausch et al. (2007) stated that, depending on the casein concentration (1 to 15%) and the pressure release rate, structures ranging from liquid (near Newtonian flow behavior) to a gel can be obtained.

The changes in the physico-chemical properties of casein micelles lead to modifications in their functionalities. Thus, acidification by glucono-delta-lactone of pressurized milk induced a faster coagulation than for unpressurized milk (Desobry-Banon et al. 1994). High-pressure treatments (above 230 MPa) induced a decrease of the rennet clotting time (Shibauchi et al. 1992; Desobry-Banon et al. 1994). The maximum buffering capacity was shifted from 4.8 to 5.0 for untreated milk to 5.2–5.4 for pressurized milks (Famelart et al. 1998). The gel strength increased with increasing high-pressure treatment (Johnston et al. 1992; Desobry-Banon et al. 1994) and a syneresis resistance was observed. High-pressure treatments appear as an interesting tool to entrap different ligands like curcumin, vitamin D₂, and α -tocopherol (Benzaria et al. 2013; Menéndez-Aguirre et al. 2011; Chevalier-Lucia et al. 2011). It increases the binding constant of these compounds to casein micelles. This could be related to the increased surface area of processed micelles since they were smaller and more numerous after high-pressure treatment.

2.1.4 Ultrasound

Ultrasonic processing could be an interesting technique for the food industry. This method consists in submitting samples to a sound wave of frequency between 16 kHz and 10 MHz. The effects of this treatment on milk proteins are not well studied, and only few publications describe the effects of this physical treatment on casein. Madadlou et al. (2009) reported decreases in turbidity and diameter of particle suggesting disruption of casein micelles after their sonication (sonodissociation). The same authors (Madadlou et al. 2010) indicated a delay in the acid gelation time and an increase of the elasticity of gels formed with sonicated caseins. The gels contained a more interconnected structure and smaller non-distinguishable particles. These results were in accordance with those obtained by Vercet et al. (2002) and Riener et al. (2009) who reported that the rheological properties of yoghurt made with milk submitted to manothermosonication were improved in comparison with untreated milk. More recently, Liu et al. (2014b, c) indicated that ultrasonic treatment improved the properties of milk coagulation by rennet. The small reduction in the size of milk particles and changes in the protein hydrophobicity caused by physical effects from cavitation when applying low-frequency ultrasounds were the possible causes of this improvement of coagulation.

2.1.5 pH decrease

The structure and charge of casein molecules depend on the pH value. Decrease in pH reduces their ionization and induces changes on intra- and intermolecular interactions. Thus, during acidification of caseins, the phosphoserine residues and carboxyl groups change their ionization state because they have an affinity for protons—their protonation depends on their pKa values. Thus, at neutral pH, casein molecules are negatively charged. During acidification, they bind protons and casein molecules become less and less negatively charged to be neutral at their isoelectric pH. At this pH, casein molecules are aggregated and their solubilities are minimal.

The description of acidification of casein micelle is more complex than the one for casein molecules alone because micellar calcium phosphate is implied. Several hundred publications describe the different changes occurring during the decrease of pH (De Kruif 1997; McMahon et al. 2009). During milk acidification, the organic and inorganic phosphate, citrate, and carboxylic residues of caseins become more protonated (or less ionized). In the same time, the aqueous phase becomes less saturated in calcium phosphate due to a dissociation of this salt. Consequently, the micellar calcium phosphate is progressively dissolved with increases of calcium and inorganic phosphate concentrations in the aqueous phase (Visser et al. 1986; Dalgleish and Law 1988, 1989; Mekmene et al. 2010). This is accompanied by a dissociation of casein molecules which are transferred in the aqueous phase. The extent of mineral and casein dissociations depends on the pH value. At pH 5.2, a part of calcium and the totality of the inorganic phosphate are solubilized. Calcium is totally solubilized at pH 3.5 (Le Graët and Brulé 1993; Mekmene et al. 2010). The pH-induced solubilizations of minerals from concentrated casein micelles are shifted toward lower pH values (Le Graët and Gaucheron 1999). In the same time, interactions between caseins themselves and between caseins and water are also affected because casein molecules are less and less

charged. During acidification, the hairy layer of κ -casein shrinks and finally collapses. Consequently, the steric stabilization of casein micelles diminishes and, as a result, micelles exhibit attractive interactions (De Kruif 1997). In the pH range from 6.0 to 5.6, casein micelles swell and dissociation of caseins reaches a maximum at pH 5.6 with a maximal dissociation of β -casein (Dagleish and Law 1988). In this pH range, a new small population of caseins corresponding to casein aggregates is observed (Silva et al. 2013). These small particles have an average diameter of about 20–35 nm and a molecular weight between 10^6 and 10^7 g.mol⁻¹. The proportion of this population smaller in size increases when the pH used to obtain the different casein suspensions is more acidic ($6.7 < 6.4 < 6.1 < 5.8 < 5.5$). In terms of size, hydration, shape, and zeta potential, the non-dissociated casein micelles seemed similar to the native ones (Silva et al. 2013). The demineralization of casein micelles by decreasing the pH from 6.7 to 5.2 resulted in a reduced granular aspect of the micelles observed by cryo-transmission electron microscopy and atomic force microscopy (Marchin et al. 2007; Ouanezar et al. 2012) and the existence of a characteristic point of inflection in SAXS profiles (Marchin et al. 2007). At pH 4.6, the charge of caseins is equal to zero and consequently their solubility is minimal. Precipitation or gelation of caseins is observed.

The micellar destructureation induced by acidification is relatively similar with the different types of acid used (lactic, chlorhydric, citric). It is the pH value which mainly governs the physico-chemical changes. However, the composition of the aqueous phase especially its ionic environment as a function of acid types is not the same and this can influence the structure and functions of acidified caseins. Another remark concerns the CO₂ use to reversibly acidify milk. With this treatment, Guillaume et al. (2004) reported some impacts on colloidal minerals, buffering capacity, and rennet coagulation which was improved.

The functional properties such as gel properties and heat stability of milk acidified at different pH values are strongly modified. Extensive information are described in different reviews and book chapters (Home 1999). From a general point of view, at acid pH, the heat stability is decreased whereas the rennet coagulation is improved with a decrease in the coagulation time (Van Hooydonk et al. 1986; Famelart et al. 1996; Liu et al. 2014a).

2.1.6 pH increase

Alkalinization is not commonly used in dairy industry and all the studies done at pH superior to 7 were performed to understand the salt equilibria, organization, and behavior of casein micelles in other pH conditions. After an increase of pH to 9, decreases in turbidity and whiteness of milk are described. Milk becomes transparent indicating a destruction of casein micelles with the presence of casein molecules in serum (Odagiri and Nickerson 1965a; Van Dijk 1991, 1992; Ahmad et al. 2009). In the same time, decreases in concentrations of calcium (especially ionic Ca) and inorganic phosphate in the aqueous phase were observed. Ahmad et al. (2009) proposed that at alkaline pH, the inorganic phosphate ion changes its ionization state from HPO₄²⁻ to PO₄³⁻. This last form of phosphate has a greater affinity for calcium, and consequently, a demineralization of casein micelles was induced. These decreases of mineral concentrations in the aqueous phase could improve the solvent quality and facilitate the dissociation of casein micelles by a diminution of cohesive interactions between the

hydrophobic regions of caseins (Vaia et al. 2006). In addition to the mineral changes, these same authors and Huppertz et al. (2008) explained that the changes in the ionization of caseins could also contribute to this micellar disruption. To our knowledge, there are no published results on the techno-functionalities of alkalized casein micelles.

2.1.7 Addition of di- and trivalent cations

Due to their negative charges at neutral pH, the phosphoserine residues and secondarily the carboxylic groups of acid residues have a strong affinity for di- and trivalent cations. A lot of results were published on the interactions between cations and molecules of casein, and it is admitted that these bindings depend on (1) the type of casein molecules (α_{s1} , α_{s2} , β , or κ); (2) their phosphorylation state; (3) their charge; (4) the considered cations which can be different in terms of number of positive charge, size, and hydration; (5) type of binding (electrostatic or coordinative); and (6) the physico-chemical environment (pH, ionic strength, temperature, presence of other compounds) (Gaucheron et al. 1997c; Gaucheron 2004a, b). The consequences of the binding of cations to casein molecules are a neutralization of their charge with an aggregation and precipitation of casein molecules if their binding sites are saturated by the added cations. The best example to show the influence of the cation type on the physico-chemical and techno-functionalities of casein molecules is the comparison between calcium caseinate and sodium caseinate. In terms of molecular organization and techno-functionalities, these two types of caseinate are different.

The description of the effects of addition of cations to milk or to casein micelles is more complex than those of addition of the same cations to molecules of caseins. Indeed, with milk, it is necessary to integrate that casein micelles are saturated in calcium phosphate (nanoclusters of calcium phosphate) and that they are in suspensions in aqueous phase also saturated in calcium and phosphate (Fig. 1). Theoretically, the mineral exchanges between aqueous and micellar phases are mainly controlled by the solubility of calcium phosphate of the aqueous phase. It is described that addition of divalent cations induces pH decrease and important modifications in the salt distribution between aqueous and micellar phases (Fig. 1) (Gaucheron 2004a, b). These modifications depend on (1) the nature of the aqueous phase, (2) the type of added cation (calcium, magnesium, iron, copper, zinc, etc.), (3) the added quantities, and (4) the eventual pH correction.

The calcium-induced modifications of salt balance in milk are the most described because this addition is largely practiced in dairy industry especially in cheese manufacture. Thus, after addition of 10 mM of calcium chloride to milk, about 80% of this ion is associated to casein micelles. In the same time, inorganic phosphate and citrate ions are transferred from the aqueous phase to the micellar phase (Philippe et al. 2003, 2005; Mekmene et al. 2009). Consequently, the structure of casein micelles was modified with decreases in their zeta potential, hydration, and heat stability (Nieuwenhuijse et al. 1988). The buffering capacity was shifted towards lower pH in comparison with a non-enriched milk due to the formation of new micellar calcium phosphate (Salaun et al. 2007). Moreover, after addition of calcium to milk, its rennet coagulation was improved with a decrease in gelation time and an increase in gel firmness. On the contrary, addition of insoluble calcium salts like calcium phosphate

does not change the physico-chemical and technological properties of enriched milks (Philippe et al. 2004; Omoarukhe et al. 2010).

Even if magnesium is a divalent cation like calcium, its addition to milk induces different effects in comparison with calcium. Modification of salt equilibria is less critical because magnesium-phosphate salt is not at saturation in the aqueous phase of milk. The low percentage of magnesium association with casein micelles can be explained by the low saturation indexes of salts like MgHPO_4 and $\text{Mg}_3(\text{PO}_4)_2$ which are below 1. Due to these characteristics, the added magnesium interacts less than calcium with casein micelles and the consequences in terms of heat stability or rennet coagulation are less important than after calcium addition (Van Dijk 1991; Philippe et al. 2005).

Addition of iron, Fe^{2+} or Fe^{3+} , to milk was also studied. Although the addition of iron is mainly performed for nutritional reason, the induced modifications of structure of the casein micelle could be used to improve techno-functionalities of milk enriched with iron. In suspensions containing only casein molecules, iron establishes coordinative links with oxygen atoms of phosphoseryl, aspartyl, and glutamyl residues (Gaucheron et al. 1996, 1997b; Raouche et al. 2009a, b). When iron is added to milk, it is mainly associated to casein micelles by interacting with organic and inorganic phosphates. The physico-chemical consequences of interactions between casein micelles and iron are decreases in zeta potentials and amount of water associated to casein micelles (Philippe et al. 2005). The technological consequences induced by the addition of iron depend on the state of oxidation of iron. The increases in times of clotting and aggregation and the decrease in the curd firmness are stronger with Fe^{2+} than with Fe^{3+} (Gaucheron et al. 1997b).

2.1.8 Addition of NaCl

The structure, charge, and state of aggregation of casein molecules depend also on the NaCl concentration. Sodium ions act as counter ions of negative charge of phosphoseryl residues and carboxylic groups. At low ionic strength, casein molecules are individualized with electrostatic repulsions between themselves. In the presence of NaCl, the electrostatic repulsions are screened and casein molecules associated together to form small aggregates. HadjSadok et al. (2008) reported changes of molecular weight and radius which increased from $2.5 \times 10^4 \text{ g}\cdot\text{mol}^{-1}$ and 3 nm in the absence of added NaCl to $2.6 \times 10^5 \text{ g}\cdot\text{mol}^{-1}$ and 11 nm in the presence of 100 mM NaCl. The functionality of casein depends also on the NaCl concentration. Among the modifications, it is reported a best solubility of casein molecules close to the isoelectric point in the presence of NaCl.

With casein micelles in milk conditions, the situation is more complex than with casein molecules. Milk contains sodium and chloride at the concentrations of 22 and 27 mM, respectively. Both ions are mainly present in the aqueous phase and partly contribute to the ionic strength evaluated to about 70 mM. When supplementary NaCl is added to milk or concentrated milks, pH slightly decreases and soluble calcium concentration increases (Le Graët and Brulé 1993; Huppertz and Fox 2006; Mekmene et al. 2009). These releases would correspond to exchanges between added sodium and calcium and protons bound directly to phosphoseryl residues of casein molecules. In the same time, the hydration of casein micelles is increased although their size and

charge remain constant (Grufferty and Fox 1985; Van Dijk 1991; Casiraghi and Lucisano 1991; Le Ray et al. 1998; Famelart et al. 1999; Gaucheron et al. 2000; Huppertz and Fox 2006). High sodium concentration leads to a decrease in electrostatic repulsions between caseins, by shielding their negative charges. The casein-casein and casein-water interactions are modified. Karlsson et al. (2005) indicated that addition of NaCl to a milk concentrate induced the formation of rough micellar structure observed by transmission electron microscopy. The techno-functionalities of casein micelles are also different according to added NaCl concentration. Famelart et al. (1999) showed that the rennet gelation was delayed in the presence of NaCl. An increase in viscosity was observed. Huppertz and Fox (2006) showed that addition of NaCl on concentrated milks induced a change in the heat coagulation time as a function of pH and a reduction of ethanol stability. Besides, addition of NaCl (up to 400 mM) shifted the maximum buffering capacity of milk from pH 5.2 (without NaCl addition) to 5.5 (Salaun et al. 2007). The solubilization of micellar phosphate induced by addition of NaCl at high concentrations could be responsible for this shift of buffering capacity. Rehydration of micellar powder was improved in the presence of NaCl compared to a rehydration in water (Schuck et al. 1994; Hussain et al. 2011a, b; Schokker et al. 2011). To avoid a bad rehydration of micellar powder, Sikand et al. (2013) proposed to add NaCl or KCl during the diafiltration step. In these cases, the authors indicated that the added salts were probably responsible for structural modifications of casein micelles with as consequence an improvement of their rehydration.

2.1.9 Removal of diffusible ions

Membrane technologies are commonly used to concentrate milk proteins (ultrafiltration) or casein micelles (microfiltration with molecular weight cut-off of 0.1 μm). The use of these technologies allows to retain a protein fraction in the retentate and the small diffusible components (salts, free ions, lactose, peptides, amino acids, etc.) are able to pass through the membrane (filtrate). If a step of diafiltration with water (in general with 4–5 vol of the retentate volume) is performed before the final concentration, the diffusible components are removed (Pierre et al. 1992; Schuck et al. 1994). In this case, the ratio of mineral concentrations in colloidal and aqueous phases increases. It is also observed a pH increase due to the removal of buffering ions like inorganic phosphate. As the aqueous phase is water, the physico-chemical properties of caseins molecules are modified especially for their pH of precipitation (their apparent pHi increases when the ionic strength decreases). The consequences are also an increase of the electrostatic repulsions between caseins more negatively charged. Famelart et al. (1996) showed that casein micelles suspended in water formed a strong gel at acid pH and coagulated with a shorter renneting time than casein micelles suspended in presence in NaCl or in milk ultrafiltrate. Another example showing the importance of the aqueous phase is the difficulty to solubilize powder of purified casein micelle in water (Schuck et al. 1994).

2.1.10 Addition of calcium chelatants

Addition of calcium chelatants (citrate, EDTA, oxalate, pyrophosphate, polyphosphate, cation-exchange resins) to milk is studied since several years

(Odagiri and Nickerson 1965b; Morr 1967a, b; Casiraghi and Lucisano 1991; Le Ray et al. 1998; Udabage et al. 2000; Pitkowski et al. 2008; Mekmene et al. 2009; Kaliappan and Lucey 2011), and there is no doubt about their demineralizing actions on the casein micelles. The chelatants act indirectly on casein micelles by chelating the ionic calcium (2 mM) present in the aqueous phase. Consequently, the degree of calcium phosphate saturation of the aqueous phase is reduced and the solubilization of micellar calcium phosphate occurs. The extent of this micellar demineralization depends on the association constants (or calcium binding capacity) between calcium and chelatants. Due to this solubilization, the micellar suspensions or milk become less turbid and white (Mizuno and Lucey 2005). This decrease of turbidity corresponds to a destruction of the micellar structure with a release of small aggregates of non-sedimentable casein molecules in the aqueous phase. Pitkowski et al. (2008) showed that these small aggregates contained 10–15 casein molecules with hydrodynamic radius of about 10 nm. The zeta potential of casein becomes more negative because interactions between phosphoserine residues and calcium are reduced.

These additions of chelatants by modifying the organization of casein micelles can prevent some important problems like deposit formation on heat exchangers and membrane surfaces. They are also interesting in the development of innovative products because the addition of chelatants improves heat stability and storage life of milk products especially concentrated milk. These salts are also used in the manufacture of processed cheese. As a consequence of their chelating actions, these molecules quantitatively and qualitatively modify the buffering capacity of dairy liquids. Quantitatively, the buffering capacity increases because the chelating molecules have acid-basic groups which are able to bind protons and resist to the decrease in pH. The maximal buffering capacity is also shifted toward lower or higher pH (in comparison with micellar suspension without chelatant) depending on pK values of chelatants (Salaun et al. 2007).

In relation with these physico-chemical modifications, the calcium chelatants alter the technological properties of casein. De Kort et al. (2011) indicated that the presence of chelatant induced an increase in viscosity due to the presence of these small aggregates. The apparent shear modulus and the resistance to syneresis increased in acid gels containing calcium-chelating agents (Johnston and Murphy 1992). This was confirmed by Ozcan-Yilsay et al. (2006) who indicated that the addition of trisodium citrate (between 10 and 15 mM) to milk improved the textural properties of acid gel and reduced the whey separation. Improvement in foaming properties was also described after addition of hexametaphosphate (Kelly and Burgess 1978), EDTA (Ward et al. 1997), or citrate (Augustin and Clarke 2008) to milk. Schuck et al. (2002) and Schokker et al. (2011) indicated that solubilization of micellar powder was improved in presence of citrate (in comparison with solubilization in water). Ye (2011) indicated that stable emulsions were formed in presence of protein concentrates having low micellar calcium content. This improvement was in relation with the mineralization and organization of casein. With a high micellar calcium content, casein are under micellar form (200 nm) and on the contrary, with a low micellar calcium content, caseins form small aggregates (30–50 nm) which are able to stabilize emulsion by their presence at the oil-water interface.

2.1.11 Addition of external ligands

Some publications report the effect of addition of external molecules on the structure and functionality of caseins used as vector (in most cases, the added molecules were interesting for health). In general, there are interactions between casein molecules and the added compounds. Sometimes these new interactions affect the structure of caseins and also their functional properties. In this context, O'Connell and Fox, in a review published in 2001, reported significance and applications of phenolic compounds on the quality of milk and dairy products. They indicated that these compounds interact with casein by hydrophobic and hydrogen bindings or after chemical reactions. One of the consequences of the interactions between caffeic and ferulic acids and casein micelles was an increase of their heat stability. Kartsova and Alekseeva (2008) reported that tea catechins were also able to be bound to casein micelles. This is interesting for application of coffee/tea whiteners. The binding sites were not clearly identified, but it was suggested that proline residues were involved in these interactions. Recently, Haratifar and Corredig (2014) indicated that this association increased the time of milk gelation after rennet addition. For the authors, this effect was in relation with a modification of the enzymatic cleavage leading to a slower release of caseinomacropptide after rennet addition. For the authors, the accessibility of the enzyme to κ -casein was probably limited. Harbourne et al. (2011) reported that addition of tannic and gallic acids modified the formation of acid gel. In the presence of phenolic compounds, the gelation time was faster. These additions induced also a higher storage modulus and a decrease in the water mobility, and no effect on the syneresis was determined. Titapiccolo et al. (2010) showed that (1) the rennet gelation of casein micelles was accelerated in the presence of different nonionic surfactants and (2) higher elastic modulus were obtained in their presence. By varying the size of the surfactant (Tween 20 or Tween 80) as well as the colloidal state of casein, these authors showed that the surfactant had a direct effect on the casein micelles.

2.2 Chemical modifications

2.2.1 Reaction with sugars

It is an interesting method to improve the functional properties of food proteins (Liu et al. 2012) and especially casein. This can be done through a chemical combination of a free aldehyde function of sugar with ϵ -amino groups of lysyl residues. Sugar can be lactose (reaction of lactosylation) but also glucose, sucrose, mannose, and other (reaction of glycation). All casein molecules are concerned by these reactions because all contain lysyl residues (Swaisgood 1992). These reactions occur naturally or are induced especially during heat treatment or storage (Siciliano et al. 2000; Le et al. 2013). Glycation can be followed by evaluation of the number of the free amino groups of lysyl residues and more precisely by mass spectrometry (Steffan et al. 2006; Siciliano et al. 2013). Courthaudon et al. (1989) reported that glycation of casein with glucose, fructose, galactose, mannose, maltose, or lactose improved their solubility especially close to their isoelectric point (but it remained unchanged at neutral and alkaline pH and decreased at low pH values). Increases of viscosity were also described by these authors. Colas et al. (1988) showed that

glycation of casein with galactose, glucose, fructose, lactose, and maltose resulted in an increase of voluminosity of casein. Closs et al. (1990) described an improvement of the foaming properties (capacity and stability) when caseins were glycated. They explained this change by an increase in the flexibility of glycated casein allowing a more rapid absorption at the water-air interface (in comparison to non-glycated caseins). Darewicz et al. (1999a) studied the impact of glycation and enzymatic dephosphorylation on β -casein. In this study, ten glucose and nine lactose were bound to casein while all phosphate groups were removed. After these modifications, they concluded that glycation (1) shifted the pHi of casein toward more acidic pH values and (2) decreased the solubility at acid pH. In another publication, the same team (Darewicz et al. 1999b) reported structural changes deduced by modification of absorption spectra in UV and a better heat stability of β -casein after its glycation. Corzo-Martinez et al. (2010) worked on the characterization and improvement of rheological properties of glycated sodium caseinate with galactose, lactose, and dextran. They showed a higher reactivity to form glycoconjugates for galactose compared to lactose and dextrans, due to its smaller molecular weight. Reaction with galactose and lactose increased the viscosity of caseinate. In addition, the flow characteristics of glycated caseinates were modified, passing from a Newtonian behavior to a shear-thinning behavior. On the contrary, glycation with dextran did not improve the rheological properties of caseinate, probably due to the limited extent of the reaction. Similarly, Oliver et al. (2006) reported that glycated sodium caseinate with ribose, glucose, fructose, lactose, and inulin have increased viscosities compared to unmodified sodium caseinate with a non-Newtonian behavior. According to these authors, the main cause of increased viscosities was related to an increase of bound water to the glycated proteins. Preparations of glycated caseinate with maltodextrin were described by O'Regan and Mulvihill (2009). The reactions were performed at 60 °C and 79% of relative humidity for 4 days. The conjugates have an improved solubility compared to unmodified caseinate, particularly around their isoelectric point. Moreover, oil/water emulsions were more stabilized with the glycated caseinate compared to unmodified caseinate.

It is also possible to induce reaction between caseins and lactose (reaction of lactosylation). In a general way, the extent of lactosylation depends on several factors such as (1) intensity of heat treatments, (2) type and structure of the considered protein (van Boekel 1998; Scaloni et al. 2002), (3) lactose/protein ratio (Evangelisti et al. 1994), and (4) environmental conditions like pH. The literature describing the reaction of lactosylation and its effects on casein micelles is relatively poor. In contrast, reaction between lactose and purified casein molecules (and also whey proteins) is better described, especially with α_{S1} - and β -caseins because they contain important amounts of lysyl residues (Swaisgood 1992). The consequences of the lactosylation of casein molecules are multiple and complex. It is reported a modification of enzymatic proteolysis of lactosylated proteins. Thus, Dalsgaard et al. (2007) showed that lactosylation of α -, β -, and κ -caseins, β -lactoglobulin, or lactoferrin affected negatively their hydrolysis by plasmin, especially for the globular proteins. In the same way, Bhatt et al. (2014) indicated that a high level of lactosylation of lysyl residues present on β -casein affected the plasmin-induced hydrolysis. In contrast, lactosylation of caseins did not affect significantly their cleavage by cathepsin D and chymosin.

2.2.2 Chemical reticulation

An alternative to modify casein structure is its chemical reticulation. One of the most known chemical agents is glutaraldehyde. Silva et al. (2004) showed that this bifunctional compound was able to link lysyl residues to form aggregates with high molecular weight. However, no intensive research was made with this chemical agent due to its toxicity. Another reagent is the genipin, a natural molecule extracted from the fruits of *Gardenia jasminoides* (Endo and Taguchi 1973; Song et al. 2009). As its toxicity seems limited, this molecule was investigated as a cross-linking agent in many biological applications (Chaubaroux et al. 2012). In recent works, Silva et al. (2014) and Nogueira Silva et al. (2015) have characterized the casein micelles cross-linked by genipin. After reaction, micellar suspension was blue in color. These authors showed that reticulated casein micelles were (1) formed via cross-linking with lysyl and arginyl residues, (2) smaller in size, and (3) more negatively charged and have a smoother surface than native casein micelles. Light scattering measurements showed that the reaction between genipin and casein molecules was mainly intra-micellar with the determination of only one population of particles. The viscosity of the modified micellar suspensions was reduced. These reticulated casein micelles were not dissociated in presence of citrate and urea or at the air-solution interface where they formed rather a solid than a viscoelastic gel. In terms of techno-functionality of these type of reticulated casein micelles, no results are reported in the literature. It is probable that some similarities with casein micelles reticulated by transglutaminase would be determined (“Reticulation” section). Another chemical compound is also described named cinnamaldehyde able to cross-link proteins like chitosan (Nipun Babu and Kannan 2012). To our knowledge, no result is reported on milk protein but such compound could be interesting to reticulate casein molecules or casein micelles.

2.2.3 Succinylation/acylation

The reactions of succinylation and acylation concern the free amino groups of lysyl residues of casein molecules with as consequence modifications of their physico-chemical properties, especially their charge.

Succinylation The reaction of succinylation is performed by addition of succinic anhydride to caseins. The result of this reaction is a covalent attachment of succinate groups containing one negative charge per group. One of the consequences of this reaction is an increase of the net negative charge of the caseins inducing a shift of pHi of caseins toward a lower pH value. In addition, increases of electrostatic repulsions due to the increase of the negative charges of caseins were induced (Vidal et al. 2002). Hoagland (1966) indicated that succinylated β -casein remained soluble in the presence of 25 mM added calcium whereas unmodified β -casein was totally precipitated. According to Lieske (1999), there is also a calcium depletion for the succinylated casein micelles, a decrease in their surface hydrophobicity, and an increase of their diameter. Lakkis and Villota (1992) reported spectral changes of fluorescence and circular dichroism of caseins after their succinylation.

As consequence of this reaction, the techno-functional properties of caseins are also modified. As the negative charge of caseins was increased, succinylated caseins were

more soluble at acidic pH values (Girerd et al. 1984). Vidal et al. (1998) studied the influence of the succinylation on the ability of caseins to form acid and rennet gels. They indicated a delay in the time of gelation and a decrease in the firmness of rennet gel. These modifications were related to a greater resistance of succinylated casein micelles to their aggregation. Lieske et al. (2000) also reported that succinylation of milk proteins increased the rennet gelation time and decreased the gel firmness. They explained these effects by (1) changes in the distribution of calcium between micellar and aqueous phase, (2) changes in the release of CMP during proteolysis, and (3) changes in the charge of casein micelles with release of individual casein molecules in the aqueous phase. Recently, Yang et al. (2014) reported that the solubility, emulsifying activity, and absorption at water-oil interface of succinylated caseins were enhanced, while their capacity to stabilize foam was reduced.

Acetylation or acylation The reaction of acetylation is performed by addition of acetic anhydride to caseins. The reaction results in a covalent attachment of acetate groups to amino groups of lysyl residues. Hoagland (1966) indicated that for a concentration of 25 mM of added calcium, unmodified β -casein was totally precipitated whereas the acylated β -casein was soluble at 44%. Vidal et al. (2002) reported an increase of micellar hydration without effect on the size and zeta potential of acylated casein micelles. In terms of techno-functionality, the acetylation of casein increased their heat stability (Creamer et al. 1971) and solubility at acid pH (Santos and Tomasula 2000).

2.2.4 Phosphorylation

Casein molecules are naturally phosphorylated, but it is interesting to add supplementary phosphorus to change their physico-chemical and functional properties. The chemical phosphorylation, performed in presence of phosphorus oxychloride (POCl_3), results in a covalent linking of phosphorus atoms to proteins (Matheis 1991). After their phosphorylation, caseins are more electronegative. As a consequence of this supplementary phosphorylation, the pHi and surface hydrophobicity of caseins were reduced (Medina et al. 1992; Sitohy et al. 1995). Due to the increase of the electronegativity of caseins after their phosphorylation, their foaming and emulsifying properties and also their solubility were improved (Girerd et al. 1984; Medina et al. 1992; Sitohy et al. 1995). Nevertheless, Matheis et al. (1983) showed that addition of 7.4 phosphate groups per mole of caseins increased their viscosity and capacity of water holding, but decreased their solubility and emulsification capacity.

2.3 Enzymatic modifications

2.3.1 Dephosphorylation

Dephosphorylation of phosphoserine residues can occur naturally but phosphatase activity in milk is relatively limited (Molina et al. 2007). The dephosphorylation of casein can also be deliberately induced by addition of acid or alkaline phosphatases. However, studies with these enzymes are not always easy due to their residual proteolytic activity which can induce misinterpretation of the results. In addition, the

extent of dephosphorylation depends on substrate and hydrolysis conditions (enzyme used, pH, temperature, presence of inhibitors). The effects of dephosphorylation were mainly studied on purified casein molecules and secondarily on casein micelles. Lorient and Linden (1976) showed a preferential dephosphorylation of κ - and β -caseins with purified milk alkaline phosphatase. According to Li-Chan and Nakai (1989), dephosphorylation reached 97, 70, and 90% for whole casein, α_{s1} -casein, and β -casein, respectively. Nevertheless, Molina et al. (2007) indicated levels of dephosphorylation of 71.6, 89.2, and 73.7% for whole casein, α -casein, and β -casein, respectively.

As phosphate groups are negatively charged, the dephosphorylation reduced the negative charge of casein. Consequently, this charge modification shifted the pHi value of caseins toward neutral pH and increased their solubility at acidic pH (Darewicz et al. 1999a). Recently, McCarthy et al. (2013) showed that dephosphorylation of β -casein increased its pH of minimum solubility from pH 5 to 5.5. As other consequences of this dephosphorylation, the charge distribution along the protein chain was also altered affecting the auto-association of caseins. Besides, the calcium binding and precipitability in the presence of calcium were reduced (Pepper and Thompson 1963; Yamauchi et al. 1967; Bingham 1976; Molina et al. 2007; McCarthy et al. 2013). The loss of phosphate groups reduced the number of calcium bridges (Aoki et al. 1985). Formation of artificial micelles with dephosphorylated caseins was difficult because of the removal of the majority of calcium-binding sites (Pepper and Thompson 1963; Yamauchi et al. 1967; Bingham et al. 1972; Pearse et al. 1986; McCarthy et al. 2013) and resulted in the formation of abnormal micelles less stable in presence of calcium and smaller in size (Pepper and Thompson 1963; Knoop and Peters 1975; Pearse et al. 1986; Hiller and Lorenzen 2009).

Changes in the functional properties of dephosphorylated caseins are also described. The effects of dephosphorylation of caseins on their rennet coagulation were reported by several authors. They determined longer coagulation times for dephosphorylated casein (Yamauchi and Yoneda 1978; Reimerdes and Roggenbuck 1980; Pearse et al. 1986) than native ones. Moreover, the curd tensions were lower for rennet gels of dephosphorylated casein (Yamauchi and Yoneda 1978; Reimerdes and Roggenbuck 1980). The rennet gel was made up of smaller units of dephosphorylated casein unable to be self-associated into dense and uniform network. Consequently, a weak gel was formed (Van Hekken and Strange 1994). The micro-structure of acid curds made with native and dephosphorylated casein micelles showed also major differences (Li-Chan and Nakai 1989). Lorenzen and Reimerdes (1992) showed that dephosphorylation of α_s - and β -casein stabilized the creaming behavior of oil/water emulsions. By molecular dynamics, Cassiano and Area (2003) reported that the affinity of totally dephosphorylated β -casein for water/lipid interface was lower than β -casein which was able to be inserted in the water/lipid interface.

2.3.2 Deamidation

The enzyme catalyzing the deamidation of glutamine in glutamic acid (with production of ammonium) is a protein glutaminase. This enzyme was discovered in the culture supernatant of *Chryseobacterium proteolyticum* (Yamaguchi and Yokoe 2000; Yamaguchi et al. 2001), and its action was investigated on several food proteins (Motoki et al. 1986; Gu et al. 2001; De Jong and Koppelman 2002; Yong et al.

2004, 2006). On skim milk proteins, Miwa et al. (2010) showed that deamidation increased the electronegativity of caseins due to the formation of supplementary carboxyl groups leading to a reduction of the pHi toward lower pH. Turbidity decreased until the milk became translucent. All these observations suggested that deamidation induced dissociation of casein micelles due to increase in the electrostatic repulsions between caseins and breaking of salt bridges. The particle size analysis and observations by transmission electron microscopy confirmed this deconstruction of casein micelles by showing small particles in highly deamidated skim milk (Miwa et al. 2010).

Due to these physico-chemical changes, deamidation is an interesting method to improve solubility, emulsifying, foaming, and gelation properties of different food proteins (Hamada 1994). The solubility, viscosity, and emulsifying capacity of milk were improved with an increase of the deamidation degree (Miwa et al. 2010). Recently, Miwa et al. (2014) studied the effect of milk enzymatic deamidation on the textural and microstructural properties of yoghurts, and they highlighted a decrease in their firmness. In addition, syneresis on the surface of the yoghurt decreased while the surface appeared to be smooth and glossy. Authors attributed these structural modifications to the presence of more hydrophilic caseins with a low capacity to self-associate, thereby decreasing the cohesive forces between casein.

2.3.3 Reticulation

Transglutaminase, used to reticulate food proteins, has a microbial origin and is recognized as safe. This enzyme cross-links covalently proteins between the γ -carboxamide group of glutamine residues and the ϵ -amino group of lysyl residues (Jaros et al. 2006). This reaction is influenced by various parameters such as temperature, pH, pressure, and type of milk proteins. Thus, Lorenzen et al. (1998) and Lorenzen (2002) showed that the degree of protein cross-linking decreased in the following order: sodium caseinate > ultrafiltered skim milk powder > skim milk powder > whey protein isolate. These authors found that a decrease of only 5% of free amino groups was necessary to observe a complete oligomerization of caseins. Compared to sodium caseinate, the reaction of casein micelles with transglutaminase formed fewer amounts of polymers suggesting that casein molecules in sodium caseinate were more accessible and reactive for the reticulation than in casein micelles (Bönisch et al. 2004). Tang et al. (2005) indicated that the reactivity with transglutaminase of different casein molecules in sodium caseinate was in the following order: κ -casein > α -casein > β -casein. With casein micelles, the rate of cross-linking was different and decreased in the following order: κ -casein > β -casein > α -casein (Sharma et al. 2001; Smiddy et al. 2006; Hinz et al. 2007). By using dynamic light scattering, Mounsey et al. (2005) and Moon et al. (2009) found that the cross-linking induced by transglutaminase did not influence the micellar sizes suggesting intramicellar cross-linkings rather than intermicellar.

As expected, these cross-linking reactions led to modifications of functional properties of caseins such as solubility, emulsifying capacity, foaming, and gelation properties (Motoki et al. 1984; Nio et al. 1986; Faergemand and Qvist 1997; Nonaka et al. 1997; Faergemand et al. 1998; Ozer et al. 2007). Cross-linking of casein micelles improved their intra-micellar stability. Thus, cross-linked casein micelles were able to form nanogel particles more stable to heat-induced coagulation than normal casein

micelles (O'Sullivan et al. 2002; Smiddy et al. 2006; Huppertz et al. 2007; Huppertz and de Kruif 2008). Due to the positive improvement of the functional properties of caseins, several patents describing the use of transglutaminase for the development of innovative dairy products have been published (Han et al. 2003; Kumazawa and Miwa 2005; Timmer-Keetels et al. 2012). The scientific literature is also rich of articles concerning the yogurt production using transglutaminase knowing that cross-linking of milk proteins might represent an interesting method for improving texture. Thus, Schey (2003) reported texture improvements of fermented dairy products by enzymatic cross-linking with transglutaminase with an increased gel strength, a reduced syneresis, a dry and smooth surface for set-style yogurt, or an improved viscosity and creaminess for stirred yogurt.

Other enzymes named tyrosinase and laccase could also cross-link milk proteins (Huppertz 2009), but no precise results concerning the modifications of casein structures and functions are described in the literature.

2.3.4 Deglycosylation

κ -Casein is a hydrophilic and negatively charged protein. Due to these characteristics, this casein is located on the micellar surface and stabilizes the micellar structure by steric hindrance and electrostatic repulsions (Horne 1986; Walstra 1990; Holt and Horne 1996; Dziuba and Minkiewicz 1996). These properties are partly due to the presence of different sugar residues like N-acetylneuraminic acid (NANA). Minkiewicz et al. (1993) studied the influence of this carbohydrate by using neuraminidase which can liberate up to 85% of NANA linked to κ -casein. The authors have compared the deglycosylated casein against native casein and indicated no significant difference in their structure, size distribution, and property to be coagulated by rennet. In contrast, they determined smaller heat stability for modified casein. Difference in the rennin-gel structure for deglycosylated casein was also observed with the observation of larger cluster and pores in comparison with native casein. With the same objective, Cases et al. (2003) deglycosylated also casein micelles by neuraminidase and studied the physico-chemical properties and their ability to form acid gel. They indicated that partial deglycosylation (~85%) did not significantly affect neither the partition of molecules of casein between aqueous and micellar phases nor their charge and hydration. In the same time, they showed a shorter acid gelation time and a higher final firmness for the deglycosylated casein micelles. This enhancement could be due to the increase of hydrophobic sites on the micellar surface after their deglycosylation.

More recently, Jacobsen et al. (2014) found that after treatment with N-glycosidase-F, yoghurt gel firmness increased. This effect was stable over a period of 28 days. Moreover, a sensory panel described that the yoghurts have an improved thickness.

2.3.5 Proteolysis

Proteolysis is also an interesting method to modify casein structure in order to enhance their functional properties. The relationship between enzymatic hydrolysis and structural change of casein is studied since several years (Lee et al. 1987; Haque 1993; Panyam and Kilara 1996; Chobert et al. 1998; Slattery and Fitzgerald 1998; Gastaldi et al. 2003; Banach et al. 2013; Wang et al. 2013; Luo et al. 2014). From a general point

of view, proteolysis of caseins induces (1) decrease in their molecular weight, (2) increase in the number of ionized groups (NH_3^+ and COO^-), and (3) conformational

Table 4 Methods modifying the charge of casein

Methods	Main biochemical consequences	Electrical charge of caseins
Heat treatment	<ul style="list-style-type: none"> – Blockage of lysyl residues by lactose – Covalent binding of β-lactoglobulin – Precipitation of calcium phosphate 	Less negative
Cooling	<ul style="list-style-type: none"> – Solubilization of micellar calcium phosphate – Solubilization of β-casein 	ND
High pressure	<ul style="list-style-type: none"> – Disruption of casein micelles 	ND
Ultrasounds	<ul style="list-style-type: none"> – Disruption of casein micelles 	ND
pH decrease (6.7 to 4.6)	<ul style="list-style-type: none"> – Protonation of casein – Decrease of cations-casein interactions 	Less negative
pH increase (6.7 to 9.0)	<ul style="list-style-type: none"> – Increase of the casein ionization – Insolubilization of calcium phosphate 	More negative
Addition of divalent cations	<ul style="list-style-type: none"> – Direct association of added cation to casein – Association of cation-inorganic phosphate to casein micelles – Increase of ionic strength 	Less negative
Addition of NaCl	<ul style="list-style-type: none"> – Solubilization of micellar calcium – Increase of ionic strength 	No effect
Micro- or ultrafiltration combined with diafiltration	<ul style="list-style-type: none"> – Removal of diffusible ions (decrease of shielding charge) 	More negative
Addition of calcium chelant	<ul style="list-style-type: none"> – Decrease in casein-calcium interaction – Solubilization of micellar calcium phosphate 	More negative
Addition of external compounds	<ul style="list-style-type: none"> – Binding to casein by hydrophobic and hydrogen bonding 	ND
Glycation/lactosylation	<ul style="list-style-type: none"> – Blockage of lysyl residues 	More negative
Chemical reticulation by genipin	<ul style="list-style-type: none"> – Blockage of lysyl residues 	More negative
Succinylation	<ul style="list-style-type: none"> – Blockage of lysyl residues 	More negative
Acetylation	<ul style="list-style-type: none"> – Blockage of lysyl residues 	More negative
Chemical phosphorylation	<ul style="list-style-type: none"> – Addition of phosphate 	More negative
Dephosphorylation	<ul style="list-style-type: none"> – Removal of organic phosphate from phosphoseryl residues 	Less negative
Deamidation	<ul style="list-style-type: none"> – Release of ammonia from glutamine transformed in glutamic residues 	More negative
Reticulation by transglutaminase	<ul style="list-style-type: none"> – Cross-link between lysyl and glutamine residues 	More negative
Deglycosylation of κ -casein by neuraminidase	<ul style="list-style-type: none"> – Release of NANA 	No effect
Proteolysis of κ -casein by chymosin	<ul style="list-style-type: none"> – Release of caseinomacropeptide (106–169) negatively charged 	Less negative for peptide 1–105

Charge of casein molecules or casein micelle is determined by evaluation of their zeta potential

ND not determined

change with exposure of hydrophobic groups. As results of this proteolytic degradation, size, isoelectric point, and molecular flexibility of caseins alter their techno-functionalities. Generally, the solubility at the isoelectric point of caseins increases with hydrolysis (Chobert et al. 1988; Chobert 2012). For the other techno-functionalities, there is no clear relationship with proteolysis. Proteolysis of caseins can improve as well as reduce their emulsifying and foaming properties. This difficulty is due to the complexity of the proteolysis. Indeed, its intensity (number of produced peptides) and its quality (nature of produced peptides) depend on several factors like (1) quality of substrates (milk proteins, casein micelles, purified casein molecules, etc.); (2) environmental conditions; (3) time of hydrolysis; (4) type/specificity of enzyme used such as bovine and camel chymosin, trypsin, chymotrypsin, plasmin, papain, pronase, alcalase, neutrase, pepsin, thermolysin, pancreatine, plant enzymes, microbial enzymes, etc.; and (5) enzyme/substrate ratio. Among all these enzymes modifying the structure-function of casein micelle, one of the most remarkable is chymosin because it can destabilize casein micelle mainly with the cutting of the 105–106 peptidic bond of κ -casein. This destabilization occurs when 90% of this bond is cut and corresponds to the release of caseinomacropeptide with concomitant decreases in electrostatic repulsion and steric hindrance between casein micelles. It can also be interesting to use it but in a controlled way to initiate destabilization of casein micelles without observing coagulation. Thus, Gastaldi et al. (2003) studied the effects of controlled κ -casein hydrolysis (19, 35, and 51%) on the physico-chemical properties of casein micelles and their rheological properties of acid milk gels. Decreases in zeta potential, hydrodynamic diameter, and micellar hydration with the level of κ -casein hydrolysis were observed. In the same time, these authors determined increases in pH of gelation, loss tangent, and serum-holding capacity of acid gels with increasing the degree of κ -casein hydrolysis.

3 Conclusion

For several decades, the scientific community has been working to describe and understand the complexity of casein micelles in terms of composition, structure, and functional properties. For that purpose, different approaches in chemistry, biochemistry, biology, biophysics, and physics have been used. Today, the plasticity of casein is admitted and a lot of results describing the different structures of native and modified molecules of casein and casein micelles have been published. The structure of the molecules of casein alone or of caseins organized under micellar form can be modulated according to their physico-chemical environment or through their direct modifications performed by chemical or enzymatic actions. The consequences of these structural modifications induce techno-functional modifications relatively well described. Among all data reported in this article, the charge is one key factor responsible for the structure-function of casein molecules or casein micelles. Table 4 reports the methods described in this paper to modify positively or negatively this parameter.

One of the consequences of the charge modification is a change of the hydrophilicity/hydrophobicity balance affecting the techno-functionality of caseins. In spite of this intense research, it is necessary to improve our knowledge on structures, organizations, and techno-functionalities of these neo-formed micellar structures. It is a real challenge for scientific community but also for the dairy industry. Today, the

number of food applications for modified casein micelle is low but could be very high. As written in this review, physical, chemical, and enzymatic methods can be used to modify casein micelles. New technologies or combination of methods could be interesting for creating new and innovative micellar functionalities. Due to its abundance and its relative ease to isolate casein, their potential to be used in food as described in this paper and non-food applications could be enlarged. Non-food uses of caseins include glue, wool, bone, film, and component for TV tubes (de Kruif 2003). Different publications also relate the use of casein micelles as vehicles for bioactive substances (Livnev 2010).

Conflict of interest F. Gaucheron and C. Broyard declare that they have no conflict of interest.

Compliance with ethics requirement statements This article does not contain any studies with human or animal subjects performed by any of the authors.

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