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Review

Studies of casein micelle structure: the past and the present

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Abstract – At the heart of the milk system are the colloidal casein-calcium-transport complexes termed the casein micelles. The application of physical chemical techniques such as light, neutron, and X-ray scattering, and Electron Microscopy (EM) has yielded a wealth of experimental detail concerning the structure of the casein micelle. From these experimental data bases have arisen two conflicting models for the internal structure of the casein micelle. One model emphasizes protein submicellar structures as the dominant feature, while the other proposes that inorganic calcium phosphate nanoclusters serve this function. In this study, these models are critically examined in light of the two current primary dogmas of structural biology which are: protein structure gives rise to function and that competent and productive protein-protein interactions (associations) will lead to efficient transit through the mammary secretory apparatus. In this light an overwhelming argument can be made for the formation of proteinaceous complexes (submicelles) as the formative agents in the synthesis of casein micelles in mammary tissue. Whether these submicelles persist in milk has been questioned. Recently we have carried out studies on casein micelles and submicelles using Atomic Force Microscopy (AFM) and high resolution Transmission Electron Microscopy (TEM) to gain insights on the nature of protein-protein interactions in submicelles and micelles from a structural biology perspective. The results provide experimental evidence that protein-protein interactions are important in the formation and stabilization of casein micelles.

milk / micelle / casein / structure / protein-protein interaction

摘要 – 酪蛋白胶束结构研究的回顾与展望。酪蛋白胶束是由酪蛋白与钙盐形成胶体, 酪蛋白胶束是乳系统的核心。采用物理化学方法, 如光、中子、X-射线散射及电子显微镜技术等方法对酪蛋白胶束结构给予了详细的分析。现有的资料对酪蛋白内部结构的解释已经形成了两个互相矛盾的模型, 一种是强调酪蛋白结构的亚胶束模型, 而另一种是认为无机磷酸钙和酪蛋白形成的纳米簇结构。在本研究中基于两种目前公认的结构生物学理论, 即蛋白的结构赋予其功能性和高效的蛋白-蛋白之间相互作用 (凝聚) 导致其通过乳腺分泌器官进行有效的转移过程, 对这些模型进行了严格的验证。目前更多的理论观点认为在乳腺组织中的酪蛋白胶束是以蛋白质复合物也就是亚胶束结构存在。但是亚胶束结构观点受到了质疑。近来我们采用原子力显微镜 (AFM) 和高分辨率透射电镜 (TEM) 从结构生物学性质的角度对酪蛋白胶束和亚胶束中蛋白质之间的相互作用进行了研究。结果证明了蛋白-蛋白的相互作用是胶束形成和稳定的重要因素。

乳 / 胶束 / 酪蛋白 / 结构 / 蛋白-蛋白相互作用

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Résumé – Structure de la micelle de caséines : études passées et actuelles. Les micelles de caséines, complexes colloïdaux caséine/calcium, sont au cœur de l'organisation moléculaire du lait. L'application de techniques physico-chimiques comme la diffraction de lumière, de neutrons et de rayons X et la microscopie électronique a permis d'obtenir une mine de renseignements expérimentaux concernant la structure de la micelle de caséines. Ces résultats ont abouti à l'élaboration de deux modèles opposés sur la structure interne de la micelle de caséines. L'un d'eux met en avant des structures sub-micellaires des caséines comme caractéristique prédominante, tandis que l'autre propose une organisation sous forme de nano-clusters (amas) de phosphate de calcium inorganique. Dans cette étude, ces modèles sont examinés et discutés à la lumière des deux dogmes élémentaires de la biologie structurale actuelle, à savoir que la structure protéique détermine la fonction, et que des interactions (associations) protéines/protéines adéquates conduiront à un acheminement efficace au sein de l'appareil sécréteur de la glande mammaire. À la lumière de ces éléments, on peut suggérer que la sub-micelle est l'élément de base dans la synthèse des micelles de caséines dans le tissu mammaire. La question reste de savoir si ces sub-micelles persistent dans le lait. Nous avons conduit récemment des études sur les micelles et les sub-micelles à l'aide de la microscopie à force atomique (AFM) et la microscopie électronique à transmission haute résolution (TEM) pour avoir une visualisation de la nature des interactions protéines/protéines dans les sub-micelles et les micelles du point de vue de la biologie structurale. Les résultats obtenus démontrent que les interactions protéines/protéines sont importantes dans la formation et la stabilisation des micelles de caséines.

lait / micelle / caséine / structure / interaction protéique

1. INTRODUCTION

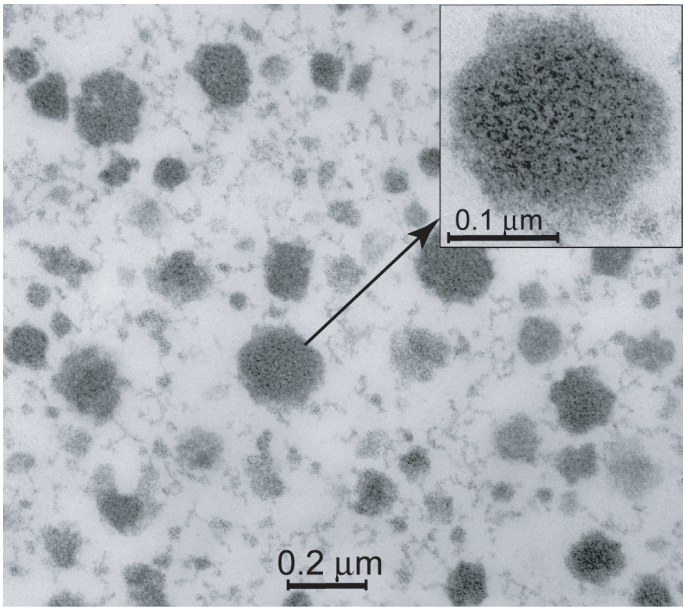
The virtual image of milk, which would be constructed by most people, is that of a creamy white fluid. The lubricity and taste of milk are related to this perception and are based upon three unique biological structures: the colloidal calcium-protein complexes (the casein micelles), the milk fat globules with their limiting membrane, and the milk sugar: lactose [51]. The complexity of these structures is necessitated by the fact that milk is in essence predominantly water. It is the accommodation of these ingredients to an aqueous environment that forms the basis for the structure of milk at the molecular level and calls for the unique secretory process: milk synthesis [51].

Although these colloids have been the subject of extensive research for many years [15, 26, 28, 30, 36, 47, 48, 50, 52, 54, 62, 68] the structural details of the casein micelles on the molecular level remain elusive [24]. Biochemical and physical studies of these colloids have focused on: the size and properties of the colloids, their protein and mineral composition, and stepwise reconstitution of the micelles. Conflicting models for the structure of the casein micelles have arisen from differing interpretations of the core data bases developed in these studies. This manuscript will focus on the recent advancement in the field of structural biology and protein-protein interactions will be applied to attempt to discern the biologically competent route for the formation of the casein micelles.

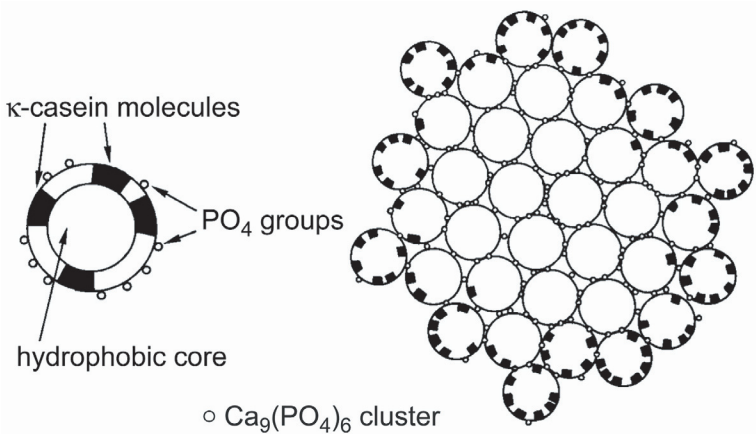
2. BRIEF REVIEW OF PROPOSED MODELS FOR CASEIN MICELLES

2.1. Protein composition of skim milk

The dominant constituent in skim milk is the casein micelle (Fig. 1a). It is this unique supramolecular aggregate imparts the opalescence characteristic of skim milk. The chief function of the micelle is to fluidize the casein molecules and solubilize the calcium and phosphate [16]. In general, when milks that contain > 2% protein are analyzed, the accompanying inorganic phosphate and calcium levels found would



(a)



(b)

Figure 1. Transmission Electron Micrograph of bovine milk casein micelles (a) fixed with glutaraldehyde and stained with uranyl acetate and lead citrate (pH 7.0). Inset at the upper right shows an enlarged single micelle. (b) The submicellar model for casein micelles showing proteinaceous structures and surface arrangement of κ-casein; reprinted from Schmidt [54].

Table I. Casein distribution (%) in various milks.

	α_{s1}-	α_{s2}-	β-	κ-
Goats	5 to 17	6 to 20	50	15
Cows	38	10	40	12
Human	Trace	Trace	70	27

by themselves, yield insoluble precipitates (apatite or brushite depending upon the pH). Conversely in the absence of these salts, the casein components, as a result of their open structures, have a high viscosity. The formation of the casein colloidal complexes, micelles, averts these two problems.

Four major casein components in cow's milk, α_{s1}-, α_{s2}-, β- and κ-casein, have been well characterized in the past [59]. Caseins have been found to be homologous to these proteins in their gene and primary protein sequences in all species examined to date [23]. However, the proportions of the various caseins vary widely. β-Casein is the primary casein in human milk and in goats' milk it comprises 40% to 50% of the casein, for example. In goats' milk there is also a high degree of variance in casein proportions among animals, which appears to be genetically controlled (Tab. I). Despite the variations in casein components all species competently form colloidal casein micelles for the transport of calcium and phosphate. At the ultra structural level, the casein micelles of most species appear quite similar.

The α_{s1}-, α_{s2}- and β-caseins are precipitated by calcium (calcium-sensitive) binding to their phosphoserine residues at the concentrations of protein and calcium found in most milks. However, κ-casein is not only soluble in calcium (calcium-insensitive), but also interacts with and stabilizes the calcium insoluble caseins to initiate formation of the stable colloidal state. It has been generally recognized by studies [7, 10, 13, 29] using numerous chemical, enzymatic and immunological techniques that while the majority of the κ-casein reside on the surface of the casein micelles, other caseins might also occur there as well. In all models for casein structure, κ-casein is thought to predominate on the micellar surface (Fig. 1b). In milk clotting in the neonate stomach, the enzyme chymosin (rennin) specifically cleaves one bond in κ-casein to initiate aggregation of the micelles. It has been clearly demonstrated recently [55] by κ-casein gene null mutation experiments in mice that κ-casein is essential for the assembly of the casein micelles and for lactation to occur in vivo.

2.2. The submicelle theory of casein structure

For many years the most accepted theory [15, 54] of the structure of the casein micelle was that it was composed of spherical aggregates of the caseins (submicelles) held together by calcium-phosphate linkages (Fig. 1b). The submicelle hypothesis has been historically supported by biochemical and biophysical studies [39, 40, 42, 57] on the individual casein components, reconstitution of micelles from their component caseins [27, 41, 56], as well as electron microscopy of the micelles themselves [8] and partially decomposed micelles. Early studies on the purified caseins demonstrated that in the absence of calcium they formed rather large aggregates (submicelles) and that these aggregates formed colloidal complexes in the presence of added calcium. By gradual dialysis and direct addition of EDTA to remove calcium from casein micelles, Lin et al. [45] proposed possible emergence of casein micellar framework or subunits as later substantiated by physical techniques and freeze fracture electron microscopy [41]. These particles had physical properties similar to the aggregates found in whole casein preparations in the

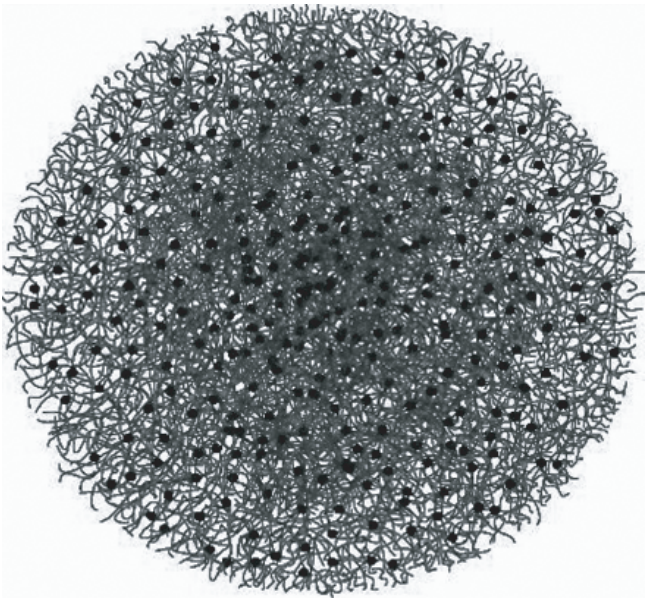
absence of calcium and were considered as submicellar in nature. Based on these data, Schmidt proposed the "submicelle" model [54] in Figure 1b. In summary this model stipulates protein-protein interactions, traps calcium phosphate and forms the colloidal complexes.

In recent years this theory has been challenged by concepts [30, 35] arising from the study of the casein-calcium-phosphate interactions, the micelles themselves and physical chemical studies of the individual proteins at interfaces.

2.3. Casein micelle models with an internal gel matrix

Two more recently proposed models for the casein micelle have emerged that refute the notion of discrete submicellar structures within the micelle. The first model to depart from the submicelle theory was that of de Kruif and Holt [12]. During a series of studies on casein-calcium-phosphate interactions Holt and coworkers [31, 33] discovered that the phosphopeptide fraction of β-casein could bind to and stabilize calcium-phosphate aggregates resulting in the formation of nanoclusters of a discrete size and composition; without the peptides the calcium phosphate structures would grow randomly and precipitate. This discovery led de Kruif and Holt to propose that such nanoclusters are the centerpiece of casein micelle structure [14]. The formation of nanoclusters with a radius of 2.3 nm would drive micelle formation by randomly binding phosphoproteins causing an inverted micelle, and then more proteins could coat this new hydrophobic surface and in turn, bind more calcium phosphate until a size limited colloid is formed. There are about 800 of these amorphous calcium phosphate nanoclusters in an average sized casein micelle (~100 nm in diameter), see Figure 2a. This nanocluster model is supported by the earlier rheomorphic theory of casein structure [32] and the recent SAXS and USAXS data by Pignon et al. [53]. In this view, the unstructured proteins form about the amorphous inorganic species and their function of binding to the calcium-phosphate gives rise to their structure, hence no specific protein secondary structures or protein-protein interactions are invoked, except that a surface position for κ-casein is required.

The casein micelle model proposed by Horne [35] (Fig. 2b) considered the surface chemistry of the individual caseins and concluded that protein-protein interactions were indeed important, but in essence the model retains the rheomorphic concept. In this view the amphiphilic nature of the caseins causes them to act more as block copolymers of alternating charge and hydrophobicity, that is, a charged phosphopeptide loop and a hydrophobic train for β-casein, the reverse for κ-caseins, and an N-terminal hydrophobic train, followed by a charged loop and a final C-terminal hydrophobic train for α_{s1}-casein. Individual secondary structural elements of the each casein are ignored and only gross hydrophobic interactions are considered. In this model, the growth of the calcium phosphate nanoclusters begins the process of micelle formation, but it is limited by binding to the phosphopeptide loop regions of the caseins. Once bound to the amorphous inorganic matrix, further protein-protein interactions are related to the hydrophobic blocks and polymerization proceeds by repeating the entire process. Micelle formation leads to an internal gel-like structure with embedded nanoclusters of calcium and phosphate, and the reaction of κ-casein which contains only one phosphoserine residue limits micellar growth by acting as a dead end capping unit in analogy with the growth of synthetic polymers. Note that this model shows only monomeric κ-casein molecules.



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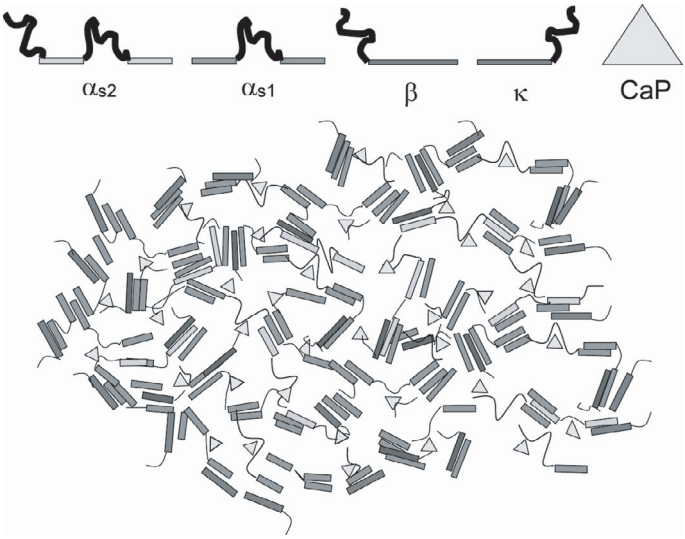


(b)

Figure 2. (a) The nanocluster model for casein micelles. Casein monomers are thread-like, while the dark circles represent calcium phosphate nanoclusters; reprinted from [12] with permission of Springer, NL. (b) The model of Horne – dual bonding model of casein micelles using casein monomers as indicated. Protein-protein interactions occur between hydrophobic regions (rectangular bars) while the protein hydrophilic regions (loops) bind to calcium phosphate clusters (triangles). κ-Casein is monomeric and on the surface; reprinted with permission from Elsevier.

Interestingly, these two distinctly different views of the internal structure of the casein micelles-submicelles versus gel matrices arise essentially from the same biochemical and physical chemical data bases. For a further exposition on their similarities and differences see the excellent review of Walstra [67].

3. SYNTHESIS AND SECRETION OF CASEINS

3.1. Cell physiology

The evolution of the mammary gland, presumably from external sweat glands, has yielded a great variety of exterior appearances in many species [51], but at the tissue level there is a common organizational theme as shown in Figure 3a. Mammary secretory cells are epithelial in nature and are arranged in alveoli which are connected to ductal tissue. The secretory epithelial cells (SEC) are surrounded by a layer of myoepithelial cells, which are able to contract and expel milk into the ducts in response to the hormone oxytocin. The alveoli are highly vascularized to ensure a constant flow of the metabolic precursors needed for milk synthesis and secretion. Finally the vascularized alveoli are embedded in an extracellular matrix. This matrix not only supports the cells, but also through cell-cell interactions is responsible for the full expression of the genes that control milk synthesis [51].

3.2. Protein synthesis and secretion: overview

Adaptation of milk components to their ultimate aqueous environment begins during secretion. Lipid and protein synthesis are partitioned from the start. Amino acids and their metabolic precursors are actively transported into the SEC and assembled into proteins on the ribosomes of the highly developed rough endoplasmic reticulum [51]. All milk proteins of mammary origin have conserved leader sequences which cause insertion of the nascent proteins into the lumen of the endoplasmic reticulum (ER) shown in Figure 3b. The proteins are then transported through the Golgi apparatus [15, 51] as shown in Figure 3b; presumably the globular proteins of milk are folded during this period. In the Golgi apparatus, the caseins, which are the major milk proteins in most species, appear to be spherical complexes of about 10 nm in diameter. The caseins are phosphorylated by a calcium-activated membrane bound kinase to begin the process of calcium transport [3]. A membrane associated ATPase delivers calcium to the vesicles [5]. The gradual intercalation of calcium, casein, and phosphate into the submicellar structures leads to the formation of casein micelles and insures the effective transport of these vital minerals. This process can be visualized in Figure 4 (top) where small submicellar particles are seen in the secretory vesicles nearest the trans Golgi. Through the binding of calcium and the accretion of phosphate, the colloidal casein micelles are formed and finally secreted by reverse pinocytosis (Fig. 4, bottom). Overall this view strongly supports the involvement of proteinaceous submicellar structures in the synthesis and secretion of casein micelles.

3.3. Protein synthesis and secretion: details in the ER lumen

The process of casein secretion within the lumen of the ER has not been studied in specific detail [24]; however reference to recent information on cell biology and protein folding in other systems may shed more light on the issues of casein micelle formation. The issue of quality control of protein folding has become a widely researched area in cell biology [49].

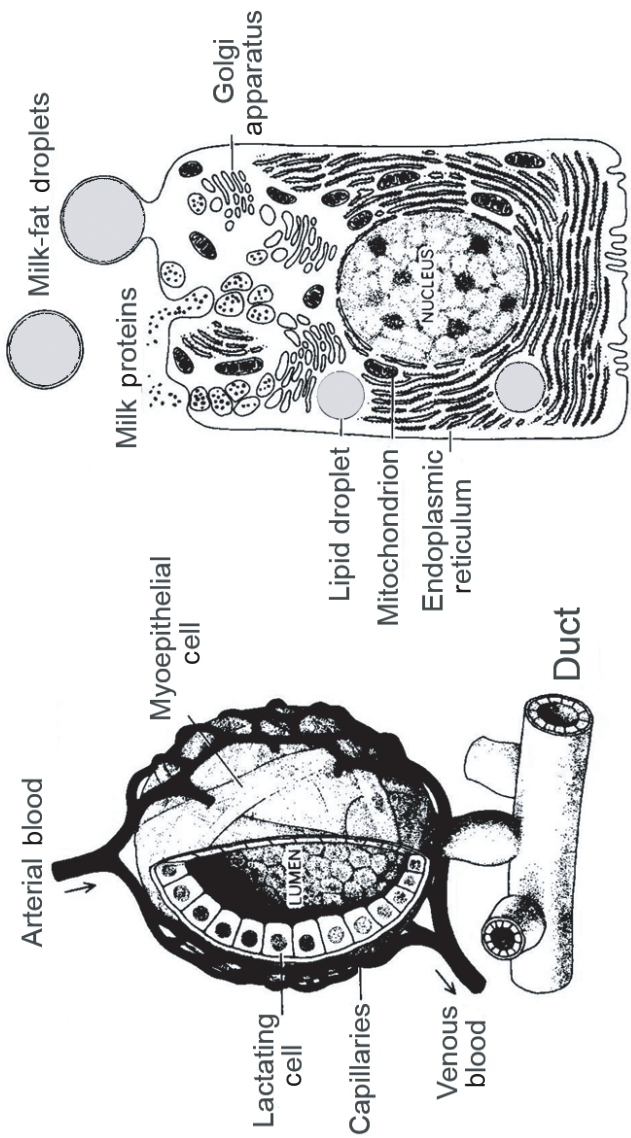


Figure 3. Cell physiology of lactating mammary gland. A single alveolus (a) consisting of lactating epithelial cells (SEC) surrounding the lumen. A typical lactating cell (b) indicating active secretion of protein and lipid by distinct mechanisms; reprinted with permission of Scientific American and S. Patton.

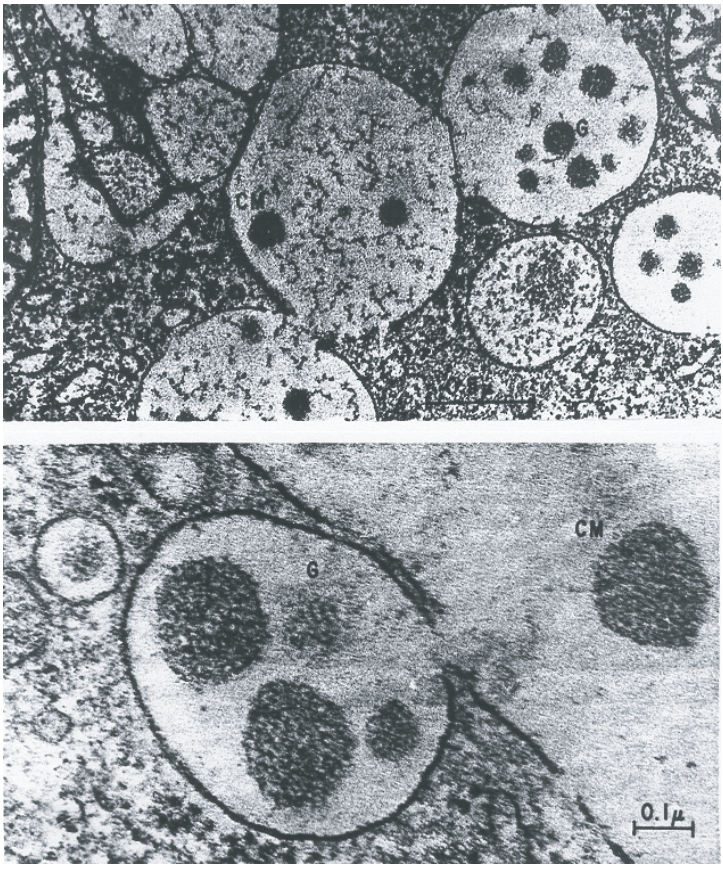


Figure 4. Formation of casein micelles (CM) within Golgi vesicles (G) and depicting the aggregation of small submicellar particles into larger micelles (top). A Golgi vesicle (G) about to discharge its contents into the alveolar lumen (bottom); a casein micelle (CM) is already present in the lumen; reprinted from Farrell et al. [17].

The process of endoplasmic reticulum associated degradation (ERAD) has been found to occur in many secretory systems. As noted above conserved leader sequences of casein cause insertion of the nascent proteins into the lumen of the ER. Before the newly secreted proteins can traffic beyond the ER they must fold into their final conformations and multicomponent systems must assemble. Failure to properly associate or fold leads to the unfolded protein response, tagging and removal by the ERAD system. Overall the environment of the ER lumen would be conducive to the signature functional property of the caseins (self-association) so initial casein-casein interactions would naturally occur here. While the ER lumen serves as a calcium storage center, the free calcium ion concentration fluctuates between 1 and 3 $\mu mol \cdot L^{-1}$, a concentration well below the binding constants for the unphosphorylated caseins. Presumably, proper association of the caseins helps them to escape degradation and move on to the Golgi for processing. It has been our

contention that conserved sequences of the individual caseins give rise to selected secondary structural elements and that these elements lead to self-association of the caseins without classical protein folding [20, 21], that is to say, caseins contain little or no tertiary structure and proceed directly from secondary structure formation to quaternary structures exhibiting both rigid and flexible elements. This is in line with the basic tenet of structural biology that protein structure gives rise to biological function (the Anfinsen hypothesis [2]). Recent development in the field of protein folding has shed light on understanding protein self-assembly and aggregation as delineated by Jaenicke and Lilie [37]. Self-associations are those on-line and productive reactions which lead to competent biological protein assemblies such as amyloid [38], whereas non-productive aggregations lead to often mis-assembled protein complexes. The latter would be targeted for the ERAD process. The question now arises as to which of the many known in vitro reactions of the caseins studied to date are important in this context. It may be of interest to examine those "productive" self-associations which may lead to the formation of casein micelles and those "non-productive" aggregations formed through various protein-protein interactions.

The self-association of bovine β-casein has been studied by many (for example [36]). The most commonly accepted mechanism is that the β-casein molecules form rather spherical polymers of limited size following a critical micelle pathway. Interestingly the dimensions of this polymer are rather fixed, but its molecular weight is highly dependent on ionic strength and temperature. In species such as human where β-casein is the predominate protein, this process could be viewed as a prominent on-line self-association in the ER lumen. It must be noted that the unphosphorylated form of the human β-casein has nearly the same propensity for self-association as its phosphorylated forms [6]. The weight average molecular weight of bovine β-casein at 37 °C in the absence of calcium is 1 250 000 as shown in Table II. This is almost three times the estimated values for the submicellar structures observed in Figure 4 and could potentially lead to ER stress and ERAD tagging.

Studies on the polymerization of α_{s1}-casein had previously shown [1] that the molecule exhibits a progressive consecutive association to dimers, tetramers, hexamers, etc., and this process is highly dependent on pH and ionic strength. Most of the early studies [54] on the polymerization of α_{s1}-casein were conducted at or below 25 °C; because of its hydrophobic nature it was expected that polymerization would be accentuated at 37 °C. This clearly is not the case at 37 °C as shown in Table II; all three major genetic variants depolymerize [46] and behave essentially as dimers. The dimer formation is centered on its C-terminal half with a strong selective interaction between residues 136 and 160 of each monomer, and there appears to be no involvement of N-terminal hydrophobic train of α_{s1}-casein at normal ionic strength and 37 °C. In addition, the latter region is positively charged and could participate in phosphate binding. This appears to contradict one of the modes of crosslinking postulated by Horne [35] as part of the casein gel network. Finally it has been shown [4] that both native and dephosphorylated α_{s1}-caseins undergo similar aggregation and precipitation reactions. Thus in the ER lumen the α_{s1}-casein molecule would not be greatly polymerized and the higher order polymers observed in vitro are the products of an aggregation process, which would likely be "non-productive".

κ-Casein is the calcium-soluble stabilizing protein of the casein micelles; it is also the only casein whose disulfide bonds play a significant role in casein structure. As isolated from milk, the protein displays a

Table II. Weight average molecular weights of selected caseins and mixtures by analytical ultracentrifugation at 37 °C[a].

Casein or mixture	Weight average molecular weight	Weight average polymeric size	Rotor speed (rpm)
α_{S1}-Casein[b]	56 000	Dimer	12 000
β-Casein[c]	1 250 000	52 mer	3000
RCM κ-casein[d]	3 040 000	160 mer	3000
1.5 α_{S1}-:1 RCM κ-[d]	316 000	15 mer	3000
4 α_{S1}-:1 RCM κ-[c]	92 400	Tetramer	6000
4 β-:1 RCM κ-[c]	1 010 000	43 mer	3000
1 β-:1 α_{S1}-[c]	213 000	Nonamer	3000
RCM whole casein[c]	110 000	Hexamer	6000

[a] All data were obtained at 37 °C, pH 6.75 in 25 mmol·L^{-1} disodium piperazine-N,N′-bis(2-ethane sulfonic acid) with 80 mmol·L^{-1} KCl to mimic milk salt conditions in the mammary gland in the absence of calcium; the rotor speeds were appropriate to the weight average molecular weight as previously described [22, 46]. The protein SH groups were reduced and carboxymethylated (RCM).
[b] [46].
[c] [24].
[d] [22].

unique laddered disulfide bonded pattern in SDS gel electrophoresis with sizes ranging from dimer to octamer and above [18]. In analogy with the use of cleavable disulfide reporter groups, it can be stated that the nearest neighbor to a κ-casein molecule is another κ-casein molecule as shown in the Schmidt model (Fig. 1b). In essence, the monomeric κ-casein molecules depicted in the Horne model (Fig. 2b) are not found.

The source of the laddered disulfide pattern of κ-casein is unknown. In most secretory systems, the enzyme protein disulfide isomerase (PDI) occurs on the inner membrane of the ER and acts as both a chaperone and a catalyst for the rearrangement of disulfide bonds. However this enzyme may not be responsible for the linear polymer pattern. For this pattern to form, the κ-casein monomers would have to be in queue at or near the PDI. The κ-casein's ability to polymerize may inhibit the action of PDI and thus allow it to retain the SH character in the ER. Therefore, the studies on the polymerization of reduced κ-casein that demonstrate an association involving a critical micelle model similar to that of β-casein [54] may be relevant. However, as was the case for α_{s1}-casein, these studies were primarily conducted below 25 °C. At 37 °C both reduced κ-casein by DTT or β-mecaptoethanol [61] and reduced carboxymethylated κ-casein (RCM-κ) [22] have been found to form large stranded amyloid fibrils. In the former case, Thorn et al. [61] showed that α_s- and β-casein can effectively inhibit the reduced κ-casein fibril formation. The latter [22], as demonstrated in Table II, are clearly aggregations and not a part of competent casein secretion. So the κ-casein must either be SH capped or self associate with other caseins for the successful transit through the ER lumen. At a ratio of 1.5 α_{s1}-:1 RCM κ-casein, amyloid formation is inhibited and moderate molecular weight complexes of 316 000 are formed at 37 °C (Tab. II). Increasing the ratio of α_{s1}-casein to 4:1 substantially reduces the complexes to 92 400. Bovine β-casein, while limiting amyloid formation, does not have the same effect of reducing the weight average molecular weight as α_{s1}-casein (Tab. II). Since the κ- and β-caseins

share a similar self-association mechanism, it would appear that in mixed associations the RCM κ-casein can be inserted interchangeably into the self-association reaction of β-casein; but the resulting overall size is somewhat smaller than that of β-casein alone (Tab. II). Such large complexes might not allow the associated proteins to escape ERAD and move on to the Golgi apparatus. In fact Chanat et al. [9] have studied the transport of caseins from the ER to the Golgi apparatus in mammary epithelial cells. Their data suggest that for animals with a high casein content, α_{s1}-casein must interact with the other caseins for efficient transport to the Golgi. In cells that completely lack α_{s1}-casein the accumulation of β-casein (or κ-β-mixtures?) is observed in the ER. In the long term this causes ER stress, activates the ERAD system and impedes secretion. Recent work by Shekar et al. [55] demonstrated that κ-casein is essential for the formation of casein micelles as well as for lactation to occur.

To test the efficacy of α_{s1}-casein at reducing the size of β-casein aggregates in vitro, 1:1 mixtures of the two proteins were studied by analytical ultracentrifugation at 37 °C. The weight average molecular weights of the complexes were speed dependent, increasing with decreasing speed, indicating strong hydrophobic interactions; see Table II. The weight average molecular weight of the 1:1 complexes was 213 000 which represents a six fold reduction of the β-casein aggregate or a four fold increase over that of the α_{s1}-casein alone. This result is of importance because there are few studies of the mixed associations of these two caseins at 37 °C and conditions close to that of the ER. Here the α_{s1}-casein acts to diminish the size of either the β- or κ-casein aggregates; in this sense it may be considered a molecular detergent for the other caseins. Thus these in vitro data confirm the in vivo observations that α_{s1}-casein can reduce aggregated species and allow the associated particles to escape the ER. For human milk, the small amount of α-caseins present may help reduce the aggregates of β-casein; also the net casein content in human milk is only 17% of that in bovine milk (Tab. I) so smaller aggregates would be favored. Finally, RCM-derived whole bovine casein, with the standard ratios of the four caseins, has a weight average molecular weight of 110 000 at 37 °C under the same conditions of pH and in the absence of calcium. From all of these biological and physical chemical studies it would appear that for the competent synthesis and secretion of casein: preformed casein complexes of the size of the putative casein submicelles must form through protein-protein interactions – triggered by conserved protein sequences – and emerge from the ER for efficient transit via secretory vesicles to the Golgi apparatus.

3.4. Protein synthesis and secretion: details in the Golgi vesicles

From the discussion above it seems most likely that the individual casein molecules undergo significant self-association in the ER and are then transported in vesicles to the cis face of the Golgi apparatus. In this region three significant events occur in the process of casein secretion. The first event is most likely an increase in calcium concentration accomplished by an ATPase-driven pump [5]. The second most likely next step is the phosphorylation of the associated caseins by a membrane associated casein kinase which uses calcium-ATP as substrate and is specific for Ser residues preceded at the n + 2 position by Glu or a serine phosphate (SerP) residue [23]. The casein kinase [3] responsible for this reaction has not been purified, but in Golgi preparations the enzyme requires a surprisingly high calcium ion concentration ($K_M \sim 20$ mmol·L^{-1}) so that at

the time of phosphorylation, calcium ions may be almost immediately bound to the caseins ($K_D \sim 5$ mmol·L^{-1}). The third and often overlooked step is that one of the byproducts of the kinase reaction, ADP, retains bound calcium, so that when this is converted by membrane associated diphosphatases to phosphate and AMP [17], both calcium and phosphate are released near the interior membrane surface where the casein proteins are still being phosphorylated. Studies by West and Clegg [69] showed that phosphorylation of casein is still proceeding in large Golgi vesicles as is most likely calcium transport. Thus both calcium and phosphate may automatically be bound to the submicellar complexes, as seen in Figure 4, prior to micelle formation. In general, when milks that contain > 2% protein are analyzed, the accompanying inorganic phosphate and calcium levels found would yield insoluble precipitates (apatite or brushite) in the absence of casein. But the question arises are the concentrations of these compounds ever high enough or concentrated enough to form nanoclusters in the Golgi vesicles? Additionally, would the energy gained by coating these precipitating clusters be sufficient to depolymerize the preformed casein complexes in the manner suggested by the Horne model? Veis [65] has suggested that, in general, mineralization in mammalian systems such as collagen and dentin matrices is directed and controlled (assembled) by the structural proteins present; inorganic direction appears to be limited to simpler systems such as the crystalline shells of corals.

Based on the average composition of the colloidal caseinate [15] the average concentrations of calcium and phosphate within the colloidal complexes are 18.7 and 15.2 mmol·L^{-1}, respectively. In turn the average concentration of casein within the colloidal complexes is 1 mmol·L^{-1} but the average casein molecule has 6.5 phosphate groups thus the concentration is 6.5 mmol·L^{-1} SerP. Moreover all four caseins have selected areas of positive surface [59] which may bind phosphate after calcium binds to the protein as suggested for α_{s1}-casein [46]. In all of the above models, the binding of phosphate has been largely neglected. The prominent positive patches of the caseins are: 1–10 of α_{s1}- (+6); 165–199 of α_{s2}- (+11); 97–113 of β- (+6) and 97–116 of κ-casein (+6); these too average out to 6.5 mmol·L^{-1}. If each serine phosphate binds one calcium which in turn binds one inorganic phosphate, then this would result in a double layer. Additionally if each positive charge in the above mentioned areas then bound one phosphate and one calcium in a second double layer, then the possible concentrations of unbound calcium and phosphate within the micelle is further reduced. It can be seen in a molecular dynamics study [19] that both the smaller calcium ions and the larger chloride ions bind to the peptide and then associated ions tend to form a charged double layer about the peptide chain. In this case there is limited attraction between the aqueous calcium and chloride ions, but complexes between calcium and phosphate would be more numerous. In vivo the formation of such a double layer of calcium phosphate would reduce the unbound (uninfluenced) concentrations of these two ions to 5.7 and 2.2 mmol·L^{-1} respectively. This is far from the concentrations used by Holt and coworkers [31, 33] to form nanoclusters in vitro (37 mmol·L^{-1} calcium and 30 mmol·L^{-1} phosphate and 3 mmol·L^{-1} phosphopeptide). In this same context the average submicelle would have 65 negative charges [46] due to clusters of SerP groups and 65 positive charges due to clusters of basic amino acids, in total it could carry 260 ions as a simple double layer; the standard nanocluster has about 355 ions at its core [31, 33]. The coalescence of two submicelles with their bound (influenced) ions then would terminate any possible calcium phosphate

growth and begin micelle formation. It may be that nanoclusters then have an interesting and informative chemistry but actually represent a process which is an inorganic aggregation similar to amyloid formation by κ-casein rather than an on-line productive biological process. Thus from the point of view of the synthesis and secretion of casein micelles the Schmidt model may be representative of the biological process, although the stoichiometry of the inorganic "cement" is probably incorrect based upon the latest physical chemical data [11, 13] which indicates a type of apatite is the most likely candidate for the molecular structure within the micelles.

4. STUDIES ON THE STRUCTURE OF CASEIN MICELLES

From the above discussion it is clear that proteinaceous submicelles (here defined as associated casein species with an average size of 10 nm, not necessarily spherical, Figs. 1a and 4) play a major role in the on-line formation of casein micelles in mammary tissue. Additionally it would also appear that α_{s1}-casein acts as a type of detergent to limit the size of these submicellar particles in order to defeat the unfolded response and escape the ERAD system. The formation of these controlled aggregates (productive association) allows for and facilitates transfer from the ER to the Golgi apparatus. Once present in the Golgi, micelle formation occurs. Past research on understanding the detailed structure of the micelles has centered on using electron microscopy, neutron scattering and X-ray scattering techniques. Atomic Force Microscopy (AFM) [25, 64] has only recently been applied to the study of the casein micelles.

4.1. Electron microscopy

Electron microscopy (EM) represents a powerful tool for elucidation of biological ultrastructures as seen in Figures 1a and 4. The main problem with this technique lies in the fixatives and metal staining used to accentuate the particular features they react with and visualize, usually at the expense of other features. In contrast, when uranyl oxalate is used as a positive stain for proteins [48] a more uniform distribution of material is seen because the stain is binding to the caseins, particularly the SerP and accentuating the protein distribution. However, more details can be visualized from our studies of casein micelles and sodium caseinate (Figs. 1a and 6). Similar strand-like protein structures with a "knot" about 10 nm (diameter) can be found in skim milk (Fig. 1a) and sodium caseinate, a common commercial casein product without calcium (Fig. 5), the latter perhaps exemplifies the prevalent existence of casein-casein interactions even after the complete destruction of the micelles. In addition, Figure 4 shows what could be "submicellar" casein aggregates in rat Golgi vesicles. Clearly, EM images are influenced by the stains used in the experiments. One is tempted to employ the scientific dialectic here and say that neither the thesis (submicelles) nor the antithesis (no submicelles) is correct, but that synthesis is needed. Walstra [67] has proposed that the submicelles reemerge in EM representations of products such as cheese. Work on a variety of cheeses [63] demonstrates the dynamic nature of the submicellar structures of the cheese protein matrix.

4.2. Micelle dissociation studies

Studies on micelle dissociation were among the first to indicate the existence of submicelles and Schmidt [54] drew heavily on these and on reconstitution studies in framing the model shown in Figure 1b. Clearly the Walstra hypothesis on micelle equilibria is at play in these experiments (Fig. 5). Those components in

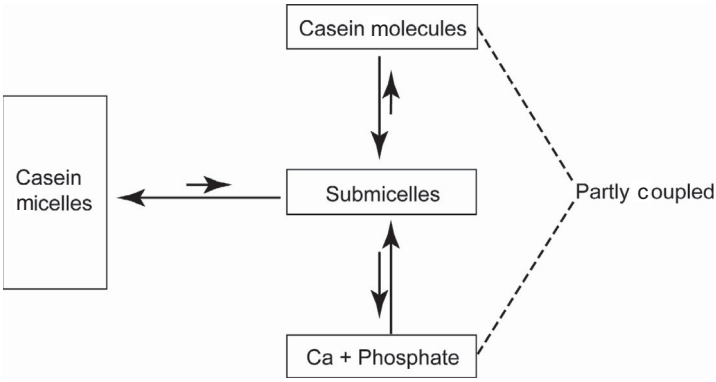


Figure 5. Proposed scheme of the most important dynamic equilibria between casein micelles and milk serum from Walstra [67]; reprinted with permission from Elsevier.

rapid equilibrium will quickly exchange and yield one result, while those slow to equilibrate will accentuate another feature. It has also been suggested that calcium binding to caseinates must precede phosphate binding [66]. Temperature plays another role in that the aggregation of α_{s1}-casein is, as noted above, accentuated at lower temperatures.

Finally, Holt [31] studied the effect of κ-casein on micelle dissociation; he expected that added κ-casein would cause dissociation of the micelles, and it did not. The experiment conducted was similar to what Talbot and Waugh termed micelle transformation [60]. In all previous studies of κ-casein content versus size, the more κ-casein present, the smaller the micelles. Addition of purified κ-casein to micelles causes a shift to smaller sizes not complete dissociation. It should also be noted that the purified κ-casein used in these experiments represents an SH-capped laddered polymer and not a reactive reduced monomeric species as discussed above (Tab. II).

4.3. Scattering studies

Both small-angle X-ray (SAXS) scattering [34, 43, 53] and small angle neutron (SANS) scattering [28, 58] have been applied to the casein micelles. These techniques both provide information on the electron density of the sample relative to the solvent. Because of technical limitations, regarding the wavelength of the radiation relative to the total particle size, SAXS methodologies in essence provide a viewing window on the micelles. The data then must be interpreted in terms of the density of the average particles observed within the micelles. To circumvent this problem it has been common to study first the sodium caseinate, which is totally contained within the experimental window and so determine its scattering density. Comparison of the density difference is then made with that observed for the window on the micelle. When this is done carefully, good inferences into the nature of the particles within the overall micelle structure can be made. Using these concepts and enhanced experimental techniques, the two SANS studies came to very similar conclusions that the micelles have within them particles with electron densities (scattering centers) similar to those found for the sodium caseinate (caseins only). For the SAXS data the electron density difference for the sodium caseinate is extremely low relative to globular proteins (9.9 e·nm^{-3} for

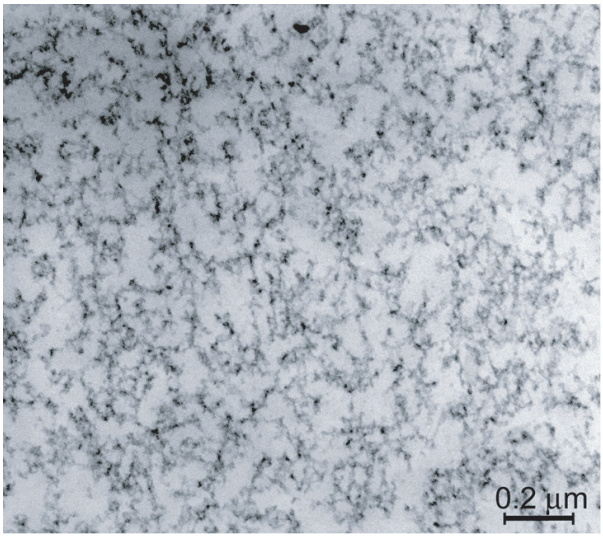


Figure 6. Transmission Electron Micrograph of sodium caseinate in imidazole buffer (pH 7.0), fixed with glutaraldehyde and stained with uranyl acetate and lead citrate.

casein versus 67 e·nm^{-3} for α-lactalbumin) and the particles within the micelles have this same low electron density; similar calculations can be done for the SANS data. Where these calculations differ is in the mathematical models used to fit the data for the proteins; basically the scattering centers within the micelles display a good deal of heterogeneity leading Hansen et al. [28] to conclude a polydisperse distribution of submicelles, while Stothart and Cebula [58] postulated submicelles of more closely packed nature. Kumosinski et al. [43] fitted their data for sodium caseinate to a somewhat lopsided sphere within a sphere, basically a spherical hydrophobic core and a loose hydrophilic shell reminiscent of Schmidt's submicelle model given in Figure 1b. However, the data could only be fitted well for the reformed micelles when there was significant overlap among the casein molecules contained in adjacent submicelles. These studies arrived at the same conclusion: proteinaceous submicellar structures exist within the casein micelles. Where they differ are on the possible arrangement of the casein monomers within the clusters and the possibility of overlap.

Holt et al. [33] studied calcium phosphate nanoclusters with both SANS and SAXS. These clusters also gave the best fit to the shell core model used by Kumosinski et al. [43], and this led Holt to speculate that the calcium phosphate "clusters rather than putative submicelles could be solely responsible for the heterogeneous structure revealed by electron microscopy, neutron scattering and X-ray scattering". On the surface this interpretation appears plausible, but it is not. First the calcium phosphate nanoclusters studied by Holt et al. [33] yielded an electron density of 700 to 900 e·nm^{-3}, nearly 100 times that of the caseins as noted above. Secondly the SAXS data by Kumosinski et al. [43] were for reformed casein micelles in a non-phosphate buffer thus nanoclusters were absent. The agreement between the SANS data with calcium phosphate and the SAXS data without is therefore all the more striking indicating the presence of submicellar

structures of a proteinaceous nature and ruling out calcium phosphate nanoclusters as the central feature of the casein micelle.

Considering the nature of the submicelles which may not be globular within the micelle, the overlap of the structures makes good sense particularly if the calcium phosphate double layers surround them at the time of coalescence in the Golgi vesicles. This would allow for the rearrangements seen in the alveolar lumen as well as the clustering of the κ-caseins into disulfide linked polymeric chains.

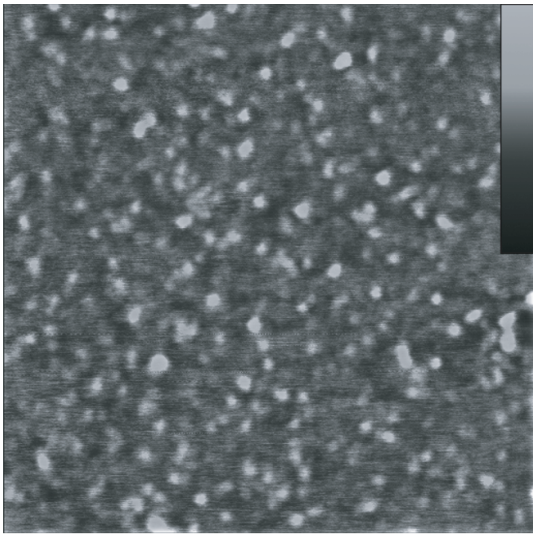
4.4. AFM studies

Atomic Force Microscopy techniques [25, 44, 64] have recently been applied to study the effects of pH and pressure on the casein micelles. To understand the important role of protein-protein interactions in the formation and stabilization of the micelles, we investigated native casein micelles using AFM in tapping mode in comparison to sodium caseinate which may serve as a model for submicelles (proteinaceous particles). The results were shown in Figure 7. Under dilute native micelle concentration, the particle size distribution centered on ~10 nm in diameter (Fig. 7a). It should be noted that these particles were persistently found in other caseinate systems such as calcium caseinate. We speculate this category of particles might be the "basic" necessary but not sufficient proteinaceous unit formed through protein-protein interactions; they may or may not undergo binding calcium and assembling into micelles successfully. The particle size distribution in sodium caseinate, on the other hand, exhibited nearly double sized particles with severe overlapping and clustering. Clearly, protein-protein interactions are the dominating feature in this system. Our TEM results seemed to suggest a similarity among the "random strands" (protein aggregates) in skim milk (Fig. 1a), the proteinaceous particles secreted in the Golgi (prior to calcium binding, Fig. 4), and the sodium caseinate system (Fig. 6). This is consistent with the findings by Gebhardt et al. [25] in their work on pressure-induced dissociation of casein micelles. It is, therefore, reasonable to assume that these 20 nm sized particles might be attributable to the much debated term "submicelles". We recognize that further research is needed to fully understand the driving force in the formation and stabilization of casein micelles.

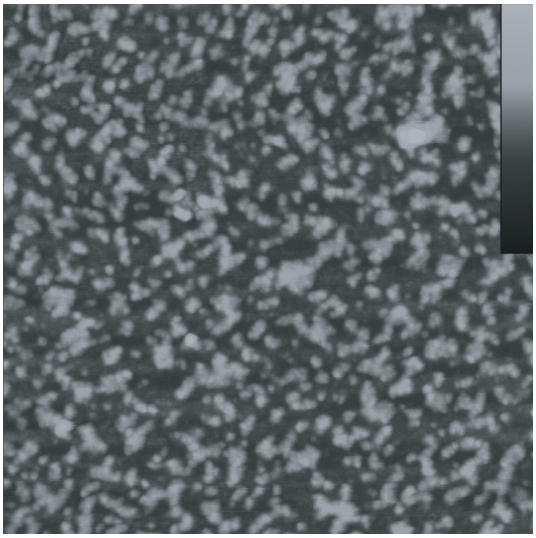
5. CONCLUSIONS

The common attribute that defines the vertebrate class mammalia is the production of milk, as the primary nutrient for the neonate, by mammary tissue. While the morphology and physiology of the mammary gland varies considerably from species to species, at the ultrastructural level the mammary epithelial cells have a common cellular motif. This cellular motif is quite adaptable and by the regulation of its elements, each species can respond to a variety of nutritional circumstances and efficiently produce a milk with a composition suited to the requirements of its neonate. This is particularly true for the calcium-phosphate-transport complexes, the casein micelles. Despite a wide variety of genetic influences that can alter the ratios of the individual caseins, casein micelles are formed in a biologically competent fashion to allow the secretion of the completed micelles. The combination of past research on the details of this biological process and recent developments from the studies of protein-protein interactions in the field of protein science leads to the following conclusions:

- Selective and productive protein-protein interactions (electrostatic and



(a)



(b)

Figure 7. Atomic Force Microscopy images of native micelles (7a) and sodium caseinate (7b). Original protein concentration applied onto the mica was 0.3 mg·mL^{-1}. Mica was washed three times to remove unbound proteins with water before imaging. The scan scale is 500 nm × 500 nm and 1 μm × 1 μm, and the height bar is 5 nm and 10 nm for 7a and 7b, respectively.

hydrophobic, etc.) are the driving force in the formation of casein micelles.

- On transport to the Golgi apparatus, the pre-formed proteinaceous particles (submicelles) are phosphorylated (rather slowly) and calcium and phosphate intercalated into these particles.
- Casein association/aggregation occurs in the Golgi vesicles through the coupling of the "submicelles" like (~20 nm) complexed with calcium and phosphate-casein micelles.
- It is likely that casein micelle formation is a hierarchal process-originated from a basic protein-protein interaction unit (~9.0–11 nm), which may or may not lead to the successful formation of micelles.

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