Casein Micelles for Bioactives

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Caseins and casein micelles are the most prevalent amphiphilic proteins that are widely used to make stabilised emulsions. Caseins can adsorb at the oil–water interface, thus having a high surface activity during homogenisation, processing and storage by preventing coalescence in emulsions under different conditions, such as pH, temperature, structure elasticity and aggregation. Because of these properties, casein is now used to deliver different hydrophobic bioactive in emulsion-based drug delivery systems.

casein micelles encapsulation bioactives microencapsulation nano emulsion hydrogels

1. Introduction

Bioactive food components have received remarkable attention in developing functional foods and nutraceuticals due to their countless physiological health benefits. However, these bioactive components are rapid to inactivation and degradation by light, pH and temperature [1][2]. This rapid degradation can be dodged or slowed down by the encapsulation process till the absorption of these components at the targeted sites. Various encapsulation procedures have been projected to make bioactive components fully functional by preventing their chemical degradation during preparation, storage and transport [3]. There are four delivery systems (lipid-based, carbohydrate-based, hybrid system, protein-based) proposed based on processing conditions, physicochemical stability, sensory and nutritional properties of bioactive components [4][5].

Moreover, the choice of a reasonable protein for a specific transporter relies on the properties of the particle (e.g., size, charge, surface qualities and biodegradability), properties of the bioactive compound to be encapsulated (e.g., polarity, solubility and stability), and environmental conditions (e.g., pH, ionic quality, solvent properties and temperature) [6]. Though various proteins have been widely used as delivery vehicles, milk proteins (caseins and whey) are exotic encapsulation particles due to their elastic structural and functional properties. They have efficient bioactive binding abilities, better encapsulation efficiencies and controlled and target release of bioactive components [3]. As compared to whey proteins, casein micelles are recognised as a natural vehicle for bioactive components since casein proteins have a porous structure with cavities and are recognised as GRAS (Generally Recognized as Safe) [7]. Casein micelles have unique structural and physicochemical properties, such as binding with ions and small molecules to form macromolecules, exceptional stabilising characteristics, self-assembling, emulsifying and water-binding abilities. The porous structure and unique functional properties make them

appropriate for the transport of bioactive components; therefore, they have been used in traditional and new drug delivery systems [8].

Furthermore, casein micelles are amphiphilic, which then can act as a nano-vehicle for both hydrophobic bioactive components such as vitamin (D 2, D 3, E, K) and/or hydrophilic macromolecules such as whey protein and polysaccharides. The vulnerability of caseins to proteolysis [9] guarantees the high discharge of bioactives by a proteolytic enzyme in the gastric tract. The cellular uptake investigation of casein micelles revealed that casein spheres could enter the plasma layer in an independent energy fashion due to the proline-rich peptide sequence in casein [10]. Moreover, caseins have various preservation capabilities essential for the safety of sensitive encapsulated bioactive components, thereby controlling these bioactive agents' biosafety and bioavailability. The casein spheres could significantly advance as one of the best nutraceuticals and drug delivery systems due to its protein matrix rich in surface reactive groups, hollow structure and innovative cell-penetrating properties [8][11].

Although much work has been done regarding caseins as a delivery system for pharmaceuticals, functional foods and nutraceuticals [12][13][14][15], still some areas such as induced structural modification of casein micelles, by altering secondary processing parameters, need to be explored. A recent review by Nascimento and colleagues [16] presented an overview of casein-based hydrogels. Ranadheera [17] examined casein and casein micelles' unique properties as capsules, emulsions, hydrogels and film coatings and observed that different processing parameters can alter casein micelles' techno-functionalities, consequently facilitating the encapsulation of food bioactive components inside casein micelles by binding at its hydrophobic and hydrophilic domains. Thus, this review provides updated and most recent studies about casein micelle as a delivery vehicle with particular attention to deliver bioactives in functional foods and nutraceuticals, along with detailed facts on how pH and temperature affect the incorporated food bioactive component's binding and release properties.

2. Casein Micelles and Its Structure

Another characteristic of caseins is the proline residues, specifically in β caseins, which disrupt the casein micelle structure and give a non-globular nature to caseins with an open structure. These proline-rich caseins carry numerous properties like resistance to heat denaturation, favouring the elastic conformations in solution, great structural flexibility against environmental stresses, specific proteolytic cleavage and targeted drug delivery [18].

Numerous studies have been undertaken on how caseins interact with each other in the past. In 1920, it was considered that caseins undergo self-association as well as with other caseins. The association of caseins within a micelle depends on pH, ionic strength and temperature [19]. Von and Waugh [20] were the first to perform a thorough study about caseins interactions and the complexes they may form when calcium concentrations, temperature and pH are varied [20]. However, there have been several divergent opinions and debates about the critical forms of relationships that dictate casein structure [18].

Hydrophobic interactions occur when two opposing surfaces come close together by the exclusion of water. Only the interactions of β caseins with other caseins are typically hydrophobic, which results in β casein dissociation

from casein micelles when hydrophobic interactions are minimised in milk upon cooling [21]. Typically, when β casein is cooled, up to 30% of it dissociates from the casein micelles, while the remainder remains attached to the micelles. However, when milk is heated at 30 °C, all dissociated β caseins reassociate with the micelles. This happens to β caseins associated with other caseins rather than attaching to calcium phosphate nanoclusters [22]. Although certain β caseins will dissociate from the casein micelle, this does not seem to disrupt the casein micelles structure. As other caseins do not form hydrophobic interaction, so there is no dissociation upon cooling. According to amino acid concentrations, 28% of κ casein, 30% of α s2 casein, 32% of α s1 casein and 34% of β casein residues are hydrophobic, or about 1 in 3 [23][24]. Hydrophobic Clustering Analysis (HCA) was carried out by Horne, 2017 to explore the hydrophobic residues along the caseins sequence. The sole purpose of 2D-HCA was to show that all caseins contain segments that might interact hydrophobically with other caseins [18].

Several models of casein micelles have been reported based on the characteristics and interactions of caseins. The oldest model was the core coat model proposed by Waugh, 1958 as in **Figure 1** a. According to this model, casein micelles composed of variable-sized cores of insoluble slats of α or β casein covered by a coat of κ caseins [25]. Later, a submicelle model was projected by Schmidt, 1982 shown in **Figure 1** b, which suggested that casein micelles were distinct subunits composed of colloidal calcium phosphate crosslinkages [26]. Walstra in 1984 proposed a submicelle model according to which casein micelles are the assembly of roughly spherical subunits or submicelles held together by hydrophobic interactions and calcium phosphate bridges [27].

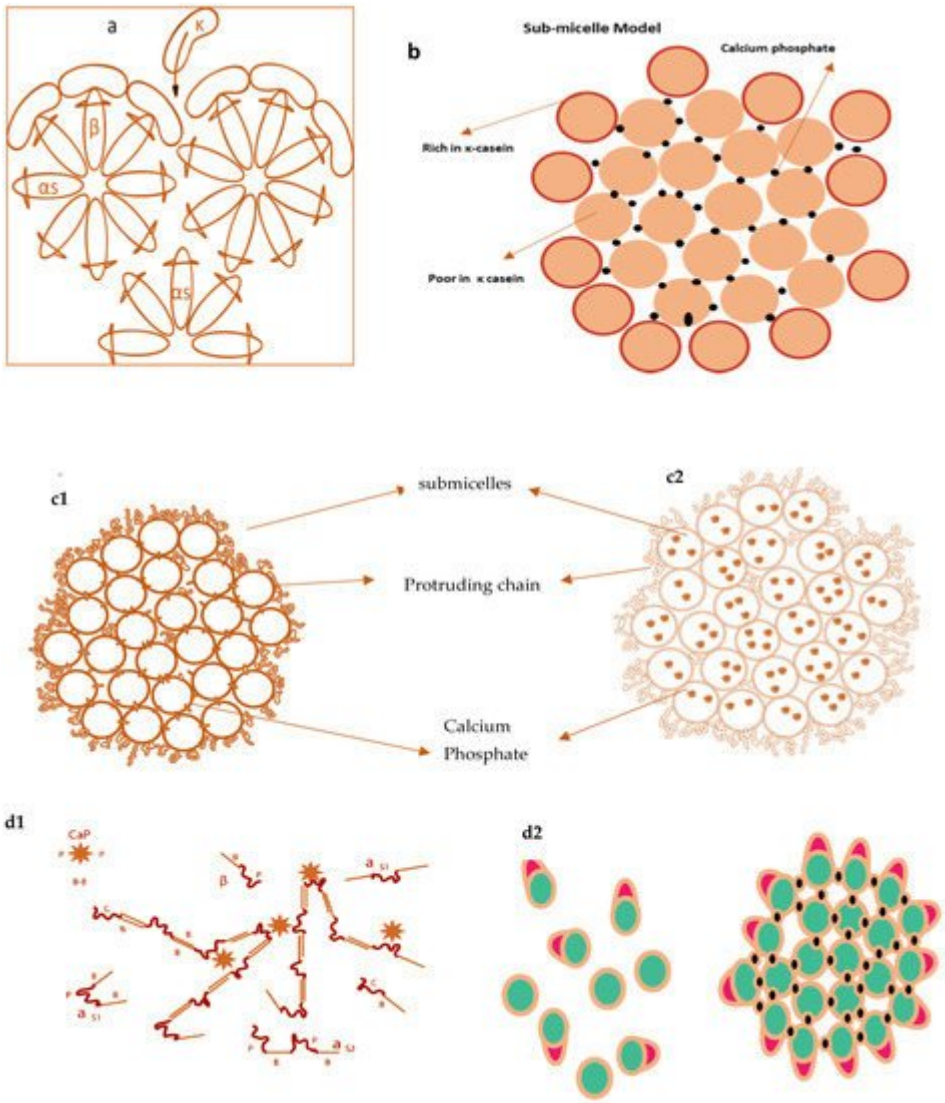

Figure 1. Casein micelles model by Waugh 1958 (**a**), models by Schmidt in 1982 (**b**), model proposed by Walstra in 1990 (**c1**) & 1999 (**c2**), (differs in calcium phosphate location), Dual binding model by Horne (2003) (**d1**) and interpretation of Schmidt's model in 2005 (**d2**).

3. Factors Affecting Techno-Functionalities of Casein Micelles

After discussing the numerous mechanisms by which caseins interact during micelle formation, it is important to explore how such interactions can be engineered to change the functional properties of casein micelles. The structures and techno-functionalities of casein micelles could be modified by various intrinsic and extrinsic factors as shown in **Table 1** . However, this review will focus just on temperature and pH effects.

Table 1. Intrinsic and Extrinsic Factors to Modify Casein Micelles Structure and Functionalities.

Physical Methods	Biochemical Effect	Charge on Casein	Chemical Methods	Biochemical Effect	Charge on Casein	Enzymatic Methods	Biochemical Effect	Charge on Casein
Temperature (High) [28][29][30] Temperature (Low) [31][32]	Blockage of lysyl residues by lactose β-lactoglobulin covalent association Calcium phosphate precipitation and solubilisation B casein solubilisation	Reduced negative charge Not determined	Reaction with sugar Glycation [33] Lactosylation [34]	Blockage of lysyl residues Blockage of lysyl residues	More negative More negative	Dephosphorylation [35][36]	organic phosphate removal from phosphoseryl residues	Reduced negativity
pH (Acid) [37][38][39][40] pH (alkaline) [41][42]	Protonation of casein Decrease of cations casein interactions Increase of the casein ionisation Insolubilisation of calcium phosphate	Reduced negativity More negative	Chemical Reticulation [43][44]	Blockage of lysyl residues	More negative	Deamidation [45][46]	- Release of ammonia from glutamine transformed into glutamic residues	More negative
Pressure [47][48][49][50]	Casein micelles distruptions	Not determined	Phosphorylation [51]			Reticulation [52][53]	Lysyl and glutamine crosslinking	Enhanced negativity
Ultrasound [54][55][56][57]	Casein micelles disruptions	Not determined	Glycosylation			Deglycosylation [58][59]	- Release of NANA	No effect
Addition of cations (di & trivalent) [60][61]	Direct association of added cation to casein Association of cation-inorganic phosphate to casein micelles Increase in ionic strength	Less negative	Succinylation [46] Acetylation [62]	Lysyl residues inhibition	More negative More negative	Proteolysis [63][64]	- Release of caseino macropeptide negatively charged between 106 to 169 peptides	Reduced negativity between 1–105 peptides
Adding salt [65][66]	Micellar calcium	No change						

Physical Methods	Biochemical Effect	Charge on Casein	Chemical Methods	Biochemical Effect	Charge on Casein	Enzymatic Methods	Biochemical Effect	Charge on Casein
	solubilisation Ionic strength enhancement							
Removal of diffusible ions	Diffusible ions removal	More negative ions						
Calcium chelatants addition [67][68]	Casein and calcium association reductions Micellar calcium phosphate solubilisation	More negative ions						
External ligands addition [69]	Hydrophobic and hydrogen interactions to caseins	ND						

The heating of proteins induces conformational changes, exposing the hydrophobic sites. Owing to the absence of a tertiary structure, casein micelles are heat stable. However, distinct changes have been noted concerning the frequency of the heat. Several biochemical modifications are identified, including deamidation of asparagine and glutamine residues, proteolysis [29], and reticulation between amino acids, which results in protein polymerisation, disulphide bridge breakdown and exchange of free thiols on cysteine residues. During heat treatment, the mineral fraction, especially calcium phosphate, becomes less soluble in the aqueous phase, which may interact with casein micelles [70]. When the temperature is less than 95 °C for a few minutes, the changes in salt equilibria are reversible. In comparison, prolonged exposure to high temperatures (for example, 120 °C for 20 min) results in irreversible alterations to the casein micelles and salt distribution. Casein phosphoseryl residues may be partly hydrolysed at temperatures greater than 110 °C [30]. There are limited and old dated reports describing the physicochemical changes in casein micelles induced by cooling.

Koutina and colleagues [31] reported that calcium and phosphorus concentrations in the soluble phase were more significant at 4 °C than at 40 °C due to the increased solubility of calcium phosphate at lower temperatures. Simultaneously, reduced hydration of casein micelles and release of β casein from the micellar structure has been observed [22]. Indeed, temperature reduction alters protein interactions, which allows the transfer of β casein into the aqueous system. These modifications are reversible, and the prior clustering may be restored after heating; however, the native framework is not fully restored because β casein would not revert to its original location [32]. Liu and colleagues [32] confirmed that the volume of soluble casein, hydration and apparent voluminosity of casein micelles reduced as the temperature increased demonstrating that casein micelles structure and mineral in milk

were temperature-dependent between 10 °C and 40 °C. However, the mineral system reaction is prompt during this heating, while casein micelle re-equilibration occurs gradually during cooling. This method could be opted to obtain purified β casein and obtain remained novel casein micelles (less mineralised, depleted in β casein and more hydrated) with innovative techno-functionalities [71].

At pH 5.6, casein micelles enlarge and dissociation of caseins approaches a plateau, with β casein dissociation reaching a maximum [39]. A new limited group of caseins similar to casein aggregates is found in this pH spectrum of 5.6 to 6 [40]. These smaller units range in diameter from about 20 to 35 nm and have a molecular weight of 106 and 107 g·mol −1 . As the pH value decreases (6.7, 6.4, 6.1, 5.8, 5.5), the proportion of these smaller particles increases. The non-dissociated casein micelles seemed to be close to native casein micelles in size, hydration, appearance and zeta potential [40]. Demineralisation of casein micelles by reducing the pH from 6.7 to 5.2 resulted in a reduction of micelles' granularity as determined by cryo-transmission electron microscopy, atomic force microscopy [72], and by the presence of a distinctive point of inflection in SAXS profiles [73]. At pH 4.6, caseins have no charge and therefore have negligible solubility and got precipitate. Acidification causes a similar degree of micellar destruction regardless of the type of acid used (lactic, citric), as physicochemical modifications primarily depend upon pH. However, the composition of the aqueous phase, especially its ionic state, varies according to the acid form, which has an impact on the structure and functionality of acidified caseins [74].

4. Casein Micelles–Based Delivery Systems

The scientific community has spent many decades attempting to characterise and comprehend the complexity of casein micelles in terms of composition, structure and functional properties. As discussed in the previous section, casein micelles may be modified under various temperature and pH conditions to alter their techno functionalities. However, other physical, chemical or enzymatic methods have also been used to alter the technological functionalities of casein micelles and these innovative micellar functionalities have been utilised in various functional foods and nutraceuticals as carriers for bioactive compounds. The bioactive's low absorption and efficacy are associated with deprived bioavailability upon taking through the oral route and their vulnerability to degradation (chemical, physical and enzymatic) during different processing, storage and transportation. These factors require the protection of these bioactive. In this context, casein micelles were exploited to form microparticles, nanoparticles and hydrogels for targeted delivery of bioactive food compounds at the site of action, as illustrated in **Table 2** [75][76][77][78][79].

Table 2. Casein Micelles-Based Capsules and Hydrogels in Delivering Food Bioactives.

Casein Type	The Technique Used for Preparing Loaded Reassembled Casein Micelles	Bioactive	Encapsulation Mechanism	References
Micellar casein	• Casein–emodin complex formation by vortex	Emodin	Microencapsulation	[80]

Casein Type	The Technique Used for Preparing Loaded Reassembled Casein Micelles	Bioactive	Encapsulation Mechanism	References
	• Heat and Ultrasound treatments • Spray-drying microencapsulation • In Vitro digestion evaluation			
β casein micelle	• Drug loaded β caseins dispersion • Freeze drying • Making and description of gastro-resistant Nanoparticle in Microparticle Delivery Systems • pH 2 and 6.5 • In Vitro drug release	Antiretroviral (ARV) combinations of Darunavir, efavirenz and ritonavir encapsulation in β caseins and further within Eudragit L100	Co-encapsulation, Nanoparticle-in-microparticle delivery system (NiMDS)	[81]
Casein gels	• Casein gel production at pH 1 and 9 • Spray-dried gel and tablet • Oven-dried gel and tablets • Controlled release under various compression methods	Caffeine	Gels	[82][83]
β casein micelle Sodium Caseinate	• β casein preparation in 7.4 phosphate buffer • Blending of protein and resveratrol • Production of polysaccharide conjugates by Millard reaction Resveratrol loading at pH 7.5	Resveratrol	Encapsulation Emulsions	[84][85][86]
β casein depleted Casein micelles	• Centrifugation • Lyophilisation • Mixing by shaker • Ultracentrifugation • Enzymatic crosslinking	Linoleic acid	Nanoencapsulation	[87]

Casein Type	The Technique Used for Preparing Loaded Reassembled Casein Micelles	Bioactive	Encapsulation Mechanism	References
Caseins	• Acidification • Homogenisation at high pressure • Curcumin/casein/soy polysaccharide complex at pH 10.0 • In Vitro digestion evaluation • CUR pharmacokinetics of CUR/CN/SSPS in mice	Curcumin	Nanoencapsulation	[88]
Casein Micelle	• Chemical acidification • Crosslinking by transglutaminase	Jaboticaba extract	Hydrogels	[14]
Sodium casienate/Carrageenan	• Primary and multilayered emulsion preparations • Microbeads preparation by gelation in an atomiser	β carotene	Emulsions/Gels	[89]
Casein micelles	• Mineral arrangement restoration and spray-drying • Homogenisation at high pressure • pH and temperature-induced opening	β carotene	Nanoencapsulation	[11][90][91][92]
Re-assembled casein micelles (r-CM) Sodium caseinate (CNP)	• Binding at pH 7.4 and temperature 74 °C • Centrifugation • EGGC binding r-CM and CNP • Encapsulation efficiency determination	Epigallocatechin gallate (EGGC), folic acid	Nanoencapsulation	[93]
Casein micelles	• Preparation of casein-PAAm	Polyacrylamide	Hydrogels	[94]

Casein Type	The Technique Used for Preparing Loaded Reassembled Casein Micelles	Bioactive	Encapsulation Mechanism	References
	hydrogels by free radical polymerisation			
Casein micelles	• Spray-drying pH-shifting • High-pressure treatment	curcumin	Nanoencapsulation	[95][96][97] [98][99][100]
Reassembled Casein micelles	• Restoration of mineral composition and ultrahigh-pressure homogenisation	Vitamin D_3	Nanoencapsulation	[91][92][101] [102][103]
Micellar Casein	• A shift in pH and ultrasonication	Fish oil	Emulsions	[104]
Micellar casei Re-assembled casein micelle from micellar casein	• A shift in pH and ultrasonication	Vegetable oil (Lactobacillus and Bifidobacteria	Nanoencapsulation Microencapsulation	[104][105]
Casein micelles	• Mineral composition restoration • Homogenisation with high pressure	Omega-3	Nanoencapsulation	[104]
β Casein micelles	• Lyophilization	Celecoxib	Nanoencapsulation	[106]
Casein micelles + konjac glucomannan (KGM)	• Enzyme-induced casein KGM hydrogels preparation • Ageing in refrigeration	Docetaxel	Gel	[107]
Casein micelles	• Skim milk natural conditions • Thermally treated commercial skim milk	Vitamin A	Nanoencapsulation	[102][108]
Casein micelles	• Mineral composition restoration and homogenisation at high pressure • Re-assembly of casein micelles	Vitamin D_2	Nanoencapsulation	[101]

Casein Type	The Technique Used for Preparing Loaded Reassembled Casein Micelles	Bioactive	Encapsulation Mechanism	References
Casein micelles		Rosemary Extract	Nanoencapsulation	[109]
Casein micelle		Lactoferrin	Nanoencapsulation	[110]
Casein micelle	• Spray-drying crosslinked with genipin	Alfuzosin	suspension	[111]
Casein micelle	• Spray-drying crosslinked with genipin	Flutamide	Microencapsulation	[111]

In the nutraceuticals industry, both the hydrophobic and hydrophilic properties of casein micelles have been exploited [112]. The hydrophobic molecules present several bonding options when binding to the caseins, for example, hydrogen bonding, van der Waals forces and hydrophobic interactions [9]. A hydrophobic molecule of vitamin D 2 has been encapsulated by Semo and colleagues [8], within casein micelle by using sodium caseinate. However, the pH of the solution was changed to 6.7 according to natural milk pH. Caseins were able to encapsulate vitamin D 2 efficiently due to hydrophobic domains and self-assembled micelle structure. Moreover, vitamin D 2 was found 5.5 times more in casein micelles than in serum.

It has also been stated that casein interactions with polyphenols alter the conformation of caseins, resulting in a decrease in the number of α helices and β sheets [113], so in a casein –polyphenol mixture, the antioxidant activity decreased slightly, indicating a major influence of casein on polyphenol activity. This reduction was more evident in casein that had been incubated with catechin or epicatechin. However, MALDI-TOF mass spectra of incubated caseins did not reveal any stable adduct between the individual caseins, neither with catechin/epicatechin nor with cocoa polyphenols derived from cocoa [113].

All these findings offered help for future utilisation of casein micelles to make complexes with other polysaccharides/lutein/resveratrol to enhance their emulsifying and stabilising properties to acts as a carrier for polyphenols.

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