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Comprendre les comportements des micelles de caséines dans des environnements variés, de leur équilibre minéral à leurs propriétés colloïdales et fonctionnelles : émulsion et coagulation présure

Fanny Lazzaro

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Fanny LAZZARO • 27 octobre 2017

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sous le label de l'Université Bretagne Loire
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Comprendre les comportements des micelles de caséines dans des environnements variés, de leur équilibre minéral à leurs propriétés colloïdales et fonctionnelles : émulsion et coagulation présure

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Presented by:

Fanny Lazzaro

A comprehensive investigation of the behaviors of casein micelles in multiple environments, from their mineral balance to their colloidal and functional properties: focus on emulsion and rennet coagulation functionalities

Public Defense on Friday, 27th October, 2017, in front of the following jury

Jury:

Philippe CAYOT	Professor, University of Burgundy	Reviewer
Sylvie DESOBRY BANON	Professor, ENSAIA, University of Lorraine	Reviewer
Thomas CROGUENNEC	Professor, AGROCAMPUS OUEST	Examiner
Joëlle LEONIL	Research Director, UMR INRA-AO STLO	Examiner
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Ecole Doctorale : VieAgroSanté

Présenté par: **Fanny Lazzaro**

Comprendre les comportements des micelles de caséines dans des environnements variés, de leur équilibre minéral à leurs propriétés colloïdales et fonctionnelles : émulsion et coagulation présure

soutenue le « 27 octobre 2017 » devant la commission d'Examen

Composition du jury:

Philippe CAYOT	Professeur, Université de Bourgogne	Rapporteur
Sylvie DESOBRY BANON	Professeur, ENSAIA, Université de Lorraine	Rapporteur
Thomas CROGUENNEC	Professeur, AGROCAMPUS OUEST	Membre
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A mio nonno,

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LIST OF ABBREVIATIONS

A.U.: Arbitrary Unit

AMF: Anhydrous Milk Fat

ANRT: French National Association of Research and Technology (Agence Nationale de la Recherche et de la Technologie)

AsFlFFF: Asymmetrical Flow Field Flow Fractionation

CA(s): Casein Aggregate(s)

Ca: Calcium

$CaCl_2$: Calcium chloride

CaP: Calcium Phosphate

Cit: Citrate

Cl: Chloride

CMP: Caseinomacropeptide

CNIEL: French Dairy Interbranch Organization (Centre National Interprofessionel de l'Economie Laitière)

Cryo-TEM: Cryo-Transmission Electron Microscopy

CSIRO: Commonwealth Scientific and Industrial Research Organisation

CTRL: Control suspension of casein micelles (in part B)

DLS: Dynamic Light Scattering

d_{mean}: Mean diameter

E^*: Complex modulus

E': Elastic modulus

E'': Viscous modulus

$E1^{ec}$, $E2^{ec}$, $E3^{ec}$, $E4^{ec}$: Milkfat-in-water emulsion produced to assess the emulsifying capacities of the casein aggregates in suspension (EC = emulsifying capacity)

$E1^{st}$, $E2^{st}$, $E3^{st}$, $E4^{st}$: Milkfat-in-water emulsion produced to assess the emulsion-stabilizing capacities of the casein aggregates in suspension (ST = Stabilizing)

FG: Fast Green FCF

Firmness: Maximal firmness of the gels determined within 60 min after the chymosin addition

H^+: Proton

HCl: Hydrochloridryc acid

IMCU: International Milk Clotting Units

INRA: French National Institute for Agricultural Research (Institut National de la Recherche Agronomique)

IPR: Institute of Physics of Rennes (Institut de Physique de Rennes)

K: Potassium

MALLS: Multiple Angle Light Scattering

Mg: Magnesium

$MgCl_2$: Magnesium chloride

MPC: Milk Protein Concentrate

MW: Molecular Weight

n_a: SAXS parameter corresponding to the number of casein micelles (population A)

Na: Sodium

Na_3Cit: Tri sodium citrate

NaCas: Sodium Caseinate

NaCl: Sodium chloride

NaN_3: Sodium azide

NaOH: Sodium hydroxide

n_b: SAXS parameter corresponding to the number of dense regions per casein micelle (population B)

n_c: SAXS parameter corresponding to the number of population-C scatterer per casein micelle

n_{cCaP}: SAXS parameter n_C, considering the scatterers being calcium phosphate nanoclusters

n_{cPi}: SAXS parameter n_c, considereing the scatterers being protein inhomogeneities

NPC: Native PhosphoCaseinate

NR: Nile Red

NTA: Nanoparticle Tracking Analysis

OH^-: Hydroxide ions

PC: Principal Component

PCA: Princinpal Component Analysis

Pi: inorganic Phosphate

PI: Protein Inomogeneities

Po: organic Phosphate

r_a: SAXS parameter, mean radius of the casein micelle (population A)

r_b: SAXS parameter, mean radius of the dense regions (population B)

r_c: Creaming ratio

r_c: SAXS parameter, mean radius of the population-C scatterers

RCT: Rennet Clotting Time

R_h: Hydrodynamic radius

r_{NTA}: Mean radius of the casein micelles and small aggregates determined by nanoparticle tracking analysis

r_{rms}: Root mean square radius

RSD: Relative Standard Deviation

$S1_d$, $S2_d$, $S3_d$, $S4_d$: Suspensions of casein aggregates dialyzed (d = dialyzed)

SANS: Small Angle Neutron Scattering

SAXS: Small Angle X-ray Scattering

SD: Standard Deviation

SDS: Sodium Dodecyl Sulfate

SMCF: Drying; Concentrate, Matrix, Functionality (Sechage, Concentré, Matrice, Fonctionalité)

TIPIL: Transfers and Interactions in Processes and Dairy Industrie (Transferts et Intéractions dans les Procédés de l'Industrie Laitière)

UMR STLO : Science and Technology of Milk and Egg laboratory (Unité Mixte de Recherche Science et Technologie du Lait et de l'Oeuf)

VAS: Life Agronomy Health (Vie Agro Santé)

Vma: SAXS parameter, mean volume of the casein micelles (population A)

Vmb: SAXS parameter, mean volume of the dense regions (population B)

Vmc: SAXS parameter, mean volume of the population-C scatterers (population C)

ζ: Zeta potential

γ: Interfacial tension

$\Gamma_{s/l}$: Ratio of small (< 50 nm) over large particles (> 50 nm), determined on cryo-transmission electron microscopy images

$\Delta\rho_a$: SAXS parameter, contrast of the casein micelle (population A)

$\Delta\rho_b$: SAXS parameter, contrast of the dense regions (population B)

$\Delta\rho_c$: SAXS parameter, contrast of the population-C scatterers (population C)

σ_a: SAXS parameter, polydispersity of the population of casein micelle (population A)

σ_b: SAXS parameter, polydispersity of the population of dense region (population B)

σ_c: SAXS parameter, polydispersity of the population of population-C scatterers (population C)

τ: Turbidity

Φ: Phase shift

SOME DEFINITIONS OF CONCEPTS

In order to avoid confusion, our definitions of some concepts often mentioned in the study are given hereafter:

Colloidal phase: caseins, minerals and water associated.

Colloidal properties: properties of associated caseins (casein micelles and smaller aggregates) in terms of physical characteristics (ʒ, overall size) and composition (contents in caseins and hydration). This term also corresponds by extension to some properties of the suspensions of casein micelles (and aggregates) such as turbidity (τ), soluble proteins and viscosity.

Mineral content of casein micelles, which also participates to its colloidal stability, is considered in more details through the term “mineral balance”. This dissociation of terms is chose to avoid confusion and repetitions. Indeed, the mineral fraction of the casein micelles plays a central role in this project given that this is the direct targeted of the environmental modifications applied. Colloidal minerals are therefore considered apart from the colloidal properties of casein micelles.

Diffusible: refers to permeates of milk or casein micelles suspensions obtained after their ultrafiltration on a membrane having a 10 kDa molecular weight (MW) cut-off.

Functional property or functionality: properties of dairy constituents leading to the formation of food products, especially dairies, such as emulsifying or rennet coagulation abilities.

Internal organization: the way caseins, minerals and water associate together through various mineral-protein and protein-protein interactions to form casein micelles.

Mineral balance: quantitative and qualitative distributions of minerals in diffusible (or soluble) and colloidal phases of casein micelles suspensions.

Soluble: refers to the supernatant of milk or casein micelles suspensions obtained after ultracentrifugation (typically 100 000 g for 1 h or 80 000 g for 2 h) or to their rennet whey.

Soluble proteins: milk proteins remaining in the supernatant after ultracentrifugation of milk or casein micelles suspensions (typically 100 000 g for 1 h or 80 000 g for 2 h).

Structure: relates to the contributions of the models defined by Bouchoux et al. (2010) and Ingham et al. (2016). The structure takes into account the levels of organization of caseins and minerals (overall aggregate, dense regions, CaP nanoclusters and/or protein inhomogeneities).

General introduction

GENERAL INTRODUCTION

Milk and dairy products, staple food for humans, are centerpieces of all world cultures and are positively viewed in terms of nutritional and taste qualities. Dairy products and their processes also take an important place in the international economy. Besides medium growth of total world milk production (+ 2 % in 2015, International Dairy Federation, 2016) and regions such as Europe or north America where the market becomes saturated, the dairy product demand remains strong. In 2015, the global dairy consumption was estimated at 111.3 kg per person and should increase by 12.5 % by 2025 (FAO & OCDE, 2016). This estimation considers growing population (more than 1.9 billion peoples supplementary in the world until 2050) and the development of emerging regions, such as Asia and Africa that will potentially increase their dairy consumption in the future. In this context, food and dairy products will have to meet these new consumer's expectations, which have different cultural and taste habits than occidental ones. The industry and the research in the dairy field have to bring new knowledge for the development of adapted and still competitive products. The stake is to guarantee the future of the dairy industry in preserving their quality, the cost-efficiency of the processes, the environment but also in valorizing the products and coproducts through the optimization, the improvement and the development of the technological functionalities of milk constituents.

Major milk constituents are of different nature (water, fat, carbohydrate, protein and minerals) and numerous dairy products properties derive from the processing of proteins. Caseins represent 80 % of the milk proteins and assemble together via protein-protein and mineral-protein interactions with calcium phosphate (CaP) to form highly hydrated colloids of around 200 nm in diameter called casein micelles. Casein micelles are dynamic assemblies that constantly exchange materials (caseins, minerals and water) with their surrounding environment. It was largely demonstrated that environmental modifications, such as variations in temperature, pressure or pH and removal or additions of salts and chelating agents, affect these dynamic balances and enable to change the compositional and colloidal properties of the casein micelles. However, even if it seems generally admitted that such changes would cause the enhancement or depreciation of their functional properties, this aspect is poorly documented and understood.

In this context of understanding and improving the casein micelles functionalities, the present Ph.D. project aimed to contribute to fill this knowledge gap in establishing relationships between the mineral composition, the colloidal and the functional properties of the casein micelles. The focus was placed on the study of emulsion and rennet coagulation functionalities. The strategy consisted in modifying purified casein micelles in targeting specifically their mineral balance.

This was achieved through the modification of individual or multiple environmental factors: variations in pH and addition of salts and a chelating agent. The suspensions of modified casein micelles produced were then characterized using common (e.g. atomic absorption and ionic chromatography, soluble proteins, dynamic light scattering (DLS), chymoGRAPH®) or advanced biophysical techniques (cryo-transmission electron microscopy (cryo-TEM), and small angle X-ray scattering (SAXS)) to assess their colloidal and functional properties.

This Ph.D. project, titled “A comprehensive investigation of the behaviors of casein micelles in multiple environments, from their mineral balance to their colloidal and functional properties: focus on emulsion and rennet coagulation functionalities“, started with the willingness of French private industries and public research to collaborate in order to reach this objective. The French dairy interbranch organization (CNIEL) and the Science and Technology of Milk and Eggs (STLO) laboratory of the French National Institute of Agronomical Research (INRA) represent the two parts, respectively. Other French and international collaborations were established during the project to meet the multidisciplinary requirements of the study.

The organization of the present manuscript is conventional. First, the **“literature review”** section provides an overview on the casein micelles knowledge, in particular on the different models proposed over the past 50 years. The internal organization of the casein micelles remains a scientific challenge besides decades of studies and hundreds of scientific publications. The literature review also reports the scientific knowledge acquired on the environmental modifications of interest for our study, in terms of their influences on the mineral balance, the colloidal and functional properties of the casein micelles. The general and detailed objectives of the Ph.D. project and the experimental strategies employed are presented in a second section named **“objectives and strategies”**. Further details are provided about the collaborations established and the originalities of this project. The choice was made to present **the detailed materials and methods, their associated results and discussion** under the form of published (Part A) or to be submitted scientific articles (Part B**). Part A focuses on the study of the emulsion functionality while Part B, divided in two chapters, focuses on the study of the rennet coagulation functionality** of the modified casein micelles. Finally, the section **“general discussion and conclusion”** reminds and links the main results presented in each part of the manuscript, demonstrates how these results answer the different research questions and the general objective, discusses the results limitations and suggests recommendations for their application or further outlooks.

INTRODUCTION GENERALE

Le lait et les produits laitiers, aliments de base pour l'Homme, se trouvent au cœur des différentes cultures de notre monde et sont perçus de manière positive, que ce soit en termes de qualités nutritionnelles ou gustatives. Les produits laitiers et leurs procédés ont également une place importante dans l'économie mondiale. La demande en produits laitiers demeure forte malgré une production mondiale de lait en croissance moyenne (+2 % en 2015, International Dairy Federation, 2016) et la saturation des marchés économiques dans des régions telles que l'Europe et l'Amérique du Nord. En 2015, la consommation mondiale de produits laitiers s'élevait à 111,3 kg par personne. Celle-ci devrait augmenter de 12.5 % d'ici à 2025 selon les prévisions de la FAO & OCDE publiées en 2016. Cette estimation prend en compte la croissance de la population (plus de 1,9 milliards de personnes supplémentaires dans le monde en 2050) ainsi que le développement de régions émergentes, telles que l'Asie et l'Afrique, qui verront certainement augmenter leur consommation en produits laitiers dans le futur. Dans ce contexte, la nourriture et les produits laitiers devront satisfaire les attentes des ces nouveaux consommateurs, possédants des habitudes alimentaires différentes de celles des régions occidentales.

L'industrie et la recherche laitières se doivent d'apporter de nouvelles connaissances pour le développement de produits adaptés et compétitifs. L'enjeu est de garantir le futur de la filière en préservant la qualité des produits, la rentabilité des procédés, l'environnement, mais aussi en valorisant les produits et coproduits au travers de l'optimisation, de l'amélioration et du développement des fonctionnalités technologiques des constituants laitiers.

Les principaux constituants du lait sont de différentes natures (eau, matière grasse, carbohydrates, protéines et minéraux) et de nombreux produits laitiers dérivent de la transformation des protéines. Les caséines représentent 80 % des protéines laitières. Elles s'assemblent via des interactions inter protéiques et minérales, avec le phosphate de calcium (CaP), pour former des colloïdes fortement hydratés d'environ 200 nm de diamètre, appelés micelles de caséine. Les micelles de caséine sont des assemblages dynamiques qui échangent de la matière (caséines, minéraux, eau) avec leur environnement de manière constante. Il a été largement démontré que des modifications environnementales, comme des variations de température, de pression ou de pH, et comme le retrait ou l'addition de sels et d'agents chélatants, affectent les équilibres dynamiques des micelles de caséine et permettent des modifications de leurs compositions ou de leurs propriétés colloïdales. Bien qu'il soit généralement admis que de tels changements sont à l'origine de l'amélioration ou de la

dépréciation des propriétés fonctionnelles des micelles de caséines, cet aspect est cependant peu documenté et compris.

C'est dans ce contexte de compréhension et d'amélioration des fonctionnalités des micelles de caséine que se place ce projet de thèse. Son objectif est de combler le manque de connaissances par l'établissement de relations entre la composition minérale, les propriétés colloïdales et fonctionnelles des micelles de caséine. L'accent a été placé sur l'étude des fonctionnalités « émulsion » et « coagulation présure ». La stratégie mise en place lors de cette étude a consisté à induire des changements dans les propriétés de micelles de caséine purifiées en ciblant plus spécifiquement leur contenu minéral. Cette stratégie fut réalisée grâce aux modifications, de manières individuelles ou combinées, de plusieurs facteurs environnementaux : variations de pH et additions de différents sels et agent chélatant. Les suspensions de micelles de caséine modifiées ainsi produites ont ensuite été caractérisées par des techniques biophysiques courantes (par exemple : absorption atomique et chromatographie ionique, dosage des protéines solubles, diffraction dynamique de la lumière (DLS), ChymoGRAPH®) ou plus avancées (cryo-microscopie électronique à transmission (cryo-TEM), et diffraction des rayons-X aux petits angles (SAXS) afin d'évaluer leurs propriétés colloïdales et fonctionnelles.

Ce projet de thèse, intitulé « Comprendre les comportements des micelles de caséines dans des environnements variés, de leur équilibre minéral à leurs propriétés colloïdales et fonctionnelles : émulsion et coagulation présure », a débuté suite à la volonté d'industries privées et de la recherche publique françaises de collaborer dans le but d'atteindre ce même objectif. Le Centre National Interprofessionnel de l'Economie Laitière (CNIEL), et le laboratoire de la Science et Technologie du Lait et de l'œuf (STLO) de l'Institut National de la Recherche Agronomique (INRA), représentent respectivement les deux parties. D'autres collaborations françaises et internationales ont été établies au cours de ce projet afin de répondre aux exigences multidisciplinaires de cette étude.

L'organisation de ce manuscrit de thèse est conventionnelle. Une première section **« revue de la littérature »** fournit une vue d'ensemble des connaissances concernant les micelles de caséine, en particulier sur les différents modèles proposés au cours de ces 50 dernières années. La compréhension de l'organisation interne des micelles de caséine demeure un défit scientifique malgré des décennies d'études et des centaines de publications scientifiques. Cette revue de la littérature fait aussi état des connaissances scientifiques acquises sur les modifications environnementales d'intérêts pour notre étude, en termes d'influence sur l'équilibre

minéral, sur les propriétés colloïdales et fonctionnelles des micelles de caséines. Les objectifs généraux et détaillés du projet de thèse ainsi que les stratégies expérimentales employées sont présentées dans une seconde section intitulée « **objectifs et stratégies** ». Des détails complémentaires sont fournit au sujet des collaborations scientifiques établies et des originalités de ce projet. Le choix a été fait de présenter **les matériels et méthodes détaillés, leurs résultats et discussions associés** sous la forme d'articles scientifiques publié (partie A) ou à soumettre (partie B). **La partie A se concentre sur l'étude de la fonctionnalité émulsion tandis que la partie B, divisée en deux chapitres, concerne l'étude de la fonctionnalité coagulation présure** des micelles de caséine modifiées. Enfin, la section « **discussion générale et conclusion** » rappelle et lie les principaux résultats présentés dans chacune des parties du manuscrit, démontre comment ces résultats répondent aux différentes questions de recherche et à l'objectif général, discute les limites de ces résultats et suggère des recommandations pour leurs applications ou des perspectives à cette étude.

Literature review

LITERATURE REVIEW

1 Overall aspect of the casein micelles from bovine milk

1.1 Generalities

One of the most important component of skim milk are casein micelles, present at an overall concentration of 25 g kg^{-1}. They represent around 80 % of the protein content of milk, while whey proteins account for 20 % (Table 1) (Jenness, 1988; McSweeney & Fox, 2013). These unique supramolecular aggregates are responsible for the white turbid aspect of milk and are secreted by the Golgi apparatus within the mammary glands of every mammalian species (Farrell, Malin, Brown, & Qi, 2006). Casein micelles are naturally present in milk as polydispersed particles ranging between 80 and 400 nm (de Kruif, 1998; McSweeney & Fox, 2013; Udabage, McKinnon, & Augustin, 2003). In average, they have diameters of around 200 nm, molecular weights (MW) of 7.2 x 10^8 Da (de Kruif & Holt, 2003) and overall surface negative charge (zeta potential - ʒ) of about -20 mV in milk conditions (Dalgleish, 1984). Regarding their composition, casein micelles consist in an association of proteins (92 – 94 % in dry matter, ~ 10^4 molecules per casein micelle) and minerals (6 – 8 % of dry matter), highly hydrated (3 - 4 kg H_2O kg^{-1} proteins) (de Kruif & Holt, 2003; de Kruif, 1998; McSweeney & Fox, 2013). Four casein molecules composed the casein micelles: α_{s1}, α_{s2}, β, κ-caseins, in ratios of 4 : 1 : 4 : 1.3, respectively (Walstra, 1990). Regarding the mineral fraction (Table 2), it is essentially made of calcium (Ca) and inorganic phosphate (Pi), representing 51.4 and 30.3 %, of the total mass of colloidal minerals, respectively. Citrate (Cit) (8.6 %), potassium (K) (5.7 %), magnesium (Mg) (2.6 %) and sodium (Na) (1.4 %) account for the rest (Gaucheron, 2004).

Table 1: Average gross composition of cow milk. Composition are given in % (w / w). Adapted from Jenness, (1988).

Water		87.3
Lipids		3.9
Proteins	Caseins	2.6
	Whey proteins	0.6
Lactose		4.6
Minerals		0.7

1.2 The building blocks

1.2.1 Caseins molecules

The four casein molecules (α_{s1}, α_{s2}, β and κ-caseins) belong to the group of phosphoproteins because of the presence of 8, 9 to 11, 5 and 1 phosphoryl groups on certain of their serine residues, respectively (Farrell et al., 2004). This feature is of particular importance considering the presence of "phosphate centers" in α_s and β-caseins (Figs. 1, 2 and 3). These centers consist of the amino sequence SerP-SerP-SerP-X-SerP, where Ser corresponds to a serine residue, P to a phosphoryl group and X to any amino acid. Such sequences bring to α_s and β-caseins the ability to bind Ca, leading to the precipitation of casein molecules in Ca solutions (Dalgleish & Parker, 1980). κ-casein contains only one single SerP residue and no phosphate center (Fig. 4), which considerably limits its capacity to bind Ca. Not only this protein do not precipitate in Ca solution, but it also stabilizes the Ca-rich α_s and β-caseins. Another singularity of this protein is the presence of glycosylated sites that confers a high hydrophilicity to the C-terminal third of the molecule (residues 106 – 169, Fig. 5) (Farrell et al., 2004).

All four casein molecules also contain significant amounts of hydrophobic amino acids (Fig. 5), sometimes in the form of large hydrophobic regions (especially β and κ). The separation between the hydrophilic parts, i.e. glycosylated C-terminal residues (κ-casein) or the phosphorylated regions (β-casein), and the hydrophobic parts confers an amphipatic nature to these two caseins (Fig. 5). Finally, none of the casein possesses a lot of secondary, nor tertiary structure which means that they are able to adapt their structure to suit environmental conditions (Gaspar, Appavou, Busch, Unruh, & Doster, 2008; Holt & Sawyer, 1993). Due to this absence of elaborated structures, casein molecules are considered as rheomorphic proteins.

```
     1                                   10                                      20
H-Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln-Glu-Val-Leu-Asn-Glu-Asn-Leu-
     21                                  30                                      40
     Leu-Arg-Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys-Glu-Lys-Val-Asn-Glu-Leu-
     41                                  50                                      60
     Ser-Lys-Asp-Ile-Gly-SeP-Glu-SeP-Thr-Glu-Asp-Gln-Ala-Met-Glu-Asp-Ile-Lys-Gln-Met-
     61                                  70                                      80
     Glu-Ala-Glu-SeP-Ile-SeP-SeP-SeP-Glu-Glu-Ile-Val-Pro-Asn-SeP-Val-Glu-Gln-Lys-His-
     81                                  90                                      100
     Ile-Gln-Lys-Glu-Asp-Val-Pro-Ser-Glu-Arg-Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg-
     101                                 110                                     120
     Leu-Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-Asn-SeP-Ala-Glu-Glu-Arg-Leu-
     121                                 130                                     140
     His-Ser-Met-Lys-Glu-Gly-Ile-His-Ala-Gln-Gln-Lys-Glu-Pro-Met-Ile-Gly-Val-Asn-Gln-
     141                                 150                                     160
     Glu-Leu-Ala-Tyr-Phe-Tyr-Pro-Glu-Leu-Phe-Arg-Gln-Phe-Tyr-Gln-Leu-Asp-Ala-Tyr-Pro-
     161                                 170                                     180
     Ser-Gly-Ala-Trp-Tyr-Tyr-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser-
     181                                 190                                     199
     Asp-Ile-Pro-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser-Glu-Lys-Thr-Thr-Met-Pro-Leu-Trp-OH
```

Figure 1: Amino acid sequence of α_{s1}-casein B-8P. From Farrell et al. (2004). Sites of post-translational phosphorylation (SeP) are indicated in italicize, boldface type. The underline indicates the location of another phosphorylation site in a minor speciies of this protein (α_{s1}-casein B9-P)

```
  1                                   10                                      20
H-Lys-Asn-Thr-Met-Glu-His-Val-SeP-SeP-SeP-Glu-Glu-Ser-Ile-Ile-SeP-Gln-Glu-Thr-Tyr-
  21                                  30                                      40
  Lys-Gln-Glu-Lys-Asn-Met-Ala-Ile-Asn-Pro-SeP-Lys-Glu-Asn-Leu-Cys-Ser-Thr-Phe-Cys-
  41                                  50                                      60
  Lys-Glu-Val-Val-Arg-Asn-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly-SeP-SeP-SeP-Glu-Glu-
  61                                  70                                      80
  SeP-Ala-Glu-Val-Ala-Thr-Glu-Glu-Val-Lys-Ile-Thr-Val-Asp-Asp-Lys-His-Tyr-Gln-Lys-
  81                                  90                                      100
  Ala-Leu-Asn-Glu-Ile-Asn-Gln-Phe-Tyr-Gln-Lys-Phe-Pro-Gln-Tyr-Leu-Gln-Tyr-Leu-Tyr-
  101                                 110                                     120
  Gln-Gly-Pro-Ile-Val-Leu-Asn-Pro-Trp-Asp-Gln-Val-Lys-Arg-Asn-Ala-Val-Pro-Ile-Thr-
  121                                 130                                     140
  Pro-Thr-Leu-Asn-Arg-Glu-Gln-Leu-SeP-Thr-SeP-Glu-Glu-Asn-Ser-Lys-Lys-Thr-Val-Asp-
  141                                 150                                     160
  Met-Glu-SeP-Thr-Glu-Val-Phe-Thr-Lys-Lys-Thr-Lys-Leu-Thr-Glu-Glu-Glu-Lys-Asn-Arg-
  161                                 170                                     180
  Leu-Asn-Phe-Leu-Lys-Lys-Ile-Ser-Gln-Arg-Tyr-Gln-Lys-Phe-Ala-Leu-Pro-Gln-Tyr-Leu-
  181                                 190                                     200
  Lys-Thr-Val-Tyr-Gln-His-Gln-Lys-Ala-Met-Lys-Pro-Trp-Ile-Gln-Pro-Lys-Thr-Lys-Val-
  201                     207
  Ile-Pro-Tyr-Val-Arg-Tyr-Leu-OH
```

Figure 2: Amino acid sequence of α_{s2}-casein A-11P. From Farrell et al., (2004). Seryl residues (SeP) identified as phosphorylated are indicated in italicized, boldface type. Residues that have been determined to be partially phosphorylated or that potentially mey be phosphorylated according to CN kinase specificity are underlined.

```
  1                                   10                                      20
H-Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-SeP-Leu-SeP-SeP-SeP-Glu-
  21                              ↓   30                                      40
  Glu-Ser-Ile-Thr-Arg-Ile-Asn-Lys-Lys-Ile-Glu-Lys-Phe-Gln-SeP-Glu-Glu-Gln-Gln-Gln-
  41                                  50                                      60
  Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr-
  61                                  70                                      80
  Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln-Thr-
  81                                  90                                      100
  Pro-Val-Val-Val-Pro-Pro-Phe-Leu-Gln-Pro-Glu-Val-Met-Gly-Val-Ser-Lys-Val-Lys-Glu-
  101                ↓        ↓       110                                     120
  Ala-Met-Ala-Pro-Lys-His-Lys-Glu-Met-Pro-Phe-Pro-Lys-Tyr-Pro-Val-Glu-Pro-Phe-Thr-
  121                                 130                                     140
  Glu-Ser-Gln-Ser-Leu-Thr-Leu-Thr-Asp-Val-Glu-Asn-Leu-His-Leu-Pro-Leu-Pro-Leu-Leu-
  141                                 150                                     160
  Gln-Ser-Trp-Met-His-Gln-Pro-His-Gln-Pro-Leu-Pro-Pro-Thr-Val-Met-Phe-Pro-Pro-Gln-
  161                                 170                                     180
  Ser-Val-Leu-Ser-Leu-Ser-Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Gln-Lys-Ala-Val-Pro-Tyr-
  181                                 190                                     200
  Pro-Gln-Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu-Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-
  201                                 209
  Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val-OH
```

Figure 3: Amino acid sequence of β-casein A²-5P. From Farrell et al., (2004). Sites of post-translational phosphorylation (SeP) are indicated in italicized, boldface type. The arrows indicate the points of attack by plasmin responsible for β-CN fragments (γ-CN and proteose peptones) present in milk.

```
*1                                    10                                      20
H-Glu-Glu-Gln-Asn-Gln-Glu-Gln-Pro-Ile-Arg-Cys-Glu-Lys-Asp-Glu-Arg-Phe-Phe-Ser-Asp-
  21                                  30                                      40
  Lys-Ile-Ala-Lys-Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-Ser-Arg-Tyr-Pro-Ser-Tyr-Gly-Leu-
  41                                  50                                      60
  Asn-Tyr-Tyr-Gln-Gln-Lys-Pro-Val-Ala-Leu-Ile-Asn-Asn-Gln-Phe-Leu-Pro-Tyr-Pro-Tyr-
  61                                  70                                      80
  Tyr-Ala-Lys-Pro-Ala-Ala-Val-Arg-Ser-Pro-Ala-Gln-Ile-Leu-Gln-Trp-Gln-Val-Leu-Ser-
  81                                  90                                      100
  Asn-Thr-Val-Pro-Ala-Lys-Ser-Cys-Gln-Ala-Gln-Pro-Thr-Thr-Met-Ala-Arg-His-Pro-His-
  101                ↓                110                                     120
  Pro-His-Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys-Thr-Glu-Ile-Pro-
  121                                 130                                     140
  Thr-Ile-Asn-Thr-Ile-Ala-Ser-Gly-Glu-Pro-Thr-Ser-Thr-Pro-Thr-Thr-Glu-Ala-Val-Glu-
  141                                 150                                     160
  Ser-Thr-Val-Ala-Thr-Leu-Glu-Asp-SeP-Pro-Glu-Val-Ile-Glu-Ser-Pro-Pro-Glu-Ile-Asn-
  161                            169
  Thr-Val-Gln-Val-Thr-Ser-Thr-Ala-Val-OH
```

Figure 4: Amino acid sequence of κ-casein A-1P. From Farrell et al., (2004). The arrow indicates the point of attack by chymosin (rennet). The * indicates pyroglutamate as the cyclized N-terminal. The site of post-translational phosphorylation (SeP) is indicated in italicized, boldface type; residues that may potentially be phosphorylated are underlined.

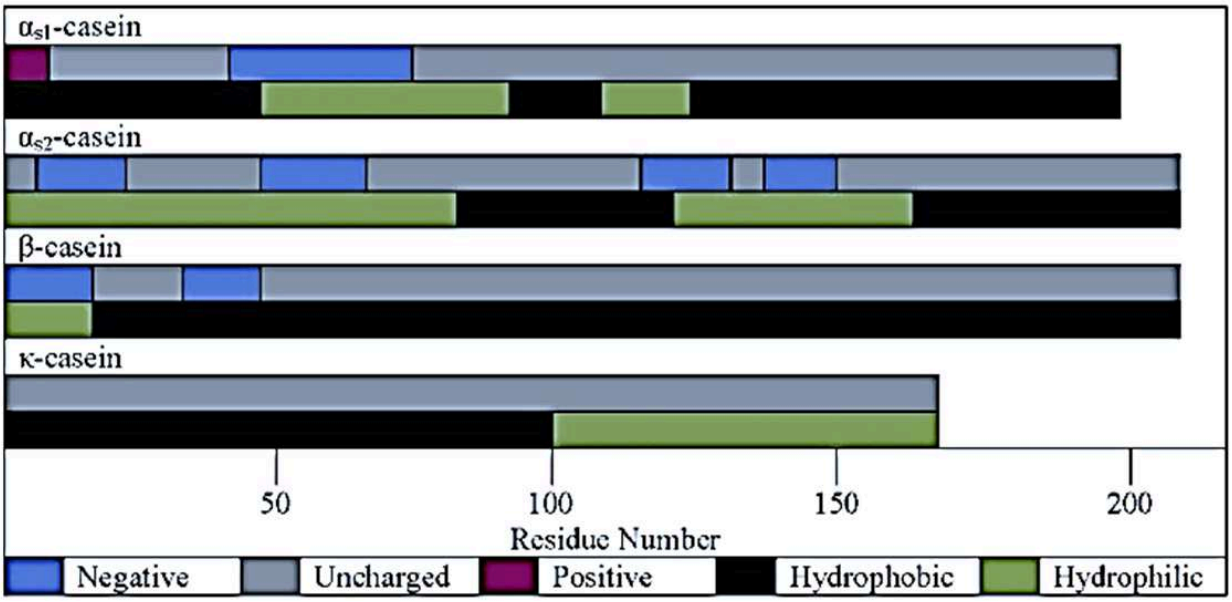

Figure 5: Schematic diagram of the location of the mostly hydrophilic, hydrophobic, charged and uncharged regions along the casein chains at pH 6.6. From Ranadheera et al., (2016)

1.2.2 Minerals

The milk mineral fraction essentially consists of macro-elements (> 10 mg L^{-1}) that are Ca, Mg, Na and K and Pi, Cit and Cl (Cashman, 2002a; Gaucheron, 2004, 2005; Lucey & Horne, 2009). Other trace elements (< 1 mg L^{-1}), such as zinc, copper or iron, will not be discussed in the present review considering their low amounts and / or the absence of interactions with caseins (Cashman, 2002b).

These macro-elements are differently distributed within the diffusible (or soluble) and colloidal phases of milk (Table 2). Theoretically, the diffusible (or soluble) ions are not associated with any proteins while colloidal ions are part of the casein micelles. Experimentally the diffusible ions are measured out in milk ultrafiltrates (ultrafiltration or dialysis on 10 – 15 kDa MW cut off membrane), while soluble ions correspond to the ones in milk ultracentrifugal supernatants (centrifugation at 80 000 g for 2 h or 100 000 g, 1 h) or in rennet whey. The colloidal concentrations are deduced by subtracting the diffusible (or soluble) to the total ion concentrations (Gaucheron, 2005). The separation method used slightly influence the results of both diffusible and colloidal ion concentrations. Na, Cl and K are essentially present as diffusible forms while Ca, Pi, Mg and Cit belong to both diffusible and colloidal categories (Table 2).

Table 2: Mineral composition of milk and distribution between the colloidal and diffusible phases. Pi corresponds to the phosphorus not covalently bound to casein molecules. Total phosphorus comprises Pi and Po (organic phosphate - phosphoryl groups bound to serine residues). From Lucey & Horne, (2009)

Mineral	Total concentration (mmol kg^{-1})	Colloidal concentration (%)	Diffusible concentration (%)
	Cations		
Calcium	26-32	69	31
Magnesium	4-6	47	53
Sodium	17-28	5	95
Potassium	31-43	6	94
	Anions		
Total phosphate	30-32		
Inorganic phosphate	19-23	53	47
Citrate	7-11	14	86
Chloride	22-34	5	95

Diffusible cations and anions are present as free ionic forms and also associate together via electrostatic bindings to form complexes. The extents of association depend on their reciprocal affinities and can be estimated considering the composition of the phases and the association constants of each ion between themselves (Holt, Dalgleish, & Jenness, 1981; Mekmene & Gaucheron, 2011; Mekmene, Le Graët, & Gaucheron, 2009). Briefly, Ca and Mg exist as free forms but mostly form complexes with Cit, Pi and Cl. The other monovalent cations (Na, K and Cl) exist mainly as free ions.

Regarding colloidal ions, Ca has the ability to associate directly with α_s and β-caseins, such as Mg (but in a smaller extent), because of the presence of the "phosphate centers". The more phosphate centers in the casein sequence, the higher ability to bind cations ($\alpha_{s2} > \alpha_{s1} > \beta >$ κ-casein, Dalgleish & Parker, 1980; Holt et al., 1981; Parker & Dalgleish, 1981). However, not all of the colloidal cations are directly bound to caseins as they also associate with colloidal anions to form CaP nanoclusters. Although the term "CaP" refers only to the two principal components of nanoclusters, they also contain Mg, Cit and organic phosphate (Po). The formation of these entities occurs consequently to the large insolubility of the CaP salts. The physical nature and composition of the nanoclusters are still controversial, e.g. the CaP nanoclusters were described as apatite (Pyne & McGann, 1960), brushite (Holt, Hasnain, & Hukins, 1982; Le Graët & Brulé, 1993) or amorphous types (McGann et al., 1983; Schmidt, 1982). The synthetic preparation of CaP nanoclusters (claimed to have the same properties as the micellar clusters), by mixing β-casein phosphopeptide to a salts solution simulating the salt composition of milk, led to the formation of essentially amorphous nanoclusters of 2.3 nm in radius and 61 kDa in MW. The authors considered that their composition was of 57.4 % Ca, 4.4 % of Mg atoms; 5.7 % of Cit and 28.3 % of Pi molecules per nanoclusters. In total, one nanocluster would be surrounded by 49 phosphate centers, which represents 4.3 % of the number of entities per nanoclusters (de Kruif & Holt, 2003; Holt, Timmins, Errington, & Leaver, 1998). Native nanoclusters were also isolated by enzymatic digestion of casein micelles (Ono, Takagi, & Kunishi, 1998) and the authors estimated a MW of 18 kDa and only 4-5 phosphate centers per nanoclusters. Little & Holt (2004) demonstrated that the size and composition of this particular feature did not depend on the environmental conditions (pH, temperature, peptide concentration, salt composition and rate of reaction) under which they formed.

1.3 The assemblies of the building blocks

Despite extensive studies within the past 50 years, the organization of the casein molecules and minerals within the casein micelles is still unclear and remains a matter of debate. Numerous models, more or less complete in defining the role and the arrangement of each constituent were suggested in the literature. The extensive description of all these models is not the aim of the present manuscript. Therefore, this review focuses on models selected according to their relevance to our study.

In a first step, the focus is put on the surface of the casein micelle due to the existence of a consensus between most of the literature models. Then, the models based on protein-protein and protein-minerals interactions are presented. Finally, the latest contributions of electronic microscopy and scattering experiments regarding the internal structuration of the casein micelle are reported.

1.3.1 The surface of the casein micelle

κ-casein differs from the three other casein molecules due to its limited capacity to bind Ca, and due to the presence of the hydrophilic part in its structure. These specificities are responsible for the position of this casein at the surface of the micelles, leading to a consensus between most of the models. However, due to relatively low proportion of κ-caseins compared to other caseins (13 %), Dalgleish (1998) argued that this molecule may not completely cover the surface of the micelles. Because of its strong affinity for water, the C-terminal part extends in the surrounding aqueous phase (Fig. 6), forming a so-called “hairy layer” of 5-10 nm thickness all around the colloids (Horne, 1986). This hairy layer is responsible for the high stability of the casein micelles through predominant steric hindrance and some electrostatic repulsive interactions (de Kruif & Zhulina, 1996; Horne & Davidson, 1986).

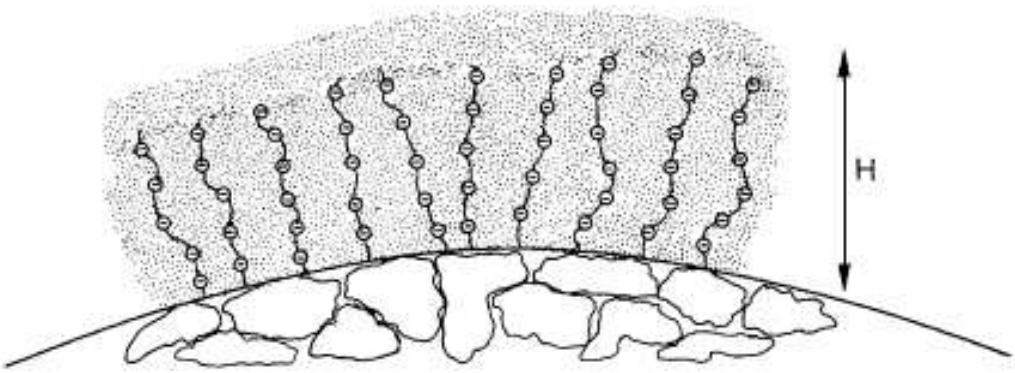

Figure 6: Schematic representation of the κ-casein hairy layer. The small dots represents the salts ions in solutions that interacts with the charge casein chains. H letter defines the thickness of the layer (between 5 and 10 nm). From de Kruif, (1999).

1.3.2 The core of the casein micelle

1.3.2.1 The role of the attractive and repulsive interactions

1.3.2.1.1 Submicelle model

The submicelle model was introduced by Waugh in 1958 and updated over the years by Schmidt, (1982), Slattery & Evard, (1973) and Walstra, (1999) (Fig. 7). The model was first described as an association of small aggregates named “submicelles”, nearly spherical, of 20 nm in diameter and containing 25 – 30 monomers of all four caseins (Slattery & Evard, 1973; Waugh, Creamer, Slattery, & Dresdner, 1970) (Figs. 7A and B). The caseins hold within the subunits through hydrophobic interactions, with the non-polar portion of each monomer orientated radially inward. The phosphate centers of the Ca sensitive caseins and the hydrophilic glycosylated portions of κ-casein are near the surface of the submicelle. However, the distribution of the different caseins is asymmetric, resulting in hydrophilic and hydrophobic patches on the subunits surface. κ-casein patches (hydrophilic) are surrounded by water while α_s and β-caseins patches (hydrophobic and Ca-sensitive) serves as anchors for CaP to link several submicelles together. This results in the constitution of a porous assembly. Further growth is prevented by a large percentage of hydrophilic surface (Slattery & Evard, 1973) (Fig. 7B). Schmidt (1982) proposed that the proportion of κ-casein varies between the submicelles with κ-casein rich subunits located on the exterior of the overall assembly (Fig. 7C). Walstra, (1999), in another contribution, redesigned the submicelle model in placing CaP nanoclusters inside of the casein building blocks (Fig. 7D). His main argument for this change was related to the low rate of exchange between Ca in the nanoclusters and in the diffusible phase of the casein. Therefore, the presence of CaP nanoclusters in the submicelles was more likely than CaP nanoclusters being located between neighboring submicelles

The main criticisms of this model concern the position of the CaP nanoclusters that differs from the authors: either it links the submicelles or it is located in their middle. Criticisms also underlined the lack of justification for the presence of two different kinds of κ-casein subunits (Dalgleish, 2011; Horne,2006)

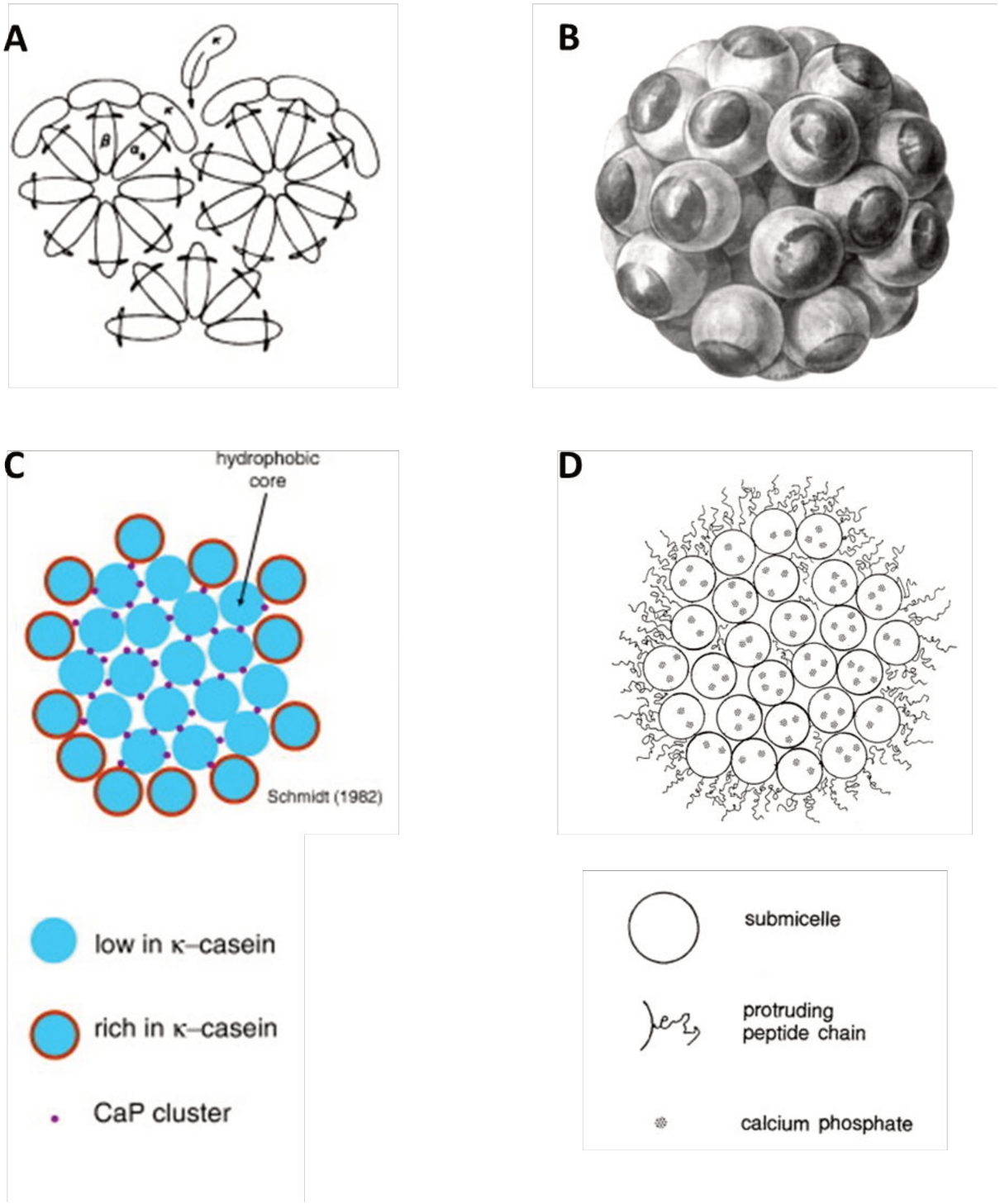

Figure 7: Evolution of the submicelle model. A. Model proposed by Waugh (1958). B. Model proposed by Slattery & Evard (1973), dark patches correspond to hydrophilic moeities of κ-caseins while light areas belongs to αs and β-caseins. C. Model proposed by Schmidt (1982) consisting of submicelles rich and low in κ-casein. D. Model proposed by Walstra (1999), note the position of the CaP nanoclusters (calcium phosphate represented by grey dots).

1.3.2.1.2 Nanocluster model

In this model, the formation of a casein network is suggested without postulating the existence of submicelle building blocks. Instead, an emphasis is put on the formation and the role of the CaP nanoclusters. The construction of this model is based on Holt and coworkers' observations (1998), indicating that the phosphate centers of β-casein can bind and stabilize CaP nanoclusters. Indeed, the complexation of CaP by phosphopeptide of β-casein resulted in the formation of nanoclusters of distinct size and composition, that would have grown and precipitate in the absence of caseins. During the formation of the micelle, it is assumed that α_s-caseins would also participate in the formation of such nanoclusters. de Kruif & Holt, (2003), defined the CaP nanoclusters as cross-linking agents that bind several Ca-sensitive proteins, forming and holding the 3D network together (Fig. 8).

The imperfections of this model resides in the absence of indication of how the κ-casein reach its position or how it controls the casein micelle size (Horne, 2006).

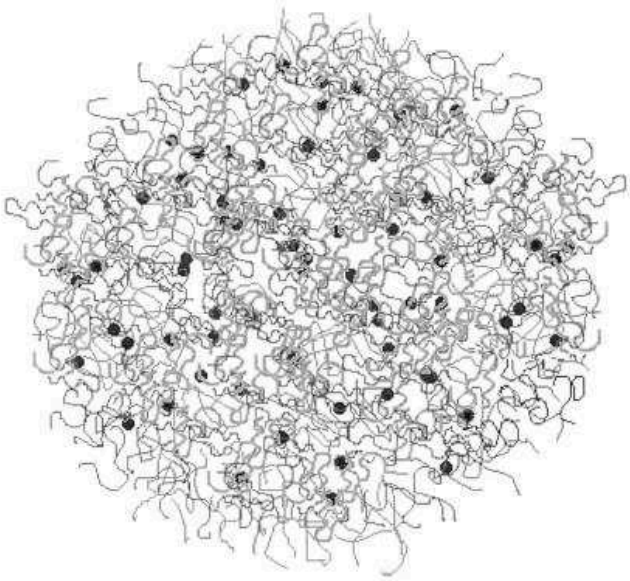

Figure 8: The nanocluster model. This model shows a more or less dense protein matrix containing CaP nanoclusters (•) cross-linking the Ca-sensitive α_s and β-caseins (De Kruif & Holt, 2003).

1.3.2.1.3 Dual binding model

The dual binding model proposed by Horne (1998), can be considered as a mix between the submicelle and the nanocluster models. Indeed, this model equally considers the protein-protein interactions (centerpieces of the submicelle model) and the CaP-protein interactions (centerpieces of the nanocluster model). Horne (1998) considered that the assembly of the casein molecules in casein micelles “is governed by a balance of attractive hydrophobic interactions and electrostatic repulsions”. According to this author, the micellar CaP plays simultaneously the roles of a cross-linker between the Ca-sensitive caseins and of a neutralizing agent. It reduces the level of protein negative charge in binding the phosphoseryl residues, which allow the protein interactions between the hydrophobic chains to dominate (Fig. 9). Thus, two different types of linkage are responsible for the integrity of the micellar network: i) the bond clusters formed by the hydrophobic protein-protein interactions and ii) the CaP nanoclusters that bridge several caseins. Horne also considered κ-casein as a terminator for both types of growth. This protein has limited ability to bind Ca (absence of phosphate center) and can only use its unique hydrophobic anchor to bind one other protein. This molecule is therefore unable to create bridges essential to form a network. According to Dalgleish (2011), this model presents the only inconvenient that it gives no details of the actual internal structuration of the casein micelle.

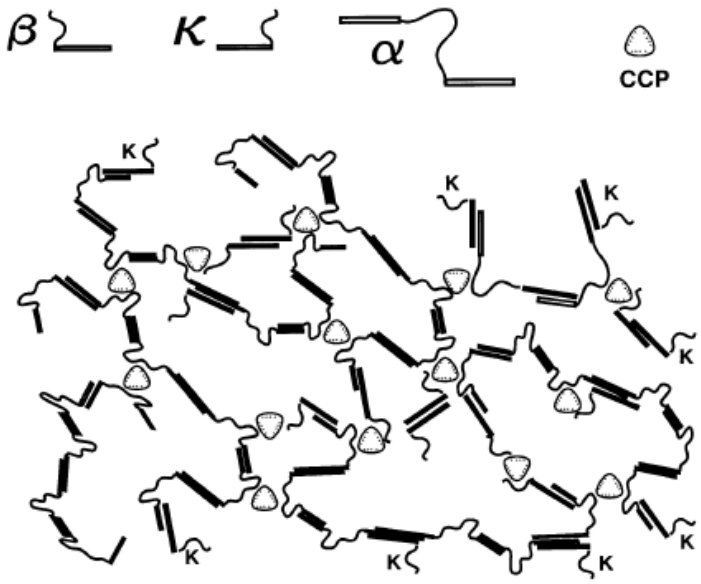

Figure 9: The dual binding model (Holt, 1998). Two different types of linkage are responsible for the formation of the casein network: the interactions between CaP (abbreviated as CCP in the figure) and α_s and β-caseins and the hydrophobic regions of the caseins. κ-casein act as a chain terminator and do not bind CaP.

1.3.2.2 Internal structure of the casein micelles

Electron microscopy techniques and scattering methods revealed to be interesting tools in probing the interior of the casein micelles. The use of these methods for the characterization of the casein micelles partially support the assembly models mentioned above and also bring further information on the internal structure of the casein micelles.

1.3.2.2.1 The contribution of the electron microscopy

Preparation processes such as metal coating, negative staining or sample fixation induced strong modifications of the structure of the casein micelle and lead to artefacts (McMahon & McManus, 1998). Cryo-preparation method seems to be the softer technique regarding the integrity of the colloid, although a slight flattening deformation have been reported by Trejo, Dokland, Jurat-Fuentes, & Harte, (2011). Therefore, only the cryo-TEM results are mentioned in this section.

The works of Marchin, Putaux, Pignon, & Léonil, (2007), McMahon & Oommen, (2008), McMahon & McManus, (1998) and Trejo et al., (2011) reported the existence of small electron dense regions, not greater than 12 nm in size and generally of 2-3 nm, and evenly distributed throughout the micellar structure (Fig. 10). This result was in agreement with the presence of clusters of CaP, which corresponds to the nanocluster and dual binding models. However, it did not support the submicelle model described in section 1.3.2.1, given than those submicelles would be of larger size. Tomographic reconstructions performed by Trejo et al. (2011), highlighted the presence of water-filled cavities (20 - 30 nm) and channels (5 nm) which were never considered by the 3 assembly models above mentioned (Fig. 11). The presence of such features was implied in McMahon & Oommen, (2008). These authors described the casein micelle as a lattice-type structure consisting of protein chains interlocked by CaP nanoclusters, resulting in an open, sponge-like colloidal supramolecule.

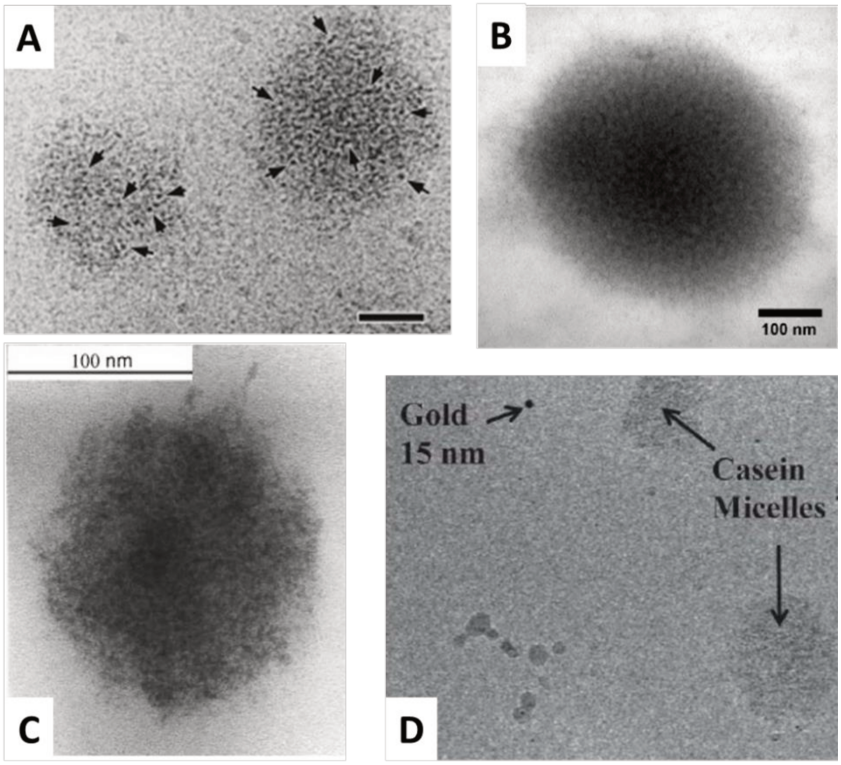

Figure 10: Casein micelles observed by cryo-TEM. A. Arrows are pointing at dark spot suggesting the presence of CaP nanoclusters, the scale bar is 50 nm (Marchin et al., 2007). B. (McMahon & Oommen, 2008) C. (McMahon and McManus, 1998). D. (Trejo et al., 2011)

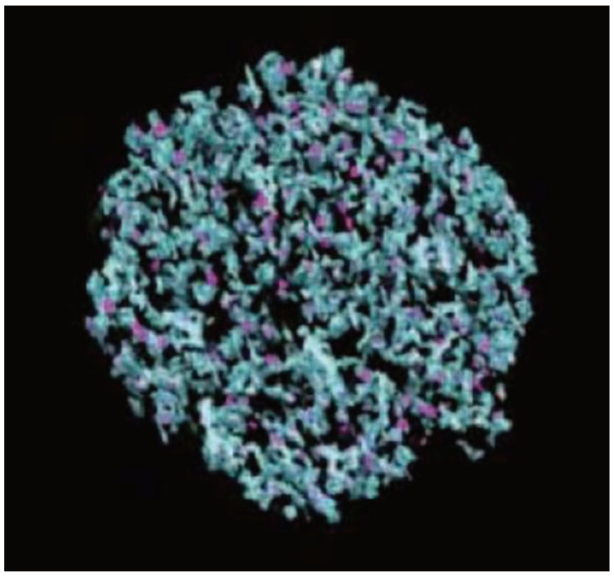

Figure 11: Isosurface representation of a 35 nm slab through the native casein micelle tomogram (Trejo et al., 2011). Water channels and cavities are distinguishable within the micelle

1.3.2.2.2 The contribution of the scattering techniques

Scattering techniques provide fewer methodological perturbations in the characterization of the casein micelle as the samples were, in worse cases, only diluted in their own diffusible phase. In the case of small angle neutron scattering (SANS) studies, performing of contrast variation studies requires the dispersion of the sample in H_2O / D_2O solvent mixture, but the effect of D_2O on the native structure of the casein micelle were demonstrated negligible. However, obtaining quantitative results from the scattering curves request an effort of modeling and oblige the user to formulate a priori hypotheses based on other analytical results. The different scattering studies performed in the past 20 years did not lead to an unique model of the internal structure of the casein micelle. The following parts focuses on the two latest released models of Bouchoux et al. (2010) and Ingham et al. (2016).

The sponge-like model (Bouchoux et al., (2010), considering three contributions

Bouchoux et al., (2010) also refers to the “sponge-like” definition in suggesting a more elaborated model consisting in a three level structure organization of the casein micelle. This model was based on results obtained by osmotic compression coupled to synchrotron SAXS observations of casein micelles in suspension (Fig. 12). Briefly, the first level corresponds to the whole casein micelle (~ 100 nm diameter) and is responsible for the intensity signal in the low q region (up to 6 x 10^{-3} $Å^{-1}$) of the SAXS pattern (Fig. 13). The greater contribution of the work of Bouchoux et al., (2010) resides in the definitions of coexisting soft and hard regions within the casein micelles (second level). Soft regions are filled with solvent and would be equivalent to the water channels emphasized by McMahon & Oommen, (2008) and Trejo et al., (2011). These features disappear under compression causing the solvent to be expelled out of the casein micelles while hard regions remains incompressible.

Soft regions do not contribute to the SAXS intensity signal while hard regions (10 - 40 nm) are responsible for the presence of a shoulder in the intermediate q region (6 x 10^{-3} to 2 x 10^{-2} $Å^{-1}$) (Fig. 13). Hard regions are composed of proteins and CaP nanoclusters (2 – 3 nm), the latest constituting the third structural level in the high q range (7 - 8 x 10^{-2} $Å^{-1}$) according to Bouchoux et al. (2010) and numerous other authors (Gebhardt, Burghammer, Riekel, Roth, & Müller-Buschbaum, 2008; Gebhardt, Takeda, Kulozik, & Doster, 2011; Marchin et al., 2007; Mata, Udabage, & Gilbert, 2011; Shukla, Narayanan, & Zanchi, 2009) (Fig. 13).

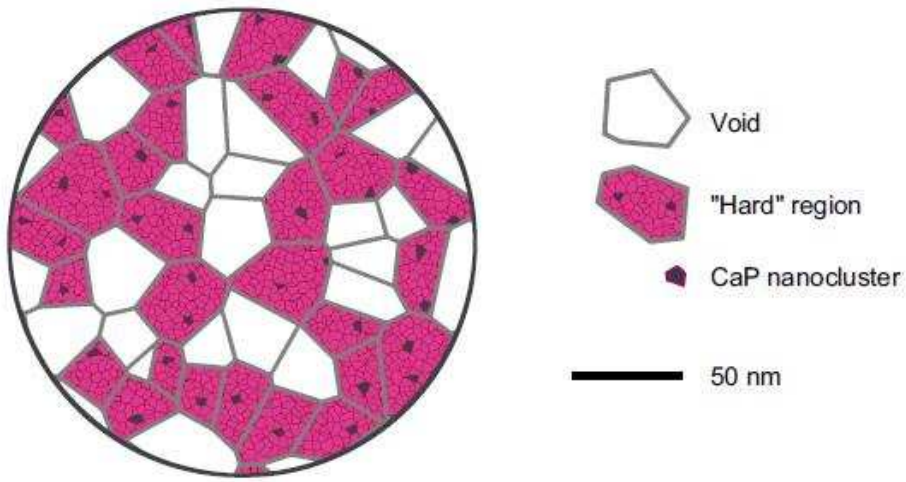

Figure 12: Schematic representation of a cross-section of the casein micelle according to the sponge model (Bouchoux et al., 2010). The micelle is made of hard and soft regions. The soft regions contains solvent while the hard regions are made of proteins and CaP nanoclusters.

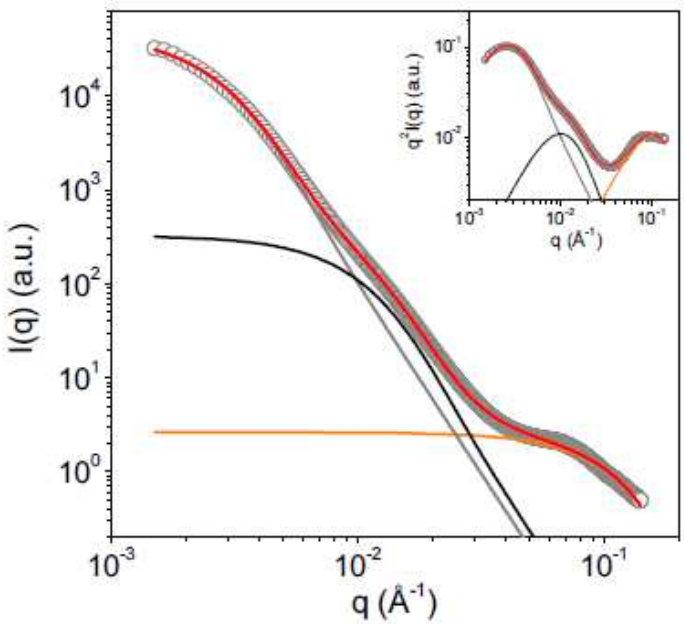

Figure 13: SAXS pattern of a 25 g kg^{-1} suspension of pure casein micelle in milk ultrafiltrate obtained on synchrotron facilities (Bouchoux et al. 2010). Open circles are the experimental data; red line corresponds to the best-fit form factor model defined in Bouchoux et al. The contributions of each structural level are displayed in grey (casein micelle), black (hard region) and orange (CaP nanocluster).

The model of Ingham et al., (2016), considering four contributions

In a recent study, Ingham et al. (2015) question the general attribution of the high q feature to the CaP nanoclusters. The use of resonant X-ray scattering technique on cow skim milk causes the emerging of a prominent peak at q = 0.035 $Å^{-1}$, that is not observed in conventional SAXS scattering for casein micelles in suspension (Fig. 14). Briefly, resonant X-ray scattering enables to enhance the scattering of Ca atoms compared to the rest of the casein micelle by selecting the electron beam energy (~350 eV) corresponding to the Ca L2 / L3 absorption edges. The peak emerging at q = 0.035 $Å^{-1}$ therefore corresponds to scattering objects containing Ca, i.e. CaP nanoclusters. The higher q feature (7 - 8 x 10^{-2} $Å^{-1}$) observed in regular SAXS experiment is only weakly present in resonant X-ray scattering (Fig. 14). Consequently, this feature is not primarily due to the CaP nanoclusters. Ingham et al., (2015) attribute this higher q feature to the presence of protein inhomogeneities suggested by de Kruif (2014) and de Kruif, Huppertz, Urban, & Petukhov, (2012). This discovery led to the rethinking of the sponge-like model into a four contribution model consisting in the whole casein micelle (I ; 1 – 4 x 10^{-3} $Å^{-1}$) containing soft and hard regions (II ; 1 – 2 x 10^{-2} $Å^{-1}$), themselves formed by CaP nanoclusters (III ; 3.5 x 10^{-2} $Å^{-1}$) and protein inhomogeneities (IV ; 7 x 10^{-2} – 1 x 10^{-1} $Å^{-1}$).

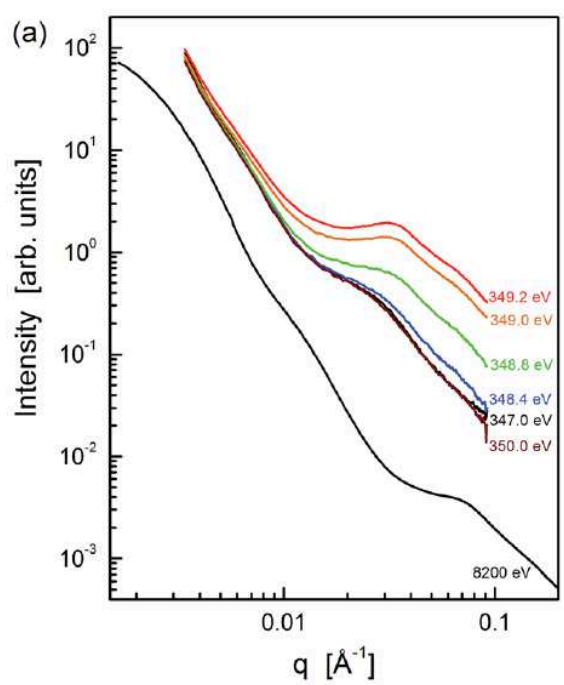

Figure 14: Resonant X-ray scattering pattern of skim milk (Ingham et al., 2015). The energy beam varied near the Ca L2-edge as labelled (color lines). The black line corresponds to typical synchrotron SAXS data collected at 8200 eV.

2 Impact of environmental modifications on the mineral balance, the colloidal and functional properties of the casein micelles

2.1 Generalities

Minerals and casein molecules are in dynamic balance between the colloidal and the diffusible phases. This balance depends on the element concentrations and the solubility of the minerals salts. The term “dynamic” means that exchanges constantly occur between the diffusible and the colloidal phases, and that any changes in environmental conditions, such as composition, concentration, pH, temperature or pressure, leads to shifts in the mineral balance. Figure 15 schematically represents this balance and considers only Ca, Pi and Cit given that other minerals play less significant roles, although present in noticeable amount in milk. It has been reported in section 1.2.2, that in spite of uncertainties on the structural arrangement of the casein micelles, all the casein micelle models consider the colloidal minerals as key components for the integrity of the structure. Therefore, any modifications of the mineral content by shifts in the balance lead to the modifications of the casein micelles properties.

The objective of this section is to review the impact of some environmental modifications on i) the mineral balance, ii) the content and colloidal properties of the casein micelles and on iii) some of their functional properties. Tables 3, 4, 5 and 6 and Figure 16 summarize the consequences of such modifications on casein micelles.

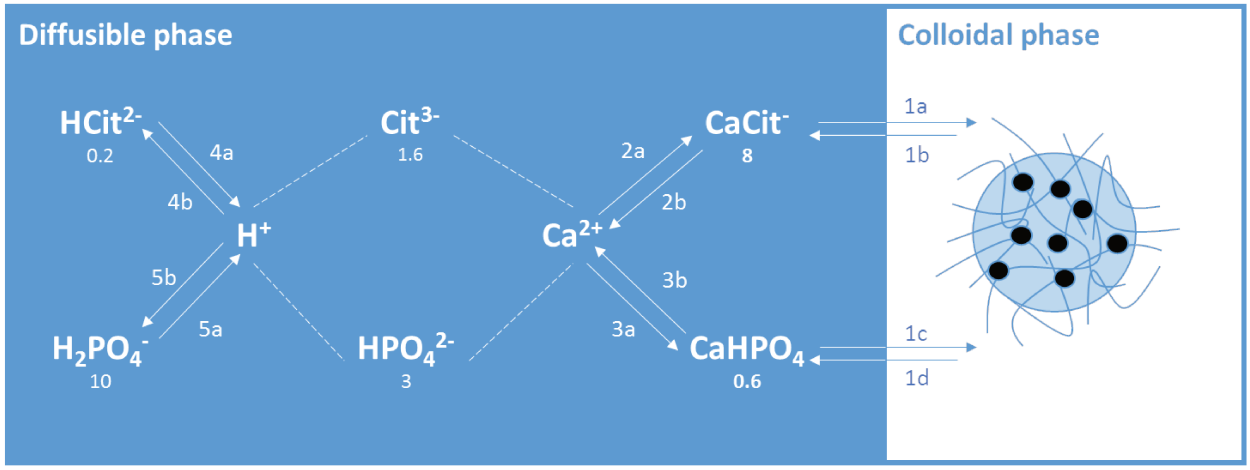

Figure 15: Schematic representation of the mineral balance between the colloidal and diffusible phases of milk. Concentrations of the different ions associations in milk are indicated in mmol kg^{-1}. Adapted from Brulé, (1981), Gaucheron, (2005).

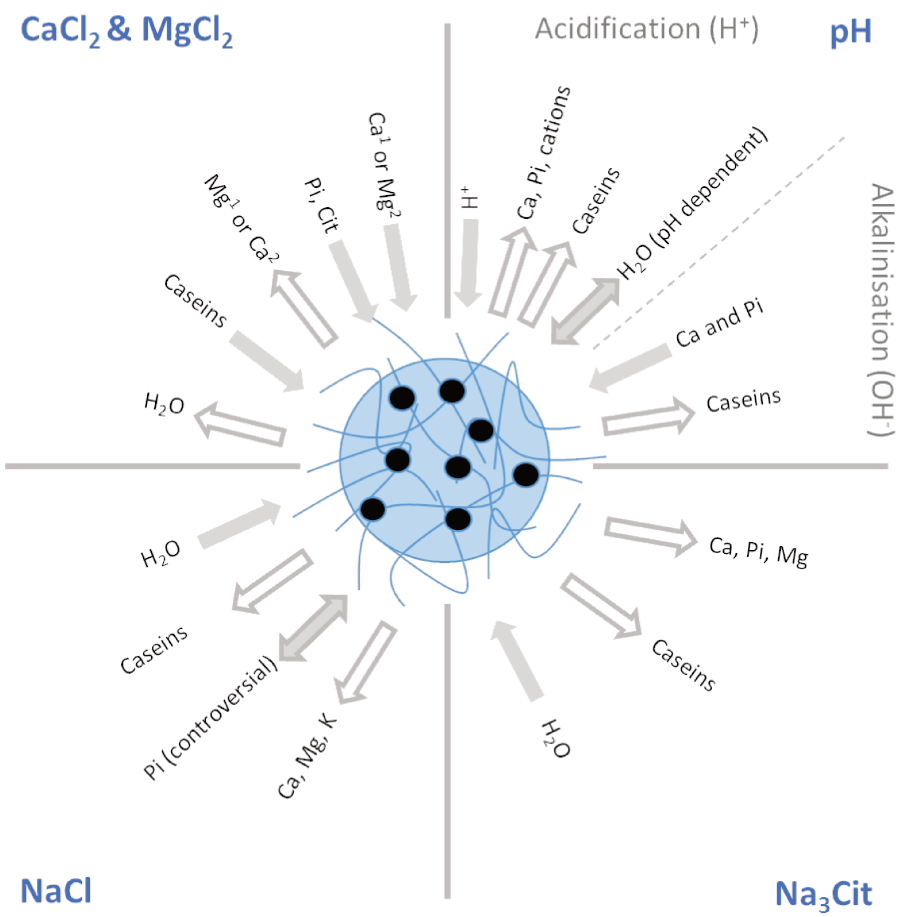

Figure 16: Impact of environmental modifications on the shifts in caseins, minerals and water balances in casein micelle suspension. Adapted from on Gaucheron, (2004)

2.2 Mineral and colloidal properties

2.2.1 pH variations

Milk acidification occurs in a large number of dairy processing, particularly in yogurt and cheese making where the pH of milk is lowered by lactic bacteria that convert lactose into lactic acid. A decrease in milk pH can also be induced by direct addition of chemicals such as strong acids like hydrochlorydric acid (HCl) (Dalgleish & Law, 1988; Le Graët & Brulé, 1993; Post, Arnold, Weiss, & Hinrichs, 2012; Silva et al., 2013; van Hooydonk, Boerrigter, & Hagedoorn, 1986a, 1986b; Zoon, van Vliet, & Walstra, 1989) or nitric acid (Le Graët & Gaucheron, 1999). These acids immediately lower the pH and cause the precipitation of the caseins at pH 4.6. Lactic acid, a weaker acid, has been used by Choi, Horne, & Lucey, (2007) and Daviau, Famelart, Pierre, Goudédranche, & Maubois, (2000). The slow hydrolysis of glucono-delta-lactone (GDL) causes a progressive decrease in pH. This mean of acidification has kinetic similarities with bacterial acidification and leads to the formation of casein gels. GDL has been used by numerous authors (Dalgleish & Law, 1988, 1989; Famelart, Lepesant, Gaucheron, Le Graët, & Schuck, 1996; Gastaldi, Lagaude, Marchesseau, & Fuente, 1997; Karlsson, Ipsen, & Ardö, 2007; Moitzi, Menzel, Schurtenberger, & Stradner, 2011; Olivares, Achkar, & Zorrilla, 2016).

Mechanistically, the in situ production of protons (H^+) or their addition from external sources have the same consequences. H^+ associate with Cit^{3-} and HPO_4^{2-} to form $HCit^{2-}$ and $H_2PO_4^-$, respectively (Fig. 15, arrows 4b and 5b). The depletion in Cit^{3-} and HPO_4^{2-} is counterbalanced by the disruption of $CaCit^-$ and $CaHPO_4$ that lead to the subsequent liberation of ionic Ca (Fig. 15, arrows 2b and 3b). The impoverishment of the diffusible phase in $CaCit^-$ and $CaHPO_4$ is compensated by the solubilization of micellar salts (Fig. 15, arrows 1b and 1d). The release of colloidal Ca and Pi from the casein micelles consequently to a pH decrease has been largely agreed (Fig. 16 and Table 3) (Choi et al., 2007; Dalgleish & Law, 1989; Daviau et al., 2000; Famelart et al., 1996; Gastaldi et al., 1997; Karlsson et al., 2007; Silva et al., 2013; van Hooydonk, Boerrigter, et al., 1986b; Zoon et al., 1989). Some authors also reported increases in concentrations of Mg (Dalgleish & Law, 1989; Silva et al., 2013) and Cit (Le Graët & Gaucheron, 1999), Na and K (Le Graët & Brulé, 1993) in the diffusible phase (Table 3). Added or newly formed H^+ also associate with phosphoseryl residues and carboxyl groups, resulting in the decrease of the net negative charge of the caseins. This neutralization induces stronger interactions between the casein chains and decreases their solubility, which leads to the precipitation or gelation of the caseins at their isoelectric pH (4.6 in milk conditions).

The changes in the mineral and protein interactions induced modifications of the physical properties of the casein micelles (Fig. 16, Table 3). Post et al. (2012) reported an increase of the negative charge at the surface of the casein micelles (decrease in their zeta-potential) with a decrease in pH. Famelart et al. (1996), Gastaldi et al. (1997) and van Hooydonk, Boerrigter, et al. (1986a) observed a pH dependency of the hydration of the sedimentable casein micelles. A decrease was observed from pH 7 to 6.3 - 6.0 then an increase up to pH 5.4 - 5.5 followed by another decrease. Lowering the pH caused also the release of all type of caseins in ultracentrifuge supernatants as mentioned by Dalgleish & Law (1988), Gastaldi et al. (1997), Post et al. (2012) and Silva et al. (2013). Regarding the evolution the micellar size, results are controversial. Famelart et al. (1996), Moitzi et al. (2011) and Silva et al. (2013) observed a decrease in the size of the casein micelles due to their complete disruption while Daviau et al. (2000) and van Hooydonk, Boerrigter, et al. (1986a) attributed the increase in micellar size or voluminosity, respectively, to the swelling of the colloids.

Alkalinisation of milk was much less studied than acidification and was mostly induced by addition of sodium hydroxide (NaOH) (Day, Raynes, Leis, Liu, & Williams, 2017; Huppertz, Vaia, & Smiddy, 2008; Vaia, Smiddy, Kelly, & Huppertz, 2006). Mechanistically, the addition of hydroxide ions (OH^-) “deprotonates” $HCit^{2-}$ and $H_2PO_4^-$ and forms supplementary Cit^{3-} and HPO_4^{2-} (Fig. 15, arrows 4a and 5a). Those two ions associate with Ca^{2+} which leads to the formation and the precipitation of $CaCit^-$ and $CaHPO_4$ salts (Fig. 15, arrows 2a and 3a), possibly as nanoclusters in the casein micelles (Vaia et al., 2006). A decrease in diffusible concentrations of Ca and Pi have been observed by Vaia et al. (2006) (Fig. 16, Table 3). The mineral precipitations would have a stabilizing effect on the structure of the casein micelles in creating more cross-binding sites between the proteins. However, increasing the pH also increases the ionization of acidic side groups of the caseins while basic side groups ionization decreases, resulting in the augmentation of the net negative charge of the caseins. In addition, the increase in “solvent quality”, in other terms an increase in the purity of the diffusible phase due to the decrease in mineral concentrations, diminishes the cohesive interactions between the hydrophobic regions of the caseins (Vaia et al., 2006). The increase in repulsions between the protein chains leads to the disruption of the casein micelles, indicated by a decrease in τ (Huppertz et al., 2008; Vaia et al., 2006) and an increase in soluble caseins (Post et al., 2012) (Fig. 16, Table 3). Day et al. (2017), observed a small increase in casein micelle size corresponding to swelling, likely caused by an increased net protein charge (Table 3).

Table 3: Effect of variation in pH on the mineral balance and the colloidal properties of the casein micelles.

Environmental modifications		Properties impacted	Variations	references
Variations in pH	Acidification	Diffusible minerals	Increase in Ca and Pi,	Choi et al. (2007) Daviau et al., (2000) Famelart et al. (1996) Gastaldi et al., (1997)*
			Mg,	Dalgleish et al., (1989) Silva et al., (2013)
			Cit,	Le Graët & Gaucheron, (1999)
			Na and K	Le Graët & Brulé, (1993)
		Soluble caseins	Increase of all type of caseins	Dalgleish & Law, (1988) Gastaldi et al., (1997) Post et al., (2012) Silva et al., (2013)
			β-casein being the major	Dalgleish & Law, (1988)
		Charge	Increase in zeta-potential (loss of negative charge)	Post et al., (2012)
		Hydration	pH 7 to 6.3,6.0: decrease ~6.3 to ~5.4: increase ~5.4 to 2: decrease	Famelart et al., (1996) Gastaldi et al., (1997) van Hooydonk et al., (1986)
		Turbidity	Decreased	Silva et al., (2013)
		Size	***Controversial*** Decrease	Famelart et al., (1996) Moitzi et al. (2011) Silva et al., (2013)
			Increase	Daviau et al., (2000); van Hooydonk, Boerrigter, et al., (1986)
	Alcalinization	Diffusible minerals	Decrease in Ca and Pi	Vaia et al., (2006)
		Soluble caseins	Increase	Post et al., (2012)
		Turbidity	Decrease	Huppertz et al. (2008) Vaia et al., (2006)
		Size	***Controversial*** Increase (SAXS and DLS)	Day et al., (2017)
			Disruption of the casein micelle (based on turbidity measurement)	Huppertz et al., (2008) Vaia et al., (2006)

2.2.2 Addition of tri sodium citrate (Na_3Cit)

Cit salts belong to the family of Ca chelating agents, such as EDTA, oxalates, pyrophosphates or polyphosphates. The effect of these chemicals on milk properties has been extensively studied (Broyard & Gaucheron, 2015). The present literature review specifically focuses on the action of Na_3Cit.

Addition of Cit to milk systems results in the complexation of free Ca^{2+} leading to the formation of $CaCit^-$ (Fig. 15, arrow 2a). The solubilization of $CaHPO_4$ from the casein micelle (Fig. 15, arrow 1d), as well as the release of Ca^{2+} previously bound to caseins, counterbalance the Ca^{2+} impoverishment. A decrease in diffusible Ca and Pi concentrations has been observed by numerous authors (Le Ray et al., 1998; Mizuno & Lucey, 2005; Mohammad & Fox, 1983; Morr, 1967; Ozcan-Yilsay, Lee, Horne, & Lucey, 2007; Vujicic, deMan, & Woodrow, 1968), and confirmed the micellar CaP solubilization (Fig. 16, Table 4). Udabage, McKinnon, & Augustin, (2000), also observed an increase in diffusible Mg concentration.

These changes in the mineral balance induce noticeable changes of the casein micelles properties (Fig. 16, Table 4). The pH of the suspensions increase (Le Ray et al., 1998; McCarthy et al., 2017; Vujicic et al., 1968) given that Cit ions also associate with H^+ (Fig 16, arrow 4b). The decrease in micellar CaP led to the disruption of the casein micelles, as indicated by the increase of soluble caseins (de Kort, Minor, Snoeren, van Hooijdonk, & van der Linden, 2011; Johnston & Murphy, 1992; Le Ray et al., 1998; McCarthy et al., 2017; Mohammad & Fox, 1983), the decrease in τ (de Kort et al., 2011; McCarthy et al., 2017; Mizuno & Lucey, 2005), the increase in viscosity (de Kort et al., 2011; McCarthy et al., 2017; Mohammad & Fox, 1983; Vujicic et al., 1968) and the decrease in casein micelle size (McCarthy et al., 2017; Udabage et al., 2000). An increase in hydration of sedimentable caseins was also reported by Le Ray et al., (1998), Morr, (1967) and Udabage et al., (2000).

Table 4: Effect of Na_3Cit addition on the mineral balance and the colloidal properties of the casein micelles.

Environmental modification	Properties impacted	variations	references
Addition of Na_3Cit	pH	Increase in pH	Le Ray et al., (1998) McCarthy et al., (2017) Vujicic et al., (1968)
	Diffusible minerals	Increase in Ca and Pi	Le Ray et al., (1998) Mizuno & Lucey, (2005) Mohammad & Fox, (1983) Morr, (1967), Ozcan-Yilsay et al., (2007) Vujicic et al., (1968)
		And increase in Mg	Udabage et al., (2000)
	Soluble caseins	Increase of all types of caseins	de Kort et al. (2011) Johnston & Murphy, (1992) Le Ray et al., (1998) McCarthy et al., (2017) Mohammad & Fox, (1983)
	Hydration	Increase	Le Ray et al., (1998) Morr, (1967) Udabage et al., (2000)
	Turbidity	Decrease	de Kort et al., (2011 McCarthy et al., (2017) Mizuno & Lucey, (2005)
	Viscosity	Increase	de Kort et al., (2011) McCarthy et al., (2017) Mohammad & Fox, (1983) Vujicic et al., (1968)
	Size	***Controversial*** Decrease	McCarthy et al., (2017) Udabage et al., (2000)
		Increase (voluminosity)	de Kort et al., (2011)

2.2.3 Addition of a monovalent salt: sodium chloride (NaCl)

The addition of NaCl to milk or casein micelles suspensions causes an increase in ionic strength. Na ions would be able to displace micellar cations such as H^+ and Ca^{2+} bound to caseins. Indeed, a decrease in pH was observed by numerous authors (Karlsson et al., 2007; Karlsson, Ipsen, Schrader, & Ardö, 2005; Le Graët & Brulé, 1993; Le Ray et al., 1998; van Hooydonk, Hagedoorn, & Boerrigter, 1986; Zhao & Corredig, 2015), as well as the solubilization of micellar Ca (Aoki, Umeda, & Nakao, 1999; Famelart et al., 1996; Grufferty & Fox, 1985; Le Graët & Brulé, 1993; Le Ray et al., 1998; van Hooydonk, Hagedoorn, et al., 1986; Zhao & Corredig, 2015) (Fig. 16, Table 5). Some of these authors also reported a concomitant increase in the concentration of diffusible Pi while others observed an increase in diffusible concentration of Ca only. Therefore, controversy remains between the facts that Na ions are only responsible for the substitution of Ca bound to protein residues and that they also induce CaP nanocluters solubilization.

The consequences on the properties of the casein micelles are a decrease in casein micelle charge (Zhao & Corredig, 2015) and an increase in hydration of sedimentable caseins (Grufferty & Fox, 1985; Karlsson et al., 2007; Le Ray et al., 1998) (Table 5). These changes were accompanied, in some cases, by an increase in soluble caseins (Famelart et al., 1996; Zhao & Corredig, 2015), while Le Ray et al., (1998) and van Hooydonk, Hagedoorn, et al., (1986) reported no evolution. The size and voluminosity of the casein micelles increased (Karlsson et al., 2005; van Hooydonk, Hagedoorn, et al., 1986; Zhao & Corredig, 2015) and the τ of the suspensions decreased (Zhao & Corredig, 2015) suggesting internal rearrangements of the colloids (table 5).

Table 5: Effect of NaCl addition on the mineral balance and the colloidal properties of the casein micelles.

Environmental modification	Properties impacted	variations	references
Addition of NaCl	pH	Decrease	Karlsson et al. (2007) Karlsson et al. (2005) Le Graët & Brulé, (1993) Le Ray et al., (1998) van Hooydonk et al. (1986) Zhao & Corredig, (2015)
	Diffusible minerals	***Controversial*** Increase in Ca and Pi	Aoki, et al. (1999) Famelart et al., (1996) Zhao & Corredig, (2015)
		Or increase in Ca only and Pi constant	Grufferty & Fox, (1985) Le Graet & Brulé, (1993) Le Ray et al., (1998) van Hooydonk et al., (1986)
		and increase in Mg and K	Le Graët & Brulé, (1993)
		Or Ca and Pi constant	Karlsson et al., (2007)
	Soluble caseins	Increase of all proteins	Famelart et al., (1996) Zhao & Corredig, (2015)
		Constant	Le Ray et al., (1998) van Hooydonk et al., (1986)
	Charge	Increase in zeta-potential (decrease in charge)	Zhao & Corredig, (2015)
	Hydration	Increase	Grufferty & Fox, (1985) Karlsson et al., (2007) Le Ray et al., (1998)
	Turbidity	Decrease	Zhao & Corredig, (2015)
	Size	Slight increase	Karlsson et al., (2005) Zhao & Corredig, (2015)
		Increase (voluminosity)	Karlsson et al., (2005) van Hooydonk et al. (1986)

2.2.4 Addition of calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$)

This section considers both actions of $CaCl_2$ and $MgCl_2$ given that the two divalent cations cause similar modifications of the casein micelle properties. Ca^{2+} and Mg^{2+} associate with Cit^{3-} and HPO_42^- to form $CaCit^-$, $MgCit^-$, and $CaHPO_4$, $MgHPO_4$ salts, respectively (Fig.15, arrows 2a and 3a). These salts at saturation in milk precipitate, probably in the casein micelle (Fig. 15, arrows 1a and 1c). The impoverishment in Cit^{3-} and HPO_4^{2-} is compensated by the dissociation of $HCit^{2-}$ and $H_2PO_4^-$ with the subsequent release of H^+ (Fig. 15, arrows 4a and 5a) that causes a decrease in pH (Le Ray et al., 1998; Philippe, Gaucheron, Le Graët, Michel, & Garem, 2003; van Hooydonk, Hagedoorn, et al., 1986). An increase in micellar mineral content (Ca or Mg, Pi and Cit) has been confirmed by several authors (Fig. 16, Table 6) (Philippe et al., 2003; Philippe, Le Graët, & Gaucheron, 2005; van Hooydonk, Hagedoorn, et al., 1986). Divalent cations are also able to replace one another in the casein micelles, leading to the solubilization of colloidal Mg upon the addition of $CaCl_2$ (Philippe et al., 2003) or the solubilization of colloidal Ca upon the addition of $MgCl_2$ (Le Ray et al., 1998) (Fig. 16, Table 6). Finally, the cations directly associate with negatively charged residues reducing the charge of the casein micelles (Lombardi et al., 2016; Philippe et al., 2003, 2005). These mineral modifications induce decreases in soluble casein concentrations and hydration of sedimentable caseins micelles (Le Ray et al., 1998; Philippe et al., 2003, 2005) (Fig. 16, Table 6). The τ of the suspensions increased while the casein micelles size was reported constant by Philippe et al., (2003, 2005). Lombardi et al., (2016) observed and increase in casein micelles size while van Hooydonk, Hagedoorn, et al., (1986) reported a decrease in their voluminosity (Table 6).

Table 6: Effect of $CaCl_2$ and $MgCl_2$ additions on the mineral balance and the colloidal properties of the casein micelles

Environmental modification	Properties impacted	variations	references
Addition of $CaCl_2$ or $MgCl_2$	pH	Decrease	Le Ray et al., (1998) Philippe et al. 2003 van Hooydonk et al., (1986)
	Colloidal minerals	Increase in Ca ($CaCl_2$) or in Mg ($MgCl_2$), Pi, Cit	Philippe et al., (2003) Philippe et al., 2005 van Hooydonk et al., (1986)
	Diffusible minerals	$CaCl_2$: increase in Ca, and in Mg (solubilization of micellar Mg) $MgCl_2$: increase in Mg and in Ca (solubilization of micellar Ca)	Philippe et al., (2003) Le Ray et al., (1998)
	Soluble proteins	Decrease	Le Ray et al., (1998) Philippe et al., (2003, 2005)
	Hydration	Decrease	Le Ray et al., (1998) Philippe et al., (2003, 2005)
	Turbidity	Increase	Philippe et al., (2003)
	Charge	Increase in zeta potential (decrease in charge)	Lombardi et al., (2016) Philippe et al., (2003, 2005)
	Size	Constant	Philippe et al., (2003, 2005)
		Increase	Lombardi et al., (2016)
		Decrease (voluminosity)	van Hooydonk et al., (1986)

2.3 Impact of environmental modifications on some functionalities of casein

One of the food science great principle is that the assembly behavior of food components in the nano-range determines the microstructure and therefore the food functional properties at macroscopic scale. Regarding specifically caseins, the way they assemble to form casein micelles or casein aggregates (CAs) depends on their interactions. According to the principle above mentioned, changes in the casein micelles assemblies induced by the environmental modifications also impact their functional properties.

One of the major functional properties of the micellar caseins is probably their ability to form gel following acidification and / or addition of rennet. Their ability to form and stabilize emulsions and foams is also of great interest. In this part, emphasis is placed between the environmental modifications and the behavior of the casein micelles to form emulsions and rennet gels.

2.3.1 Formation and stability of casein based emulsions

2.3.1.1 Generalities

Emulsions consist in two immiscible liquids, oil and aqueous phases, with one of the phase dispersed as droplets in the other. Figure 17A is a schematic representation of emulsion in which the oil phase is milkfat (typical dairy emulsion).

The mixture is thermodynamically unstable, i.e. the two phases separate with time (McClements, 2005). Regarding specifically dairy emulsions, instability is caused by gravity creaming, flocculation and droplet coalescence (Fig. 18). Therefore, emulsions are considered stable when no changes are detected in the size distributions, in the aggregation state or in the spatial arrangement of the droplets over the times-scale of observation (Dickinson, 2003). The stability time scale is subjective to users and may vary from hours to months.

Emulsions stable for longer times can be formed using emulsifying agents, such as small molecules, carbohydrates, di or tri glycerides or proteins. They facilitate the emulsions formation in decreasing interfacial tension: the lower the interfacial tension, the greater the extent to which droplets can be broken up during shearing. Emulsifiers such as proteins also stabilize the droplets in forming protectives layers at their surfaces (Fig. 17) that prevent or decrease flocculation or coalescence through steric and electrostatic stabilization, classically. Protein emulsifying agents are assessed according to their emulsifying capacity, i.e. their ability to

facilitate the blending of the immiscible phases and their stabilizing capacity, i.e. their ability to stabilize emulsions.

Milk proteins have been extensively studied for the preparation of food emulsion. Indeed, their amphiphilic nature, which allow them to adsorb and spread at the oil / water interface, made them good emulsifying agents (Dalgleish, 1996; Dickinson, 2001; Leman, Kinsella, & Kilara, 1989). Although whey proteins also possess good emulsifying and emulsion stabilizing properties, this review is limited to the study of casein based oil-in-water emulsion.

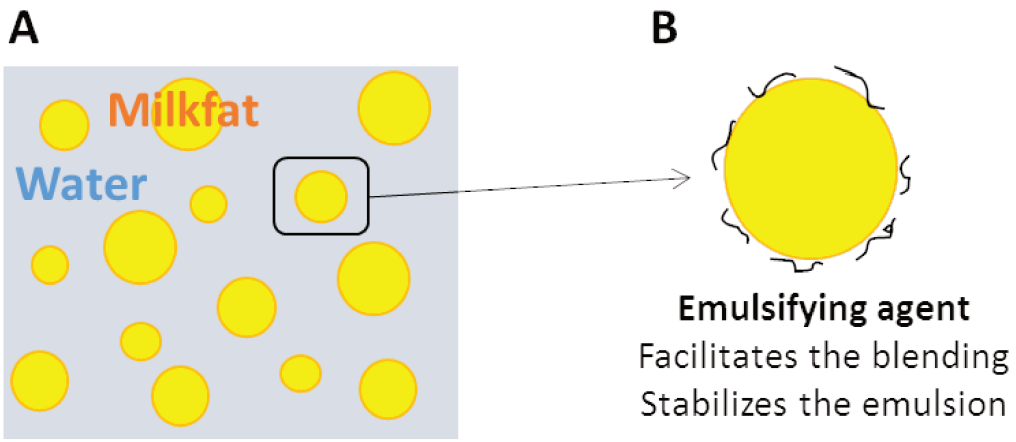

Figure 17. A. Schematic representation of a milkfat in water emulsion. B. Focus a droplet stabilized by emulsifying agents.

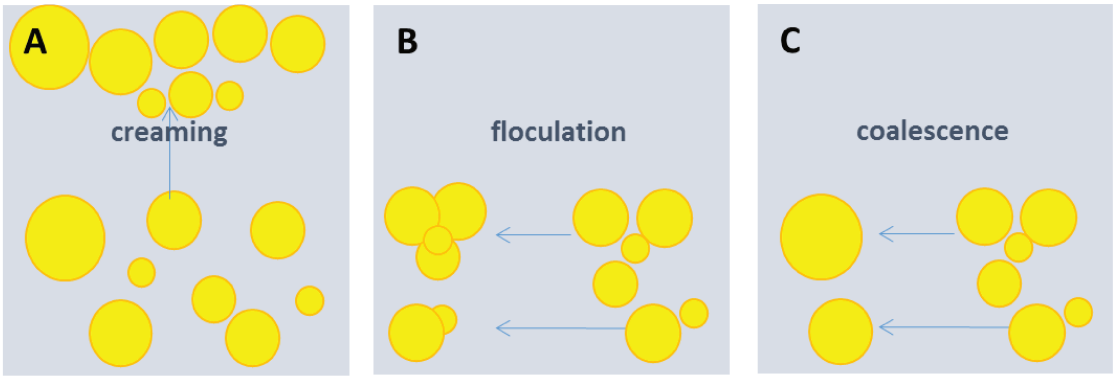

Figure 18. Schematic representation of the mechanisms responsible for the instability of milkfat-in-water emulsions. A. Gravity creaming, B. Floculation and C.Coalescence of the milkfat droplets.

2.3.1.2 Caseins, casein micelles and sodium caseinate

The relationships existing between environmental modifications of the casein micelles, their mineral balance, their colloidal and emulsion properties were not detailed in the literature. Caseins have been mainly studied for their emulsifying and emulsion-stabilizing properties in considering their aggregation state, i.e. individual form (molecules of α_{s1} and β and κ-caseins) or in more or less aggregated form (native casein micelle and caseinates).

Casein molecules combine the presence of hydrophobic and hydrophilic regions, the absence of secondary structure and relatively low MWs. These properties bring them the availability to spread rapidly and cover the oil-water interface. β-casein had higher surface activity and lower the interfacial tension more quickly than α_{s1} and κ-caseins. Pure α_{s1} and β-caseins produce emulsions of similar droplet-size distribution whereas κ-casein has poorer and less reproducible emulsifying capacities due to its tendency to aggregate via disulphide linkages (Dickinson, 1999). The surface charge density of the protein coated droplets lies in the order: α_{s1}- > β- > κ-caseins while the surface viscosities of the caseins at the oil / water interface lies in the following order β- < α_{s1}- < κ-caseins. Therefore, κ-casein had superior abilities in preventing coalescence and led to more stable emulsions (Dickinson, Murray, & Stainsby, 1988).

Regarding the aggregated states of caseins, native casein micelles also adsorb at oil/water interfaces during emulsification. However, much higher concentration of casein micelle are required to achieve similar droplet size comparing to casein molecules (Euston & Hirst, 1999). The emulsifying capacity of the caseins under micellar form is much less important than in individual forms. However, casein micelles form denser and thicker layers at the interface and enhance the emulsions stability (Dalgleish, 2006; Euston & Hirst, 1999).

Sodium caseinate (NaCas) is obtained in eliminating colloidal CaP from native casein micelle by acidic precipitation of the caseins at their isoelectric point. The decrease in pH lead to the solubilization of the micellar CaP and the disruption of the casein micelle. The casein precipitate is washed to remove the diffusible phase rich in CaP, and re suspended in reincreasing the pH to neutral values. NaCas consists of CAs of mainly 20 nm in diameter and small proportions of larger aggregates of 65 nm in diameter (Lucey, Srinivasan, Singh, & Munro, 2000; Pitkowski, Durand, & Nicolai, 2008). This ingredient is widely used as an emulsifier in food formulations (Mulvihill & Murphy, 1991). NaCas is more surface active and emulsify higher volumes of oil phase compared to casein micelles (Courthaudon et al., 1999; Mulvihill & Murphy, 1991). Its adsorption behavior is different from pure casein monomers as competitive adsorption between β and α_{s1}-caseins occurs in caseinate stabilized emulsions (Srinivasan, Singh, & Munro, 1999

and 1996). However, the aggregated state confers better emulsion-stabilizing capacities compared to casein monomers, although less important than highly aggregated casein micelles (Mulvihill & Murphy, 1991).

2.3.2 Formation of rennet gels from casein micelles

2.3.2.1 Generalities

One of the basic steps common to all cheese manufacture is coagulation, i.e. the separation of liquid whey and gelled curd from milk by destabilization of the protein fraction. Coagulation is made possible by the use of a complex of enzymes, rennet, which is naturally produced in the stomach of ruminant mammals. Rennet consists mainly of a mixture of chymosin, that is the main active ingredient, and pepsin, that both belong to the groups of aspartic proteinase (Beermann & Hartung, 2012; Moschopoulou, 2011). The proportions of enzymes are not standardized since they depend on several factors such as the kind, the age, and the feeding regimes of the animal from which the rennet is extracted. However, chymosin is the main active ingredient and specifically hydrolyze κ-casein at the Phe(105)-Met(106) bond, while pepsin is non-specific and hydrolyzes all peptide bonds involving aromatic amino acids (Phe, Tyr and Trp) (Moschopoulou, 2011). Pure chymosin can also be produced by genetically modified microorganisms.

Rennet coagulation consists of three overlapping stages (Fig. 19). The primary stage corresponds to the cleavage of κ-caseins at the micellar surfaces (specific hydrolysis at the Phe(105)-Met(106) bond) and the subsequent release of the caseinomacropeptides (CMP) from the paracaseinates (Dalgleish, 1993; de Kruif, 1992) (Fig. 19, A). This hydrolysis step causes the destabilization of the casein micelles in reducing their net negative charge. In milk conditions, about 90 % of hydrolysis need to be achieved before aggregation of paracaseinates occurs (Lucey, 2002; Zoon, van Vliet, & Walstra, 1988), which corresponds to the second stage (Fig. 19, B). Finally, the formation of the casein gel network and its reorganization occurs, which constitutes the third and last coagulation stage (Fig. 19, B). The time elapsed from the addition of rennet to the detectable onset of gelation is defined as rennet clotting time (RCT) while the strength of the gel can be characterized by its firmness. The RCT mainly depends on the rate of the enzymatic reaction and the aggregation of the paracaseinates (first and second steps), while the firmness depends on the organization and the strength of the gel (third step).

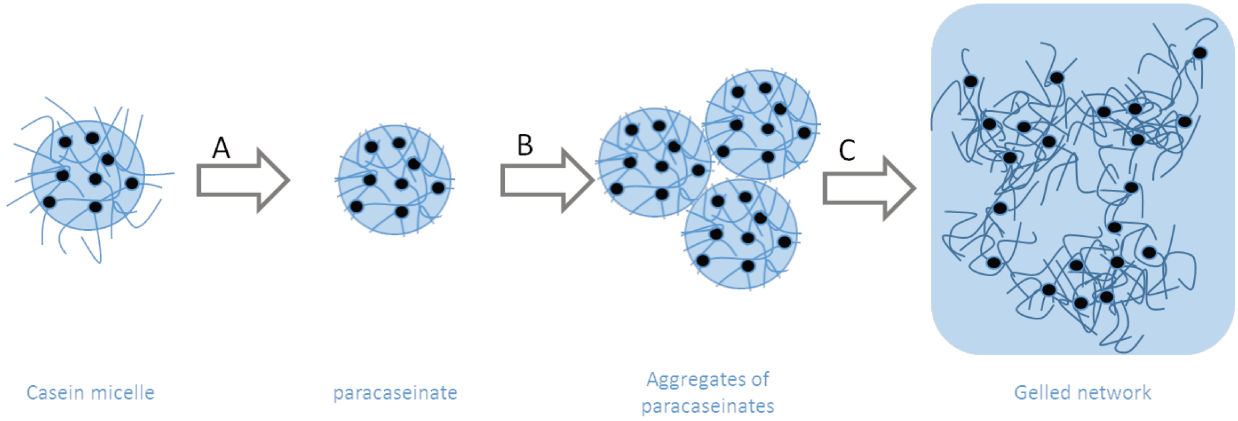

Figure 19. Schematic representation of the mechanism responsible for the rennet coagulation of casein micelles. A. hydrolysis of CMPs, B. aggregation of paracaseinates and C. gel formation and reorganization.

2.3.2.2 Some consequences of the modification of the mineral fraction on rennet gels properties

The impact of environmental modifications of the casein micelle on its rennet coagulation properties have been extensively studied since several decades to understand and control this biochemical event in cheese making. The studied systems (e.g. fresh milk, reconstituted powder or concentrated liquids, suspension of casein micelles in different media), the way the environmental modifications were applied (e.g. type and concentration of salts or chelating agents, pH correction) and the methods of gel characterization (e.g. manual, rheological and optical methods) varied a lot so that the results obtained by the different authors are sometimes difficult to compare or are even controversial. The following part only reports the principal tendencies described in the literature. However, a higher level of details concerning the impact of the environmental modifications is considered in Part B, chapters 1 and 2, of this manuscript throughout the discussion of the P.h. D. project results. The following part is limited to the effects of variations in pH, additions of Na_3Cit, NaCl, $CaCl_2$ and $MgCl_2$ on the rennet coagulation properties of the casein micelles.

Regarding variations in pH, acidification favors the proteolysis reaction (the pH optimum for chymosin activity is between 5.1 and 5.5) and reduces the rate of hydrolysis required to observe casein aggregation. The RCT is therefore reduced (Choi, Horne, & Lucey, 2007; Daviau, Famelart, Pierre, Goudédranche, & Maubois, 2000; Famelart, Lepesant, Gaucheron, Le Graët, &

Schuck, 1996). A reduction in pH has a quadratic effect on the gel firmness (Choi et al., 2007; Daviau et al., 2000; Zoon, van Vliet, & Walstra, 1989). Neutralization and alkalinization above milk pH drastically reduced the enzyme activity and lead to the complete inhibition of coagulation. Casein micelles are also strongly modified (see section 2.2.1). The additions of $CaCl_2$ and $MgCl_2$ to milk or casein micelles have similar impact on the rennet coagulation properties of the casein micelles. They cause a decrease in RCT and are responsible for the formation of stronger gels (Cooke & McSweeney, 2014; McMahon, Brown, Richardson, & Ernstrom, 1984; Sandra, Ho, Alexander, & Corredig, 2012; Udabage, McKinnon, & Augustin, 2001; van Hooydonk, Hagedoorn, & Boerrigter, 1986; Zoon, van Vliet, & Walstra, 1988). The divalent cations screen the negative charges at the micellar surface and cause a collapse of the κ-casein layer, therefore less accessible for the enzyme inducing a reduction of the enzymatic reaction rate. However, the screening effect also lead to the reduction of the electrostatic repulsions which favors the aggregation of the paracaseinates (Dalgleish, 1983). On the reverse, NaCl addition increases RCT and decreases gel firmness (Bulca, Wolfschoon-Pombo, & Kulozik, 2016; Famelart, Le Graët, & Raulot, 1999; Grufferty & Fox, 1985; Karlsson, Ipsen, & Ardö, 2007; Sbodio, Tercero, Coutaz, & Revelli, 2006; Zhao & Corredig, 2015; Zoon et al., 1989). The reduction in RCT was attributed to the reduction of the enzymatic reaction (van Hooydonk, Hagedoorn, et al., 1986). Regarding the addition of Na_3Cit, Udabage, McKinnon, & Augustin, (2001) observed a complete inhibition of the coagulation of milk.

Objectives and strategies

OBJECTIVES AND STRATEGIES

1 Generalities

Substantial work has been carried out during the past 50 years to understand the complexity of casein micelles, in terms of composition (caseins, minerals and water), internal organization and structure, colloidal and functional properties. The essential elements to remember from the literature review are the estimable knowledge revealed about the plasticity of casein micelles, in spite of remaining uncertainties on their internal organization. Only few environmental modifications were described in the previous chapter but it clearly established the links existing between their individual variations, the shifts in the mineral balance and their impact on the colloidal properties of casein micelles.

Regarding the functional properties, the impact of environmental modifications was not properly studied in regards to the formation and stability of casein-based emulsions. However, the caseins aggregation state largely influences these functionalities. Early research performed on rennet coagulation already demonstrated that casein micelles affected by different environmental changes, in an individual manner, resulted in the formation of gels with various properties. It confirms the existence of relationships between the environment, the mineral balance, the colloidal and the functional properties of casein micelles. and paves the way for further investigations or for the understanding of other casein micelles functionalities.

2 Scientific objectives

The present Ph. D. project enter into this path with the general objective to establish and understand the relationships that exist between the environmental modifications, the mineral balance of casein micelles, their colloidal and functional properties (emulsion and rennet coagulation functionalities).

More precisely, this objective divides in four research questions (Fig. 1):
I – What are the consequences of environmental modifications of the casein micelles on their mineral balance?
II – How do these modifications influence the colloidal properties of casein micelles?
III – What effects do these modifications have on the functionalities of casein micelles?
IV – Which relationships link the mineral balance, the colloidal and the functional properties of casein micelles?

3 Strategies

Figure 2 shows the five global steps of the experimental strategy applied in order to answer these research questions:
1 – Casein micelle and environmental modifications: different suspensions of casein micelles were prepared and modified according to one or several environmental factors.
2 – Mineral balance: mineral concentrations and distributions in diffusible and colloidal phases of these suspensions were determined to answer question I.
3 – Colloidal properties: the modified casein micelles suspensions were characterized in terms of colloidal properties (e.g. size, soluble proteins, zeta potential, internal structure). This step brought answers to question II.
4 – Functional properties: the modified casein micelles suspensions were used in model dairy systems to assess their emulsion and rennet coagulation functionalities and answer question III.
5 – Results interlinking. Depending on the functionality studied, the direct comparison between results, their discussions and / or the use of statistical tools (experimental designs, principal component analysis (PCA), modelling) enabled to answer question IV.

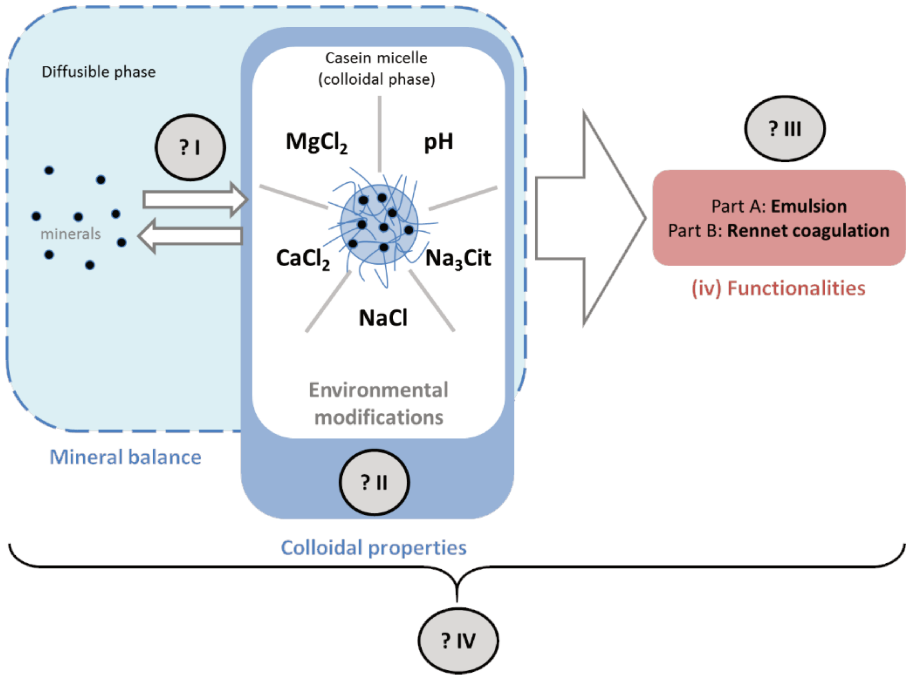

Figure 1: Project objective and research questions. What are the possible links between the environmental modifications and the properties of the casein micelles?

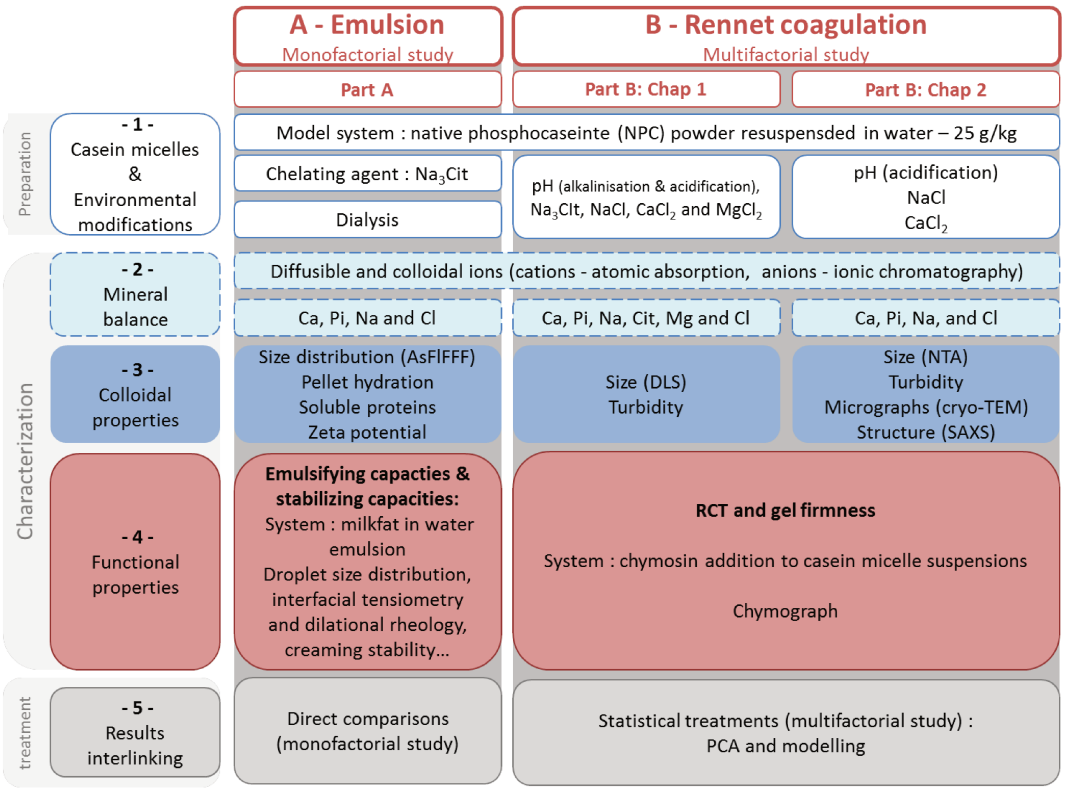

Figure 2: Experimental strategies. Similarities and adaptation of the strategic approaches in regards to the functionalities studied.

3.1 Raw material

Casein micelles are the centerpieces of the present Ph.D. project and their mineral content was the direct target of the environmental modifications. Therefore, the focus was place on two families of milk components: the caseins and the minerals.

The choice was made to work with a model system of native phosphocaseinate (NPC; purified casein micelles) powder suspended in water at a casein concentration of 25 g kg^{-1}

. This system presented the advantages of:

- reducing the complexity of milk in picking only the components of interest. Other major milk components such as milkfat, lactose and whey proteins were not included in the present study.

- standardizing the raw material necessary for the study. The same batch of NPC was used throughout the entire project.

3.2 Functionalities coupled to environmental factors

Caseins are responsible for numerous functional properties in food products, such as gelation (rennet or acid coagulation), interfacial (emulsion and foams), texturization, thermal stabilization or organoleptic (flavor, opacity) properties, just to mention a few. The present project was restricted to the study of emulsion and rennet coagulation functionalities after discussion with the French dairy industrials (CNIEL) participating in this project. Furthermore, the behaviors of casein micelles are different regarding the formation mechanism of emulsions and rennet gels. The choice of these two functionalities brings diversity to the present study. Each of these functionalities was considered according to a specific approach while keeping as much as similarities as possible (Fig. 2).

3.2.1 Emulsion functionality

3.2.1.1 A monofactorial approach

As mentioned in the literature review section, the effects of environmental modifications on the emulsion properties of the casein micelles were never reported as such, in the literature. However, it is admitted that the state of aggregation of the caseins have a significant influence on these properties, but only extreme aggregation states were studied (caseins, caseinates and native casein micelles).

Therefore, the choice was made to modify the casein micelles environment in performing a single factor study (Fig. 2A). A Ca chelating agent was added at four concentrations to induce gradual disaggregation of casein micelles leading to the preparation of four different types of casein suspensions. Among numerous chelating agents possible, Na_3Cit was chosen due to the facts that Cit^{3-} ions are of crucial importance in the mineral balance and that Cit^{3-} and Na^+ are already parts of the natural composition of milk. Furthermore, Na_3Cit is authorized as an additive in many food and dairy products by the CODEX Alimentarius. Another important element of the experimental strategy was a dialysis step of the suspensions against an identical aqueous solution saturated in Ca and Pi, at pH 7.0. The aim of this step was to control the diffusible phase of the suspension in evacuating the added Na and Cit ions so that the modified casein micelles were compared in exact same ionic environments.

The differently modified casein micelles were then used as emulsifying and stabilizing agent in the formation of model milkfat-in-water (30:70 v / v) emulsions. The different casein micelle suspensions and their corresponding casein-based emulsions were characterized in terms of mineral balance, colloidal and emulsion properties.

3.2.1.2 Collaboration strategy

Some colloidal characterizations of the modified casein micelles were performed in collaboration with F Violleau, from the Engineering School PURPAN, Toulouse (Fig. 3). Asymmetrical flow field flow fractionation (AsFlFFF) technique is more resolutive than widely used DLS techniques in regards to the size-measurement of polydispersed particles in suspension. Indeed, prior analysis, the suspension components are separated according to their size and density. The use of AsFlFFF permitted to obtain the size distributions profiles and MW of the modified casein micelles and other smaller aggregates produced by their disaggregation.

A second collaboration was established with A. Saint Jalmes, from the Institute of Physics of Rennes (IPR) (Fig. 3). Interfacial tensiometry and dilational rheology analyses were performed in order to assess the properties of the modified casein micelles at the milkfat / water interface. Such results were essential to understand the behaviors of casein micelles and aggregates in the formation and stability of emulsions.

3.2.1.3 Approach originalities

The main originality of the "emulsion functionality" study resides in the gradual modification of the casein micelles by mean of environmental changes. Considering the research questions mentioned before, the effects of the addition of Na_3Cit on the mineral balance (question I) and the colloidal properties (question II) of the casein micelles were already well described in the literature. Therefore, this part of the project brings original knowledge in response to questions III and IV. The results are presented and discussed in Part A of the present manuscript.

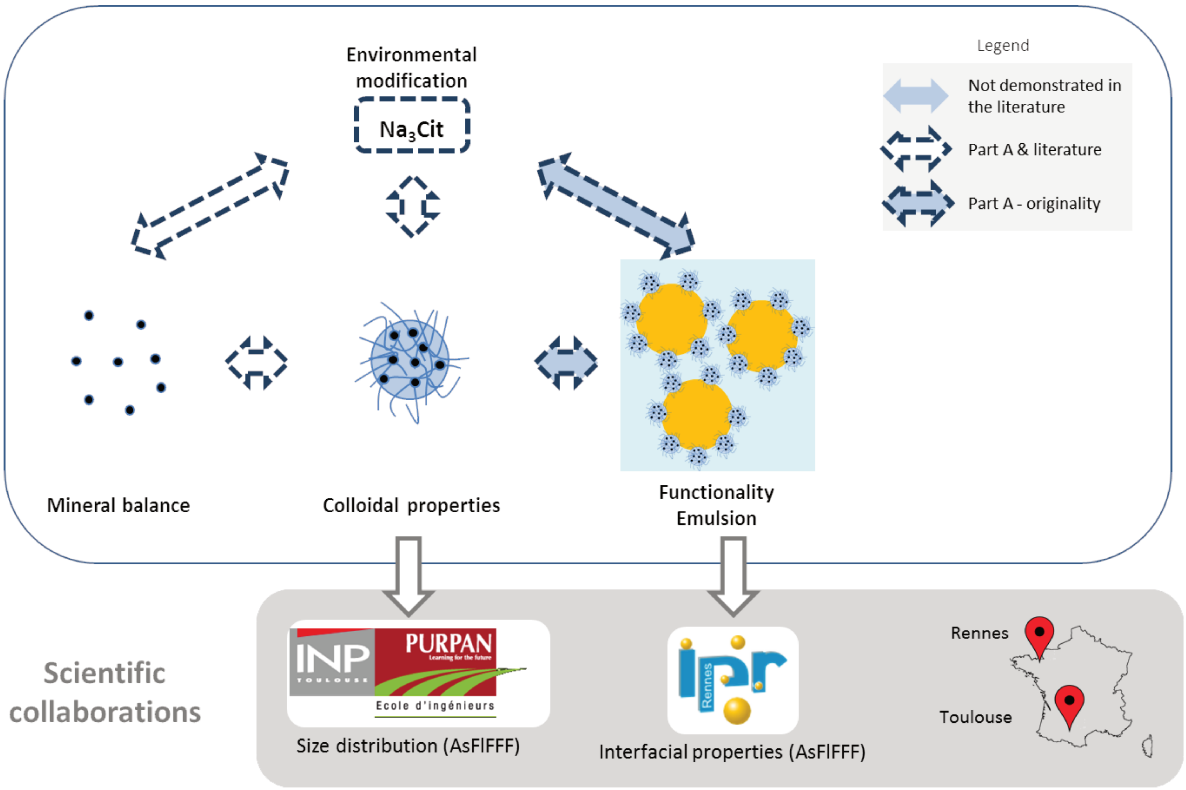

Figure 3: Contributions of Part A and scientific collaborations. Double arrows symbolize the relationships between the environmental modifications and / or the different casein micelle properties.

3.2.2 Rennet coagulation functionality

3.2.2.1 A multifactorial approach

Unlike the studies concerning the understanding of the emulsion functionalitiy, the individual influences of environmental modifications on casein micelles and rennet coagulation properties were extensively studied. However, cheese manufacture often implies simultaneous modifications of several environmental factors (e.g. pH decrease coupled to Ca addition) with the result that fundamental single factor studies are often far from real practices. The choice was made to perform a multifactorial study, i.e. to assess the consequences of simultaneous environmental modifications on the casein micelles properties. The environmental factors were selected according to three principles:

- their individual impacts on the rennet coagulation properties were already described in the literature, even partially;

- all chemicals added to reach the expected modifications were already part of the milk mineral fraction;

- all chemicals added (HCl, NaOH, Na_3Cit, NaCl, $CaCl_2$ and $MgCl_2$) were authorized as food additives by the CODEX Alimentarius.

The modified casein micelle suspensions were then used to form gels under the action of pure chymosin.

The complexity of such multifactorial approach was manage in proceeding in two steps (Fig. 2B):

Part B, chapter 1 - A first study was performed in order to model the conditions under which modified casein micelle suspensions coagulate following chymosin addition. Five factors - variations in pH (acidification and alkalinization), additions of Na_3Cit (chelating agent), NaCl (monovalent ions), $CaCl_2$ and $MgCl_2$ (two different divalent ions) – were considered through the definition of a fractional experimental design. Attention was paid on the relationships existing between the multifactorial modifications of the casein micelles environment, the mineral balance and the rennet coagulation properties (Fig. 4).

Part B, chapter 2 - An in-depth study focused on the conditions under which coagulation happened. The experimental approach was restricted to three factors – variations in pH (acidification only), additions of NaCl and $CaCl_2$ – and a full experimental design was carried out. This study brought knowledge on the relationships existing between the mineral balance, the

colloidal properties of the modified casein micelles and the properties of the rennet gels. Emphasis was put on the consequences of structural modifications determine by advance biophysical techniques (SAXS and cryo-TEM).

In both studies, statistical tools were used to define the experimental designs, to established correlations between the results obtained and to produce predictive models.

3.2.2.2 Collaboration strategy

Some colloidal characterizations were performed in the framework of collaborations with two Australian institutions, during a three-month stay:

- cryo-TEM observations were performed in collaboration with E. Hanssen, L. Ong and S. Gras from the University of Melbourne (Fig. 4). The micrographs obtained and their treatment enabled to bring substantial knowledge on the size and the structure of the modified casein micelles in suspension and the presence of aggregates released from casein micelles.

- Nanoparticle tracking (NTA) and SAXS analyses were carried out with the help of J Raynes, R. Williams and A. Logan from the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Werribee (Fig. 4). These analyses enabled to determine the size and structure of the modified casein micelles, respectively.

A third collaboration was established with A. Bouchoux from the INRA, Toulouse, regarding the modeling and interpretations of the SAXS scattering patterns.

V. Lechavalier and S. Pezenec from the UMR STLO, INRA, Rennes were of great help in regards to the establishement of the experimental designs and the statistical treatments of the data.

The use of a ChymoGRAPH® made possible the characterization of the rennet coagulation properties of the casein micelles in suspension in determining the RCT and the gel firmness. The company Chr Hansen provided this apparatus for the duration of the entire P.h. D. project.

3.2.2.3 Approach originalities

The main originality resides in the multifactorial approach implemented in this part of the Ph. D. project. Although the individual effects of the selected environmental factors were already described in the literature, no knowledge was available regarding their simultaneous effects that

can be opposite (e.g. $CaCl_2$ addition and acidification). This part of the project brings original knowledge in response to each of the four questions mentioned in the scientific objectives section of the present chapter: Part B - chapter 1 reports the results of the five-factor approach and specifically answer questions I and III, while Part B - chapter 2 (three-factor approach, in-depth study) answers questions II and IV.

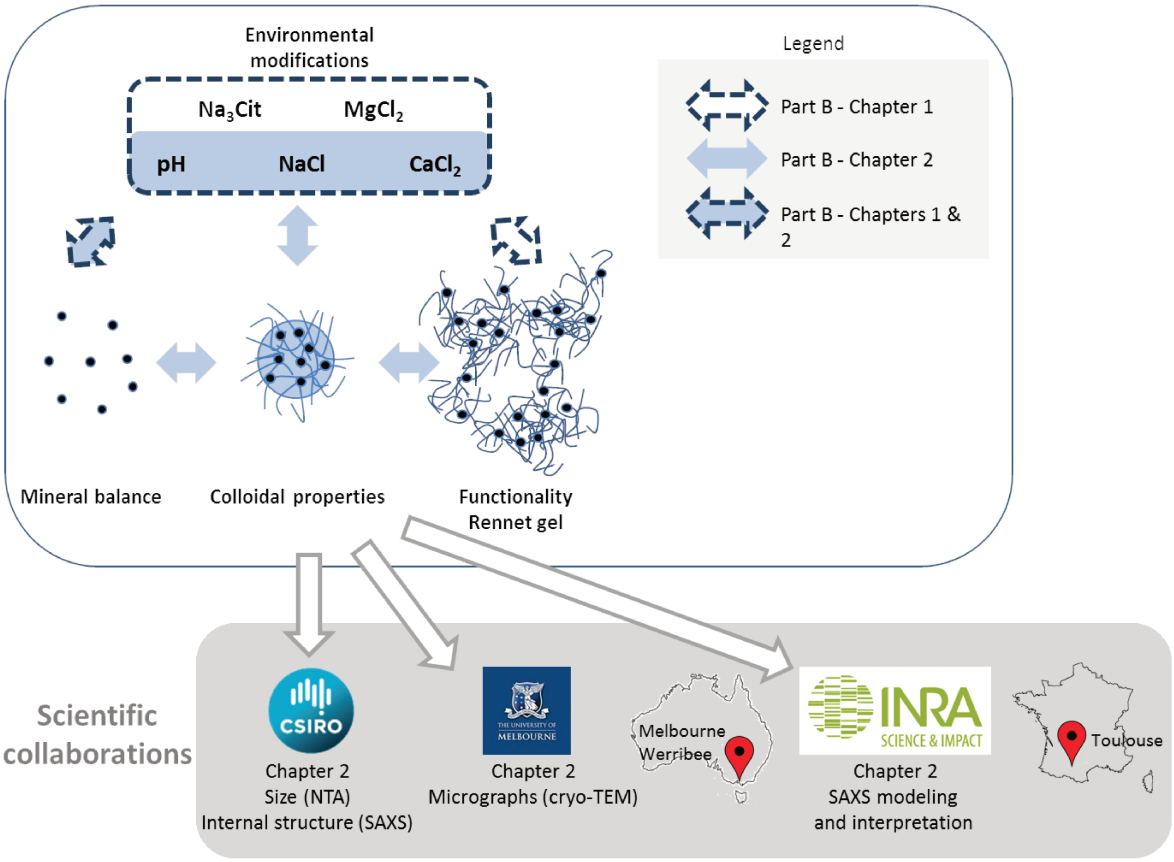

Figure 4: Part B - Contributions of each chapter and scientific collaborations. Double arrows symbolize the relationships between the environmental modifications and / or the different casein micelles properties.

OBJECTIFS ET STRATEGIES

1 Généralités

Un travail considérable a été réalisé au cours des 50 dernières années afin de comprendre la complexité des micelles de caséine, en termes de composition (caséines, minéraux et eau), d'organisation interne et de structure, de propriétés colloïdales et fonctionnelles. Les éléments essentiels à retenir de la revue de la littérature concernent les connaissances révélées à propos de la plasticité des micelles de caséines, en dépit des incertitudes demeurant au sujet de leur organisation interne. Seules quelques modifications environnementales ont été décrites dans la section précédente, mais ces descriptions établissent clairement les liens existants entre leurs variations individuelles, les déplacements d'équilibre minéral et les impacts sur les propriétés colloïdales des micelles de caséines.

En ce qui concerne les propriétés fonctionnelles, l'impact des modifications environnementales n'a pas été clairement étudié par rapport à la formation et la stabilité des émulsions laitières produites à partir de caséines. Cependant, l'état d'agrégation des caséines influence largement ces fonctionnalités. Des recherches réalisées sur la coagulation présure ont démontré que les micelles de caséines affectées par différents changements environnementaux, de façon individuel, conduisent à la formation de gels possédants des propriétés variées. Ces études confirment l'existence de relations entre l'environnement, l'équilibre minéral, les propriétés colloïdales et fonctionnelles des micelles de caséines. Elles ouvrent la voie à d'avantage d'investigations ainsi qu'à une meilleure compréhension des fonctionnalités micellaires.

2 Objectifs scientifiques

Ce projet de thèse emprunte cette voie avec l'objectif général d'établir et de comprendre les relations existantes entre les modifications environnementales, l'équilibre minéral des micelles de caséines, leurs propriétés colloïdales et fonctionnelles (fonctionnalités émulsion et coagulation présure).

Plus précisément, cet objectif se divise en quatre questions de recherche (Fig. 1) :

I – Quelles sont les conséquences des modifications environnementales des micelles de caséine sur leur équilibre minéral ?

II – Comment ces modifications influencent-elles les propriétés colloïdales des micelles de caséine ?

III – Quelles sont les effets de ces modifications sur les fonctionnalités des micelles de caséine ?

IV – Quelles relations lient l'équilibre minéral, les propriétés colloïdales et fonctionnelles des micelles de caséine ?

3 Stratégies

La Figure 2 montre les 5 étapes globales de la stratégie expérimentale mise en place dans le but de répondre à ces questions de recherche :

1 – Micelles de caséine et modifications environnementales : différentes suspensions de micelles de caséine ont été préparées et modifiées selon un ou plusieurs facteurs environnementaux.

2 – Equilibre minéral : les concentrations des minéraux et leurs distributions entre les phases colloïdales et diffusibles de ces suspensions ont été déterminées pour répondre à la question I.

3 – Propriétés colloïdales : les suspensions de micelles de caséine modifiées ont été caractérisées en termes de propriétés colloïdales (ex. taille, protéines solubles, potentiel zêta, structure interne). Cette étape apporte des réponses à la question II.

4 – Propriétés fonctionnelles : les suspensions de micelles de caséine modifiées ont été utilisées dans des systèmes laitiers modèles afin d'évaluer leurs fonctionnalités émulsion et coagulation présure et de répondre à la question III.

5 – Interconnexion des résultats : selon la fonctionnalité étudiée, les comparaisons directes entre les résultats, leurs discussions et / ou l'utilisation d'outils statistiques (plans d'expérience, analyse en composante principale (PCA), modélisation) ont permis de répondre à la question IV.

3.1 Matières premières

Les micelles de caséine se trouvent au cœur de ce projet de thèse et les modifications environnementales ciblent directement leur contenu minéral. De ce fait, cette étude s'est focalisée sur deux familles de composants principaux du lait : les caséines et les minéraux.

Le choix a été fait de travailler avec un système modèle de poudre de phosphocaséinate natif (NPC, micelles de caséine purifiées) resuspendue dans l'eau à une concentration de 25 g kg^{-1}.

Ce système présente plusieurs avantages :

- il diminue la complexité du lait en conservant uniquement les composés d'intérêts pour cette étude. D'autres composés laitiers majeurs, comme la matière grasse, le lactose ou les protéines sériques, ne font pas partie de cette étude.

- il permet de standardiser la matière première nécessaire à cette étude. Le même lot de NPC a été utilisé pour l'ensemble du projet.

3.2 Fonctionnalités couplées aux facteurs environnementaux

Les caséines sont responsables de nombreuses propriétés fonctionnelles dans les produits alimentaires, tel que la gélification (par présure ou acide), des propriétés interfaciales (émulsions et mousses), de texture, de stabilisation thermique ou organoleptiques (saveur, opacité). Ce projet de these a été restreint à l'étude des fonctionnalités émulsions et coagulation présure suite à la concertation des industriels Français participants à ce projet (CNIEL). De plus, les comportements des caséines sont de différentes natures dans les mécanismes de formation des émulsions et des gels présure. Le choix de ces deux fonctionnalités apporte plus de diversité à cette étude. Chacune de ces fonctionnalités a été considérée selon une approche spécifique tout en conservant le plus de similarités possible (Fig. 2).

3.2.1 Fonctionnalité émulsion

3.2.1.1 Une approche mono factorielle

Comme mentionnés dans la section « revue de la littérature », les effets des modifications environnementales sur les propriétés émulsifiantes n'ont jamais été reportés en tant que tel dans la littérature. Cependant, il est admis que l'état d'agrégation des caséines a une influence significative sur ces propriétés, mais seuls les états d'agrégation extrêmes (caséines, caséinates, et micelles de caséine natives) ont été étudiés.

Le choix à donc été fait de modifier l'environnement des micelles de caséines par une étude mono factorielle (Fig. 2A). Un agent chelatant du Ca a été ajouté à quatre niveaux de concentration différents pour provoquer la désagrégation graduelle des micelles de caséine afin de préparer quatre différents types de suspensions. Parmi de nombreux agents chélatants possible, Na_3Cit a été choisi étant donné que les ions Cit^{3-} sont d'une importance majeure dans l'équilibre minéral des micelles de caséines et que Cit^{3-} et Na^+ font partie de la composition ionique naturelle du lait. De plus, Na_3Cit est un additif autorisé dans de nombreux produits alimentaires par le CODEX Alimentarius. Un autre élément important de cette stratégie expérimentale est l'étape de dialyse des suspensions contre une solution aqueuse saturée en Ca et Pi, à pH 7.0. Le but de cette dialyse a été de contrôler la phase diffusible des suspensions en évacuant les ions Na^+ et Cit^{3-} ajoutés de façon à ce que les micelles de caséines modifiées soient comparées dans des environnements ioniques identiques.

Par la suite, les différentes suspensions de micelles de caséine modifiées ont été utilisées en tant qu'agents émulsifiants et stabilisants pour la formation d'émulsions modèles matière-grasse-laitière-dans-eau (30:70 v / v). Les différentes suspensions de micelles de caséine et leurs émulsions correspondantes ont été caractérisées en termes d'équilibre minéral, de propriétés colloïdales et de fonctionnalités émulsion.

3.2.1.2 Stratégie de collaboration

Certaine caractérisation colloïdale des micelles de caséine modifiées ont été réalisées en collaboration avec F. Violleau, de l'école d'ingénieur PURPAN, Toulouse (Fig. 3). La technique de fractionnement par couplage flux force asymétrique (AsFlFFF) est plus résolutive, en ce qui concerne la mesure de taille de particules polydispersées en suspension, que les techniques de DLS plus largement utilisées. En effet, avant analyse, les composés en suspensions sont

séparés selon leur taille et leur densité. L'utilisation de l'AsFIFFF a permis d'obtenir des profils de distribution de taille et de poids moléculaire des micelles de caséine modifiées et ainsi que des plus petits agrégats produits par leur désagrégation.

Une seconde collaboration a été mise en place avec A. Saint Jalmes, de l'Institut de Physique de Rennes (IPR) (Fig. 3). Des analyses de tensiométrie interfaciale et de rhéologie dilationnelle ont été effectuées dans le but d'évaluer les propriétés des micelles de caséines modifiées à l'interface matière grasse / eau. De tels résultats ont été essentiels pour comprendre les comportements des micelles de caséine et des agrégats dans la formation et la stabilité des émulsions.

3.2.1.3 Originalités de cette approche

L'originalité principale de l'étude de la fonctionnalité émulsion vient de la modification graduelle des micelles de caséine par des changements environnementaux. En considérant les questions de recherché énoncées précédemment, les effets de l'addition de Na_3Cit sur l'équilibre minéral (question I), et les propriétés colloïdales (question II), des micelles de caséine étaient déjà décrits dans la littérature. En revanche, Cette partie du projet de thèse apporte des connaissances originales en réponse aux questions III et IV. Les résultats sont présentés et discutés dans la partie A de ce manuscrit.

3.2.2 Fonctionnalité coagulation présure

3.2.2.1 Une approche multifactorielle

Contrairement aux études concernant la compréhension de la fonctionnalité émulsion, les influences individuelles des modifications environnementales sur les micelles de caséines et leur propriétés de coagulation présure ont été largement étudiées. Cependant, la fabrication de fromage implique des modifications simultanées de plusieurs facteurs environnementaux (ex : une diminution de pH couplée à l'addition de Ca) avec pour conséquence le fait que les études scientifiques mono factorielles sont souvent éloignées des pratiques réelles. Le choix à donc été fait de réaliser une étude multifactorielle, c'est-à-dire d'évaluer les conséquences des modifications simultanées de plusieurs facteurs environnementaux sur les propriétés des micelles de caséines. Les facteurs environnementaux ont été sélectionnés selon trois principes :

- Leurs impacts individuels sur les propriétés de coagulation présure étaient déjà décrits dans la littérature, même partiellement.

- Tous les produits chimiques ajoutés pour atteindre les modifications attendues faisaient déjà parties de la fraction minérale du lait.

- Tous les produits chimiques ajoutés (HCl, NaOH, Na_3Cit, NaCl, $CaCl_2$ et $MgCl_2$) sont autorisés en tant qu'additifs alimentaire par le CODEX Alimentarius.

Les suspensions de micelles de caséine modifiées ont ensuite été utilisées pour former des gels sous l'action de chymosine pure.

La complexité d'une telle approche multifactorielle a été gérée en procédant en deux étapes (Fig. 2B) :

- Partie B, chapitre 1 – Une première étude a été réalisée dans le but de modéliser les conditions dans lesquelles les suspensions de micelle de caséines modifiées ont coagulé suite à l'addition de chymosine. Cinq facteurs – variation de pH (acidification et alcalinisation), addition de Na_3Cit (agent chelatant), NaCl (ions monovalents), $CaCl_2$ et $MgCl_2$ (deux ions divalent différents) – ont été considérés au travers d'un plan d'expérience fractionnaire. Une attention particulière a été portée sur les relations existant entre les modifications multifactorielles de l'environnement des micelles de caséines, leur équilibre minéral et leurs propriétés de coagulation présure (Fig. 4).

- Partie B, chapitre 2 – Une étude approfondie, focalisée sur les conditions dans lesquelles la coagulation a eu lieu. L'approche expérimentale a été restreinte à l'étude de 3 facteurs – variation de pH (acidification seule), addition de NaCl et $CaCl_2$ – et un plan d'expérience complet a été réalisé. Cette étude a apporté des connaissances sur les relations existant entre l'équilibre minéral, les propriétés colloïdales des micelles de caséines et les propriétés des gels présure. L'accent a été porté sur les conséquences des modifications structurales des micelles de caséine déterminées par des techniques biophysiques avancées (SAXS et cryo-TEM).

Des outils statistiques ont été utilisés dans le cadre de ces deux études afin de définir les plans d'expérience, d'établir les corrélations entre les résultats obtenus et pour produire des modèles prédictifs.

3.2.2.2 Stratégie de collaboration

Certaine caractérisations colloïdales on été réalisées dans le cadre d'une collaboration avec deux institutions Australiennes, au cours d'un séjour de 3 mois :

- Des observations par cryo-TEM ont été réalisées en collaboration avec E. Hanssen, L. Ong et S. Gras de l'Université de Melbourne (Fig. 4). Les micrographes obtenus et leurs traitements ont permis d'apporter des connaissances conséquentes sur la taille et la structure des micelles de caséine modifiées en suspension et sur la présence d'agrégats libérés par ces micelles de caséine.

- Des analyses du suivit individuel de particules (NTA) et SAXS ont été réalisées avec l'aide de J. Raynes, R. Williams et A. Logan du Commonwealth Scientific and Industrial Research Organization (CSIRO), Werribee (Fig. 4). Ces analyses ont permis de déterminer la taille et la structure des micelles de caséines modifiées, respectivement.

Une troisième collaboration a été établie avec A. Bouchoux de l'INRA, Toulouse, concernant la modélisation et l'interprétation des données SAXS.

V. Lechevalier et S. Pezenec de l'UMR STLO, INRA, Rennes, ont été d'une grande aide en ce qui concerne l'établissement des plans expérimentaux et les traitements statistiques des données.

L'utilisation d'un ChymoGRAPH® a rendu possible la caractérisation des propriétés de coagulation présure des micelles de caséine en suspension en déterminant le RCT et la fermeté des gels. L'entreprise Chr Hansen à mis à disposition cet appareil pour la durée du projet de thèse.

3.2.2.3 Originalités de l'approche

L'originalité principale vient de l'approche multifactorielle utilisée dans cette partie du projet de thèse. Bien que les effets individuels des facteurs environnementaux sélectionnés soit déjà décrit dans la littérature, aucune connaissance n'était disponible en ce qui concerne leurs effets simultanés qui peuvent être opposés (ex : ajout de $CaCl_2$ et acidification). Cette partie du projet apporte des connaissances originales en réponse à chacune des quatre questions de recherche mentionnées dans la section « objectifs scientifiques » de ce chapitre. Le premier chapitre de la partie B fait état des résultats concernant l'approche cinq-facteurs et répond spécifiquement aux questions I et III, tandis que le second chapitre de cette même partie du manuscrit (approche trois-facteurs, étude approfondie) répond aux questions II et IV.

Part A:

Emulsion functionality

PART A:

GRADUAL DISAGGREGATION OF THE CASEIN MICELLE IMPROVES ITS EMULSIFYING CAPACITY AND DECREASES THE STABILITY OF DAIRY EMULSIONS

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Abstract

The casein micelle is a highly aggregated colloid consisting of phosphoproteins and minerals, in particular calcium and phosphate. Its properties are affected by physico-chemical changes which provide possibilities for the development of new casein aggregates (CAs) with novel functionalities. The aim of this study was to investigate the emulsifying and emulsion-stabilizing capacity of gradually demineralized CAs in model dairy emulsions. Tri sodium citrate was used to remove calcium and inorganic phosphate from pure casein micelles in order to produce four suspensions of differently demineralized CAs. Two types of milkfat-in-suspension (30:70 v / v) emulsions were then prepared to study the emulsifying and emulsion-stabilizing capacity of these CAs separately. Casein micelles were progressively demineralized (from 24 to 81

calcium reduction) and dissociated with the increase in Na_3Cit concentration. Three distinct populations of particles (micelle-like aggregates, sodium caseinate-like aggregates and casein monomers) were present in every suspension in different proportions. The smaller CAs had better emulsifying capacity and similar surface activity according to interfacial studies. The state of aggregation of the CAs was thus the main factor that controlled their emulsifying capacity. However, the emulsions formed with these smaller aggregates were less stable against creaming and flocculation, but still resisted coalescence under our storage conditions (21 days at 50℃). The properties of the interfacial casein layers did not depend on the aggregation state of the CAs used to form the emulsions. The differences in instability were attributed to the nature of the non-adsorbed CAs and storage conditions.*

Keywords: milk, citrate, mineral, calcium phosphate, functionality, casein aggregate

1 Introduction

The casein micelle consists of a highly aggregated particle of 150 to 200 nm diameter constituted of proteins (i.e. the four casein molecules α_{s1}, α_{s2}, β, κ), and minerals, mainly calcium phosphate (CaP), that ensure its colloidal stability (Dalgleish & Corredig, 2012; Holt & Horne, 1996; Holt, Carver, Ecroyd, & Thorn, 2013; Marchin, Putaux, Pignon, & Léonil, 2007; Schmidt & Payens, 1976; Trejo, Dokland, Jurat-Fuentes, & Harte, 2011; Walstra, 1990). The casein micelle has a key role in food products, especially dairy products, as it often contributes to their functional properties (i.e. the ability to form and / or stabilize networks such as gels, foams and emulsions, etc) (Foegeding & Davis, 2011).

The colloidal properties of the casein micelle (structure, composition, charge, hydration, etc) can be modified by controlling environmental factors such as pH, salt and chelating agent addition, temperature, etc (de Kort, Minor, Snoeren, van Hooijdonk, & van der Linden, 2011; Gaucheron, 2004; Silva et al., 2013). However, only a few studies have described the link between the colloidal organization and the functional properties of the modified casein micelle (Broyard & Gaucheron, 2015). Of all their functional properties, the capacity of the casein micelle to emulsify and stabilize oil in water emulsions is of great interest for the food industry, especially for the dairy industry. Indeed, many dairy products are edible emulsions (e.g. cream and ice-cream, infant formulae, etc) (Barbosa-Cánovas, Kokini, Ma, & Ibarz, 1996; Guzey & McClements, 2006).

Emulsions consist of mixtures of two immiscible liquids (such as oil and water), one of the liquids being dispersed as droplets in the other (McClements, 2005). These systems are thermodynamically unstable. The two phases will separate as a result of creaming, flocculation (agglomeration) and / or coarsening (fusion by coalescence or Oswald ripening) of the droplets. It is crucial to control both their formation and their stability during manufacture and storage to ensure the final quality of food emulsions.

One way to improve the formation and the stability of emulsions is to use emulsifying agents that adsorb at the oil-water interface and lower its tension. This results in the formation of smaller droplets that are less prone to creaming. The adsorbed layer formed by the emulsifying agents at the droplet surface can also protect the emulsion against flocculation and coalescence. Emulsifying agents can be assessed according to two main characteristics: their ability to facilitate the blending of the emulsion phases (i.e. emulsifying capacity) and their ability to stabilize the emulsion (i.e. emulsion-stabilizing capacity). Caseins are known to adsorb at the

interface, either in individual or aggregated form (Dickinson, 1999), and are therefore able to fulfill the role of emulsifying agent.

The emulsifying and stabilizing capacity of caseins is associated with their chemical nature and conformation at the interface and also depend on their aggregation state. Poorly aggregated casein systems such as CasNa (30 to 50 nm diameter – formed by extreme acid demineralization of native casein micelle) (Pitkowski, Durand, & Nicolaï, 2008) have enhanced emulsifying properties but are less effective for the stabilization of emulsions than highly aggregated casein micelles (Courthaudon et al., 1999; Mulvihill & Murphy, 1991). However, little information is available on the emulsifying properties of the intermediate aggregation states of casein micelles. Ye (2011) contributed to this information by studying different milk protein concentrates (MPCs) containing both casein and whey proteins as well as lactose in soya oil-based emulsions. Demineralization of the MPCs was induced by cation exchange but did not control the diffusible phase.

The aim of our study was to investigate the effects of the gradual disaggregation of pure casein micelles on their colloidal properties and on their emulsifying and stabilizing capacities in model dairy emulsions. Tri sodium citrate (Na_3Cit), a chelating salt, was used to remove calcium (Ca) and inorganic phosphate (Pi) from the casein micelle and to produce four suspensions of differently demineralized casein aggregates (CAs). Dialysis was performed on each suspension to control their diffusible phases. The CAs in these suspensions were characterized physico-chemically and used to form two types of emulsion to study their emulsifying and emulsion-stabilizing capacities separately. In addition, emulsions containing large droplets were produced to facilitate the creaming during storage and foster the appearance of flocculation and coalescence.

2 Materials and methods

2.1 Chemicals

All chemicals used for this study, (hydrochloridryc acid) HCl and Na_3Cit (Carlo Erba reagent, Val de Reuil, France), sodium azide (NaN_3) (Riedek-de Haën, Seelze, Germany), sodium hydroxide (NaOH), sodium dodecyl sulfate (SDS), D(+)-saccharose (saccharose) (VWR international, Leuven, Belgium), calcium chloride dihydrate ($CaCl_2.2H_2O$) (Sigma-Aldrich, St. Louis, USA), sodium di-hydrogen phosphate 2-hydrate ($NaH_2PO_4.2H_2O$) (Panreac, Barcelona, Spain), Fast Green FCF (FG) (Sigma-Aldrich, St. Louis, USA) and Nile Red (NR) (5H-Benzo α-phenoxazine-5-one, 9-diethylamino, Sigma-Aldrich, St. Louis, USA) were of analytical grade.

2.2 Materials

Purified casein micelles were used to monitor our system (native phosphocaseinate NPC). They were supplied by Gillot SAS (Saint Hilaire de Briouze, France) and obtained by microfiltration (0.1 µm pore size membrane) of raw skimmed milk followed by diafiltration against milli-Q water and spray dried according to Pierre, Fauquant, Le Graët, & Maubois (1992) and Schuck et al. (1994) on Bionov facilities (Rennes, France). The powder comprised 96 % (w / w) proteins - especially caseins (97 %) (w / w). Residual whey proteins (3 %) (w / w), lactose and diffusible Ca were present in the powder. Anhydrous milkfat (AMF, melting point 32°C) was supplied by Corman (Limboug, Belgium).

2.3 Preparation of different CA suspensions

Casein micelle powder was suspended in milli-Q water at a concentration of 28 g kg^{-1} and NaN_3 (1.6 g kg^{-1}) was added for conservation (Fig. 1A). To ensure good resuspension of the powder, the suspension was stirred at 900 rpm for 6 h at 40°C in a water bath and then for 16 h at room temperature. The rehydration of the casein micelle powder was checked by laser light diffraction as defined by Schuck, Dolivet, & Jeantet (2012). The results expressed in volume showed that more than 90 % of the particles were of size of casein micelles (150 nm diameter). This suspension was used to prepare four CA suspensions (S1, S2, S3 and S4). In S2, S3 and S4 varying amounts of a stock solution of Na_3Cit (0.85 mol kg^{-1} in milli-Q water, pH 7.0) were added to reach final concentrations of 4, 13 and 34 mmol kg^{-1}, respectively. S1 was kept as a control

suspension (without addition of Na_3Cit). These suspensions were stirred for 30 min and then diluted with milli-Q water to reach an intermediate casein concentration of 25 g kg^{-1}. The pH was then adjusted to 7.0 with HCl 1 M. S1, S2, S3 and S4 were left overnight at room temperature and the pH of each suspension was readjusted if necessary.

S1, S2, S3 and S4 were then dialyzed against an aqueous solution saturated in Ca and Pi (5 mmol kg^{-1} $NaH_2PO_4.2H_2O$ and 5 mmol kg^{-1} of $CaCl_2.2H_2O$, pH was adjusted to 7.0 using 1 M NaOH). The aim of the dialysis was to remove the added Cit and the ions solubilized from the casein micelle. This provided an identical ionic environment for all the CAs in the four different suspensions. Using a solution saturated in Ca and Pi also provided the advantage of limiting any further demineralization that might have been induced by classical dialysis against pure water. This was performed in two steps: first, the suspensions were individually dialyzed (in separate baths) for 27.5 h at room temperature against a total volume of 44 times each suspension volume, and the baths were changed four times. The second step was a combined dialysis (in the same bath) of the four CA suspensions for 15 h at room temperature against a volume 11 times the total suspension volume. The molecular weight (MW) cut-off of the dialysis membrane was between 12 and 14 kDa (Spectra / Por, Rancho Dominguez, Canada). The last dialysis bath was then filtered on a 2.5 µm filter paper and used to dilute the suspensions to reach a final casein concentration of 19.7 ± 0.6 g kg^{-1}. The final pH was 6.98 ± 0.04. The dialyzed CA suspensions, named $S1_d$, $S2_d$, $S3_d$ and $S4_d$, were prepared in duplicate.

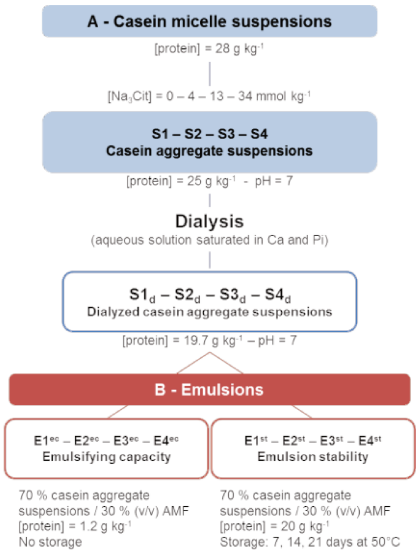

Figure 1. Preparation of CA suspensions and emulsions. d, ec and st represent «dialyzed», «emulsifying capacity» and «stability», respectively.

2.4 Recovery of the diffusible phases of the CA suspensions

The diffusible phases of each CA suspension were obtained by ultrafiltration for 30 min at 20°C on Vivaspin 20 concentrators (MW cut-off 10 kg mol^{-1}, Vivascience, Palaiseau, France). They were used for the determination of diffusible cation and anion concentrations in the CA suspensions, as well as for the dilution of the CA suspensions and the emulsions for determination of the zeta potentials (ʒ) and sizes.

2.5 Preparation of the two types of emulsion

Two types of emulsion (E^{ec} for "emulsifying capacity" and E^{st} for "stability") were prepared with each of the four CA suspensions in order to evaluate the emulsifying and emulsion-stabilizing capacity of the CAs (Fig. 1B).

Emulsions E^{ec} were prepared with the CA suspensions diluted at a protein concentration of 1.2 g kg^{-1} with milli-Q water and then added to the 60°C melted AMF at a 30:70 (v / v) ratio. The mixture was emulsified at 50°C in a water bath using a Polytron PT 3100 (Kinamatica AG, Littau, Switzerland) at 29,000 rpm for 5 min. Working at a limited protein concentration (1.20 g kg^{-1} compared to our emulsification system) highlighted the differences between the CAs by producing emulsions with different droplet sizes.

E^{st} emulsions were prepared following the same procedure except that the CA suspensions were kept at a protein concentration of about 20 g kg^{-1}. In this case, the choice of an excess protein concentration produced emulsions with similar droplet sizes, necessary for the study of the stabilizing capacity of the CAs. E^{st} emulsions were divided into several samples and stored in transparent, cylindrical, hermetically sealed tubes at 50°C for three weeks. The temperature of 50°C was chosen in order to prevent the formation of fat crystals in the emulsion that could affect their physical stability (Lopez, Bourgaux, Lesieur, & Ollivon, 2007). Each week, one sample was analyzed by laser light diffraction, electrophoretic light scattering, multiple light scattering and confocal microscopy to follow the evolution of the emulsion. Two replicate emulsions were made for each type of emulsion.

2.6 Analysis

2.6.1 Mineral composition and distribution

Total cations (Ca, magnesium - Mg, sodium - Na, potassium - K) and diffusible cations and anions (Pi, Cit, chloride - Cl) were determined in the CA suspensions and in their diffusible phases, respectively. Total anions were determined in the diffusible phases of the CA suspensions previously acidified at pH 4.6 with a 10 % (v / v) acetic acid solution. Cation concentrations were measured by atomic absorption spectrometry (Varian 220FS spectrometer, Les Ulis, France) as described by Brulé, Maubois & Fauquant (1974). Anion concentrations were determined by ion chromatography (Dionex ICS 3000, Dionex, Voisin le-Bretonneux, France) as described by Gaucheron, Le Graët, Piot & Boyaval (1996). Colloidal concentrations were deduced by subtracting diffusible from total ion concentrations. The Ca demineralization rates corresponded to the percentage of solubilized Ca compared to total Ca initially present in the suspensions prior to dialysis.

2.6.2 Protein content

Protein content was determined in the CA suspensions and in their respective ultracentrifuged supernatants to deduce the non-sedimentable casein concentrations (soluble caseins). The Kjeldahl method (IDF standard 20-1,2014) was used to determine the total nitrogen concentration in the samples, and a conversion factor of 6.38 was used to convert nitrogen to protein concentration. Measurements were performed in duplicate.

2.6.3 Pellet hydration and sedimentable protein concentrations

Twenty grams of CA suspension were ultracentrifuged at 20°C for 1 h at 100 000 g (Sorvall Discovery 90 SE, Hitachi, Courtaboeuf, France) and the ultracentrifuged pellets were recovered. Hydration was deduced according to the weight loss after drying the ultracentrifuged pellets of each sample mixed with Fontainebleau sand in an oven at 105°C for 8 h (FIL-IDF Standard 26A, 1993).

Sedimentable protein concentrations were deduced from the proportion of pellets and hydration data by considering that ultracentrifuged pellets consisted mainly of proteins and water (mineral weights were disregarded). Measurements were performed in duplicate.

2.6.4 CA sizes and proportions in the CA suspensions (AsFlFFF)

The MWs and hydrodynamic radii (R_h) of the CAs were determined in suspensions $S1_d$, $S4_d$ (extreme points), and $S2_d$ (intermediate point) using asymmetrical flow field-flow fractionation (AsFlFFF) coupled to multi angle laser light scattering (MALLS) as described in Guyomarc'h, Violleau, Surel & Famelart (2010) with slight modifications. A solution saturated in Ca and Pi (similar to the solution used for the dialysis step) was filtered through 0.1 µm filter paper. This filtered solution was used as the eluent for the AsFlFFF separation, and for the ten-fold dilution of the samples.

During the AsFlFFF run, the laminar flow was fixed at 1 mL min^{-1} and only the cross flow varied. The first focusing-injection step (10 min) consisted of setting up the cross flow at 1.5 mL min^{-1} for 1 minute. Then 30 µL of sample were injected while the cross flow was maintained at 1.5 mL min^{-1} for 9 min. This allowed the analytes to diffuse away from the membrane according to their R_h. The elution step then started with a 5 min plateau at a cross flow rate of 1 mL min^{-1} followed by a linear decrease of 5 min to reach 0.15 mL min^{-1} for 25 min. The cross flow was finally stopped to eliminate all the particles that might have remained in the AsFlFFF channel. Under our operating conditions, the AsFlFFF worked in normal mode, which means that larger particles were retained in the channel for longer times than smaller ones, providing that all particles had similar density.

The AsFlFFF was connected to an 18 angle DAWN-DSP MALLS detector (Wyatt Technology, Santa Barbara, CA, USA) (λ = 633 nm), an Optilab Rex Refractometer (Wyatt Technology, Santa Barbara, CA, USA) (λ = 685 nm), and an Agilent 1100 UV detector (λ = 280 nm). The UV signal was used as the source data for measurement of protein concentrations and a calculated extinction coefficient of 9.009 L g^{-1} cm^{-1} was determined and used (bovine serum albumin at 280 nm in the eluent). Astra software version 6.0 was used to analyze the UV and Rayleigh ratio data and determine the MW and R_h values. In this study, it was assumed that the different CAs were spherical and homogenous in composition. R_h were determined between 20 and 28 min (population A) via Berry formalism of a Debye plot. R_h cannot be calculated directly between 14 to 20 min (populations B and C) because of the low Rayleigh ratio signal in this time range. For suspensions $S1_d$ and $S2_d$, the R_h values between 20 and 24 min were therefore fitted with a first order exponential model that was extrapolated between 14 and 20 min (populations B and C). This treatment was not possible on $S4_d$ because of the low Rayleigh ratio signal, and the values of R_h determined for S4d calculated between 20 and 28 min were not accurate enough for their extrapolation (see section 3.1.2).

Similar tests were performed to determine MW values, with slight modifications. The MW values of $S1_d$ and $S2_d$ were fitted with exponential models of first order between 18 to 20 min and the models were extrapolated between 14 to 18 min. In contrast to R_h, the $S4_d$ Rayleigh ratio was high enough to determine MW. For this suspension, the MW values were fitted between 15.5 and 16.5 min (population B) and the model was extrapolated between 14 and 15.5 min (population C).

2.6.5 Zeta potential (ʒ)

The electrophoretic mobility of CAs (in CA suspensions) and milkfat droplets (in emulsions) were measured by electrophoretic light scattering using a Zetasizer 3000 HS (Malvern Instruments, Worcestershire, UK). CA suspensions and emulsions were diluted in their corresponding diffusible phases. Diluted CA suspensions were filtered through a 0.45 µm pore size membrane to eliminate possible dust particles prior to analysis.

Henry's equation:

$$ʒ = (3 \eta \mu / 2 \varepsilon f(Ka)) \quad (1)$$

where η is the viscosity and ε the dielectric constant of the solution, was applied to determine the apparent ʒ of the particles from their electrophoretic mobility µ. F(Ka) = 1.5 was used according to the Smoluchowski approximation. The measurements for the CA suspensions were performed at 20°C and the viscosity and the dielectric constant of the dissociating medium (water) were 1.00 cp and 80.4, respectively. Measurements for the emulsions were performed at 50°C with a viscosity of 0.55 cp and a dielectric constant of 70.2. Measurements were performed in triplicate.

2.6.6 Droplet size distribution in emulsions

The milkfat droplet size distributions were determined by laser light diffraction immediately after the preparation of the emulsions and after 7, 14 and 21 days of storage at 50°C, using a Mastersizer 2000 (Malvern Instruments, Worcestershire, UK) equipped with a He / Ne laser (λ = 633 nm) and an electroluminescent diode (λ = 466 nm). The refractive indices were set at 1.46 (at 466 nm) and 1.458 (at 633 nm) for milkfat and 1.33 for water. Before measurements, samples were dispersed in milli-Q water as was, or were previously diluted ten times in a solution of 1 % (w / w) SDS to separate aggregated milkfat droplets and estimate the extent of

droplet flocculation. All distributions and / or their corresponding mode values (i.e. the maxima of the size distribution) were used to compare the emulsions. Specific surface areas (area per unit mass) were used for the determination of the protein surface concentrations. Measurements were performed in triplicate.

2.6.7 Confocal microscopy of the emulsions

The microscopy observations were carried out with a Nikon Eclipse-TE2000-C1si confocal microscope (Nikon, Champigny sur Marne, France) equipped with argon and He-Ne lasers operating at 488 and 543 nm excitation wavelengths, respectively (emissions were detected between 500 and 530 nm and between 565 and 615 nm, respectively). One milliliter of emulsion was stained using 100 µL of a milkfat soluble Nile Red fluorescent dye solution (0.1 % w / w in propane diol) and 50 µL of a Fast green FCF solution (1 % w / w in water) to stain the proteins. The samples were left for 15 min at 50℃ prior to observation. Microscopy observations were performed at 50℃ using a thermal PE100-NI System plate warmer (Linkam Scientific Instruments Ltd., Tadworth Surrey, England). Images were collected with an oil immersion objective with a magnification of x 60. Characteristic images were selected from the 9 images taken for each sample.

2.6.8 Interfacial tension and dilational rheology

An oscillatory drop tensiometer (Tracker, Teclis, France) was used to measure the interfacial tension (γ) and the interfacial dilatational moduli (E*, E' and E'') at the milkfat / CA suspension interfaces, at 50℃. The CA suspensions and the last dialysis bath (control) were used to form a pendant drop of 10 µL at the tip of a syringe that was suspended in an 8 mL cuvette containing melted milkfat (50°C). Two opposite forces, gravity and the force related to γ, were exerted on the drop to induce its shape. Analysis of the shape of the drop 5 min after its formation (equilibrium state) made it possible to calculate the γ value by solving the Laplace equation (Ravera, Loglio, & Kovalchuk, 2010).

Dilational rheology was performed on our system by applying the conditions used by Silva, Saint-Jalmes, de Carvalho, & Gaucheron (2014) with slight modifications. Briefly, a sinusoidal oscillation of the drop volume of 10 % at a frequency of 0.2 Hz was applied to a 2 min old 10 µL CA suspension drop in the melted milkfat at 50℃. The volume variation engendered a controlled oscillatory compression / dilation of the droplet interfacial area A and resulted in the surface

tension oscillation as a function of time γ(t). Monitoring of γ(t) and determination of its phase shift (ϕ) compared to A(t) made it possible to calculate the complex (E*), elastic (E') and viscous (E'') moduli of the adsorbed interfacial layer. Purely elastic and solid-like interfacial layers had E′ ≫ E″ and ϕ tended to 0, whereas viscous and fluid-like interfacial layers had E″ > E′ and a large ϕ.

2.6.9 Creaming stability ratio

A transparent, cylindrical, hermetically sealed glass tube was filled with 20 mL of fresh E^{st} emulsion and placed in the measurement chamber of a Turbiscan MA2000 multiple light scattering optical analyser Turbiscan MA2000 (Formulaction, France). The tube was scanned at 50°C from top to bottom by a 850 nm light source and the back scattered light was recorded every 40 µm. Analysis of the back scattered signal as a function of the height of the tube determined the total height of the emulsion (H) and the thickness of the creamed layer (h). The creaming ratio (r_c) was defined as $r_c = H / h$. The measurements were performed on each E^{st} emulsion after 0, 7, 14 and 21 days of storage at 50°C.

2.6.10 Surface protein concentration

The method of separation of the non-adsorbed proteins from the emulsion droplets was derived from Patton & Huston, (1986). Forty-four milliliters of E^{st} emulsion were gently mixed with 5 g saccharose in 50 mL centrifuge tubes and maintained at 50°C in a water bath. The tubes were centrifuged at 200 g for 20 min at 50°C and frozen at - 20°C. The frozen tubes were cut at the interface to separate the creamed milkfat droplets at the top of the tube and the aqueous phase containing saccharose and non-adsorbed caseins at the bottom. The milkfat droplet phases were transferred to other centrifuged tubes, melted at 50°C and redispersed in 15 mL of 4 % (w / w) SDS solution. The tubes were centrifuged at 1 500 g for 20 min at 50°C, frozen at - 20°C and cut to separate the top milkfat phase from the bottom aqueous SDS phase containing the adsorbed caseins. The first bottom saccharose aqueous phase (containing the non-adsorbed caseins) and the second bottom aqueous SDS phase (containing the adsorbed proteins) were both analyzed in terms of protein concentration using Kjeldahl and micro-Kjeldahl methods, respectively. The amounts of casein adsorbed at the interfaces were related to the specific surface areas of the droplets (previously determined by laser light diffraction) to calculate the interfacial casein concentrations and the percentages of adsorbed caseins.

2.7 Statistics

Measurements were carried out on each of the replicates of suspensions $S1_d$, $S2_d$, $S3_d$ and $S4_d$, emulsions $E1^{ec}$, $E2^{ec}$, $E3^{ec}$ and $E4^{ec}$ and emulsions $E1^{st}$, $E2^{st}$, $E3^{st}$ and $E4^{st}$, except for AsFlFFF, γ and dilatational rheology measurements. The standard deviations were calculated for each determination.

3 Results

The results are presented in two steps with first a focus on the mineral balance and colloidal characteristics of the CAs in suspensions only. Their functional properties are then described when used as emulsifying agents in our model dairy emulsions.

3.1 Mineral and colloidal characterization of casein aggregate suspensions

3.1.1 Mineral characteristics

Colloidal Ca and Pi concentrations (Table 1) decreased simultaneously and in a correlated fashion (Fig. 2) in the order: $S1_d$ < $S2_d$ < $S3_d$ < $S4_d$. This progressive casein micelle demineralization, expressed as a Ca demineralization rate (Table 1), was 24, 35, 56 and 81 % for suspensions $S1_d$, $S2_d$, $S3_d$ and $S4_d$, respectively.

On the other hand, the concentration of colloidal Na (Table 1) increased in the suspensions with the increase in added Na_3Cit. This increase was correlated with the decrease in colloidal Ca concentration (Fig. 2), and therefore with the decrease in Pi concentration. The Cl ions were only diffusible in the CA suspensions (Table 1). The Na and Cl present in the saturated dialysis baths (counter ions of Pi and Ca) mainly contributed to the high colloidal and diffusible concentrations observed in the dialyzed CA suspensions.

Mg and K were not present in the CA suspensions because these ions were not present in the purified casein micelles. Diffusible ion concentrations (Table 1) were similar in the suspensions and no diffusible or colloidal Cit was found after the dialysis step. Diffusible Ca was close to zero for all suspensions.

Table 1. Distribution of mineral salts in the casein aggregate suspensions. Colloidal concentrations were determined by deducing soluble from total concentrations. The Ca demineralization rates corresponded to the percentage of solubilized Ca compared to total Ca initially present in the suspensions.

	$S1_d$	$S2_d$	$S3_d$	$S4_d$
Diffusible Ca (mmol kg^{-1})	0.0	0.0	0.0	0.0
Colloidal Ca (mmol kg^{-1})	11.8	10.3	7.5	3.0
Ca demineralization rate (%)	24	35	56	81
Diffusible Pi (mmol kg^{-1})	2.1	2.0	1.8	1.8
Colloidal Pi (mmol kg^{-1})	3.0	2.5	1.2	0.4
Diffusible Na (mmol kg^{-1})	21.5	21.1	21.2	20.6
Colloidal Na (mmol kg^{-1})	2.6	2.9	3.6	5.3
Diffusible Cl (mmol kg^{-1})	8.3	8.4	8.1	8.0
Colloidal Cl (mmol kg^{-1})	0.0	0.0	0.0	0.0

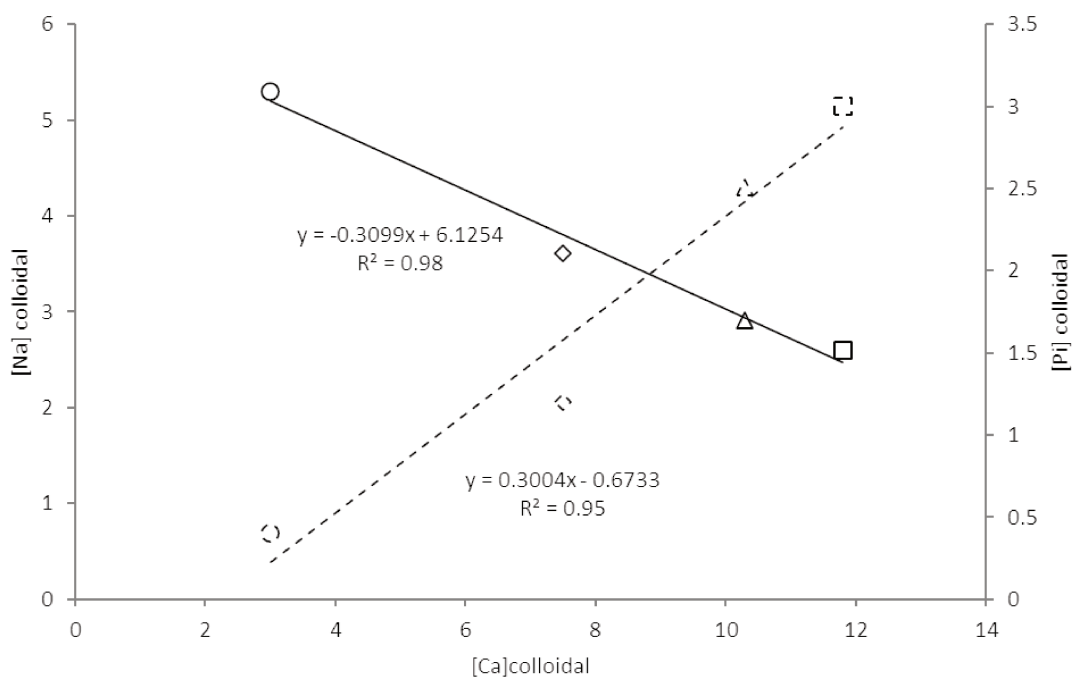

Figure 2. Correlations between colloidal Ca, Na and Pi concentrations. Colloidal Pi (_____) and colloidal Na (- - -) as a function of colloidal Ca for: $S1_d$ (○), $S2_d$ (◊), $S3_d$ (Δ) and $S4_d$ (□). Concentration are in mmol kg^{-1}.

3.1.2 Colloid characterization

Hydration of the ultracentrifugation pellets was constant for $S1_d$, $S2_d$ and $S3_d$ and slightly lower for $S4_d$ (Table 2). The concentration of sedimentable proteins decreased with the increase in the amount of Na_3Cit added and a reduction of 82 % was found when comparing suspension $S1_d$ with $S4_d$ (Table 2). The non-sedimentable casein content thus increased from 8 to 18 g kg^{-1} with the addition of Na_3Cit to the CA suspensions (Table 2).

Similar ʒ (22.7 ± 1.3 mV) were measured for each CA suspension (Table 2).

Table 2. Physicochemical properties of the different CA suspensions.

	$S1_d$	$S2_d$	$S3_d$	$S4_d$
Hydration (g of water g^{-1} of dried pellet)	3.0 ± 0.1	2.8 ± 0.1	2.6 ± 0.1	2.1 ± 0.1
Non-sedimentable casein (g kg^{-1})	8.0 ± 0.5	10.3 ± 0.5	15.0 ± 0.4	18.0 ± 0.8
Sedimentable protein (g kg^{-1})	12.9 ± 0.1	11.6 ± 0.1	8.3 ± 1.7	2.3 ± 0.3
Zeta potential of casein aggregates (mV)	-23.5 ± 1.2	-24.4 ± 2.0	-21.5 ± 3.7	-21.5 ± 2.4

The UV, Rayleigh ratio and the calculated MW and R_h of the CAs in the suspensions obtained by AsFIFFF are represented as a function of elution time, respectively (Fig. 3). For each suspension, three peaks that corresponded to three different populations of particles (A, B and C) were observed by UV:

Population A (19 - 27 min) corresponded to particles with MW from 7.5 x 10^7 to 2.5 x 10^9 g mol^{-1} in $S1_d$, $S2_d$ and R_h from 30 to 100 nm. Population A in $S4_d$ had MW ranging from 2.2 x 10^7 to 3.7 x 10^9 g mol^{-1}, and R_h between 130 and 250 nm. Differences between population A in $S4_d$ and in the other samples must be interpreted with caution because the Rayleigh ratio for this population in $S4_d$ was weak and the MW and R_h values deduced from this signal might be less accurate. Moreover, according to the UV and Rayleigh ratio signals, the largest particles of $S4_d$ suspensions were eluted simultaneously with the largest particles of other suspensions, i.e. $S1_d$ and $S2_d$ (peaks are superimposed), and therefore these particles had similar MW and R_h.

Population B particles (15.5 – 17 min) had R_h between 21 and 26 nm (evaluated on $S1_d$ and $S2_d$ only). Corresponding MW were between 1.4 x 10^7 and 3.15 x 10^7 for $S1_d$ and $S2_d$ and between 3.4 x 10^6 and 1 x 10^7 for $S4_d$. Finally, population C (14-15 min) had R_h between 18 and 22 nm

(evaluated on $S1_d$ and $S2_d$ only) and MW between 7.0 x 10^6 and 1.5 x 10^7 g.mol^{-1} for $S1_d$ and $S2_d$ and between 1.4 x 10^6 and 3.4 x 10^6 for $S4_d$. As for population A, differences between MW in $S4_d$ and in the other samples must be interpreted with caution. Again, UV signals indicated that for all suspensions, the B and C populations of particles eluted simultaneously in $S1_d$, $S2_d$, and $S4_d$. According to the quality of the Rayleigh ratios signals of the suspensions, different data treatments were applied which could explain the differences in the MW values observed.

The proportions of the different populations of particles depended on the amount of added Na_3Cit: the largest particles (A) disappeared when the Na_3Cit concentration increased, permitting the appearance of the two smallest populations (B and C). Nevertheless, the loss in surface area under the A peak was not equal to the gain in surface area under the B and C peaks due to the fact that the largest particles not only absorbed but also diffused the UV signal compared to small particles that only absorbed the UV signal.

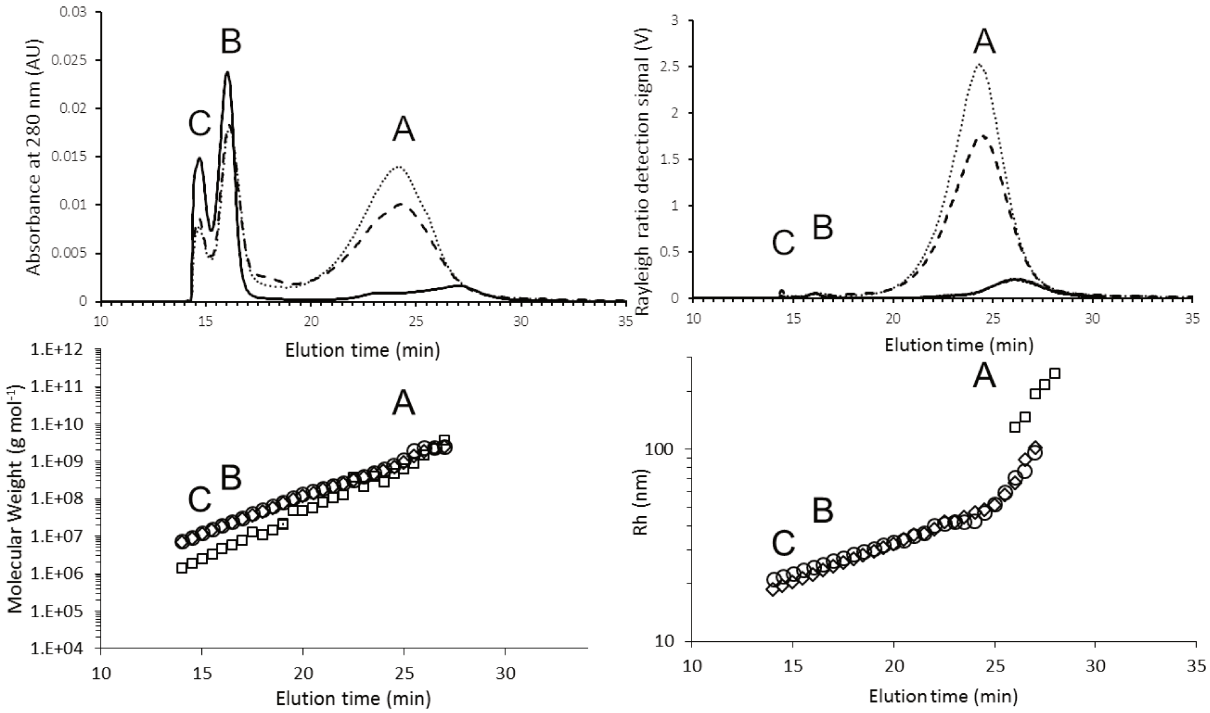

Figure 3. AsFIFFF determination of structural characteristics of casein aggregates in suspensions. The UV signal (top left), Rayleigh ratio (top right), molecular mass (bottom left) and hydrodynamic radius (bottom right), were determined for the two extreme suspensions $S1_d$ (○)(····), $S4_d$ (□)(_____) and one intermediate $S2_d$ (◊)(- - -) CA suspension as a function of the elution time. Casein micelle-like aggregates (population A), NaCas-like aggregates (population B) and protein monomers (population C) are labeled.

3.2 Functional characterization of casein aggregate suspensions

3.2.1 Emulsifying capacity of casein aggregate suspensions

The particle size distribution profiles of E1^{ec}, E2^{ec}, E3^{ec} and E4^{ec} emulsions are presented in Figure 4. Given that the size distribution profiles were monomodal, the mode values (i.e. the maximum of each peak) are represented as a function of the added Na_3Cit concentration in the CA suspensions (Fig. 4C empty symbols). The distributions shifted to smaller sizes (Fig. 4A, C) as the added Na_3Cit concentration increased in the CA suspensions and the mode values varied between 27 and 14 µm. This size range corresponded to macro emulsions. In the presence of SDS, the size distributions of the particles were smaller and narrower than in the absence of SDS (Fig. 4), revealing aggregation of the emulsion droplets. The mean diameter of the emulsion droplets decreased as a function of the increase in Na_3Cit concentration in the CA suspension (Fig 4).

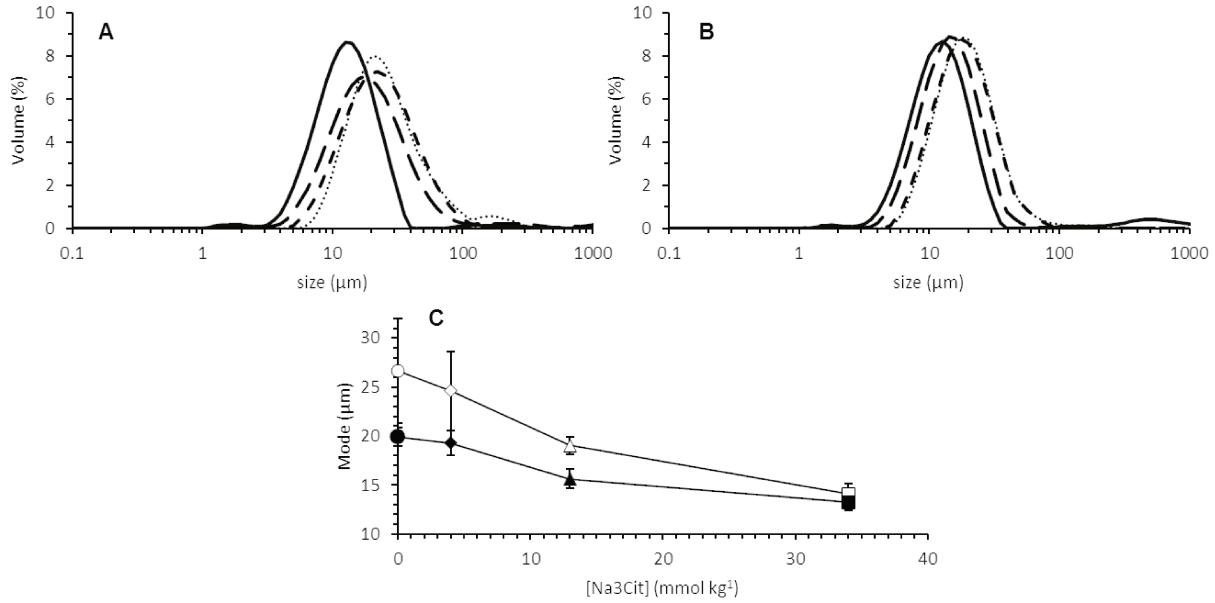

Figure 4. Size distribution profile of emulsions prepared for the determination of emulsifying capacity (E^{ec}). Emulsions E1^{ec} (○)(····), E2^{ec} (◊)(- - -), E3^{ec} (Δ)(— —) and E4^{ec} (□)(_____) were analyzed as is (A) and diluted ten times in a dissociating medium (aqueous solution of 1 % w / w SDS) (B). Evolution of the mode as a function of the concentration of added Na_3Cit are represented (C) either in the absence (empty symbols) or presence (filled symbols) of SDS.

Figure 5 shows confocal micrographs of the fresh $E1^{ec}$, $E2^{ec}$, $E3^{ec}$ and $E4^{ec}$ emulsions. Milkfat droplets (in red) were surrounded by casein aggregates (in green). Microstructural observations confirmed the decrease in the size of the emulsion droplets as a function of the increase in Na_3Cit concentration in the CA suspensions. Moreover, flocculation of the emulsion droplets was characterized in each emulsion, in agreement with particle size measurements (Fig. 4).

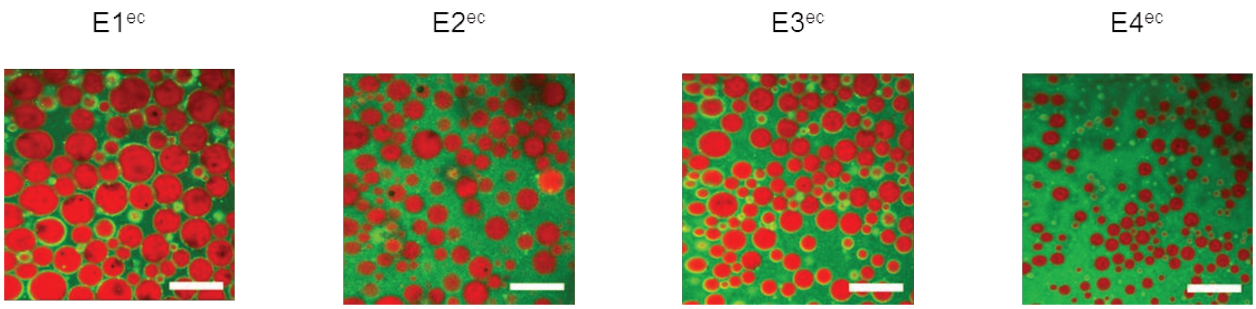

Figure 5. Confocal laser scanning microscopy images of the emulsions prepared for determination of emulsifying capacity (E^{ec}). Microscopic images were recorded at 50°C using a thermal plate warmer. Milkfat emulsion droplets (in red) surrounded by casein (in green). Scale bars measure 50 µm.

The interfacial tension (γ) at the melted milk fat / CA suspension interface was measured to evaluate the activity of the CAs at the milkfat droplet surface. Blank interfacial tension determined on the last dialysis bath of the CA suspensions was 10 mN m^{-1}. The presence of CAs decreased γ to around 5 - 6 mN m^{-1} whatever the added Na_3Cit concentration.

3.2.2 Emulsion-stabilizing capacity of the casein aggregate suspensions

The evolution of the creaming ratios (r_c) of E^{st} emulsions over time are shown in Figure 6. None of the emulsions were stable against creaming. Phase separation was easily observable after 7 days of storage and did not vary during the following 14 days. The determination of r_c indicated that the thickness of the creamed layers decreased with the increase in added Na_3Cit in the CA suspensions.

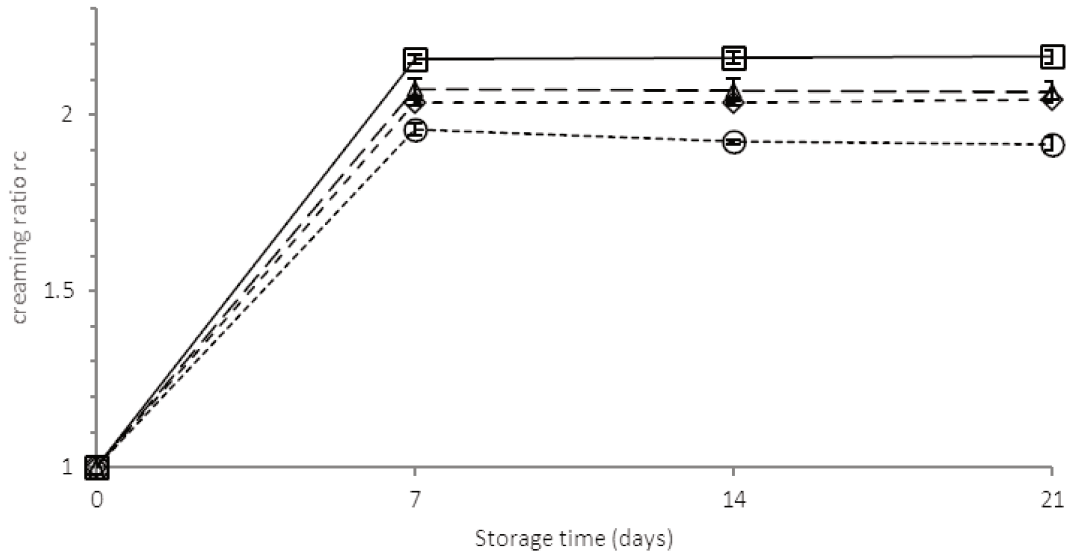

Figure 6. Time evolution of creaming ratios r_c of emulsions prepared for the determination of emulsion stability (E^{st}). Creaming ratio defined as r_c = H / h where H is the total height of the emulsion and h the thickness of the creamed layer. Standard deviation bars are represented behind the point marks.

Laser light scattering measurements and confocal microscopy observations were performed on each emulsion throughout storage at 50°C (Fig. 7). Given that the particle size distribution profiles were monomodal (data not shown), the evolution of the mode value of each emulsion as a function of time is represented. The light scattering measurements were carried out in the presence and absence of SDS. Indeed, this small surfactant is able to dissociate flocculated droplets by replacing the protein at interfaces, permitting discrimination of flocculated droplets from coalesced droplets. When droplets flocculated, the emulsion size distribution shifted to smaller sizes (smaller mode values). In contrast, the addition of SDS had no influence on the size distribution of coalesced droplets. Figure 7 shows that the size of the particles in emulsions increased with time without SDS, especially for emulsions from CA suspensions containing Na_3Cit. For example, the mode value of $E2^{st}$ increased from 12 to 33 µm after 21 days of storage and from 12 to 90 µm for $E4^{st}$. In the presence of SDS, the size distribution of the droplets did not evolve over time, the mode being 12 µm, similar to the size determined after the preparation of the emulsions (data not shown). These constant values indicated that $E2^{st}$, $E3^{st}$ and $E4^{st}$ were destabilized by flocculation but were stable against coalescence. The $E1^{st}$ emulsion, which maintained a constant mode value throughout storage, was stable against both flocculation and coalescence phenomena.

The corresponding micrographs of each emulsion at each time-point were in good agreement with laser light scattering data (Fig. 7). Each emulsion maintained the same droplet size during storage. However, some micrographs showed contrast differences, with bright milkfat droplets at the foreground of the image and dark red droplets at the back. This color variation was attributed to the appearance of 3D milkfat droplet flocs in the emulsions that coexisted on different focal planes of the micrographs. According to the microscopy observations, E3st and E4st emulsions were the most highly flocculated under our storage conditions.

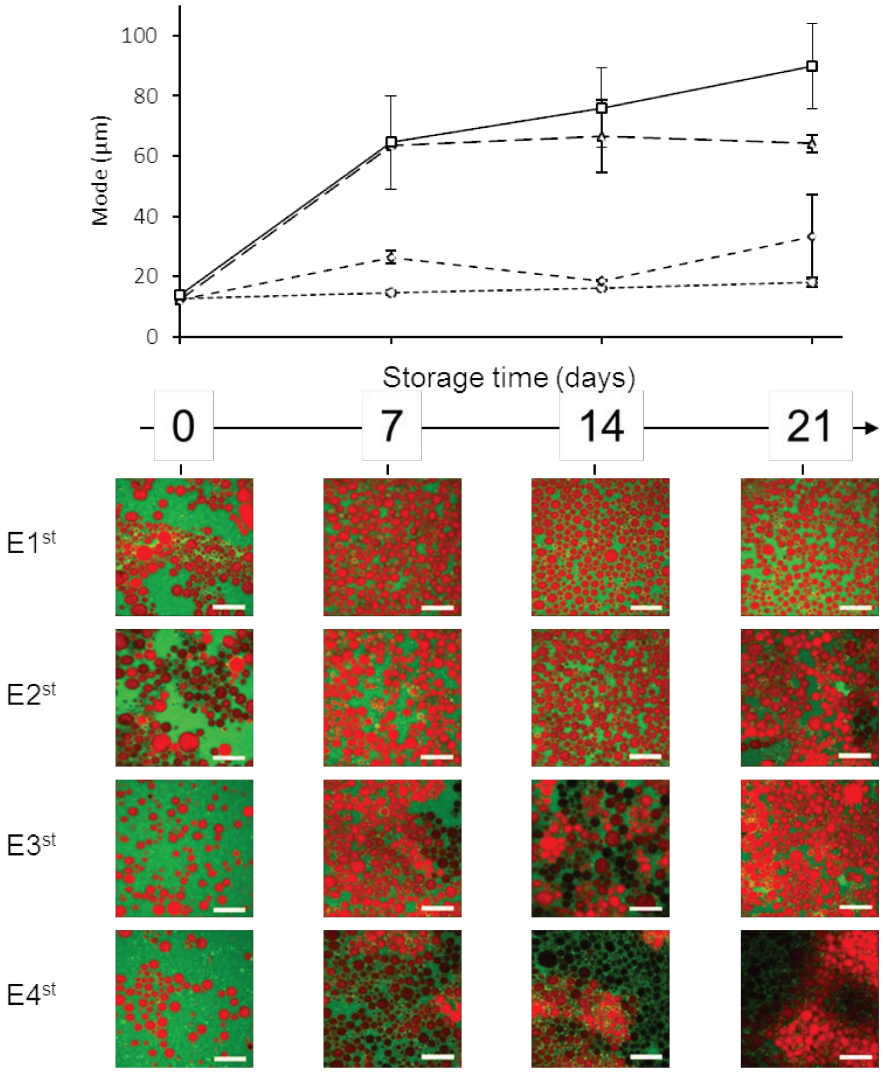

Figure 7. Microscopic evolution of the emulsions over time (Est). Droplet size (mode) and confocal micrograph evolution as a function of storage time: E1st (○)(····), E2st (◊)(- - -), E3st (Δ)(— —) and E4st (□)(_____). Microscopy images were recorded at 50°C using a thermal plate warmer. Milkfat emulsion droplets (in red) are surrounded by casein (in green). Contrast differences are attributed to the

appearance of 3D milkfat droplet flocs in the emulsion that coexisted on different focal planes of the micrographs.

ʒ of individual emulsion droplets and flocculated droplets did not evolve significantly during the 21 days of storage (23.1 ± 1.4 mV).

Around 24 ± 1 % of the total protein present in the emulsions was adsorbed at the interface, whatever the type of CA suspension used to make the emulsion, which corresponded to a casein surface concentration of around 17.4 ± 0.7 mg m^{-2}.

The interfacial dilatational moduli (E*,E' and E'') were determined at the melted milkfat / CA suspensions interface. All suspensions presented similar values: 14.6 ± 0.4, 14.4 ± 0.4 and 2.9 ± 0.2 for complex (E*), elastic (E') and viscous (E'') moduli, respectively. The contribution of E' to E* was higher than the E'' contribution, reflecting solid-like behavior of the adsorbed casein aggregate layers.

4 Discussion

The results are discussed in two stages, with a first focus on the characterization of the CA suspensions in terms of mineralization and colloidal properties. The second stage consisted of an investigation of the emulsifying and emulsion-stabilizing capacities of the CA used as emulsifying agents in two types of model dairy emulsions.

4.1 Characterization of the different CA suspensions

4.1.1 Addition of Na_3Cit resulted in progressive casein micelle demineralization

Analysis of the distribution of minerals confirmed that Na_3Cit had an influence on the mineralization of the casein micelle. By chelating the diffusible Ca, Cit ions induced the progressive removal of the colloidal Ca (Gaucheron, 2004). This was in accordance with results reported by many authors who recorded Cit chelation of Ca either by determining Ca activity (de Kort et al., 2011; Johnston & Murphy, 1992; Udabage, McKinnon, & Augustin, 2001), or diffusible Ca and / or colloidal Ca concentrations (Le Ray et al., 1998; Mizuno & Lucey, 2005; Mohammad & Fox, 1983; Odagiri & Nickerson, 1965; Ozcan-Yilsay, Lee, Horne, & Lucey, 2007; Vujicic, deMan, & Woodrow, 1968) in milk or micellar suspensions.

The simultaneous and correlated decrease in the colloidal Pi concentration (Fig. 2) was attributed to the solubilization of the colloidal CaP (Le Ray et al., 1998; Mizuno & Lucey, 2005; Mohammad & Fox, 1983). Increasing the concentration of Na_3Cit therefore led to progressive CaP demineralization of the CA suspensions.

Furthermore, the correlation observed between the colloidal concentrations of Ca and Na (Fig. 2) suggested that the negative charges induced by the Ca demineralization (presence of free phosphoseryl residues) were screened by monovalent Na ions, potentially explaining the constant zeta potential observed for each CA suspension (Table 2).

Mineral content was also modified by the casein powder resuspension and dialysis steps. Determination of colloidal and diffusible Ca in S1 (prior to dialysis – data not shown) and $S1_d$ (after dialysis) induced partial solubilization of the colloidal Ca. This limited Ca demineralization (24 %, reported in Table 1) was attributed to the resuspension of the purified casein micelle powder in water and to the dialysis step.

The dialysis step also permitted removal of the added Cit and established a similar diffusible phase in the four suspensions (Table 1). As the result, the ionic strengths of all the suspensions were taken to be similar in the four suspensions.

4.1.2 Na_3Cit demineralization resulted in disaggregation of the casein micelle

Structural modifications of the CA were observed in parallel to the micellar demineralization. The quantity of sedimentable proteins was reduced and that of non-sedimentable (soluble) proteins consistently increased (Table 2), which showed progressive dissociation of the CAs. Similar trends were reported by Udabage et al. (2001), Le Ray et al. (1998) and De Kort et al. (2011).

AsFlFFF characterization was performed in order to evaluate the sizes of the dissociated CAs. This revealed that three populations of particles of different sizes and proportions were simultaneously present in the CA suspensions (Fig. 3). Population A consisted of large CA with MW and R_h comparable to those of the casein micelle (MW between 5 x 10^7 and 1 x 10^{10} g.mol^{-1} and r_{rms} of 50 – 350 nm), as previously reported (Glantz, Håkansson, Lindmark Månsson, Paulsson, & Nilsson, 2010; Pitkowski et al., 2008). The addition of Na_3Cit induced dissociation of these aggregates and increased the proportion of population B. This population consisted of aggregates similar to sodium caseinate (NaCas) particles with MW of 4 to 9 x 10^6 g.mol^{-1} and R_h between in 10 – 20 nm, as reported by Lucey, Srinivasan, Singh, & Munro (2000). Using 50 times more Na_3Cit per gram of protein than in our study, Panouillé et al. (2004) reported slightly smaller CAs (MW = 2 x 10^5 g.mol^{-1} and R_h = 12 nm). Finally, population C corresponded to the smallest particles in our suspensions. The percentage of these small particles was also increased by the increased addition of Na_3Cit. This suggested that population C corresponded to casein monomers dissociated from the larger CAs. According to Guyomarc'h et al. (2010) and Glantz et al (2010), they could also be attributed to residual whey protein monomers.

As demonstrated by Pitkowski, Nicolai, & Durand (2007), Lin, Leong, Dewan, Bloomfield, & Morr (1972) and Marchin et al. (2007) with polyphosphate and EDTA Ca chelation, the dissociation of casein micelles by Ca chelating agents is a "cooperative process" in which the structure of the casein micelle remains intact (large aggregates) or becomes fully dissociated (small aggregates of the same size are produced). In other words, the dissociation of the casein micelle does not provide aggregates of intermediate sizes. The three populations of particles (casein micelle-like aggregates, NaCas-like aggregates, and protein monomers) and their dependence on the

amount of Na_3Cit added confirmed that the “cooperative process” can be applied to the Na_3Cit dissociation of casein micelles.

The hydration measurements of $S1_d$, $S2_d$, $S3_d$ and $S4_d$ pellets (Table 2) differed from the findings of Le Ray et al. (1998) who reported that the water content of the sedimented CAs increased with the addition of Na_3Cit. This was also supported by the voluminosity data determined by De Kort et al. (2011). Compared to our study, these authors did not monitor the diffusible phases of their suspensions. The dialysis step and thus the diffusible environment of the sedimentable CAs therefore seemed to have an impact on their hydration.

As expected, Na_3Cit demineralized and dissociated the casein micelle to different extents in order to produce four suspensions containing various CAs. The effects of a Ca chelating agent on the casein micelle seemed to be in good agreement with the use of an ion-exchange resin to sequestrate the Ca (Xu et al., 2016; Ye, 2011). Xu et al. (2016) reported a similar dissociation of the casein micelle into smaller CAs and a decrease in the total Ca content of their casein micelle suspension. These authors also reported that, beyond a level of 20 % of Ca demineralization (which is lower than the demineralization rate of our four suspensions), the dissociated caseins present in the ultracentrifuged supernatant (non-sedimentable or soluble proteins) were of similar composition to that of the native casein micelle. This suggests that the micelle-like CAs and the mixture of NaCas-CAs and the “free” casein monomers have the same composition.

This first step of our study was necessary to characterize and control our CA suspensions accurately in order to elucidate their emulsifying and emulsion-stabilizing capacity. To summarize, suspension $S1_d$ mostly contained highly mineralized and large casein micelle-like CAs. Intermediate suspensions ($S2_d$ and $S3_d$) contained a mixture of both large and small NaCas-like CAs, with a small quantity of “free” casein monomers. Finally, $S4_d$ mainly consisted of poorly mineralized small CAs, “free” casein monomers and residual traces of large CAs (Fig. 8A).

4.2 Investigation of CA capacity as emulsifying agents

4.2.1 Decreasing the size of the CA increased its emulsifying capacity

The emulsifying capacity of a protein (or a protein aggregate) can be characterized by measuring the emulsion droplet size at a particular protein concentration: the smaller the droplet,

the better the protein aggregate as an emulsifier (Euston & Hirst, 1999). Differences in emulsifying capacity can generally be attributed to the surface activity and / or to the size of the emulsifying agent: the higher the surface activity and / or the smaller the size, the greater the emulsifying capacity.

Emulsion size distribution profiles and micrographs (Figs. 4, 5) clearly indicated differences in emulsifying capacity which depended on the CA suspension used. The presence of small CAs facilitated the blending of the milkfat, making it possible to form emulsions with a smaller droplet size, and protected the emulsions against the appearance of bridging flocculation between the milkfat droplets (Fig. 8B).

The surface tension, γ, is characteristic of the surface activity of the CA, i.e. how effective CAs are at reducing unfavorable interactions between the milkfat and the suspension (McClements, 2005). For the concentrations used here, the surface tension measurements at the milkfat / CA suspension interfaces revealed that large micelle-like and small NaCas-like CAs had the same ability to reduce the unfavorable interactions between the two phases (5 – 6 mN m-1). All the samples had the same surface tension at equilibrium (obtained after 5 min) and hence the same surface coverage.

Our results, showing that there was no difference in equilibrium between our samples, differed from those of Courthaudon et al. (1999), who found that NaCas was more surface active than casein micelles. However, this strongly depends on the concentrations studied: in the study reported here we used a fairly high concentration (20 g kg^{-1}) and the interfacial layer was obtained at equilibrium by the combined adsorption of the free casein monomers, the NaCas-like CAs and the micelle-like CAs, which ruptured once adsorbed. Indeed, measurements at a concentration of 1.2 g kg^{-1} also provided the same surface tensions and rheological properties (whatever the state of aggregation – data not shown), meaning that at a concentration of 20 g kg^{-1} there was a large reservoir of proteins in the bulk, compared to the quantity that could be adsorbed. It is not possible from these measurements to simply ascribe the differences in emulsifying properties to different surface activities of the types of aggregates. Nevertheless, as we also report here, Courthaudon et al (1999), Ye (2011), Mulvihill & Murphy (1991) and Euston & Hirst (1999) established a correlation between the state of aggregation of the caseins and their emulsifying capacity.

For further analysis, it is important to note that we were not able to monitor the dynamics of adsorption at short timescales t (typically for t < 2 s). However, our results showed that the

surface tension had already decreased significantly during this short non-monitored period. There might therefore have been differences in the dynamics of adsorption between the samples at very short timescales (those having the highest concentrations of monomers reducing the surface tension more rapidly). In fact, the emulsion production process was rapid, and the associated timescale was also in the order of 1 s. Understanding the differences between emulsifying properties may therefore require monitoring of the surface coverage at such short timescales (less than 1 s). Many small, mobile casein units, such as casein monomers and NaCas-like CAs are thus available for rapid adsorption and to emulsify greater amounts of milkfat / suspension interface at high concentrations of Na_3Cit. In contrast, when not enough casein units were present in the suspension to adsorb on the generated interface rapidly (e.g. $E1^{ec}$), the milkfat droplets coalesced until all their surfaces were covered, thus making the emulsions coarser. Large micelle-like CAs also had the ability to share between two independent droplets and induce bridging flocculation (Fig. 8B).

4.2.2 Emulsions were stable against coalescence but creamed and flocculated

Destabilization of milkfat-in-water emulsions can result from three phenomena i.e. creaming, flocculation and coalescence. The E^{st} emulsions were designed to have identical droplet sizes, despite the differences in emulsifying capacity of the CA suspension used to prepare them. This approach removed the influence of the droplet size on the creaming, flocculation and coalescence phenomena. Visual observations (Fig. 6) and droplets size measurements (Fig. 7) as a function of time showed that the emulsions remained stable against coalescence throughout storage. However, emulsions were destabilized by creaming and flocculation (Fig. 7).

4.2.3 The adsorbed CAs contributed to coalescence stability whatever their state of aggregation.

The stability of the emulsions against coalescence is generally correlated with the characteristics of the CA layers adsorbed at the droplet surface. Interfacial casein concentrations and surface tension values provided information on the extent of casein adsorption at the interface, and dilational rheology determined how strongly proteins were adsorbed and interacted at the interface (McClements, 2005).

As with the surface tension data, the surface casein concentrations were also similar (17.4 mg m^{-2}) and independent of the type of CA used to form each emulsion. Our values were between

those found by Euston & Hirst (1999) on milk casein concentrate (21 mg m^{-2}) and Courthaudon et al. (1999) on casein micelles (10 mg m^{-2}). Our values were 6 to 8 times higher than the values reported for NaCas (2.3 mg m^{-2} by Euston & Hirst (1999), 3 mg m^{-2} by Dickinson, Golding & Povey (1997), 1 mg m^{-2} by Dickinson & Golding (1997) and 1.63 mg m^{-2} by Courthaudon et al (1999)). Our measurements thus fall within the highest reported values for interfacial concentration, and can be interpreted as a thick layer of adsorbed proteins, in agreement with the fact that we were using high protein concentrations, such that the interfacial properties did not depend on the concentration and that we had a large excess of proteins in bulk.

Dilatational rheology measurements demonstrated that adsorbed layers of CAs had similar solid-like behaviors (E' >> E'') whatever the state of aggregation of the casein used to form the emulsion. Large CAs spread out at the interface, and intermolecular interactions within the adsorbed layers were similar. The wide contribution of E' to E* (E >> E'') was in agreement with the literature on NaCas at diverse oil / water interfaces (Amine, Dreher, Helgason, & Tadros, 2014; Benjamins, Cagna, & Lucassen-Reynders, 1996). These two results suggested that the state of aggregation of the casein was not decisive for the stability of the emulsions against coalescence.

4.2.4 Creaming and flocculation enhanced each other

Creaming is due to the difference in density between the milkfat and the aqueous suspension phases of the emulsions. This phenomenon was enhanced by the large size of the individual milkfat droplets (12 µm). In our case, creaming (Fig. 6) and flocculation (Fig. 7) only occurred during the first week of storage, suggesting that these two concomitant phenomena influenced each other. On the one hand, creaming was intensified by the formation of milkfat droplet combination due to flocculation. On the other hand, flocculation was favored by the creaming that moved the droplets forward and encouraged their contact, which is a necessary step for the final destabilization of flocculation to occur (Dauphas, Amestoy, Llamas, Anton, & Riaublanc, 2008). However, the nature of the CA and the environment also had a role in the appearance of flocculation.

4.2.5 Unabsorbed CAs induced depletion-flocculation of the emulsion droplets

Depletion-flocculation is an instability mechanism that occurs in emulsions and is induced by the presence of unabsorbed particles. It takes place when two neighboring droplets are close

enough to exclude any unabsorbed particles from the gap that separates them. Consequently, an osmotic pressure gradient is induced that causes net attraction between the emulsion droplets (Asakura & Oosawa, 1958; Dickinson & Golding, 1997, 1998; Dickinson et al., 1997; Radford & Dickinson, 2004). This phenomenon was observed in our emulsions because of the presence of unabsorbed CAs.

The evolution of the milkfat droplet sizes in the emulsion as a function of time (Fig. 7) revealed that increases in percentage of small CAs in the emulsions augmented flocculation of the milkfat droplets. This was in agreement with the results obtained on native casein micelles, calcium-depleted casein micelles, Ca caseinate and NaCas (Dickinson & Golding, 1998; Euston & Hirst, 1999; Srinivasan, Singh, & Munro, 2001; Ye, 2011). These studies demonstrated that the depletion-flocculation process was strongly dependent on the state of aggregation of the casein. Furthermore, small CAs were of the optimum size (20 nm) to cause the greatest depletion-flocculation of emulsion droplets (Radford & Dickinson, 2004).

4.2.6 CA environment (mineral equilibrium and storage temperature) influenced the sticking of the emulsion droplets

Finally, storage temperatures higher than 37°C can induce gelation by flocculation of NaCas and β-casein emulsions if a sufficient amount of added Ca is present in the emulsion aqueous phase (Dauphas et al., 2008; Dickinson & Casanova, 1999; Dickinson & Eliot, 2003; Eliot & Dickinson, 2003). Added Ca reduces the steric repulsion between the emulsion droplets by binding to the adsorbed caseins, and high temperature encourages hydrophobic interactions between caseins and promotes sticking behavior (Dauphas et al., 2008; Dickinson & Casanova, 1999). However, no Ca was present in the diffusible phases of the emulsions as it was not present in the CA suspensions (Table 1). Moreover, the extent of flocculation increased when the colloidal Ca content of the CAs decreased. This suggested that Na ions reduced steric repulsion between the emulsion droplets in our system. This hypothesis was supported by the increased colloidal Na content in the CA suspensions (Table 1) and the constant ʒ values (23.1 ± 1.4 mV) measured on E^{st} emulsions throughout storage. Because of their highly aggregated state, large and strongly mineralized CAs were also less inclined to link with their counterparts adsorbed on separated milkfat droplets or suspended in the bulk emulsion phases.

5 Conclusion

Varying the concentration of added Na_3Cit in pure casein micelle suspensions produced four CA suspensions that were progressively demineralized and dissociated. The diffusible phases of these suspensions were monitored with a dialysis step. The use of these CAs as emulsifying agents in our model dairy emulsions revealed differences in emulsifying and emulsion-stabilizing properties. The smaller CAs had better emulsifying capacity as their presence favored the formation of emulsions with smaller droplet sizes. The surface activity of the four CA suspensions was similar and the differences in emulsifying capacity were attributed only to variation of the state of aggregation of the CAs. With regard to the stabilizing capacity of the CAs, all the emulsions were unstable under our storage conditions (21 days, 50°C). Creaming was promoted by the presence of large droplets in the emulsions and favored the occurrence of flocculated droplets. Flocculation was also enhanced by the presence of small, demineralized CAs. However, all the emulsions remained stable against coalescence during storage. This was probably due to the presence of similar quantities of adsorbed CAs at the surface of the emulsion droplets that formed protective layers with similar viscoelastic properties. Combining the results obtained on the CAs in suspension with the emulsion properties revealed that the state of aggregation of the CAs had a major impact on their emulsifying capacity and emulsion-stabilizing properties. Modulating the mineral content of the casein micelle is therefore an interesting method for the optimization of emulsion functionality. Further studies on CA composition and nanostructure, both in suspension and adsorbed at the milkfat / water interface, would improve understanding of the differences between the emulsifying and emulsion-stabilizing properties. In this case, the destabilization of the emulsions in the early stages should be studied for a better understanding of the involved phenomena. As an extension of this work, investigation of the rheology of the creamed layers of the emulsions is planned as well as the assessment of other functionalities of newly formed CAs.

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Part B:

Rennet coagulation functionality

PART B, CHAPTER 1:

RENNET COAGULATION PROPERTIES OF CASEIN MICELLES ARE AFFECTED BY THE INTERDEPENDENCE OF ENVIRONMENTAL FACTORS UPON MULTIFACTORIAL MODIFICATION OF THEIR MINERAL BALANCE.

This chapter will be submitted to Food Hydrocolloids

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Abstract

Milk minerals play decisive roles in monitoring casein micelles functionalities. Their mineral balance can be adjusted by changes in some environmental factors such as pH, salts or chelating agents additions. The individual effects of these factors on milk rennet coagulation are already well documented. Nevertheless, the manufacture of cheese often implies simultaneous modifications, and knowledge about this multifactorial complexity is still lacking.

The objective of the present study was to understand how the multifactorial modifications of the milk mineral fraction affected the rennet coagulation properties of the casein micelles.

A fractional experimental design was produced to study the impact of five factors: variations in pH, additions of Na_3Cit as well as NaCl, $CaCl_2$ and $MgCl_2$. In total, 42 suspensions of pure casein micelles in different environments were produced and characterized. Their abilities to coagulate following rennet addition were assessed by measuring two characteristics: the rennet clotting time and the gels firmness. Appropriated statistical treatments enabled to highlight the relationships

existing between the factors and the mineral balance, the size and the rennet coagulation properties of the casein micelle. The calcium phosphte content of the casein micelles was solubilized by a decrease in pH and an increase Na_3Cit concentration while only this latter factor caused the disruption of the colloids. The addition of salts caused variations in the diffusible minerals concentrations. The establishment of predictive models of the rennet clotting time and gel firmness demonstrated their dependence to variation in pH and Na_3Cit concentrations, through direct and interacting effects.

Keywords: functionality, multifactorial modifications, pH, tri sodium citrate, sodium chloride, calcium chloride, magnesium chloride, principal component analysis, predictive model

1 Introduction

Casein micelles are colloidal particles that consist of complex associations of four casein molecules (α_{s1}, α_{s2}, β and κ) and minerals, mainly calcium (Ca) and inorganic phosphate (Pi). The colloids present in milk are highly hydrated (3 - 4 g of water g^{-1} of dry matter, de Kruif & Holt, 2003), negatively charged (-20 mV, Dalgleish, 1984) and are polydisperse in size, with mean diameters varying from 50 to 400 nm (de Kruif, 1998; McSweeney & Fox, 2013; Udabage, McKinnon, & Augustin, 2003). Their average composition consist of 11.8 % of κ-casein present at the surface of the micelle and 36.5 % of α_{s1} and β-caseins, 9.1 % of α_{s2}-casein and 6 % of minerals in dry matters that compose the interior of the micelle (Walstra, 1990). The colloidal minerals form the so-called calcium phosphate (CaP) nanoclusters that play a key role in maintaining the caseins together via mineral-proteins electrostatic interactions, along with protein-protein hydrophobic interactions (Horne, 1998, 2017). Casein micelles are components of major importance for the processing of milk as they confer interesting functional properties, such as thermal stability, emulsifying and foaming properties, and gelation properties (Broyard & Gaucheron, 2015). Interestingly, the functional properties of the colloids can be tailored in modifying their mineral balance. This can be achieved by means of environmental changes such as variations in pressure, temperature, pH or removal and addition of salts and chelating agents (Broyard & Gaucheron, 2015; de Kort, Minor, Snoeren, van Hooijdonk, & van der Linden, 2011; Grufferty & Fox, 1985; Huppertz & Fox, 2006; Lazzaro et al., 2017; Le Graët & Gaucheron, 1999; Silva et al., 2013).

The present study focuses on the rennet coagulation properties of the casein micelles. The impact of the monofactorial modifications of the environmental factors on these properties have been largely studied within the past 50 years. Briefly, acidification was reported to enhance the enzymatic reaction and led to a decrease in the rennet clotting time (RCT) (Choi, Horne, & Lucey, 2007; Daviau, Famelart, Pierre, Goudédranche, & Maubois, 2000; Famelart, Lepesant, Gaucheron, Le Graët, & Schuck, 1996), while a decrease in pH had a quadratic influence on the gel firmness (Choi et al., 2007; Daviau et al., 2000; Zoon, van Vliet, & Walstra, 1989). The additions of divalent cations, through $CaCl_2$ and $MgCl_2$ supplementations, cause a decrease in RCT with formation of stronger gels (Cooke & McSweeney, 2014; McMahon, Brown, Richardson, & Ernstrom, 1984; Sandra, Ho, Alexander, & Corredig, 2012; Udabage, McKinnon, & Augustin, 2001; van Hooydonk, Hagedoorn, & Boerrigter, 1986; Zoon, van Vliet, & Walstra, 1988), while NaCl addition had opposite effects (Bulca, Wolfschoon-Pombo, & Kulozik, 2016; Famelart, Le Graët, & Raulot, 1999; Grufferty & Fox, 1985; Karlsson, Ipsen, & Ardö, 2007;

Sbodio, Tercero, Coutaz, & Revelli, 2006; Zhao & Corredig, 2015; Zoon et al., 1989). Finally, Udabage, McKinnon, & Augustin, (2001) observed a complete inhibition of the coagulation of milk following the addition of Na_3Cit. However, the reality of cheese manufacture often implies simultaneous modifications of several of these factors. Knowledge is currently lacking on the interactions between the different modifications that might affect both the mineral balance and the functional properties of the casein micelles.

The aim of the present study was to understand how the multifactorial modifications of the casein micelles environment influences their mineral balance and their rennet coagulation properties. The concomitant impacts of five environmental factors: variations in pH (acidification and alkalinisation), additions of three different salts (NaCl, monovalent cation and $CaCl_2$ and $MgCl_2$, divalent cations) and one chelating agent (Na_3Cit), on model suspensions of casein micelles were studied through the carrying out of a partial experimental design. In total, 42 different suspensions of casein micelles in different environment were produced and characterized. The results obtained were subjected to appropriated statistical analyses in order to highlight the correlations existing between the different factors and the coagulating properties of the casein micelles. The establishment of predictive quadratic models of RCT and the gel firmness enabled to assess the relative importance of each factors and their interactions on the coagulating properties of the casein micelles.

2 Materials and methods

2.1 Chemicals

Hydrochloric acid (HCl) and sodium hydroxide (NaOH) used for pH adjustment were supplied by VWR chemicals (Fontenay-sous-bois, France), and VWR international (Leuven, Belgium), respectively. NaCl, $CaCl_2$ and $MgCl_2$ salts used for the enrichment of the suspensions were supplied by Panreac AppliChem (Barcelone, Spain), VWR International (Leuven, Belgium) and Fisher Scientific (Loughborough, United Kingdom), respectively. The chelating agent (Na_3Cit) was supplied by Carlo Erba reagent (Val de Reuil, France). Sodium azide (NaN_3), supplied by Riedek-de Haën (Seelze, Germany), was used for preservative reasons. The chemicals used for this study were of analytical grade.

2.2 Materials

Native phosphocaseinate (NPC) powder was used as model ingredient of the native casein micelles. Concentrated NPC was supplied by Gillot SAS (Saint Hilaire de Briouze, France) and obtained by microfiltration (0.1 µm pore size membrane) of raw skimmed milk followed by diafiltration against milli-Q water. The concentrate was then spray dried according to the method described by Pierre, Fauquant, Le Graët, & Maubois (1992) and Schuck et al., (1994) using Bionov facilities (Rennes, France). Caseins and their associated minerals represented more than 90 % of the total solid content of the powder. Residual whey proteins (3 %) (w / w) and traces of lactose were present in the powder.

Pure commercial chymosin (CHY-MAX M 200, 200 IMCU ml^{-1}, Chr Hansen, Hoersholm, Denmark) was used to coagulate the NPC suspensions.

2.3 Experimental design

Six levels of pH (5.7, 6.1, 6.5, 6.9, 7.3 and 7.7), three levels of Na_3Cit concentration (0, 15 and 30 mmol kg^{-1}), three levels of NaCl concentration (0, 50 and 100 mmol kg^{-1}) and three levels of $CaCl_2$ and $MgCl_2$ concentrations (0, 7.5 and 15 mmol kg^{-1}) were studied. Considering each of the five factors at each level give a total of 486 different combinations, thus making impossible to carry out of the full experimental design in a reasonable time. Statgraphics Centurion XVII software (V. 17.1.10) was therefore used to defined a fractional factorial design that enabled the

establishment of quadratic models that take into account the second order interactions between the factors. Forty-two suspensions (Table 1) were selected out of the 486 combinations using a "top-down" approach and an exchange algorithm that optimized the I-efficiency criteria. A control casein micelle suspension (CTRL, Table 1), at pH 6.9 and without salt or chelating agent additions was produced for comparison. This suspension was not part of the experimental design, and did not participate in the construction of the principal component analysis (PCA) or for the establishment of the RCT and firmness models (section 2.6.6).

2.4 Preparation of the suspensions of casein micelles in different environments

The NCP powder was dispersed in milli-Q water following the method defined in Lazzaro et al. (2017) (Part A). Stock solutions of Na_3Cit (0.25 mol kg^{-1}), NaCl (2.5 mol kg^{-1}), $CaCl_2$ (0.25 mol kg^{-1}) and $MgCl_2$ (0.25 mol kg^{-1}) were prepared in milli-Q water and their pH were set to 6.9 using HCl (1 and 0.1 M) and NaOH (1 and 0.1 M). Different amounts of stock solutions were mixed together in such a way that after the dropwise addition of these mixtures to slightly concentrated NPC suspensions (28 g kg^{-1}), and after a dilution step, the final concentrations in salts and chelating agent targeted were reached (Table 1). The suspensions were stirred for 30 min and the pH variations due to the salts and chelating agent additions corrected to the desired pH (Table 1) using HCl (1 M) and NaOH (1 M). The suspensions were then diluted using milli-Q water to 24.1 ± 0.4 g kg^{-1} of casein, left overnight at room temperature and the pH was readjusted if necessary in the morning.

2.5 Recovery of the diffusible phases of the suspensions

The NPC suspensions were utlrafiltered on Vivaspin concentrators (molecular weight cut-off 10 kDa, Vivascience, Palaiseau, France) to recover their diffusible phases. The Vivaspin concentrators containing 15 mL of suspension were centrifuged for 30 min, at 20°C and 1800 g. The recovered diffusible phases were used for the determination of diffusible ions concentrations, for the dilutions of the suspensions for the turbidity (τ) and dynamic light scattering (DLS) measurements.

2.6 Analysis

2.6.1 Protein content

The total nitrogen content of each suspension was determined according to the Kjeldahl method (IDF standard 20-1, 2014). A factor of 6.38 was used to convert nitrogen to protein concentration. Measurements were performed in duplicate

2.6.2 Mineral composition and distribution

Total and diffusible cations (Ca, sodium - Na, magnesium - Mg) and anions (Pi, citrate - Cit, chloride - Cl) contents were determined as described in Lazzaro et al. (2017) (Part A). Colloidal concentrations were deduced by subtracting the concentration of diffusible ions from the concentration of total ions.

2.6.3 Turbidity measurements

Absorbance measurements were carried out at 600 nm and 20℃ using a UV-visible spectrometer (UVmc², Safas, Monaco). The casein micelle suspensions were diluted ten times in their diffusible phases and analyzed immediately. The diffusible phase of each suspensions was also analyzed. Absorbance measurements were converted into τ according to the following formula:

$$\tau = 10 \times 2.303 * \frac{OD(600nm)}{l}$$

with OD(600nm) being the optical density of the suspension (difference between the absorbance of the diluted suspension and the absorbance of its diffusible phase) ; and l the light path length (l = 1 cm).

2.6.4 Dynamic light scattering

DLS measurement were performed as described by Silva et al. (2013) to determine the hydrodynamic diameter of the casein micelles in suspensions. The suspensions were diluted ten times in their own diffusible phase and filtered on a membrane with pore size of 0.45 µm to

eliminate dusty particles. The measurements were carried out at 20°C. The size distribution profiles expressed in number were monomodal for each of the suspensions (data not shown), therefore the mean diameter (d_{mean}) was chose as a quantitative summary of the size information for the statistical analyses.

2.6.5 Rennet coagulation properties

The coagulation properties of samples were assessed using a ChymoGRAPH® (Chr Hansen, Denmark); this method is based on the physical principal from the Formagraph (McMahon & Brown, 1982). In both cases, the coagulation was determined according to the movement of stainless steel, loop pendulums immersed in the coagulating samples. Ten grams of casein micelle suspensions were weighed into the wells of the ChymoGRAPH® stainless steel block. The block, containing four suspensions in total (two wells per suspension), was immersed in a water bath for 10 min to equilibrate the temperature of the suspension to 30 °C. A sample of 400 µL of the enzyme solution (chymosin diluted ten times in milli-Q water) was pipetted into a multiple spoon apparatus to simultaneously disperse the chymosin in the suspensions. The suspensions were then stirred for 30 s with the spoons and transferred from the water bath to the oscillating plate of the ChymoGRAPH®. A Peltier module maintained the temperature of the block at 30°C. When the coagulation began, the resulting increase in viscosity and the formation of gels caused the pendulums to oscillate together with the samples. The movement of each pendulum was measured by the use of optical fibers over a period of 60 min and the data collected in ChymoGRAPH® software (V 1.0, Chr Hansen, Denmark).

The RCT corresponded to the time elapsed from chymosin addition to the detectable onset of coagulation, where coagulation was defined at the time point when the firmness of the suspensions was > 0. The maximal firmness recorded during the 60 min duration of the experiment was defined as the firmness of the gel. In the case where a suspension did not coagulate, the RCT value was arbitrary defined to 60 min, which corresponds to the longest time for which the firmness have been recorded.

The calculation of average, SD, RSD, maximum and minimum values for RCT and firmness, reported in Table 3, were determined on the set of 15 suspensions that coagulate within 60 min.

2.7 Statistical treatments

Fourty-two suspensions were produced in the framework of a fractional experimental design to study the impact simultaneous variation of five environmental factors (pH, Na_3Cit, NaCl, $CaCl_2$, $MgCl_2$) on the mineral balance, the size and the renneting properties of the casein micelles. The data set was subjected to statistical treatments by PCA. The Facto-MineR package and the R software (Lê, Josse, & Husson, 2008; R Development Core Team 2011) were used. This method was described in details elsewhere (Abdi & Williams, 2010; Jolliffe, 2014; Wold, Esbensen, & Geladi, 1987). Briefly, PCA summarizes a data set of n individuals (e.g. the 42 suspensions) described by m quantitative variables (e.g. τ, colloidal Ca, firmness) by defining a new set of p variables called principal components (PCs), with $p < m$. The PCs are orthogonal (uncorrelated) and consist of linear combinations of the original variables. The outputs are displays of the similarities of the individuals and projection of the original variables in a new p-dimensional space. In this study, PCA was used to highlight the correlations between the different measurements. pH, Na_3Cit, NaCl, $CaCl_2$ and $MgCl_2$ variables were used as illustrative variables in the PCA. All the correlations mentioned in section 3 were found to be significant ($p < 0.05$) using the paired student t-test.

In addition, the RCT and firmness experimental data were submitted to a modeling stage. Regression analysis was used to fit experimental data to the following second-order polynomial equation, that takes into account the quadratic effects and second order interactions between the experimental design factors:

$$y = constante + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} {x^2}_i + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} b_{ij} x_i x_j$$

where y was either RCT or firmness, xi were pH, Na_3Cit, NaCl, $CaCl_2$, $MgCl_2$, and bi, bii and bij were the coefficients of the models. The models were refined using stepwise regression procedure to eliminate the non-significant contributions ($p > 0.05$). Statistical analysis was performed using the software STATGRAPHICS Centurion XVII (V. 17.1.10, Statpoint Technologies, The Plains, USA).

Table 1: list and mineral composition of the suspensions studied. Concentrations in salts and chelating agent are expressed in mmol kg^{-1}. Abbreviation CTRL corresponds to control sample defined as micellar suspension without added minerals

suspension	pH	Na_3cit	NaCl	$CaCl_2$	$MgCl_2$	suspension	pH	Na_3cit	NaCl	$CaCl_2$	$MgCl_2$
1	7.7	30	50	15	15	23	5.7	0	100	0	0
2	5.7	30	0	0	7.5	24	7.7	0	0	0	0
3	5.7	0	0	7.5	0	25	6.9	0	0	0	15
4	5.7	15	100	15	0	26	7.7	30	100	0	0
5	5.7	30	100	0	0	27	7.7	0	0	7.5	15
6	5.7	30	50	15	0	28	5.7	15	0	0	0
7	7.7	30	0	7.5	0	29	7.7	30	100	15	15
8	5.7	30	100	15	7.5	30	6.5	0	0	15	7.5
9	5.7	30	50	0	15	31	7.7	30	50	0	7.5
10	7.7	15	100	0	15	32	5.7	0	0	0	15
11	7.7	0	100	0	7.5	33	6.9	0	50	0	0
12	5.7	30	0	15	15	34	7.7	0	100	0	15
13	6.9	15	50	7.5	7.5	35	5.7	0	100	15	15
14	7.7	30	100	15	0	36	6.5	30	0	0	0
15	6.9	0	100	15	0	37	5.7	0	0	15	0
16	7.7	0	0	15	7.5	38	5.7	30	100	0	15
17	5.7	0	50	15	15	39	7.7	0	100	7.5	0
18	5.7	0	100	0	15	40	5.7	15	0	15	15
19	6.5	30	0	15	0	41	7.7	30	0	15	15
20	7.7	0	100	15	15	42	7.7	15	0	15	0
21	7.7	30	0	0	15	CTRL	6.9	0	0	0	0
22	6.5	30	100	7.5	15						

3 Results and discussion

In this section, PCA (Figs. 1 and 2) was first used to appreciate the relationships existing between the multifactorial modifications of the environment of the suspensions and i) the mineral balance and the size-related properties of the casein micelles and ii) their coagulation properties, i.e. the RCT and gel firmness. Figure 1 shows the correlation circle of the variables in the planes defined by PCs 1:2 (A) and 1:3 (B), respectively. Figure 2 is a representation of the CTRL and the 42 suspensions in the plane defined by the two first PCs. The sum of the first three PCs explained 69.1 % of the dataset variability.

Regression analysis was applied in a second time to predict and understand the rennet coagulation characteristics of the casein gels (RCT and firmness) as a function of the five environmental factors (variation in pH, addition of Na_3Cit, NaCl, $CaCl_2$ and $MgCl_2$) (section 2.6.6).

3.1 Mineral and size-related properties of the modified casein micelles

3.1.1 Mineral balance between colloidal and diffusible phases

Prior any salts or chelating agent additions, the casein micelle suspensions (CTRL) contained essentially Ca and Pi, traces of Mg and no Cit (Table 2, CTRL suspension). The small amounts of Na and Cl were residual ions initially present in the NPC powder and were also due to the use of NaOH (1 M) and HCl (1 M) in order to set the pH of the CTRL suspension to 6.9 (see section 2.4). An overview of the mineral distributions within the all set of suspensions (Table 2) showed that the colloidal ions were mainly Ca and Pi, while Na, Cl and Cit ions were primarily present as diffusible forms. Addition of $MgCl_2$ led to the presence of Mg ions in both colloidal and diffusible phases.

Table 2: Distribution of the mineral salts in the suspensions. Colloidal concentrations were determined by subtracting diffusible from total ion concentrations. Concentrations are expressed in mmol kg^{-1}. Average, standard deviation (SD), relative standard deviation (RSD), minimum and maximum values were determined on the complete set of 42 suspensions. CTRL corresponds to pH 6.9, with no added salts.

	CTRL	Average	SD	RSD (%)	minimum	maximum
	supplementary	Full set of samples (42 suspensions)				
Diffusible Ca	1.4	14.0	7.9	56.5	0.0	31.7
Colloidal Ca	14.0	13.3	7.4	55.1	0.5	27.9
Diffusible Pi	1.8	3.6	2.8	76.8	0.0	8.8
Colloidal Pi	3.3	3.9	2.7	69.8	0.0	8.3
Diffusible Na	6.0	100.3	62.5	62.4	4.2	222.9
Colloidal Na	1.4	8.0	17.0	212.7	0.0	91.3
Diffusible Cit	0.0	13.5	13.1	97.6	0.0	32.0
Colloidal Cit	0.0	0.9	1.9	195.4	0.0	8.9
Diffusible Mg	0.0	6.6	6.0	91.7	0.0	15.7
Colloidal Mg	0.7	2.1	2.2	101.4	0.0	8.3
Diffusible Cl	6.0	88.8	50.5	56.9	0.3	174.7
Colloidal Cl	0.8	3.7	8.8	240.6	0.0	50.5

Figure 1.A showed that colloidal concentrations of Ca and Pi significantly and positively correlated with pH with coefficients of 0.53 and 0.67, respectively, while the diffusible concentrations of these ions were negatively impacted by this factor (significant coefficients of -0.53 and -0.69, respectively). These results confirmed that a decrease in pH induced the solubilization of CaP nanoclusters. These results were in accordance with those described by numerous authors (Choi, Horne, & Lucey, 2007; Dalgleish & Law, 1989; Daviau, Famelart, Pierre, Goudedranche, & Maubois, 2000; Famelart, Lepesant, Gaucheron, Le Graët, & Schuck, 1996; Gastaldi, Lagaude, Marchesseau, & Fuente, 1997; Le Graët & Brulé, 1993; Le Graët & Gaucheron, 1999; Silva et al., 2013; van Hooijdonk, Hagedoorn, & Boerrigter, 1986; Zoon, van Vliet, & Walstra, 1989).

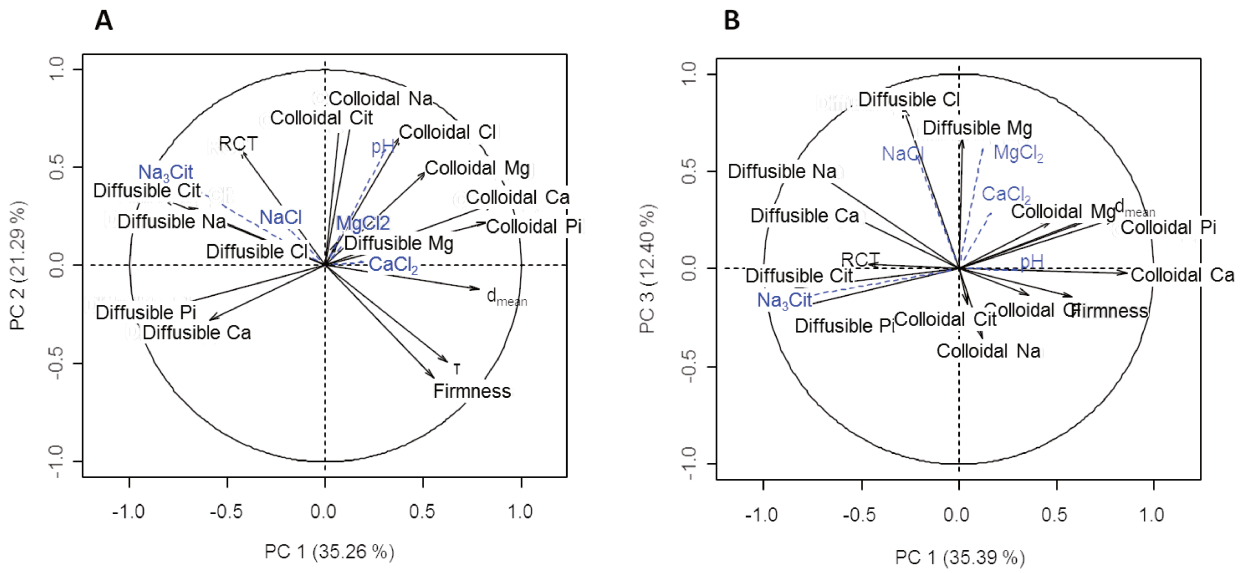

Figure 1: PCA results. A. PCA showing the correlation circle in the plane defined by the two first PCs. B. PCA showing the correlation circle in the plane defined by the first and third PCs.

The mineral content of the casein micelle was also affected by the addition of Na_3Cit, as shown by the coefficients of -0.47 and -0.60 between this factor and the colloidal concentrations of Ca and Pi, respectively (Fig. 1.A). The diffusible concentrations of these ions therefore positively correlated with Na_3Cit (0.43 and 0.57, respectively). Cit ions were responsible for shifting the mineral balance by chelating the Ca ions present in the diffusible phase, which led to the solubilization of the micellar Ca present in the CaP nanoclusters, but also bound to the phosphoseryl residues of the caseins. The dissolution of the CaP nanoclusters consequently induced the solubilization of Pi. These results were in accordance with the findings of numerous authors that also added Cit in milk or casein micelle suspensions (Le Ray et al., 1998; Mizuno & Lucey, 2005; Mohammad & Fox, 1983; Morr, 1967; Ozcan-Yilsay, Lee, Horne, & Lucey, 2007; Udabage, McKinnon, & Augustin, 2000; Vujicic, deMan, & Woodrow, 1968).

Finally, colloidal and diffusible Ca concentrations slightly increased with the addition of $CaCl_2$, as indicated by the significant correlation coefficients of 0.37 and 0.54, respectively. On its part, $MgCl_2$ influenced the colloidal and diffusible concentrations of Mg (Fig. 1.B). The addition of this salt resulted in an increase of Mg concentration in both phases, with correlation coefficients of 0.71 and 0.96, respectively. These results were in agreement with the findings of Cooke & McSweeney 2014; Le Ray et al. 1998; Philippe, Gaucheron, Le Graët, Michel, & Garem 2003; Philippe, Le Graët, & Gaucheron 2005; van Hooydonk, Hagedoorn, & Boerrigter 1986b that

reported an increase in colloidal Ca and Mg caused by the addition of $CaCl_2$ and $MgCl_2$, respectively. These authors also mentioned an increase in colloidal concentrations of Pi and Cit following the addition of the two salts due to a coprecipitation of Ca and Mg with these anions, which formed supplementary insoluble salts. However, no significant correlations were found between $CaCl_2$ and $MgCl_2$ with diffusible or colloidal Pi nor Cit, suggesting that the added divalent ions would directly associate with caseins. Such associations would occur via monoester phosphate groups on seryl and threonyl residues, carboxylic groups of aspartic acid and glutamic acid (Dickson & Perkins, 1971) and / or phenolic, sulfhydryl and imidazole groups (Gaucheron, Le Graët, Boyaval, & Piot, 1997). Philippe et al. (2005) observed that 82 % of added Ca were associated to the caseins, while only 25 % of Mg were associated with caseins. According to these authors, this difference was explained by the fact that magnesium phosphate or magnesium citrate salts were not at saturation in milk unlike phosphate salts. This could also be due to a stronger affinity of the caseins for Ca (Baumy & Brulé, 1988; Gaucheron et al., 1997). A similar observation was not obvious in the present study, probably because the effects of $CaCl_2$ and $MgCl_2$ additions on the mineral content of the casein micelle were dominated by the variations in pH and the addition of Na_3Cit.

NaCl addition presented no correlations with colloidal ions while a displacement of some micellar Ca by Na could be expected, as observed by Aoki, Umeda, & Nakao 1999; Famelart et al. 1996; Grufferty & Fox 1985; Zhao & Corredig 2015; Zoon et al. 1989. This discrepancy could arise from the facts that the amount of added NaCl was three to five times lower, and that pH deviation induced by the salt addition was controlled in the present study, compared to the works performed by the above-cited authors. The addition of NaCl was responsible for the increases in diffusible Na (0.76) and Cl (0.89) concentrations and Na_3Cit caused the diffusible concentrations of Na (0.60) and Cit (0.98) to increase (Fig. 1B). Surprisingly, $CaCl_2$ and $MgCl_2$ did not correlate with the diffusible Cl concentration although these salts contributed to the enrichment of the suspensions in this ion (Fig. 1B). The concentrations ranges defined in the experimental design (see section 2.3) could explain this absence of correlation. Indeed, the addition of the divalent cations caused a 30 mmol kg^{-1} increase in diffusible Cl only while addition of NaCl was responsible for an increase up to 100 mmol kg^{-1} of Cl. The monovalent salt therefore had a dominant effect on the concentration of diffusible Cl.

3.1.2 Size-related properties

Casein micelles were the only components that can affect τ and the DLS of the suspensions. Interpretation of the DLS measurements gave access to d_{mean} (the mean diameter of the casein micelles - see section materials and methods 2.6.4) while, according to Karlsson, Ipsen, Schrader, & Ardö (2005) and Zhao & Corredig (2016), any change in τ would be caused by a modification of the size and / or the internal structure of the micelles. The relationship existing between τ and d_{mean} was confirmed by the positive correlation coefficient (0.56, Fig. 1A.).

Table 3 reports the variations of τ and dmean within the all set of sample, with RSD values of 56 and 44.0 %, respectively. The CTRL suspension contained micelles with dmean of 121 nm in average while the smallest particles had a d_{mean} of 20 nm (suspension 36 - Table 1). The largest micelles were found in suspension 37 (Table 1) with a d_{mean} of 147 nm. The turbidities of the suspensions varied from 0.3 (suspension 36) to 39.4 (suspension 37 – Table 1).

Table 3: Size related parameters. DLS size distributions were monomodal for all the suspensions prepared for the present study (data not shown). Mean diameter values (d_{mean}) were used as quantitative data to summarize the size distributions. Average, standand deviation (SD), relative standard deviation (RSD), minimum and maximum values were determined on the complete set of 42 suspensions. CTRL corresponds to pH 6.9, with no added salts.

	CTRL	Average	SD	RSD (%)	minimum	maximum
	supplementary	Full set of samples (42 suspensions)				
d_{mean} (nm)	121	103	45	44.0	20	147
τ (cm^{-1})	15.1	14.8	8.3	56.3	0.3	39.4

As shown in Figure 1A, τ and d_{mean} were strongly affected by the addition of Na_3Cit, with correlation coefficients of -0.70 and -0.64, respectively. The size-related variables also negatively correlated with the diffusible concentrations of Na and Cit (-0.69 and -0.43 for τ and -0.68 and -0.42 for d_{mean}), which was a direct consequence of the presence of the chelating agent in the diffusible phase. Finally, τ and d_{mean} significantly correlated with the colloidal concentrations of Ca and Pi (0.36 and 0.39 for τ and 0.60 and 0.65 for d_{mean}, respectively) and with the diffusible concentration of Pi (-0.37 and -0.66, respectively). These correlations indicated that the casein micelles size, and possibly their internal structure, were related to their

CaP content. Indeed, the addition of Na_3Cit induced the solubilization of the micellar CaP (see section 3.1.1), causing the exposure of phosphoseryl residues. The resulting increase in electrostatic repulsions between caseins led to the disruption of the casein micelles and therefore a decrease in micellar size and τ of the suspensions (Mizuno & Lucey, 2005). This phenomenon was previously observed by numerous authors that also reported decreases in τ or in micellar diameter (de Kort, Minor, Snoeren, van Hooijdonk, & van der Linden, 2011; Lazzaro et al. 2017 (Part A); McCarthy et al., 2017; Mizuno & Lucey, 2005; Udabage et al., 2000). The disruption of the casein micelle caused by the Cit chelation of colloidal Ca was also confirmed by the increase of non-sedimentable proteins in ultracentrifugal supernatants (de Kort, Minor, Snoeren, van Hooijdonk, & van der Linden, 2011; Johnston & Murphy, 1992; Le Ray et al., 1998; Mohammad & Fox, 1983; Morr, 1967; Udabage et al., 2000).

As mentioned in section 3.1.1, decrease in pH also led to the solubilizations of the colloidal Ca and Pi. However, no direct correlations were found between pH and dmean or τ. Although the pH-induced solubilization of the colloidal minerals were unequivocal, the impact on the micellar size remained unclear. Indeed, Daviau et al. (2000) and van Hooijdonk et al. (1986) reported a swelling of the casein micelles during acidification of fresh skim milk, while Famelart et al., (1996), Moitzi, Menzel, Schurtenberger, & Stradner, (2011) and Silva et al., (2013), observed a decrease in the micellar diameter. On the other side, alkalinization of milk led to the small increase in micellar diameter (Day, Raynes, Leis, Liu, & Williams, 2017) or the disruption of the micelles (Vaia, Smiddy, Kelly, & Huppertz, 2006). In the present study, the results demonstrated that under multifactorial modifications, the casein micelles size and the τ of the suspensions were affected by the use of the chelating agent. The consequences of variations in pH on the disruption of the micelles certainly depended on the other factors and on the range of pH studied.

The size of the casein micelles and the τ of the suspension showed no dependences with the addition of salts. This result was in agreement with the observations of Philippe et al. (2003, 2005) that reported no evolution of the size of the casein micelle following the addition of $CaCl_2$ and $MgCl_2$. However, it was not in agreement with the observations of Karlsson et al., (2005), Lombardi et al. (2016) and Zhao & Corredig (2015) that reported either an increase of the micellar size upon the addition of $CaCl_2$ and $MgCl_2$ or NaCl and a decrease in τ in presence of NaCl. These discrepancies could arise from the facts that the pH deviations induced by the additions of salts were not corrected in these studies, or that the effects of the salts additions would be dominated by the impact of Na_3Cit addition in the present study.

3.2 Rennet coagulation properties

Table 4 gives an overview of the rennet coagulation properties (RCT and firmness) for the set of the 15 suspensions that coagulated. For the CTRL sample, the RCT and firmness were 8.4 min and 18.8 AU, respectively. In average, the RCT was of 9.3 min and varied from 1.0 to 34.2 min. The firmness of the gels ranged from 3.6 to 20.6 A.U, with an average value of 12.3 A.U.

Table 4: Rennet coagulation properties. Average, standard deviation (SD), relative standard deviation (RSD), minimum and maximum values were determined only on the set of 15 suspensions that coagulated (Firmness > 0). CTRL corresponds to pH 6.9, with no added salts.

	CTRL	Average	SD	RSD (%)	minimum	maximum
	supplementary	Set of coagulating samples (15/42 suspensions)				
RCT (min)	8.4	9.3	10.3	110.4	1.0	34.2
Firmness (A.U.)	18.8	12.3	5.9	47.8	3.6	20.6

3.2.1 Results of PCA

Results of PCA (Fig. 1A) showed a strong correlation (-0.89) between RCT and firmness variables, which indicated that rapid coagulation of the suspensions led to the formation of strong casein gels, and vice versa. The colloidal properties of the casein micelles (size and / or internal structure determined by DLS and τ) showed significant correlations with the RCT and the firmness of the gels (Fig. 1A). Thus, decrease in d_{mean} or τ corresponded to the formation of weaker gels (correlation coefficient with firmness of 0.42 and 0.66, respectively), in longer times (correlation coefficient with RCT of -0.46 and -0.55, respectively). This relationship between the firmness and the micellar size was opposite to the findings of Ford & Grandison (1986), Glantz, Håkansson, Lindmark Månsson, Paulsson, & Nilsson (2010), Logan et al. (2015) and Niki, Kohyama, Sano, & Nishinari (1994) who reported that the use of small micelles led to the formation of stronger rennet gels. Regarding the variation of RCT, the correlation was consistent with the observations of Ekstrand (1980) but opposite to the findings of Ford & Grandison, (1986) that reported a shorter RCT when small micelles were submitted to rennet coagulation. The discrepancies between these observations can arise from the fact that in the previous studies, the casein micelles were very close to their native state while in the present study the micelles underwent environmental modifications (Table 1). As mentioned in section 3.1.2, the

solubilization of the micellar CaP by addition of Na_3Cit caused the decrease in size of the casein micelles. The CaP depletion of the casein micelles was also affected by pH decrease. Interestingly, these two modifying factors also correlated with the rennet coagulating properties, as indicated by the correlations coefficients linking the variations in pH and the addition of Na_3Cit to the RCT or the gel firmness (Figure 1A). The RCT increased with an increase in pH or in Na_3Cit concentration (correlation coefficients of 0.57 and 0.53, respectively) while the firmness of the gel decreased (correlation coefficients of -0.44 and -0.57, respectively). These results were consistent with Choi et al. (2007), Daviau et al. (2000) and Famelart et al. (1996) who also observed a decrease of RCT with the pH decrease. The variation of the firmness with decreasing pH was not linear, according to Choi et al. (2007), Daviau et al. (2000) and Zoon et al. (1989) and will be discussed in the following section (3.2.2). Udabage, McKinnon, & Augustin (2001) observed an increase in RCT and a reduction of the storage modulus of the gels with the addition of Na_3Cit, and even a complete inhibition of the gelation of milk above 10 mmol kg^{-1} of added chelating agent. At this stage, the mechanisms responsible for the variations in RCT and firmness are still unclear. However the correlations underlying the implications of the micellar mineral content, size and structure (through the measurement of τ), gave potential for their better understanding. Further investigations of the colloidal properties of the casein micelles, e.g. through the characterization of their internal structure, would bring valuable information to solve this mechanistic question. Indeed, it seemed reasonable to consider that the protein-protein and protein-mineral interactions responsible for the casein micelles cohesion would also be responsible for the formation and firming of the rennet gels.

Representation of the different suspensions in the two first PCs planes (Fig. 2) showed clear separations along the Na_3Cit and pH axes between the 15 coagulating (green) and 27 non-coagulating suspensions (black). Therefore, rennet gels formation was only observable under sufficiently low pH and Na_3Cit concentration. For example, suspensions did not coagulate for pH above 6.9 (e.g. suspensions N° 29, 31, 42) and only one suspension coagulate in the presence of 30 mmol kg^{-1} of added Na_3Cit (suspension N°12). However, the presence of these coagulating and non-coagulating suspensions at given Na_3Cit concentrations (e.g. samples N°4, 13 vs 10, 42, 28) or pH values (e.g. samples N°3, 18, 12 vs 28, 9, 38) in Figure 2, made impossible to define thresholds above which the coagulation would be inhibited, given that both factors must be considered. This demonstrated the existence of an interaction between the factors pH and Na_3Cit.

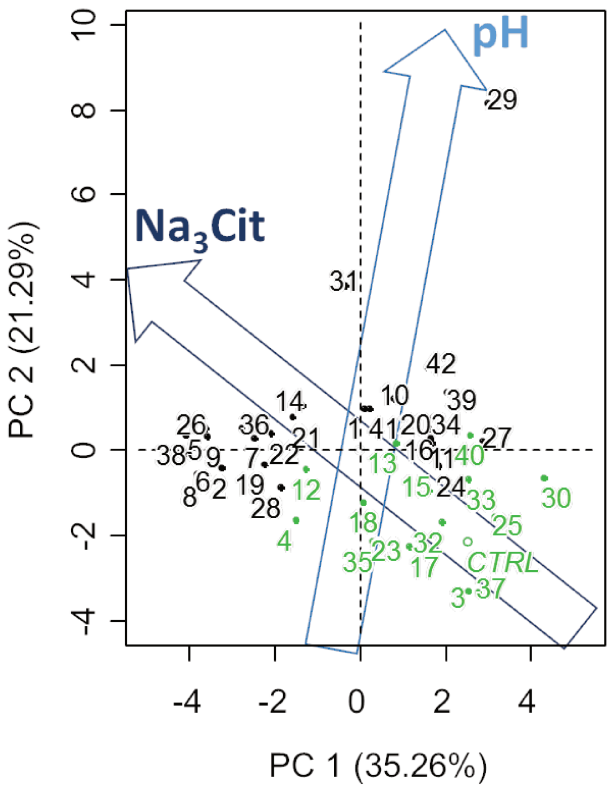

Figure 2: PCA showing the similarity map for the suspensions in the plane determined by the first two PCs. Coagulating and non-coagulating suspensions are colored in green and black, respectively. Arrows remind the directions of the pH and Na_3Cit variables in the correlation circle represented in the same PCs plane (see Fig. 1A).

3.2.2 Modeling of RCT and firmness

Given that PCA only considers the first order effects of variables between themselves, the dataset was submitted to regression analysis. This method enabled to define quadratic models of the RCT and the gels firmness as a function of the variations in pH, the addition of salts (NaCl, $CaCl_2$ and $MgCl_2$) and chelating agent (Na_3Cit). Such models made possible to define and assess the quadratic effects and second order interactions between these factors.

The following models were established to predict the RCT and the firmness of the gels, as a function of the environmental modifications carried out on the casein micelle suspensions, respectively:

$$RCT = 287.43 - 100.57\,pH - 0.08\,NaCl - 4.34\,CaCl_2 + 6.30\,Na_3Cit - 1.15\,MgCl_2 + 9.27\,pH^2 + 0.59\,(pH \times CaCl_2) - 0.80\,(pH \times Na_3Cit) + 0.02\,(NaCl \times MgCl_2)$$

$$Firmness = -151.73 + 54.42\,pH - 0.05\,NaCl + 1.35\,CaCl_2 - 1.81\,Na_3Cit + 0.08\,MgCl_2 - 4.44\,pH^2 - 0.19\,(pH \times CaCl_2) + 0.22\,(pH \times Na_3Cit) + 0.002\,(NaCl \times Na_3Cit)$$

The models explained 87.8 % and 85.8 % of the variability of the RCT and the firmness experimental data, respectively. Pareto charts (Fig. 3, A and B) show that most of the contributions were significant although having different weights in the models.

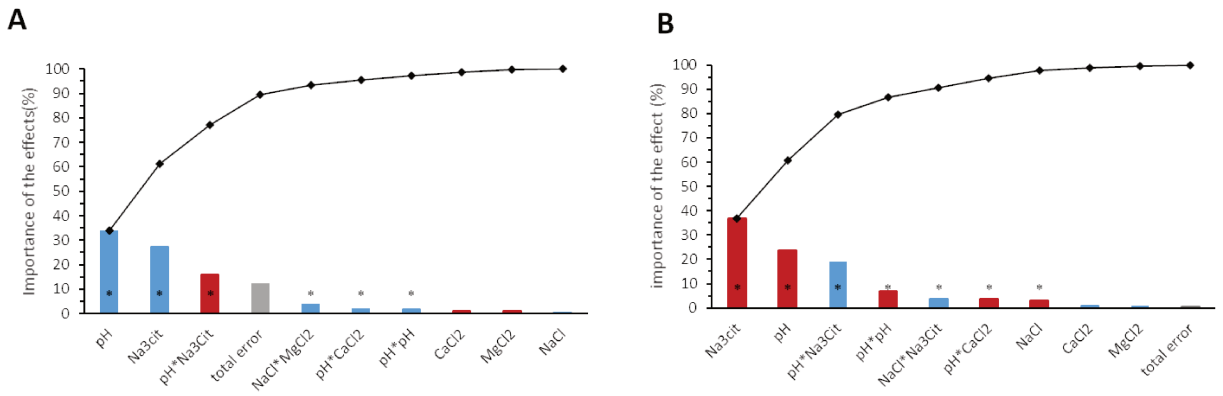

Figure 3: Pareto chart showing the importance of the each contribution in the RCT (A) and the firmness (B) models. Contributions colored in blue had positive impacts on the RCT or the firmness variations, while contributions colored in red had negative impacts. Contributions that are statistically significant ($p < 0.05$) are marked with an asterisk symbol. Dark lines represent the cumulative total of the individual values.

Variations in pH and addition of Na_3Cit were the most impacting factors and their increases caused an increase in RCT and a decrease in firmness. These findings were consistent with the results previously described and discussed from PCA (Figs. 1 and 2, section 3.2.1). The determination of a strong and significant interaction between those two factors (pH x Na_3Cit) that impacted the coagulating properties was also confirmed. The direct effects of pH and Na_3Cit and the effect of their interactions on the RCT and the firmness are observable in Figures 4A and B, respectively. The presence of interactions means that the effect of pH on RCT or firmness depended on the Na_3Cit concentration, and vice versa. In order to avoid misinterpretation of figures 4A, it is important to mention again that RCT of non-coagulating suspensions were arbitrary set to 60 min in the initial data set (see section 2.6.5). Therefore, RCT predictions with values higher than 60 min (red colored area – Fig.4A) should correspond to suspensions that did not coagulate within 60 min. On the other hand, zero or negative firmness values also corresponded to no gel formation (Fig 4B).

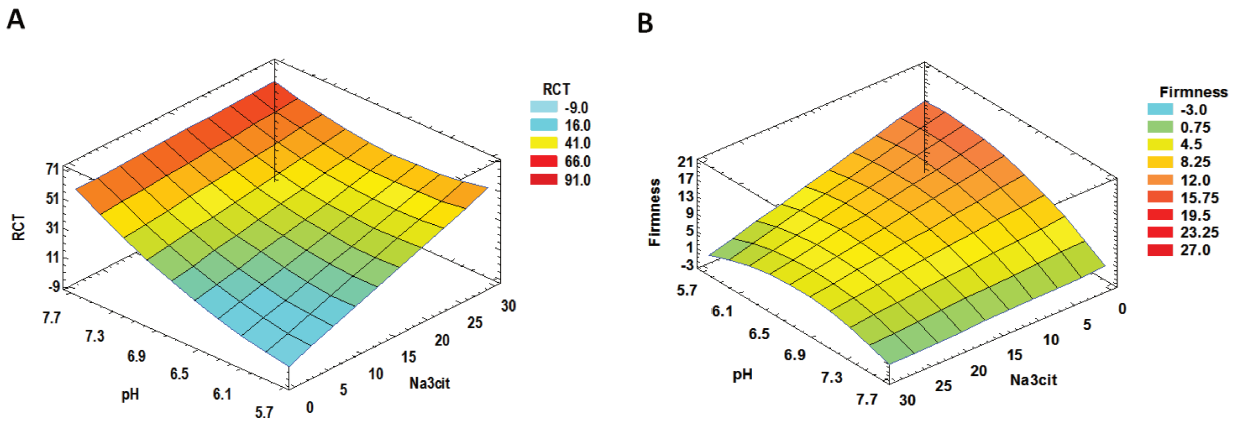

Figure 4. A: Surface response planes for the predicted RCT (A) and firmness (B) variables as a function of variations in pH and Na_3Cit concentration. RCT is expressed in min, Na_3Cit in mmol kg^{-1} and firmness in arbitrary unit (A.U). RCT > 60 min (A) and / or negative firmness (B) correspond to non-coagulating suspensions.

Other significant, but much less important interactions between pH and $CaCl_2$ (pH x $CaCl_2$), contributed to the definition of both models while an interaction between NaCl and $MgCl_2$ (NaCl x $MgCl_2$) contributed only to the RCT one (see RCT model previously described and Fig. 3A). Similarly, the interaction between NaCl and Na_3Cit (NaCl x Na_3Cit) contributed to the firmness model only (see firmness model previously described and Fig. 3B). Significant but minor quadratic effects of the pH (pH^2), i.e. the existence of minimum or maximum values at intermediate pH, were also revealed by the establishment of the models. These contributions were responsible for the slightly curved shapes of the surfaces in Figures 4A and B. These results were consistent with the observations of van Hooydonk, Boerrigter, & Hagedoorn (1986) that concluded on an optimum pH of 6.0 for the action of rennet and a minimum elastic modulus at pH 5.3 for the rennet gels. Choi et al. (2007), Daviau et al. (2000) and Zoon et al. (1989) also observed a quadratic effect of decreasing pH on the strength of rennet gels.

Interestingly, in the present study the additions of $CaCl_2$, $MgCl_2$ had no significant impacts on the RCT and firmness of the gels (Figs. 3A and B, respectively). These results were surprising given that monofactorial studies report a dependence between these factors and the rennet coagulation properties. Indeed, Cooke & McSweeney (2014), McMahon, Brown, Richardson, & Ernstrom (1984), Sandra, Ho, Alexander, & Corredig (2012), Udabage et al. (2001), van Hooydonk, Hagedoorn, & Boerrigter (1986b), Zoon, van Vliet, & Walstra (1988) reported a decrease in RCT with the addition of $CaCl_2$. The addition of $MgCl_2$ also caused a reduction in RCT, but of smaller extent than $CaCl_2$ addition (Cooke & McSweeney, 2014; van Hooydonk,

Hagedoorn, et al., 1986). The firmness of the gels was positively impacted by the addition of the divalent ions. Cooke & McSweeney (2014), Udabage, McKinnon, & Augustin (2001) and Zoon, van Vliet, & Walstra (1988) reported an increase of the elastic modulis of $CaCl_2$ added milks treated with rennet after different aging times and the first authors also observed a similar but less intense effect on the gel strength when $CaCl_2$ was replaced by $MgCl_2$. The differences between the two divalent ions were ascribed to a higher concentration of CaP nanocluster in milks supplemented in $CaCl_2$, leading the creation of thicker and stronger casein strands within the gel network.

Regarding NaCl addition, numerous authors (Bulca, Wolfschoon-Pombo, & Kulozik, 2016; Famelart, Le Graët, & Raulot, 1999; Grufferty & Fox, 1985; Karlsson, Ipsen, & Ardö, 2007; Sbodio, Tercero, Coutaz, & Revelli, 2006; Zhao & Corredig, 2015; Zoon et al., 1989) observed an increase of the RCT, while it had no significant effect in the present study (Fig. 3A). The presence of a negative and significant NaCl contribution to the model firmness indicated that this salt addition induced a decrease in the gel firmness. This result was in agreement with the observations of Bulca et al. (2016) and Zhao & Corredig (2015).

4 Conclusion

The individual effects of the variations in pH, salts and chelating agents additions on the properties of the casein micelles were consistent with the findings of previous monofactorial studies. The multifactorial approach adopted in the present study brought supplementary informations. From one side, it demonstrated that the factors did not have the same importance on the properties of the casein micelle. Regarding the mineral balance, variations in pH and addition of Na_3Cit caused the most important damages on the mineral content of the colloids: a decrease in pH and an increase in Na_3Cit concentration led to the solubilization of the micellar CaP. The mineral depletion induced by the chelating salt led to the disruption of the casein micelles, and besides, the addition of Na_3Cit was the only factor that had an impact on the micellar size and structure. Addition of NaCl, $CaCl_2$ and $MgCl_2$ only impacted the minerals concentrations in the diffusible phase of the suspensions. From the other side, the multifactorial approach enable to reveal the existence of interactions between the selected factors and their considerable effects on the rennet coagulation properties of the casein micelles. Indeed, the establishment of models of the RCT and gel firmness demonstrated that several interactions between the factors had to be considered in addition to the individual effects of the factors in order to understand and predict the variations of these characteristics. As with the mineral balance, the most impacting factors on both RCT and firmness were the variations in pH and in Na_3Cit concentration, intervening through their individual and interacting effects. Further investigations of the colloidal properties of the casein micelles and the behavior of the enzyme (chymosin) in such modified environments would be of great interest in order to identify which mechanisms were responsible of the variations in the rennet coagulation properties. As perspective, such multifactorial study could be easily extended to the study of other factors (concentration, temperature, high pressure), functionalities (emulsion, acid gelation, thermal stability) or dairy systems (milks, concentrates, isolates, powders).

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PART B, CHAPTER 2:

TAILORING THE STRUCTURE OF CASEIN MICELLES THROUGH A MULTIFACTORIAL APPROACH TO MANIPULATE AND UNDERSTAND THEIR RENNET COAGULATION PROPERTIES.

This chapter will be submitted to Food Hydrocolloids.

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Abstract:

Casein micelles consist of associations of caseins, calcium phosphate and water but their internal structure remains unsolved. Modifications of casein micelles environment are responsible for numerous changes in their properties, including functionalities.

The aim of this study was to assess the impacts of multifactorial modifications of casein micelles environment on their mineral composition, colloidal and rennet coagulation properties. Variations

in pH, NaCl and $CaCl_2$ concentrations were applied simultaneously to casein micelles suspensions through an experimental design. The structural properties of the modified casein micelles were assessed using advanced biophysical techniques. Small angle X-ray scattering results were treated according to Bouchoux et al. (2010) model and also discussed in regards to the findings of Ingham et al. (2016). Statistical treatments enabled to establish correlations between the environmental modifications and the various properties of the casein micelles.

pH decrease had major effects on their mineral balance in solubilizing calcium phosphate. NaCl increased diffusible sodium and chloride concentrations while $CaCl_2$ had slight mineralizing effects. pH decrease caused the micelles to sweell and disrupted the smallest X-ray scatterers. One of the key results was the release of dense regions (~ 20 nm) outside of the casein micelles due to the addition of NaCl. Only pH and NaCl factors affected the rennet clotting time. Modeling the firmness as a function of the structure of the casein micelles revealed that the presence of released dense regions decreased it. The casein micelles size and the number of the smallest scatterers also contributed to the firmness model through an interaction.

Keywords: internal structure, functionality, multifactorial modifications, pH, sodium chloride, calcium chloride, principal component analysis, predictive model, small angle X-ray scattering, cryo-transmission electron microscopy

1 Introduction

In milk, caseins (α_{s1}, α_{s2}, β and κ) and minerals (mainly calcium phosphate (CaP) nanoclusters) self-assemble to form colloids referred to as casein micelles. Electrostatic, hydrophobic and Van der Waals interactions hold the different components together leading to a polydisperse population of particles of 100 – 200 nm in diameter (Dalgleish & Corredig, 2012; Holt, Carver, Ecroyd, & Thorn, 2013; Holt, 2016; Holt & Horne, 1996; Horne, 2017; Thorn et al., 2005).There is a consensus that casein micelles are stabilized through electrostatic and steric repulsions induced by the presence of polyelectrolyte brushes of κ-casein at the micellar surfaces (de Kruif, 1999; de Kruif & Zhulina, 1996; Tuinier & de Kruif, 2002). Despite extensive studies performed over the last decades, the internal structure of the casein micelles, i.e. the interactions between caseins and the minerals located within the colloids, is still under debate. The recent use of small angle X-ray and neutron scattering techniques (SAXS and SANS, respectively) in parallel with cryo-transmission electron microscopy (cryo-TEM) has enabled questions around the internal structure to be partially answered, although without general agreement (Bouchoux et al., 2015; Bouchoux, Gésan-Guiziou, Pérez, & Cabane, 2010; Day, Raynes, Leis, Liu, & Williams, 2017; de Kruif, 2014; Ingham et al., 2015, 2016; Marchin, Putaux, Pignon, & Léonil, 2007; Pignon et al., 2004; Shukla, Narayanan, & Zanchi, 2009). To date, the previously most accepted submicelle model (Schmidt, 1982; Walstra, 1999) become progressively abandoned in favor of a more open structure composed of dense regions, water channels and CaP nanoclusters.

Environmental factors such as variations in pH, temperature, salts and chelating agent additions induce shifts in the mineral balance between the diffusible and colloidal phases of the casein micelles (de Kort, Minor, Snoeren, van Hooijdonk, & van der Linden, 2011; Gaucheron, 2004; Lazzaro et al., 2017 (Part A); Silva et al., 2013). These mineral modifications also involve colloidal modifications that lead to changes in the functional properties of the casein micelles, such as emulsion formation and stability, thermal stability, and ability to acid and rennet coagulations (Broyard & Gaucheron, 2015; Gaucheron, 2004). Although the minerals fraction only represents a small proportion of the milk components (0.7 % in weight), it is used to control the properties of numerous manufactured dairy products.

Of all these functional properties, this study focuses on the rennet coagulation of casein micelles, which corresponds to the first step of cheese manufacture. The rennet coagulation mechanism can be divided into three steps: first, the chymosin hydrolyzes the κ-caseins of the casein micelles by cleaving at bond Phe(105)-Met(106). The subsequent release of caseinomacropeptides (CMP) from paracaseinates causes a decrease in the colloidal stability of

the micelles. When a sufficient amount of κ-caseins is hydrolyzed, reported to be 90 % in non-modified milk (Lucey, 2002), the steric and electrostatic repulsions generated by the remaining κ-caseins are insufficient to keep the paracaseinates apart, leading to their aggregation; that is defined as the second step. Finally, this aggregation is followed by the last step, which consists of the reorganization and reticulation of the casein gel (Dalgleish & Corredig, 2012). Rennet coagulation can be assessed by two main characteristics: (i) the rennet clotting time (RCT) that corresponds to the time elapsed from the addition of rennet or chymosin to the detectable onset of gelation (the start of paracaseinates aggregation) and (ii) the firmness of the gel. The RCT mainly depends on the rate of the enzymatic reaction and the aggregation of the paracaseinates (first and second steps), while the firmness depends on the organization and the strength of the gel (third step).

Variations in pH and additions of NaCl and $CaCl_2$ are steps commonly applied in cheese manufacture to control the coagulation of milk. The separate influences of each of these parameters on the colloidal properties and on the rennet coagulation properties of the casein micelles are well described in the literature (Bulca, Wolfschoon-Pombo, & Kulozik, 2016; Choi, Horne, & Lucey, 2007; Daviau, Famelart, Pierre, Goudédranche, & Maubois, 2000; Deeth & Lewis, 2015; Famelart, Le Graët, & Raulot, 1999; Famelart, Lepesant, Gaucheron, Le Graët, & Schuck, 1996; Grufferty & Fox, 1985; Karlsson, Ipsen, & Ardö, 2007; Karlsson et al., 2007; Sandra, Ho, Alexander, & Corredig, 2012; Sbodio, Tercero, Coutaz, & Revelli, 2006; Zhao & Corredig, 2015; Zoon, van Vliet, & Walstra, 1988, 1989). Nevertheless, this monofactorial approach, although necessary to understand the dissociated effect of each parameters, does not correspond to the reality of the cheese industry. Indeed, the manufacture of cheese often uses the simultaneous variation of these parameters i.e. a multifactorial approach.

The aim of this study was to investigate the effects of the simultaneous modification of environmental parameters on the colloidal properties and on the rennet induced coagulation of the casein micelles. An experimental design was carried out involving 27 suspensions of casein micelles in water, at three different pH (5.7, 6.5, 6.9) and with different amounts of added NaCl (0, 50, 100 mmol kg^{-1}) and $CaCl_2$ (0, 7.5, 15 mmol kg^{-1}). The combination of multiple advanced biophysical techniques, such as cryo-TEM and SAXS, enable the thorough characterization of the casein micelles in suspensions, in terms of mineral balance, colloidal (including structural) and renneting properties. Finally, appropriated statistical analyses were applied to establish relationships between all these properties of the modified casein micelles, leading to the first study that combined SAXS characterizations to functional properties of the casein micelles.

2 Materials and methods

2.1 Chemicals

All chemicals used for this study, HCl (hydrochlorydric acid) (VWR chemicals, Fontenay-sous-bois, France), NaCl (PanReac AppliChem, Barcelona, Spain), $CaCl_2$ (VWR International, Leuven, Belgium), NaN_3 (sodium azide) (Riedek-de Haën, Seelze, Germany) were of analytical grade.

2.2 Materials

Experiments were carried out using native phosphocaseinate (NPC) powder suspended in milli-Q water at 24.2 ± 0.8 g kg^{-1} of protein. Concentrated NPC was supplied by Gillot SAS (Saint Hilaire de Briouze, France) and obtained by microfiltration (0.1 µm pore size membrane) of raw skimmed milk followed by diafiltration against milli-Q water. The concentrate was then spray dried according to the method described by Pierre, Fauquant, Le Graët, & Maubois (1992) and Schuck et al. (1994) using Bionov facilities (Rennes, France). Casein and their associated minerals represented more than 90 % of the total solid content of the powder. Residual whey proteins (3 %) (w / w) and traces of lactose were present in the powder.

For the coagulation experiments, Chr. Hansen (Hoersholm, Denmark) supplied commercial chymosin (CHY-MAX M 200, 200 IMCU ml^{-1}).

2.3 Preparation of the suspensions of casein micelles

An experimental design was defined in order to assess concomitant effects of variations in pH and additions of NaCl or $CaCl_2$. The range of pH and the final concentrations of added NaCl and $CaCl_2$ were selected to produce suspensions forming gels within 60 min following the addition of a set amount of chymosin. The pH values targeted were 5.7, 6.3 or 6.9, and the final concentrations of NaCl and $CaCl_2$ in the suspensions were 0, 50 and 100 and 0, 7.5 and 15 mmol kg^{-1}, respectively. A full experimental design was carried out, where 27 different suspensions of casein micelles were prepared in milli-Q water, with different salts and pH environments. The suspensions were named from A to Z and CTRL (control, NPC in milli-Q water at pH 6.9, with no added salts). The cubic representation of the experimental design is presented in Figure 1.

The dispersion of the NPC powder in milli-Q water was performed following the method of Lazzaro et al. (2017) (Part A) to produce casein micelles suspensions. Varying amounts of stock solutions of NaCl (2.5 mol kg^{-1} in milli-Q water, pH 6.9) and $CaCl_2$ (0.25 mol kg^{-1} in milli-Q water, pH 6.9) were added to the 28 g kg^{-1} casein micelle concentrated suspensions, in such a way that after the dilution step, the final concentrations in salts required were reached (Fig. 1). The suspensions were stirred for 30 min. The pH shift induced by the salts addition was corrected using HCl 1 M in milli-Q water and set to 5.7, 6.3 or 6.9 (Fig. 1). The suspensions were then diluted to 24.2± 0.8 g kg^{-1} of protein and left overnight at room temperature. The pH was readjusted in the morning if necessary.

For convenience, Tables 1, 2, 3 and Figures 6, 7, 8 report the results of different analyses on a set on 9 samples only. These selected samples correspond to the corners of the cubic experimental design (suspensions A, B, D, E, J, L, M, CTRL ; extreme points) and the center point of the experimental design (suspension T) (Fig. 1).

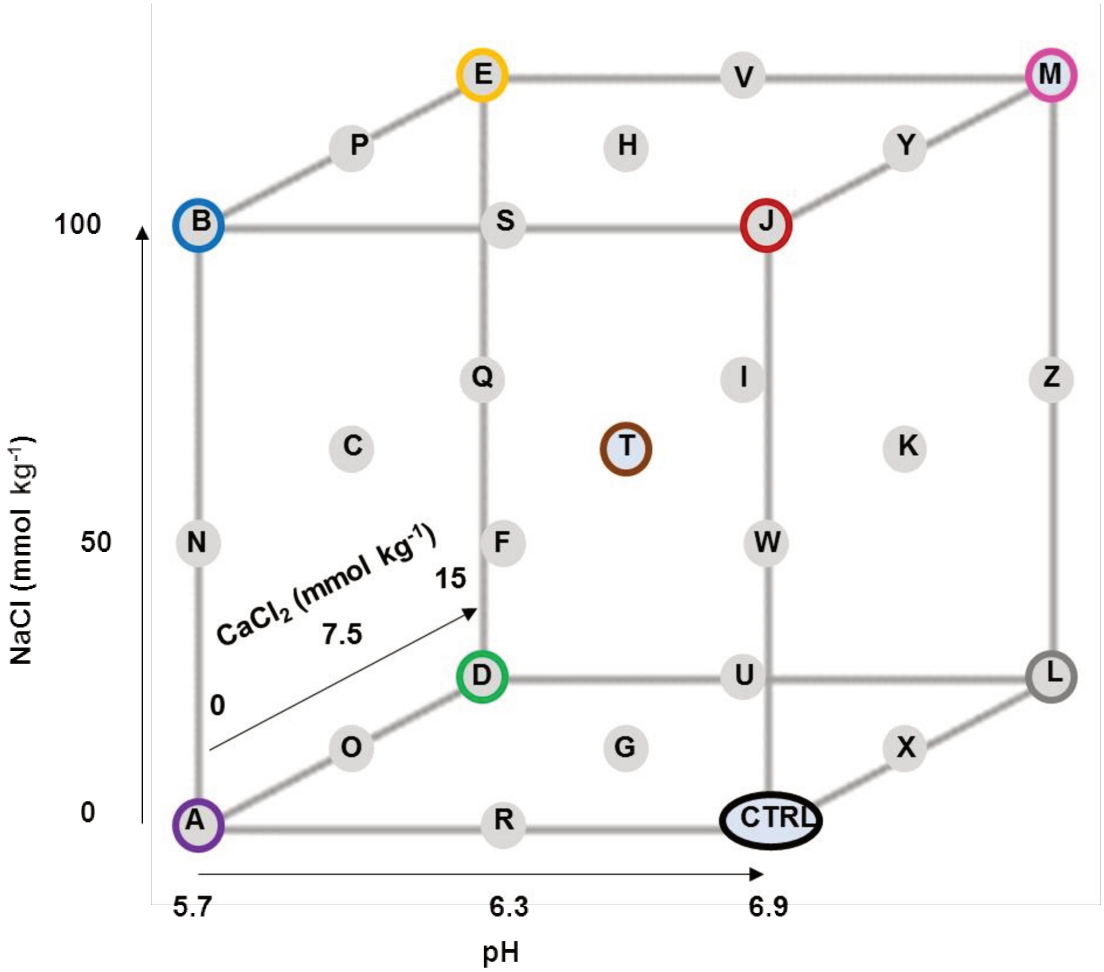

Figure 1: Cubic representation of the experimental design. Each of the 27 suspension is represented by a letter or designated as CTRL (control suspension, pH 6.9, no salts added). The pH of the suspensions was set to 5.7, 6.3 or 6.9 and the suspensions contained 0, 50 or 100 mmol kg^{-1} of added NaCl and 0, 7.5 or 15 mmol kg^{-1} of added $CaCl_2$.

2.4 Recovery of the diffusible phases of the suspensions

The NPC suspensions were ultrafiltered on Vivaspin concentrators (molecular weight cut-off 10 kDa, Vivascience, Palaiseau, France) to recover their diffusible phases. The Vivaspin concentrators containing 15 mL of suspension were centrifuged for 30 min, at 20°C and 1800 g. The recovered diffusible phases were used for the determination of diffusible ions, for the dilution of the suspension for the turbidity (τ) and nanoparticle tracking analysis (NTA) measurements and for the background determination for the SAXS measurements.

2.5 Analysis

2.5.1 Protein content

The total nitrogen content of each suspension was determined according to the Kjeldahl method (IDF standard 20-1, 2014). A factor of 6.38 was used to convert nitrogen to protein concentration. Measurements were performed in duplicate.

2.5.2 Mineral composition and distribution

Total and diffusible cations (calcium - Ca, sodium - Na) and anions (chloride - Cl, inorganic phosphate - Pi) contents were determined as described in Lazzaro et al., (2017) (Part A). Colloidal concentrations were deduced by subtracting the concentrations of diffusible ions from the concentrations of total ions. The concentrations were adjusted to account for the small differences in protein content (see section 2.5.1.)

2.5.3 Turbidity measurements

Absorbance measurements were carried out at 600 nm and 20℃ using a UV-visible spectrometer (UVmc², Safas, Monaco).The casein micelles suspensions were diluted ten times in their diffusible phases and analyzed immediately. The diffusible phase for each suspensions was also analyzed. Absorbance measurements were converted into τ according to the following formula:

$$\tau = 10 \times 2.303 * \frac{OD(600nm)}{l}$$

with $OD(600\,nm)$ being the optical density of the suspension (difference between the absorbance of the diluted suspension and the absorbance of its diffusible phase) ; and l the light path length ($l = 1\,cm$).

2.5.4 Nanoparticle tracking analysis

NTA was performed at 20°C using a Nanosight NS300 (Malvern Instruments, Malvern, United Kingdom) equipped with a Nanosight syringe pump. The principle of NTA is based on the tracking of individual particles in suspension. A large dilution (40 000 times) of the suspensions in their respective diffusible phases was necessary to meet the optimal settings of the apparatus, i.e. 20 – 100 particles per frame during the measurement combined to a dark background image. A syringe was loaded with the diluted suspension, the focus was adjusted manually, the infusion rate was set to 20, the camera level to 12 and 5 video images of 60 s recorded. The video images of the movement of particles under Brownian motion were analyzed by the NTA image analysis software (V 3.0 0064., Malvern Instruments). The screen gain was minimum and the detection threshold was set to 3 to maximize the detection of small particles (< 50 nm diameter). The particle size distributions obtained (data not shown) were fitted with a log-normal population of particles, using Schulz equation (Schulz, 1935):

$$W(R, r_{NTA}, \sigma) = \frac{R^Z}{\Gamma(Z+1)}\left(\frac{Z+1}{r_{NTA}}\right)^{Z+1} \times exp\left[-\frac{R}{r_{NTA}}(Z+1)\right]$$

Where r_{NTA} is the average radius of the particles and Z is related to the polydispersity (σ) of the particle radius (R) distribution by the expression:

$$\sigma = \left(\frac{\overline{R^2}}{r_{NTA}} - 1\right) = \frac{1}{Z+1}$$

The value of σ varied from 0.23 to 0.47 within the set of suspensions and r_{NTA} was further used in the statistical analyses.

2.5.5 Cryo-transmission electron microscopy

A thin vitrified film of casein micelles suspension was prepared similarly to the method of Chen et al. 2011. A Formvar lacey carbon film mounted on a 300 mesh copper grids (ProSciTech, Queensland, Australia) were glow discharged for 15 s and used as a hydrophilic support on

which the suspensions (4 µL) were adsorbed. After 30 s, the grids were plunged in liquid ethane using a Vitrobot (FEI Company, Eindhoven, Netherlands) to freeze the sample. The grids were observed on a Technai G2 TF30 (FEI company, Eindhoven, Netherlands) operating at 200 kV and equipped with a Gatan US1000 2kX2k CCD Camera (Gatan). Between 10 and 20 micrographs per suspension were recorded under low-dose conditions with defocus values of 4 - 6 µm. Image analysis was performed using ImageJ software (National Institute of Health, USA). Due to the large numbers of samples, this process was automated through the use of macros. In total, between 1 554 particles within suspension CTRL and 29 092 particles within suspension B were measured in three steps:

i) The region of interest was defined:

Particle detection was strictly limited to the area free from the grid structure and ice particles present as a result of sample preparation.

ii) Particle detection:

The background was subtracted from images using a 'rolling ball' algorithm and smoothed using Gaussian filtering before the threshold was applied and particles measured. Touching particles were separated using the distance transform watershed plugin (Quasi-Euclidean). Most of the very large particles (diameter > 50 nm) were often partially hidden by the grid. Therefore, the grid-obstructed particles were excluded from the detection only if more than 10 % of the area defined by the best-fitted ellipse drawn around the particle was hidden under the grid. In order to prevent any misrepresentative segmentation, which can be caused from automatic detection, all segmentation results were visualized and the overlay was saved as a separate image that was manually inspected.

iii) Shape measurement of the particles:

Feret's diameter was measured for each particle detected. Particles were defined as small (< 50 nm in Feret's diameter) or large (> 50 nm in Feret's diameter). The ratio of small to large particules was then calculated for each suspension and defined as $\Gamma_{s/l}$.

2.5.6 Small angle X ray scattering measurements, data treatment and modelling

The SAXS measurements were carried out on the suspensions and their respective diffusible phases at the SAXS / WAXS beamline of the Australian Synchrotron (Clayton, Melbourne, Australia). The beamline was equipped with a Pilatus 1 M detector (170 mm x 170 mm, effective pixel size of 172 µm x 172 µm). Two sets of measurements were performed on each sample, at different sample-to-detector lengths of 7.106 m or 0.721 m with respective photon energies of 8.2 (1.512 Å) or 18.1 keV (0.685 Å). This set up provided a q range of 1.3 10^{-3} to 1.93 $Å^{-1}$ when the data were merged. The samples were loaded in a 96 well plate from which they were drawn into a 1.5 mm glass capillary; allowing continuous flow through the X-ray beam during measurements. The data were obtained from at least 10 exposures of 2 s intervals at 20°C. The capillary was rinsed with water, followed by 8 M guanidine, water again then finally air-dried between each sample analysis.

The SAXS intensities were normalized to an absolute scale and at least ten measurements per sample were averaged to obtain the intensity profiles using ScatterBrain (V 2.71) (Australian Synchrotron, Clayton, Australia). The diffusible phases were assigned as a measure of background scatter and therefore their scattering intensities were subtracted from the corresponding scattering intensities of the suspensions. Finally, the intensities were adjusted to account for the difference in total protein content of the suspensions in Primus (V 3.2) (ATSAS, Hamburg, Germany). Igor software (V 7.0.2.2) (Wave Metrics, Lake Oswego, USA) was used to merge the data from the 7.106 and 0.721 m detector distances to obtain the final scattering intensity curves I = f(q).

The SAXS intensity curves were fitted according to the model of Bouchoux et al., (2010) with slight modifications. This model comprises three populations of particles: the whole casein micelles (population A), the dense regions (population B) and the CaP nanoclusters (population C). The intensity depends on the form factors of each population, Pn(q), approximated by the form factor of polydisperse spheres, and on prefactors a, b and c:

$$I(q) = a\,P_a(q) + b\,P_b(q) + c\,P_c(q)$$

with:

$$a = \alpha \times n_a\,(Vma\ \times\ \Delta\rho_a)^2$$

$$b = \alpha \times n_b\,(Vmb\ \times\ \Delta\rho_b)^2$$

$$c = \alpha \times n_c\,(Vmc\ \times\ \Delta\rho_c)^2$$

where n_a, n_b, n_c are the number of scatterers for each population, Vma, Vmb, Vmc their volume and $\Delta\rho_a$, $\Delta\rho_b$, $\Delta\rho_c$ their contrast, respectively. The absolute number of casein micelles is assumed to be the same in all the suspensions. This hypothesis is based on the observations of Moitzi, Menzel, Schurtenberger, & Stradner, (2011) that a decrease in pH left the number of casein micelles unmodified, even if some casein micelle materials are subdivided into individual monomers or smaller casein aggregates with a resulting decrease in micelle size and mass. In the present study, the modifications of the pH and the addition of NaCl and $CaCl_2$ applied in the experimental design are similarly not thought to be sufficient to cause the complete disruption of the micellar structure. This implies that the n_a value is not affected by the physical-chemical modifications of our samples. We chose to set it to 1 so that n_b and n_c are defined relative to one casein micelle. As a consequence, the constant α does contain both the electron scattering length and the absolute number density of casein micelles in the samples.

This model was tested on our data according to the procedure of Bouchoux et al. (2010). First, the value of the radius of each population, r_a, r_b and r_c, and the prefactors, a, b, c, were determined by fitting the model to experimental data with polydispersities set to $\sigma_a = 1/3$, $\sigma_b = 1/3$ and $\sigma_c = 0.2$. In a second step, the value of the constant α was calculated from the control sample (CTRL) using the prefactor a obtained from the fit, the size (and therefore volume) of the micelle in this case and the contrast $\Delta\rho_a$ of a native casein micelle in water, i.e. 0.018 $e^- Å^{-3}$ (ρ_{water} = 0.334 $e^- Å^{-3}$ and $\rho_{caseinmicelle}$ = 0.352 $e^- Å^{-3}$).

In a third step, the values of $\Delta\rho_a$, n_b and n_c were calculated for the 27 suspensions using the sizes and prefactors obtained through the modelling of the SAXS patterns:

1) Micelle: $\Delta\rho_a$ was simply calculated from prefactors a, sizes r_a, with $n_a = 1$.

2) Dense regions: the number of dense regions n_b was calculated from prefactors b, sizes r_b, and making the assumption that constrast $\Delta\rho_b$ is relatively insensitive to the physical-chemical modifications performed in this study. $\Delta\rho_b$ is taken as 0.035 $e^- Å^{-3}$, i.e. twice the contrast of the micelle assuming that dense regions occupy 50 % of the total volume of the casein micelle (Bouchoux et al., 2010).

3) CaP nanoclusters / Protein inhomogeneities: the number of nanoclusters n_{cCaP} is calculated from prefactors c, sizes r_c, and an estimated contrast $\Delta\rho_c$ of 0.172 $e^- Å^{-3}$ that is also assumed to be invariant from one sample to another. Note that in a recent work, Ingham et al. (2016) suggest a new interpretation and assign the high q features of SAXS data to the presence of inhomogeneous protein structures of 1 - 3 nm length scale instead of CaP nanoclusters. As our

purpose is not to take a position on this question, we decided not to restrict our analysis to the interpretation of Bouchoux et al. (2010). A number of possible protein inhomogeneities n_{cPI} was therefore calculated following Ingham's postulate, this time using an estimated contrast of 0.126 e^{-} $Å^{-3}$ (Ingham et al., 2016).

2.5.7 Rennet coagulation properties

The coagulation properties of samples were assessed using a ChymoGRAPH® (Chr Hansen, Denmark); this equipment is based on the physical principle from the Formagraph (McMahon & Brown, 1982). In both cases, the coagulation is determined according to the movement of stainless steel, loop pendulums immersed in the coagulating samples. Ten grams of casein micelles suspensions were weighed into the wells of the ChymoGRAPH® stainless steel block. The block, containing four suspensions in total (two wells per suspension), was immersed in a water bath for 10 min to equilibrate the temperature of the suspension to 30°C. A sample of 400 µL of the enzyme solution (chymosin diluted ten times in milli-Q water) was pipetted into a multiple spoon apparatus to simultaneously disperse the chymosin in the suspensions. The suspensions were then stirred for 30 s with the spoons and transferred from the water bath to the oscillating plate of the ChymoGRAPH®. A Peltier module maintained the temperature of the block at 30°C. When the coagulation began, the resulting increase in viscosity and the formation of gels caused the pendulums to oscillate together with the samples. The movement of each pendulum was measured by the use of optical fibers over a period of 60 min and the data collected in ChymoGRAPH® software (V 1.0, Chr Hansen, Denmark).

The RCT corresponded to the time elapsed from chymosin addition to the detectable onset of coagulation, where coagulation was defined as at the time point when the firmness of the suspensions was > 0. The maximal firmness recorded during the 60 min duration of the experiment was defined as the firmness of the gel.

2.6 Statistical treatments

As mentioned in section 2.3., a complete experimental design was carried out to study the combined effects of variations in pH, NaCl and $CaCl_2$ additions on the colloidal and renneting properties of the casein micelles (Fig. 1). The data set was subjected to statistical analysis by principal component analysis (PCA) using the Facto-MineR package and the R software (Lê, Josse, & Husson, 2008; R Development Core Team, 2011) and variables pH, NaCl and $CaCl_2$

were defined as illustrative variables. The principle of this multivariate statistical method has been described by Abdi & Williams (2010), Jolliffe (2014) and Wold, Esbensen, & Geladi (1987). Briefly, PCA summarizes a data set of n individuals (e.g. the 27 suspensions) described by m quantitative variables (e.g. τ, colloidal Ca, firmness) by defining a new set of p variables called principal components (PCs), with $p < m$. The PCs are orthogonal (uncorrelated) and consist in linear combinations of the original variables. The output are displays of the similarities of the individuals and correlations between the original variables in a new p-dimensional space. In this study, PCA was used to highlight the correlations between the different measurements. All the correlations mentioned in section 3 were found to be significant ($p < 0.05$) using the paired student t-test.

In addition, multiple linear regression was also applied to the SAXS variables r_a, n_b and n_{cCaP}, using the software STATGRAPHICS Centurion XVII (V.17.1.10, Statpoint Technologies, The Plains, USA) in order to predict the firmness of the gels as a function of these structural features. A model of firmness was defined that included the quadratic effects of r_a, n_b and n_{cCaP} and the second order interactions between these three factors. The full equation of the model was:

$$Firmness = constant + r_a + n_b + n_{cCaP} + r_a^2 + n_b^2 + n_{cCaP}^2 + (r_a \times n_b) + (r_a \times n_{cCaP}) + (n_b \times n_{cCaP})$$

The LS-means were calculated and differences regarded as significant for $p < 0.05$. Non-significant effects were excluded from the model, except when first order effects were participating in interaction effects.

3 Results and discussion

The results are presented and discussed in three main steps. First, PCA results were presented in global terms with respect to the entire set of data. Then, these results were interpreted to i) assess the impacts of the pH variation, the additions of NaCl and $CaCl_2$ on the mineral balance of the casein micelles and ii) establish relationships between the structure of casein micelles and their other colloidal properties. A second evaluation considers the relationships between the rennet coagulation properties and the colloidal and the structural features of casein micelles were established. PCA results enabled to explain the variation of the RCT while multiple linear regression analysis demonstrated that interactions between structural properties must be considered to understand the firmness of the rennet gels.

3.1 PCA results

The addition of the first four PCs demonstrates that these dimensions of the PCA explained almost 80 % of the variability of the experimental design (Figs. 2 and 4). More than 50 % of the data set variability was represented in the plane defined by the two first PCs, as shown in Figure 2, PC 1 and 2 describing 34 % and 24 % of this information. However, our data set can be qualified as multidimensional considering that PC 3 and 4 each express more than 10 % of this variability. PC 1 strongly correlated with variables related to the mineral content of the casein micelles, i.e. colloidal Ca and Pi, $\Delta\rho_a$ and n_{cCaP}. pH, used as an illustrative variable, was well projected along the PC 1 axis, which signified that pH variations affected strongly the mineral content of the micelle. PC 2, on its side, correlated with variables related to the structural state of the casein micelles, i.e. n_b, r_b and $\Gamma_{s/l}$ and some diffusible ions concentrations, i.e. diffusible Na and Cl. NaCl variable, also illustrative, was well projected along the PC 2 axis, reflecting its effects on these structural parameters and the mineral content of the diffusible phase. r_a, was well project on the PC 1 and PC 2 plane and equally correlated to each axis, signifying that variations in pH or in NaCl addition both impacted the casein micelles size.

Detailed explanations of the correlations between the “PC 1” and PC 2 variables” is provided in the following paragraphs of this section. Interestingly, addition of $CaCl_2$ seemed to have a minor impact on the variability of the data set, as it was only well projected along PC 4 axis (Figs. 3 and 4). PCs 1 and 2 plane (Fig. 1) did not provide a lot of information on the firmness of the gels due to the bad projection of this variable while the projection of the RCT variable was satisfactory. The firmness was well projected on the PCs 3 and 4 plane (Fig. 4) but did not

correlated directly with any of the other variables (orthogonal projections). PCA only illustrates first order correlations and do not take into consideration the possible existing interactions between the variables. The presence of such interactions was established using linear multiple regression analysis. The corresponding results are presented and discussed in part 3.3.2.

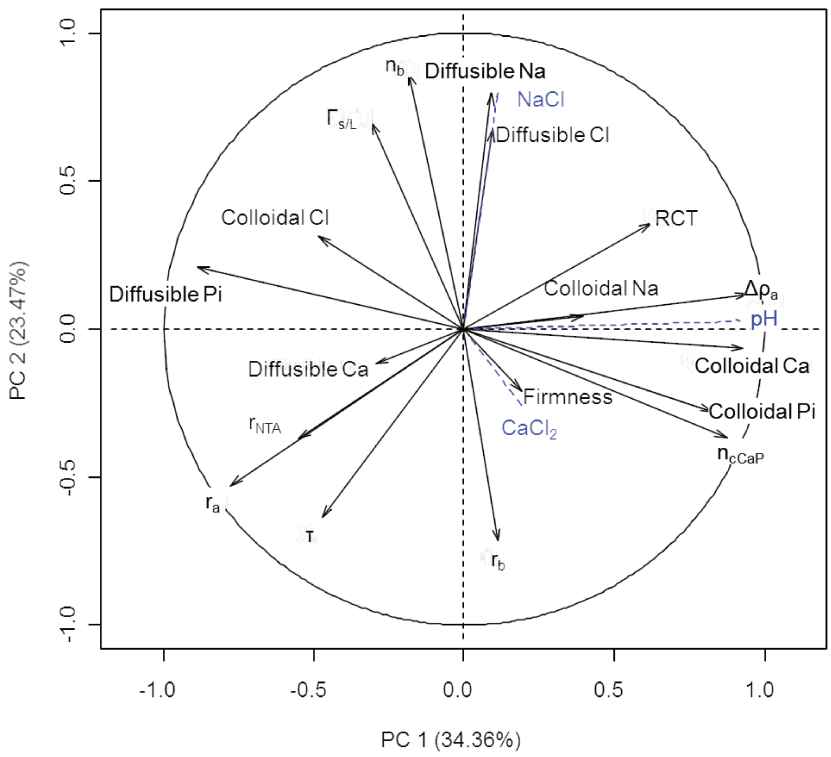

Figure 2: PCA showing the correlation circle of 21 variables in the plane delimited by the two first principal components (PCs). 57.8 % of the variability of the set of data is represented in this PCA plane.

3.2 Mineral, Colloidal and structural properties of the modified casein micelles

3.2.1 Impact of the environmental modifications on the mineral balance of the casein micelle

An overview of the mineral partition of Ca, Pi, Na and Cl contents between the colloidal and diffusible phases (Table 1) revealed that the colloidal ions consisted mainly in Ca and Pi. Na and Cl were mainly present in the diffusible phases of the suspensions when NaCl was added.

Table 1: Distribution of the mineral salts in suspensions. Colloidal concentrations were determined by subtracting diffusible from total ion concentrations. Average, standard deviation (SD), relative standard deviation (RSD), minimum and maximum values were determined on the complete set of 27 samples.

	Diffusible Ca (mmol kg^{-1})	Colloidal Ca (mmol kg^{-1})	Diffusible Na (mmol kg^{-1})	Colloidal Na (mmol kg^{-1})	Diffusible Cl (mmol kg^{-1})	Colloidal Cl (mmol kg^{-1})	Diffusible Pi (mmol kg^{-1})	Colloidal Pi (mmol kg^{-1})
full experimental design - 27 suspensions								
Average	12.4	14.8	64.8	0.2	67.4	0.5	2.4	4.6
SD	6.0	4.3	45.5	0.5	45.7	1.4	1.3	1.3
RSD (%)	48.2	29.2	70.3	300.9	67.8	287.6	55.1	28.1
minimum	2.1	3.6	6.7	0.0	0.0	0.0	0.0	1.9
maximum	22.9	20.2	135.6	2.2	138.0	6.2	5.0	7.2
Selected individual suspensions								
A	9.6	8.2	8.9	0.0	2.2	1.6	3.8	3.3
B	11.0	8.8	114.4	0.0	102.7	6.2	4.8	1.9
D	20.1	12.0	24.0	0.0	26.9	3.3	2.9	3.5
E	22.8	12.3	121.7	0.0	130.7	0.0	3.4	3.5
J	3.5	15.8	120.2	0.0	94.4	0.0	1.7	4.9
L	14.7	20.2	10.5	0.0	17.9	1.8	0.8	5.4
M	16.3	18.8	135.6	0.0	129.4	0.0	0.0	6.9
T	11.5	15.8	65.4	0.0	69.2	0.0	2.3	5.0
CTRL	2.1	18.0	6.7	0.8	0.0	0.0	1.6	4.9

Figure 2 shows strong and positive correlations between the pH and the colloidal Ca and Pi (0.85 and 0.80, respectively), and a consistent negative correlation with the diffusible concentrations of Pi (- 0.86). The diffusible Ca concentration was weakly but still significantly impacted by the pH variation, with a correlation coefficient of - 0.5 (Fig. 3). The strongest variation of this ion in the diffusible phase was attributed to the addition of $CaCl_2$, with a correlation coefficient of 0.84 (Fig. 4).

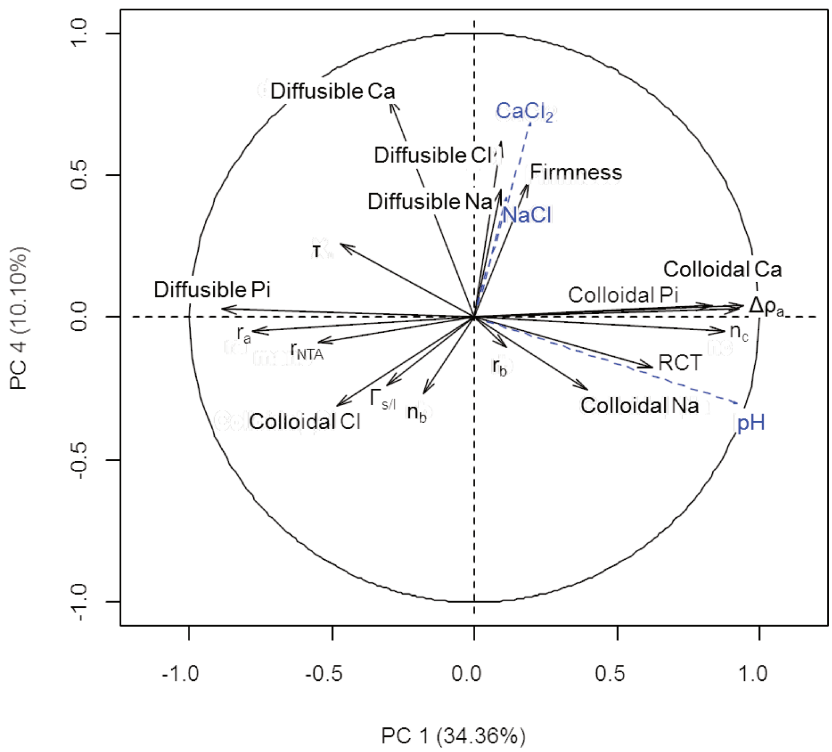

Figure 3: PCA showing the correlation circle of 21 variables in the plane delimited by the first and the fourth principal components (PCs). 44.5 % of the variability of the set of data is represented in this PCA plane.

Colloidal and diffusible concentrations of Pi were also significantly impacted by the addition of $CaCl_2$ but in a much smaller extent, as revealed by correlation coefficients of 0.42 and -0.42 respectively (Fig. 3). These results confirmed that modifications in pH and $CaCl_2$ induced opposite effects on the mineral content of the casein micelles, with a stronger influence of the pH variation compared to the $CaCl_2$ addition. The pH decrease lead to the solubilization of the CaP nanoclusters, which was reported in the literature (Dalgleish & Law, 1989; Daviau, Famelart, Pierre, Goudédranche, & Maubois, 2000; Famelart, Lepesant, Gaucheron, Le Graët, & Schuck, 1996; Le Graët & Brulé, 1993; Le Graët & Gaucheron, 1999; Le Ray et al., 1998; van Hooydonk, Boerrigter, & Hagedoorn, 1986; Zoon, van Vliet, & Walstra, 1989). Conversely, the addition of $CaCl_2$ limited the CaP solubilization presumably by shifting the Ca^{2+} equilibrium through the saturation of the diffusible phase (Moitzi et al., 2011). Added Ca would also directly associated with caseins and / or with the diffusible Pi and precipitate as CaP salts (Le Ray et al., 1998; Philippe, Le Graët, & Gaucheron, 2005; Philippe, Gaucheron, Le Graët, Michel, & Garem, 2003; Udabage, McKinnon, & Augustin, 2000). The addition of NaCl positively correlated with the diffusible Na and Cl concentrations (0.99 and 0.95, respectively) (Fig. 2) which was consistent

with the presence of NaCl in the diffusible phase. However, no significant correlations with the concentrations of colloidal ions were found (Fig. 2). In other words, the addition of NaCl had no direct effect on the mineral content of the casein micelle in our studied concentration range (0 to 100 mmol kg^{-1}). This result was in agreement with those of Karlsson, Ipsen, & Ardö (2007) who reported no change in the colloidal CaP content after NaCl addition. However, it was different from the results of Aoki, Umeda, & Nakao, (1999), Famelart et al., (1996), Grufferty & Fox, (1985), Zhao & Corredig, (2015) and Zoon et al., (1989) who reported that solubilization of Ca and occasionally Pi occurred when NaCl was added to fresh or reconstituted skim milk or casein micelles suspensions. These discrepancies could arise from the facts that in most cases the pH variations induced by the NaCl addition were not corrected and / or that the amount of added NaCl was three to five times higher than in the present study.

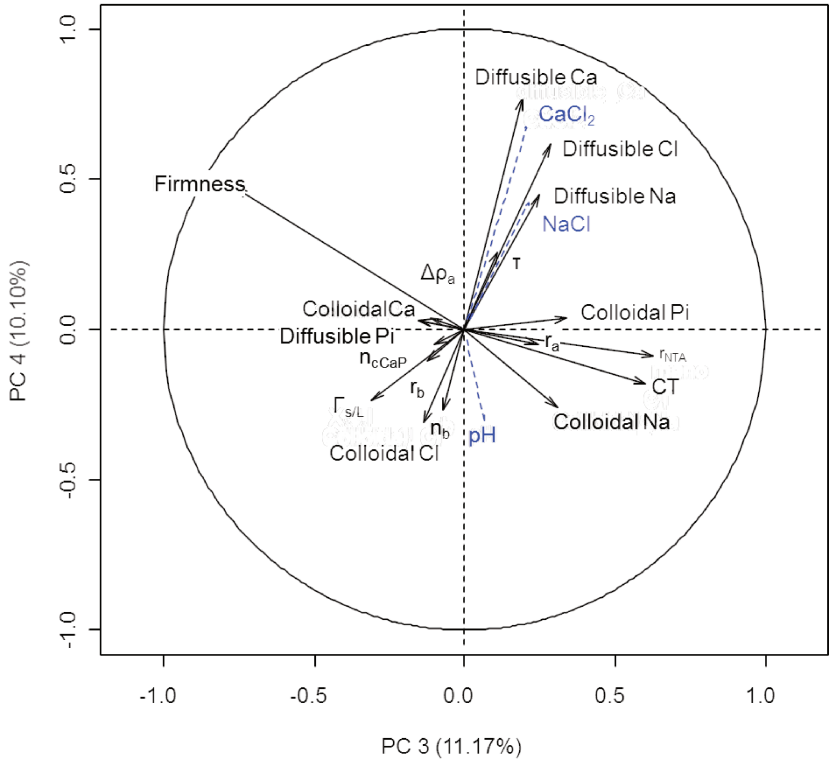

Figure 4: PCA showing the correlation circle of 21 variables in the plane delimited by the third and the fourth principal components (PCs). 21.3 % of the variability of the set of data is represented in these PCA plane.

3.2.2 Consequences of the environmental modifications on the structural properties of the casein micelle

As mentioned in the section 2.5.6 of this paper, SAXS data were treated using the sponge-like model defined by Bouchoux et al. (2010). According to these authors, the SAXS pattern of the casein micelle (Fig. 5) indicates three characteristic features at low q (up to 6 x 10^{-3} $Å^{-1}$), the scattering intensity corresponded to the presence of casein micelles (named population A). In the intermediate q regions (6 x 10^{-3} to 2 x 10^{-2} $Å^{-1}$), scattering particles corresponded to dense regions inside the casein micelles (named population B). The quality of the fits in this region of the SAXS pattern (Fig. 5) was sometimes questionable, and due to the fact that it was not possible to calculate simultaneously n_b and $\Delta\rho_b$. We therefore considered $\Delta\rho_b$ constant and n_b changeable for all the suspensions, based on the assumption that the impact of the variation in pH and addition of salts would be higher on n_b than $\Delta\rho_b$. At high q (7 - 8 x 10^{-2} $Å^{-1}$), CaP nanoclusters or protein inhomogeneties (named population C) would be responsible for the scattering intensity (Fig. 5), according to Bouchoux et al. (2010) or Ingham et al. (2016) interpretations, respectively. As mentioned in the section 2.5.6 of this paper, we decided not to rely exclusively on the interpretation of either Ingham et al. (2016) or Bouchoux et al. (2010) so the viability of both hypotheses of these studies are discussed. SAXS patterns (Fig. 5) show the variability of the intensity signal in these three different regions for the set of 9 selected samples. SAXS structure features (r_a, $\Delta\rho_a$, r_b, n_b, r_c, n_c) of the 27 suspensions were compared to the other physico-chemical variables (concentrations of colloidal and diffusible minerals, τ, r_{NTA}, $\Gamma_{s/l}$...) for each populations of scatterers A, B and C. Correlations between the variables were established through the PCA results (Figs. 2, 3 and 4). The links between the different features and the relevance of the SAXS model applied were discussed in the framework of our experimental conditions.

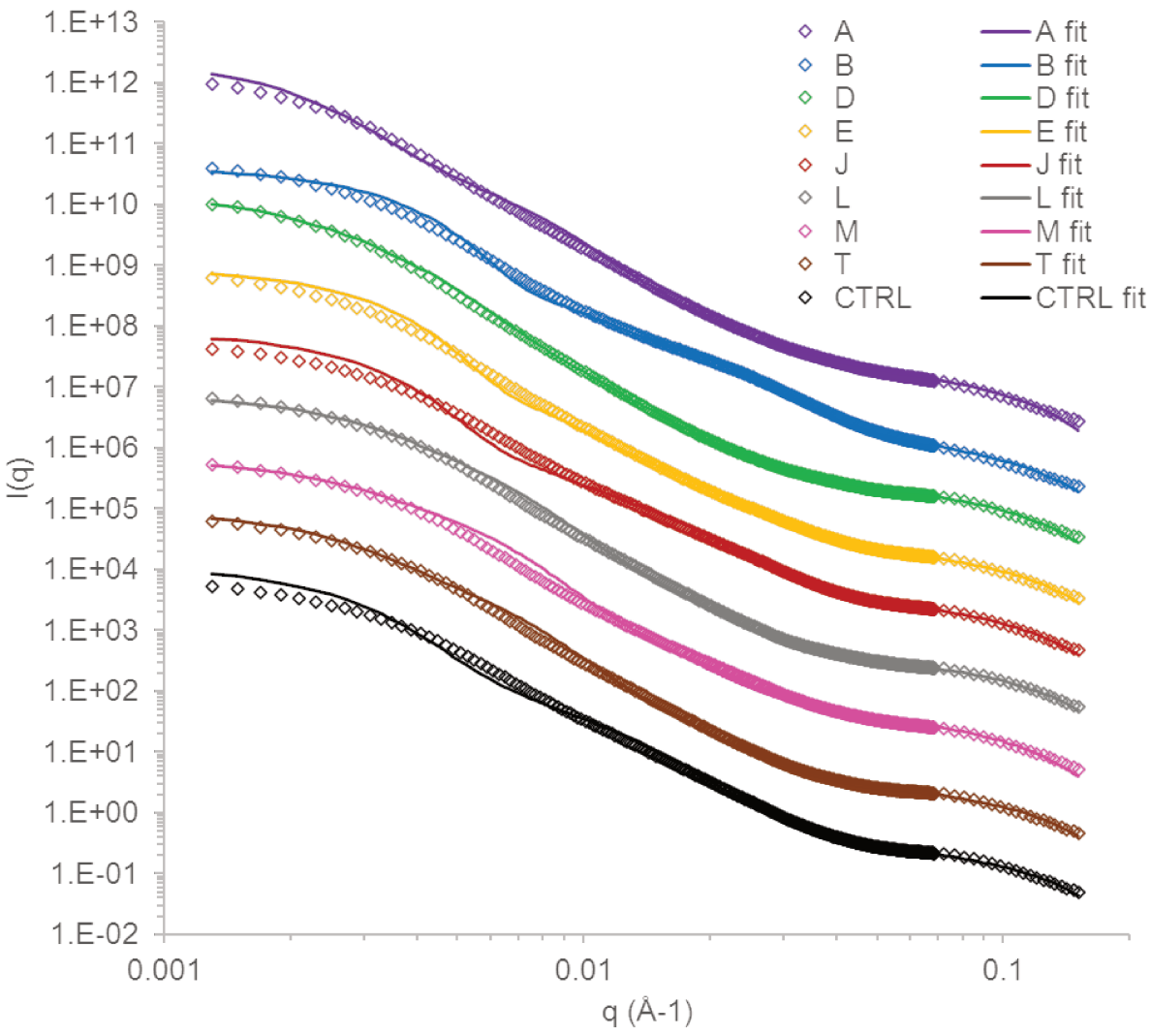

Figure 5: Fits to the SAXS data shown in log – log plot. The scattering curves were shifted along the y-axis for clarity. The scattering profile have been fitted using Bouchoux et al. (2010) model that comprises three populations of scatterers: the casein micelle (population A - up to 6 x 10^{-3} $Å^{-1}$), the dense regions (population B - 6 x 10^{-3} to 2 x 10^{-2} $Å^{-1}$) and either the CaP nanoclusters or the proteins inhomogeneities (population C – 7 - 8 x 10^{-2} $Å^{-1}$). Open diamonds show experimental data; lines are fits for which the parameter values are given in Table 2.

3.2.2.1 Population A: the casein micelle

The SAXS data treatment enabled to define two variables, r_a and $\Delta\rho_a$, describing the casein micelles radius and their contrast, respectively. First, r_a varied from 41.5 to 58.1 nm (Table 2), which was consistent with values determined in earlier characterizations of milk or casein micelles dispersions by SAXS (Bouchoux et al., 2010; Ingham et al., 2016; Pignon et al., 2004; Shukla et al., 2009).

Table 2: Size-related parameters determined by different analytical methods. Average, standard deviation (SD), relative standard deviation (RSD), minimum and maximum values were determined on the complete set of 27 samples. n_{cCaP} and n_{cPI} correspond to the number of population C scatterers per casein micelle, in case where these scatterers are considered as CaP nanoclusters or protein Inhomogeneities, respectively.

	Turbidimetry	NTA	SAXS							Cryo TEM
	τ (cm^{-1})	r_{NTA} (nm)	$\Delta\rho_a$ ($e^-.A^{-3}$)	r_a (nm)	r_b (nm)	r_c (nm)	n_b	n_{cCaP}	n_{cPI}	$\Gamma_{s/l}$
full experimental design - 27 suspensions										
Average	21.2	61.0	0.015	45.6	10.7	1.6	3.3	171.5	318.9	8.5
SD	6.8	5.5	0.002	4.2	4.2	0.0	2.8	54.0	100.4	7.8
RSD (%)	32.1	9.0	13.5	9.3	38.9	3.1	84.1	31.5	31.5	91.0
minimum	12.6	53.8	0.010	41.5	6.1	1.5	0.2	75.3	140.0	0.9
maximum	43.8	74.8	0.018	58.1	21.9	1.7	13.3	243.7	453.3	35.6
Selected individual suspensions										
A	21.4	70.8	0.011	54.7	7.6	1.7	2.1	87.0	161.9	6.7
B	14.6	53.8	0.012	45.5	6.9	1.6	13.3	75.3	140.0	35.6
D	43.8	73.4	0.012	54.5	6.1	1.6	1.6	127.4	236.9	3.0
E	18.0	59.6	0.014	46.4	7.6	1.6	4.0	121.6	226.2	6.2
J	14.0	59.3	0.016	41.8	8.7	1.6	6.6	174.2	324.0	16.8
L	13.8	59.8	0.018	43.5	12.8	1.6	1.2	231.7	431.0	6.7
M	12.6	61.8	0.017	42.3	8.3	1.6	5.1	202.6	376.9	1.7
T	24.5	62.1	0.017	44.3	12.2	1.6	1.3	203.1	377.8	6.0
CTRL	15.7	57.0	0.018	41.5	10.1	1.5	4.0	228.0	424.1	4.6

r_a positively and strongly correlated with the mean micellar radius determined by NTA (r_{NTA}) (53.8 < rNTA < 74.8 nm) and τ (12.6 < τ < 43.8 cm^{-1}) of the different suspensions (Fig. 2 and Table 2). The correlation coefficients were 0.72 and 0.70, respectively. Major differences were found when comparing this radius to the value found by Tran Le, Saveyn, Hoa, & Van der Meeren (2008) for NPC dispersed in water (212 nm). This discrepancy could arise from strong differences in the analysis conditions (e.g. higher dilution of x 40 000 for our suspensions against x 6 000 for Tran et al. (2008) and different threshold settings). PCA also reported a dependency between the micellar size and the mineral balance. Indeed, r_a showed negative

correlations with colloidal concentrations of Ca and Pi (-0.71 and -0.38, respectively) and a positive correlation with diffusible concentrations of Pi (0.38) (Fig. 2). The solubilization of the micellar CaP caused by the pH decrease resulted in the increase in micellar size due to swelling. This size increase also affected the τ of the suspensions that increased (correlation coefficient of -0.46 between pH and τ) (Fig. 2). These results were in agreement with results of Daviau et al. (2000) and van Hooydonk et al. (1986), although different compared to the observations of Moitzi et al. (2011) and Ouanezar, Guyomarc'h, & Bouchoux (2012). Indeed, these last authors reported a decrease in micellar diameter, measured by multiple angle 3D light scattering or by atomic force microscopy, respectively. In these studies, skim milk and casein micelle powders were suspended in milli-Q water or synthetic milk ultrafiltrate (lactose free saline solution), respectively, providing different ionic environments for the casein micelles than in our study. In addition, the pH ranges covered in these studies were lower than in our study and could conduct to a different behavior of the colloid in such more severe conditions. $CaCl_2$ addition had no impact on r_a (Fig. 3), which was in agreement with results reported by Philippe et al. (2005); Philippe et al. (2003) and Udabage et al. (2000). Conversely, NaCl and diffusible Na concentrations significantly correlated with r_a (-0.46, -0.43, respectively) (Fig. 2) meaning that NaCl addition slightly reduced the diameter of the casein micelles. This tendency was opposite to the findings of Zhao & Corredig (2015) and Karlsson, Ipsen, Schrader, & Ardö (2005) where an increase in the size of casein micelles was observed. However, it is possible that in these studies, the increase in size was due to the pH decrease (not corrected) induced by NaCl addition rather than the direct effect of the salt. On its part, τ negatively correlated with NaCl (-0.46) (Fig. 2), reflecting its decrease following the NaCl addition. This result was in accordance with the two above cited studies and could be caused by the decrease in micellar size and / or internal rearrangements of its structure. Diffusible Na would screen the negative charge carried out by the C-terminal part of κ-casein, causing the collapse of the hairy layer and a slight decrease the micellar size. The impact of NaCl on the size and τ of the casein micelles could also be related to the release of small casein aggregates (dense regions named population B) from casein micelles. This argument is developed further in section 3.1.2.2 of the present paper.

$\Delta\rho_a$, defined as the contrast of the casein micelles, corresponds to their electron density ($\rho_{caseinmicelle}$) relative to the electron density of the diffusible phase (ρ_{DF}). The diffusible phase consisted of water containing ions (Ca, Na, Cl and Pi) coming from the NPC powder and / or the addition of NaCl and $CaCl_2$. The contribution of these diffusible ions to ρ_{DF} ranged from 0.02 to 0.73 %, and thus, was neglected. ρ_{DF} was considered constant and equaled to the electron density of water, (0.334 e^- $Å^{-3}$). Therefore, in the present study, $\Delta\rho_a$ directly reflected the

variation of the electron density of the casein micelles. This value depended on the volume, the caseins and the CaP contents of the casein micelles. $\Delta\rho_a$ varied between 0.010 and 0.018 $e^- Å^{-3}$ (Table 2), which was of same order of the contrast of native casein micelles described by Bouchoux et al. (2010) and Ingham et al. (2016). $\Delta\rho_a$ presented a negative correlation (-0.86) with r_a (Fig. 2). It also positively and strongly correlated with concentrations of colloidal Ca and Pi (0.90 and 0.67, respectively) (Fig. 2). These results were consistent with an increase in volume of the casein micelles due to their depletion in CaP leading to the decrease in their electron density.

3.2.2.2 Population B: the dense regions

The scattering caused by population B is characterized by r_b, (radius of the dense regions) and n_b (number of dense regions per casein micelle). Both features showed variability within the full set of sample, with relative standard deviation (RSD) of 38.9 and 84.1 %, respectively (Table 2). PCA indicated a strong and positive correlation (0.73) between n_b and $\Gamma_{s/l}$ (Fig. 2) which corresponds to the ratio of small (< 50 nm in Feret's diameter) over large (> 50 nm in Feret's diameter) particles detected in cryo-TEM micrographs (section 2.5.5) (Fig. 6). Large black and homogeneous strands crossing the images were the grids that supported the suspensions and large circular spots (e.g. suspension E) or merged spots (e.g. suspension D) were individual casein micelles and aggregates of casein micelles, respectively. Differences in the granularity of the images' backgrounds were attributed to the presence of small-dissociated parts of casein micelles in the diffusible phase (Fig. 6). This feature was quantified by the ratio $\Gamma_{s/l}$.

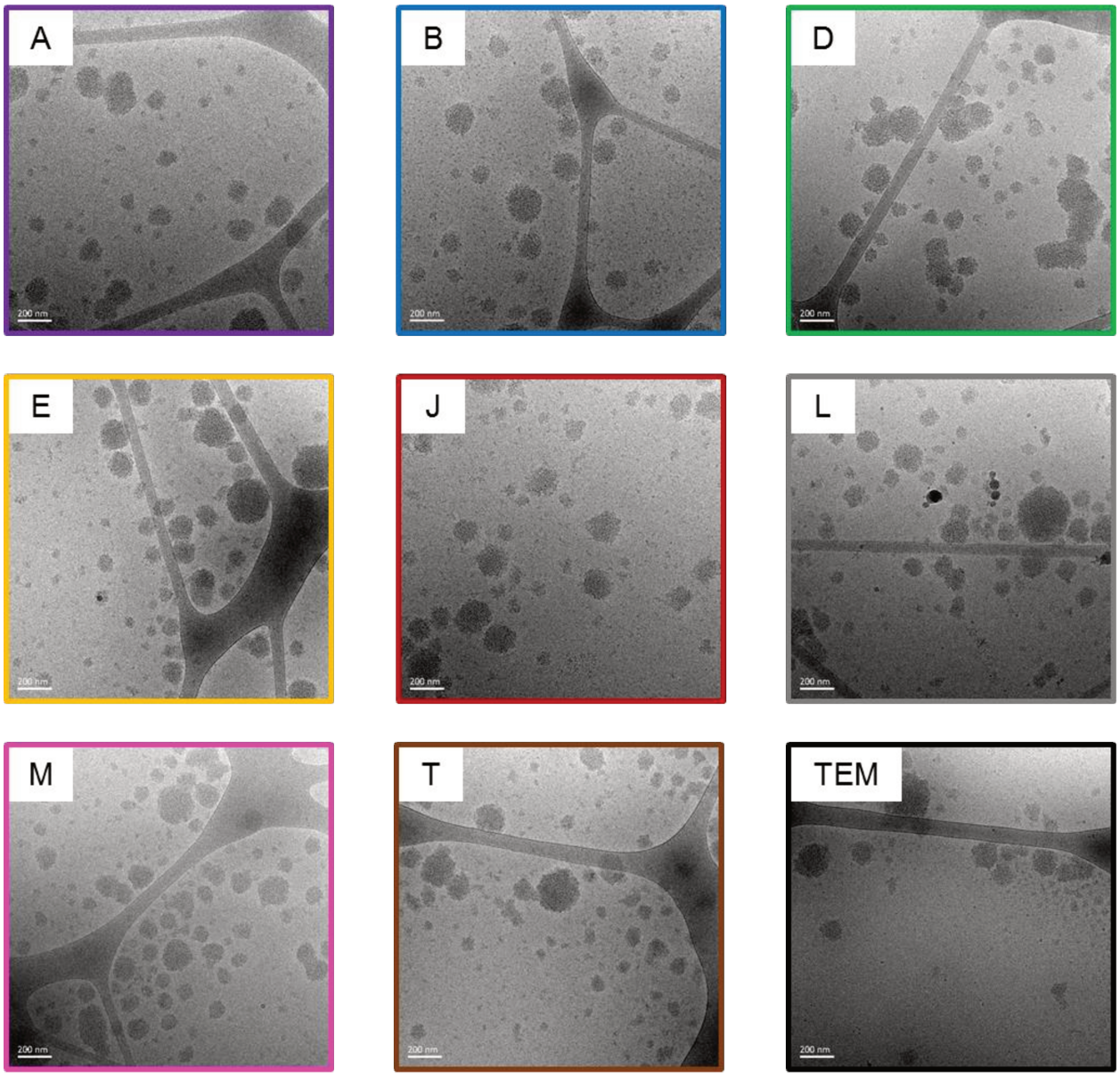

Figure 6: Cryo-TEM micrographs of the selected suspensions. Large black strands crossing the images are carbon grids; circular or merged spots are casein micelles or aggregated casein micelles; dark round spots in suspension L are ice particles that formed during storage of the grids in liquid nitrogen.

Granular backgrounds point out the presence of small casein aggregates dissociated from the casein micelles. This feature is quantified for each suspensions by a $\Gamma_{s/l}$ (ratio of small < 50 nm over large > 50 nm particles). The values of $\Gamma_{s/l}$ are reported in Table 2.

The image analysis revealed that these small particles have diameters between 5 nm (resolution limit of the microscope) and 50 nm (data not shown), which was in agreement with the size range of the population B detected by SAXS measurements (from 6.1 to 21.9 nm in radius –

Table 2). The relation linking these two variables suggested that dense regions were not only present inside, but also outside casein micelles and depended on environmental modifications. PCA also reported positive correlations between n_b and both concentrations of NaCl and diffusible Na of same value, 0.49 (Fig. 2). Therefore, the addition of NaCl increased n_b. Conversely, Ca enrichment of the suspensions with $CaCl_2$ weakly, but significantly reduced n_b (correlation coefficient of -0.39) (Fig. 7). Small particles of around 20 nm in diameter were also observed by Müller-Buschbaum, Gebhardt, Roth, Metwalli, & Doster (2007) using atomic force microscopy. These authors reported a decrease of the number of small particles in the presence of increasing Ca concentration, which is consistent with our observations. To date, Müller-Buschbaum et al. (2007) and our study are the only ones that reported the presence of dissociated aggregates outside of the casein micelles based on microscopy and SAXS observations. However, it is reasonable to assume that such small particles would not sediment by ultracentrifugation and a parallel can be established between our observations and the presence of caseins in ultracentrifuge supernatants, defined as soluble caseins. The results followed the same tendency as those observed by Famelart et al. (1999) and Zhao & Corredig (2015). These authors reported an increase in soluble casein concentration after NaCl addition. Inversely, Famelart et al. (1999), Philippe et al. (2005) and Udabage et al. (2000) observed a decrease in soluble caseins when $CaCl_2$ was added. NaCl would be responsible for the disruption and the loosening of the internal structure of the casein micelle in neutralizing negative charges on the casein chains. $CaCl_2$ would either favor the creation of new bonds between the phosphorylated caseins and / or prevent the dissociation of casein materials in limiting the solubilization of CaP nanoclusters. Furthermore, the increase in soluble casein due to the pH-induced dissociation of CaP nanoclusters have been reported by Dalgleish & Law (1989), Le Graët & Gaucheron (1999) and van Hooydonk et al. (1986). In the present study, there was no significant correlation between pH and n_b (Fig. 2). However, pH has a direct and strong effect on n_{cCaP} (the number of population C scatterers per micelle - correlation of 0.81) which correlates negatively with n_b (-0.45) (Fig. 2). $CaCl_2$ addition had no significant impact on the size of the dense regions (Fig. 7), while NaCl caused their decrease as showed by the correlations between r_b, and concentrations of NaCl and therefore diffusible Na and Cl (-0.50, -0.53 and -0.45, respectively) (Fig. 2). The decrease of population C also led to the decrease in size of the dense regions (correlation coefficient of 0.47 between n_{cCaP} and r_b) (Fig. 2). Finally, it was interesting to notice that n_b and r_b were inversely correlated (-0.59) (Fig. 2), meaning that the more dense regions, the smaller the regions.

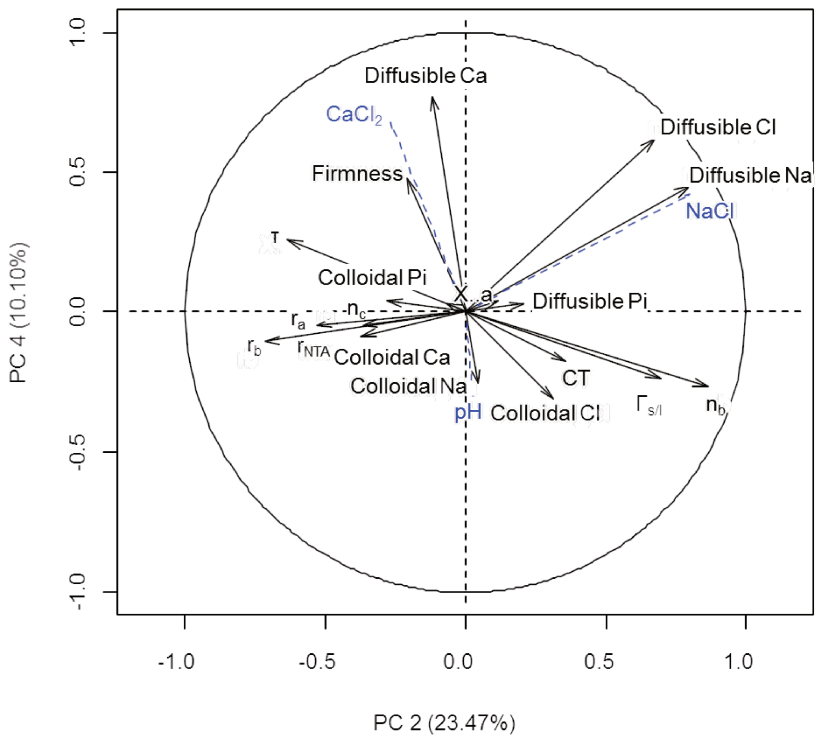

Figure 7: PCA showing the correlation circle of 21 variables in the plane delimited by the second and the fourth principal components (PCs). 33.6 % of the variability of the set of data is represented in this PCA plane. pH, NaCl and $CaCl_2$ variables are illustrative.

3.2.2.3 Population C: CaP nanoclusters or protein inhomogeneities

The mean radius of population C (r_c) ranged from 1.5 to 1.7 nm, whatever the studied suspension with a RSD of 3.1 % (Table 2). This low RSD indicated that this population has similar size in each suspension, whatever the environmental modifications applied. However, their number per casein micelle, n_{cCaP}, varied from 75 to 244. Considering this population as CaP, the number was a bit lower compared to the values of CaP nanoclusters in a native casein micelle, reported as 355 ± 20 $CaHPO_4.2H_2O$ unit by Holt, Timmins, Errington, & Leaver, (1998). It is admitted that the suspension of NPC powder in water at pH 6.9 caused ~ 20 % of the colloidal CaP to dissolve (calculation based on the colloidal and diffusible contents of CTRL sample – Table 1) and could explain this discrepancy. However, this number was consistent with 210 CaP nanoclusters per casein micelle reported by Bouchoux et al. (2010). If population C scatterers were considered as protein inhomogeneities, their number per casein micelle varied from 140 to 453, which was about 17 times lower than the value of 7 700 found by Ingham et al. (2016) (Table 2). This difference could be due to the simplest modeling of this population choose in our study, i.e. the use of a simple sphere form factor compared to a combination of a

Sorensen form factor and hard sphere structure factor used by Ingham et al. (2016). Our approach was nevertheless sufficient to properly fit the SAXS patterns in the high q-region (Fig. 5). The constant size compared to the varying number of C-scatterers per casein micelle indicated the disappearance of this population adhered to the “all-or-nothing” rule, i.e. the population C either “dissolved” completely upon the environment modifications or remained intact in the casein micelle. According to the PCA results, n_{cCaP} highly correlated with pH (0.80), concentrations of colloidal Ca and Pi (0.86 and 0.80, respectively) and with concentration of diffusible Pi (-0.84) (Fig. 2), indicating that the high-q feature disappeared with the pH-induced dissolution of colloidal CaP. Similar pH consequences on the SAXS pattern of the casein micelles were reported by Ingham et al. (2016) and Marchin et al. (2007). The disappearance of the high-q feature was also observed when colloidal CaP was removed from the casein micelles by the use of chelating agents (EDTA or Na_3Cit) (Day et al., 2017; Ingham et al., 2016; Marchin et al., 2007; Pitkowski, Nicolai, & Durand, 2007). Therefore, these correlations were consistent with the assignment of this population to CaP nanoclusters, as suggested by Holt, de Kruif, Tuinier, & Timmins (2003). However, the correlations between n_{cCaP} and the mineral content of the casein micelles did not discard the postulate defended by Ingham et al. (2016) i.e. this population of particles corresponded to protein inhomogeneities. In this case, protein inhomogeneities would be closely linked to micellar CaP. In other words, the dissolution of the CaP from the casein micelle would induce the disruption of the protein inhomogeneities. This would be in agreement with the dual binding model of Horne (1998), that considers that CaP nanocluster endorse not only the role of crosslinking agent but also neutralize the negative charges on the casein chains, allowing the proteins to form more hydrophobic interactions between themselves. As perspective, cross comparisons between the evolution of the high-q SAXS shoulder and the specific intensity variation at q = 0.035 $Å^{-1}$ observed either in SANS or resonant X-ray scattering would bring interesting information for the discussion about this possible CaP nanocluster / protein inhomogeneities dependency. Finally, n_{cCaP} correlated negatively with n_b (-0.45) $\Gamma_{s/l}$ (-0.43) and positively with r_b (0.47) (Fig. 2). These weak but significant correlations indicated that casein micelles depleted in population C released more dense regions into the diffusible phase.

3.3 Coagulation properties of the modified casein micelles

The RCT and the maximum firmness of the gels, defined here as firmness, were determined for the 27 suspensions on the firmness curves (Fig. 8). These two parameters were linked to the other colloidal and structural variables through PCA and multiple linear regression analyses.

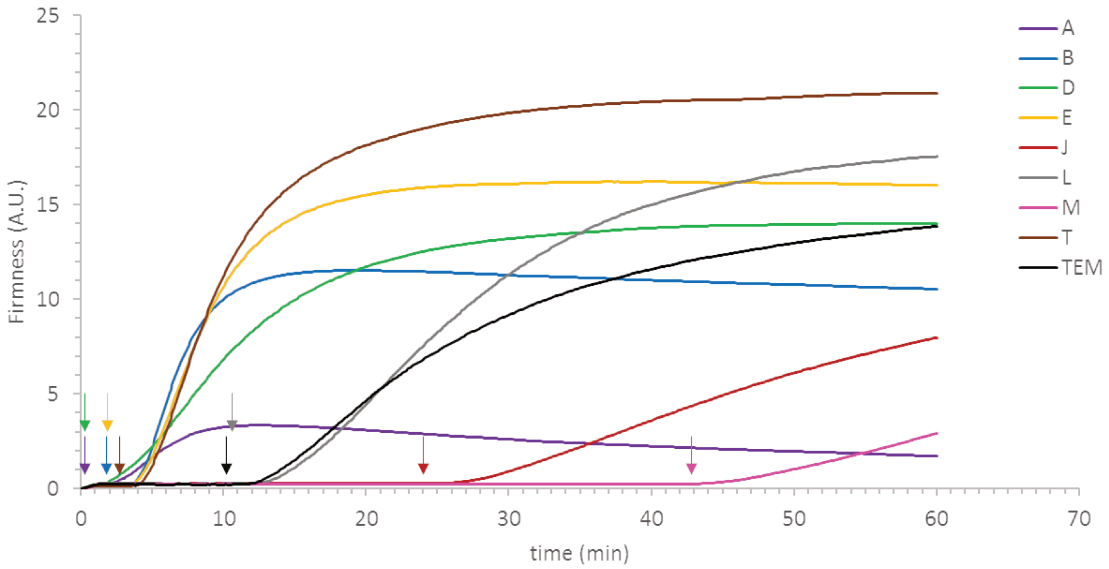

Figure 8: Firmness evolution as a function of time for the selected samples. Arrows are pointing at the RCT of each suspensions, while firmess was defined as the maximum firmness reached within the 60 min after the addition of chymosin to the suspensions. Values of the RCT and firmness are reported in Table 3.

3.3.1 Rennet clotting time

The use of rennet made the 27 suspensions clot between 1.1 and 42.4 min (RSD of 113.5 %) (Fig. 8 and Table 3). This large variability was first ascribed to the variation in pH, as RCT and pH correlated with a significant coefficient of 0.69 (Fig. 2).

Table 3: rennet coagulation properties. Average, standand deviation (SD), relative standard deviation (RSD), minimum and maximum values were determined on the complete set of 27 samples.

	Firmness (A.U.)	RCT (min)
full experimental design - 27 suspensions		
Average	14.9	8.6
SD	5.1	9.8
RSD (%)	34.0	113.5
minimum	3.0	1.1
maximum	20.9	42.4
Selected individual suspensions		
A	3.3	1.4
B	11.5	2.9
D	14.0	1.4
E	16.2	2.9
J	8.0	24.6
L	17.5	11.2
M	3.0	42.4
T	20.9	3.4
CTRL	13.9	11.0

Consequence of the pH decrease were the solubilizations of the micellar Ca and Pi (section 3.1.1). Therefore RCT also positively correlated with concentrations of colloidal Ca and Pi, and negatively with diffusible Pi (coefficients of 0.49, 0.61 and -0.61, respectively, Fig. 2). A reduction in RCT as a result of a decrease in pH was well described in the literature (Choi et al., 2007; Daviau et al., 2000; Karlsson et al., 2007; Zoon et al., 1989). This was ascribed to the enhancement of the enzyme activity and decreasing electrostatic repulsions between paracaseinates at low pH that favored their aggregation. A weaker but significant and positive correlation was also observed between the concentration of diffusible Na and RCT (0.39) (Fig. 2), meaning that increasing the concentration of this ion in the diffusible phase led to an increase in RCT. Similar effects of added NaCl were also reported by Bulca et al. (2016), Famelart et al. (1999), Grufferty & Fox (1985), Karlsson et al. (2007), Sbodio et al. (2006), Zhao & Corredig (2015) and Zoon et al. (1989) and were attributed to the decrease in the enzymatic rate due to a screening of the charges of κ-caseins and the enzyme.

The negative correlations of RCT with τ and r_a (-0.47 and –0.53, respectively) (Fig. 2) indicated that large micelles clotted more quickly than small ones, which was opposite to the findings of

Ekstrand (1980) and Ford & Grandison (1986). The increase of micellar size in the present study was a consequence of the pH decrease that caused the micelles to swell. The correlation between these two factors was probably a disguised effect of pH. In the two studies with opposite findings, the micelles were fractionated according to their size by ultracentrifugation and did not undergo any physico-chemical treatment.

3.3.2 Maximum firmness of the rennet gel

Although there was a high variability of the firmness within the set of suspensions (RDS of 34 %, Fig. 8 and Table 3), this variable did not correlate directly with the other colloidal and structural characteristics of the casein micelles suspensions. Indeed, the firmness, concentrations of $CaCl_2$ and concentrations of diffusible Ca were the only well-projected variables in plane defined by PCs 3 and 4 of the PCA (Fig. 4). Vectors representing concentrations of $CaCl_2$ and diffusible Ca were orthogonal to the firmness one, reflecting no correlations. However, PCA estimates the first order correlations between variables and does not take into account the interactions that might exist between the different features.

A more appropriated statistical approach, multiple linear regression, was used to assess the effects of possible interactions on the gels firmness. Structural SAXS features revealed to be excellent candidates for this complementary analysis for two reasons. First, they were the unique and interesting descriptors reporting information at three different structural levels: 1) the casein micelle, 40 to 60 nm in radius and previously described as population A; 2) the dense regions, 6 to 22 nm in radius and previously described as population B; 3) the CaP nanoclusters (or protein inhomogeneities), 1.5 to 1.7 nm in radius and previously described as population C. Secondly, these variables significantly correlated with all the mineral and colloidal variables determined by other techniques and constituted a way to summarize the whole set of data. Therefore, r_a, n_b and n_{cCaP} were subjected to multiple linear regression in order to define a predictive model of the firmness that considered the quadratic effects and the second order interactions between these variables. For consistency reasons, the values of n_{cCaP} used in this statistical analysis were the one determined considering population C as CaP nanoclusters. This would make no difference in the significance properties of the model as there was a proportional relationship linking n_{cCaP} for CaP nanoclusters and n_{cPI} for protein inhomogeneities.

Based on the experimental design performed, the following model equation was established to predict the maximum firmness of the rennet gels made from the suspensions:

$$Firmness = 187.3 - 3.5 \times ra - 1.2 \times n_b - 0.9 \times n_{cCaP} + 0.02 \times (r_a \times n_{cCaP})$$

where r_a was the radius of the casein micelle, n_b and n_{cCaP} were the number of dense regions and CaP nanoclusters per casein micelle, respectively. This model explained 68.5 % of the variability of the firmness and statistical analysis revealed that the interaction between r_a and n_c (r_a x n_c) and the first order effect of n_b were significant in this model. These two contributions had statistical weights of 35.3 and 27 %, respectively. Figure 9 displays each suspension in the first dimensions of the PCA. The suspensions are colored according to the intensity of their firmness (orange for weak, red for medium, black for strong). Figure 9A represents the evolution of the firmness within the set of samples, in the plane defined by PCs 3 and 4, the arrow is pointing at the direction of the firmness variable in correlation circle, in the same PCs plane (Fig. 4). The negative coefficient assigned to n_b in the firmness equation model indicated that the release of dense regions from the casein micelles led to the formation of weak gels. This direct effect is well illustrated when reading Figure 9A from the bottom right corner (e.g. suspensions G, U - poor in dense regions and strong gels) to the upper left corner (e.g. suspension B - rich in dense regions and weak gel). As mentioned in section 3.1.2.2, the release of dense regions was favored by the addition of NaCl and limited by the addition of $CaCl_2$. The influence of NaCl on the firmness has been well documented but sometimes led to conflicting results. Famelart et al. (1999) and Grufferty & Fox (1985) did not observed any modification of the moduli or the curd tension of the rennet gels upon addition of NaCl. However, our results were in agreement with those described by Bulca et al. (2016) and Zhao & Corredig (2015) who reported a decrease in the firmness or stiffness of the rennet gels with addition of NaCl. On their part, Zoon et al. (1989) observed higher moduli for 8 h aged gels supplemented in NaCl, but lower moduli only 60 min after the addition of rennet to the milk, which corresponded to the experimental conditions of the present study. The negative effect of NaCl on the firmness of rennet gels was poorly explained in the literature. A competition between Na^+ and Ca^{2+} was mentioned, as well as the screening of casein charge and in some case, the solubilization of the micellar CaP (Grufferty & Fox, 1985; Zhao & Corredig, 2015). Based on the significant correlations that linked n_b and NaCl, and the significant negative effect of n_b on the firmness of the rennet gel, we argued that the firmness

decrease observed with the addition of NaCl is due to the release of dense regions from the casein micelle. Similarly to Gaygadzhiev, Massel, Alexander, & Corredig (2012), who found that sodium caseinate (NaCas) addition to milk inhibited the aggregation of casein micelle, the soluble dense regions would adsorb on the surface of the paracaseinates formed after rennet addition, causing an increase in the steric repulsion between the renneted-altered particles. In contrast, there is a general consensus on $CaCl_2$ effect that increase the gel firmness (Deeth & Lewis, 2015; Sandra et al., 2012; Zoon et al., 1988). In this case, this improvement was attributed to the ability of Ca to preserve the number of CaP bonds between the caseins within the micelles but also within the casein gel network. It was demonstrated in section 3.1.2.2, that $CaCl_2$ addition limited the release of dense regions, which could consequently have a positive impact on the firmness through the decrease of n_b.

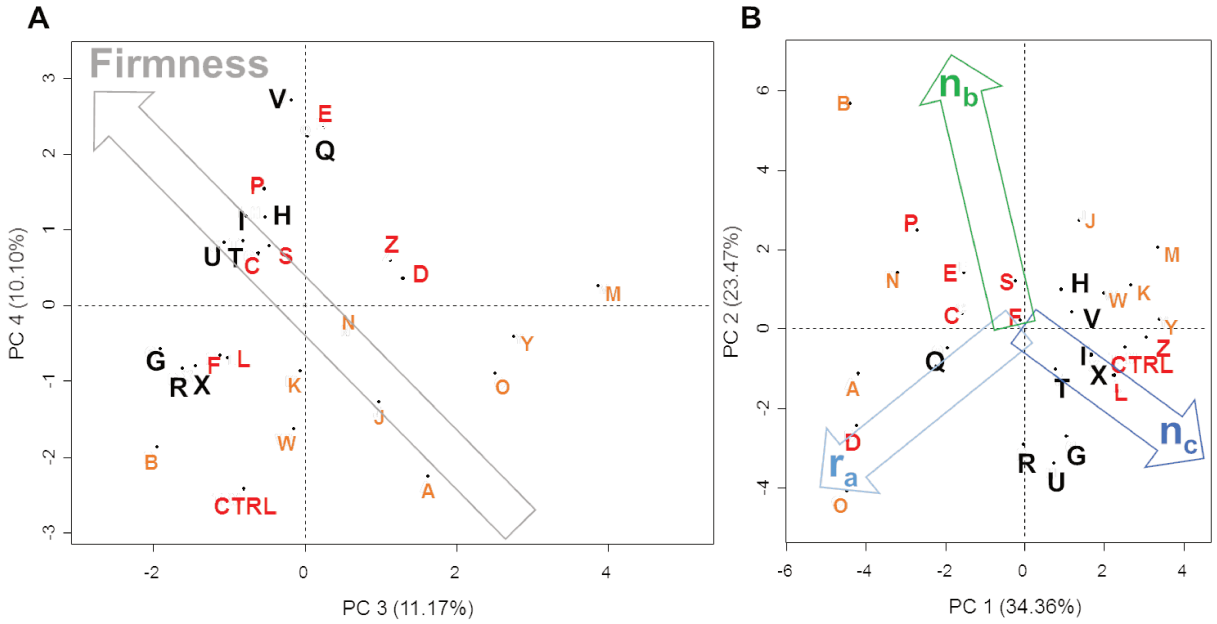

Figure 9: PCA showing the similarity maps for the suspensions determined by A. the third and fourth PCs and B. the two first PCs. Suspensions leading to gels with weak firmness properties are colored in orange, medium firmness are red and strong firmness are black. Arrows reminds the direction in the correlation circle of the firmness and SAXS variables in the same PCs planes (A. see Figures 4A and B. see Figure 2).

The effect of the interaction between r_a and n_{cCaP} was a more subtle to observe. The direct effect of r_a was readable on Figure 9B from the upper right corner (e.g. suspension M - small casein micelles) to the bottom left corner (e.g. suspensions A, D, O - large and swollen casein

micelles). There were no direct and simple consequences of r_a on the gel firmness. This can be illustrated by medium size micelles (center of the graph, suspensions B, S, V, L for instance) that can either form weak, medium or strong gels. This result was quite consistent with the observation of Dalgleish, Brinkhuis, & Payens (1981) who indicated no dependence between the size and the micellar coagulation. However, several authors reported that small casein micelles formed stronger gels (Ford & Grandison, 1986; Logan et al., 2015; Niki, Kohyama, Sano, & Nishinari, 1994). In these studies, the micelles stayed in their native states because they were simply isolated by ultracentrifugation or they were present in milk samples that were selected from cows who produced small casein micelles. On the opposite, in the present study, modifications of the environment affected the size of the casein micelles. On the other hand, the direct effect of n_{cCaP} can be read on Figure 9B from the upper left corner (e.g. suspension B – poor in population C) to the bottom right corner (e.g. suspensions L, Y – rich in population C). No direct dependency of n_{cCaP} and the firmness was observable. As mentioned in section 3.1.2.3, this population can be either CaP nanoclusters or protein inhomogeneities. In both cases, the presence of such interactions, whether they are minerals-proteins or protein-proteins interactions, would create more crosslinking points resulting in a stronger gel network. Population C was linked to the Ca and Pi contents of the casein micelles and correlated with variations in pH. Literature reports a quadratic effect of the pH on the rennet gel firmness, i.e. an increase in the gel firmness up to a maximum value followed by a decrease (Choi et al., 2007; Karlsson et al., 2007; Lucey, Johnson, & Horne, 2003; Zoon et al., 1989). The pH decrease modifies the ionization of individual amino acids containing carboxyl and phosphoseryls groups, depending on the pH values, it would either increase or decrease the electrostatic interactions between the casein chains. A simultaneous consequence of pH decrease is the solubilization of the micellar CaP that would decrease the attractive interactions between the caseins. Addition of chelating agent to milk or casein micelles suspensions also led to the solubilization of micellar CaP (de Kort et al., 2011; McCarthy et al., 2017; Mizuno & Lucey, 2005; Pitkowski et al., 2007) and caused a decrease in the firmness of the rennet gels (Choi et al., 2007). At this point, it is important to remind that the variations of r_a and n_{cCaP} were not impacted by only one factor (size fractionation, or pH, or chelating agent addition), but by three at the same time (pH, NaCl and $CaCl_2$). Therefore, the results of the firmness modelling revealed that the interaction between these variables had to be considered. The interaction between r_a and n_{cCaP} on the rennet gel firmness meant that the firmness at a given r_a depended on n_{cCaP}, and vice versa. As example to illustrate this interaction, suspensions containing medium size casein micelles (radius from 45 to 47 nm) led to the formation of weak gels if the amount of C-particles was too low (e.g.

suspension B). They formed medium strength gel (e.g. suspensions P, E, C) if their C-particle content increased or even strong gels for casein micelles rich in C-particles (e.g. suspensions X, I, T). Similarly, if too small, casein micelles rich in C-particles formed weak gels (e.g. suspensions W, K, M) but stronger gels with an increased in size (e.g. suspensions H, V, T). Large casein micelles, depleted in C-particles (e.g. suspensions A, O, D) also formed weak gels.

4 Conclusion

The multifactorial approach enable to study a large diversity of casein micelle states that would not have been reachable through one-factor studies only given that each of the factor have different influences on the casein micelles properties. pH clearly affected their mineral content while NaCl addition caused main changes in the structural organization of the colloids. $CaCl_2$ had minor impacts in the ranges of pH and concentrations studied.

Thanks to simultaneous variations, it was possible to rank the factors according to their influences on the mineral balance, the colloidal and rennet coagulation properties of the casein micelles. Variations in pH had the strongest influence on the mineral composition of the casein micelles. A decrease in pH caused the colloidal CaP to solubilize while $CaCl_2$ addition limited this solubilization. On the opposite, NaCl addition showed no impact on colloidal mineral contents but increase the diffusible concentrations in Na and Cl. The solubilization of colloidal CaP caused the micelles to swell while the addition of NaCl was responsible for the release of small particles in the aqueous phase, which decreased the micellar size. The presence of such particles, of around 25 nm in diameter, was revealed by SAXS results combined to cryo-TEM observations. These particles could be parts of the dense regions described by Bouchoux et al. (2010), and were present both inside and outside of the casein micelles. $CaCl_2$ had no effect on the casein micelles size but limited the release of the dense regions from the micellar edifices. SAXS patterns also revealed the presence of a high-q structural feature, called population-C in the present study. These scatterers kept a constant size of around 3 nm but varied in number with the environmental conditions. Their presence was strongly depended on the colloidal CaP content. This feature could be assigned to the presence of either CaP nanoclusters or protein inhomogeneities.

Regarding the rennet properties, decrease in pH had the strongest impact on the RCT, causing its reduction while NaCl supplementation led to longer RCT. In addition, the multifactorial approach combined to the thorough characterization of the different structural levels of the casein micelles enabled a better understanding of the gels firmness. The presence of released dense regions from the casein micelles directly and significantly affected the firmness. The casein micelles size and the number of the population-C scatterers (CaP nanoclusters or protein inhomogeneities) also contributed to the firmness model through an interaction.

5 Acknowledgments

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General discussion, conclusion and outlook

GENERAL DISCUSSION, CONCLUSION AND OUTLOOK

1 Review of the context and strategy

Casein micelles were the centerpieces of the present Ph.D. project. They are one of the major milk constituent and represent 80 % of the milk proteins. Caseins are the basis of numerous dairy functionalities and are widely used in food technologies, especially dairy, for their remarkable properties. Casein can be used as casein micelles during the transformation of milk into dairies but they also exist as powder ingredients, such as casein micelles concentrates or isolates, acid or rennet caseins or caseinates, which are further added in food formulations. In each case, technological processes affect the properties of caseins in order to form the desired products. As examples, acid precipitation followed by NaOH neutralization are applied to milk to form NaCas from casein micelles. This ingredient is recognized for its enhanced emulsifying properties. Rennet is added to milk to destabilize casein micelles and form gels, which corresponds to the first step of cheese manufacture.

Often, the properties of the final products are tailored in modifying some environmental factors, such as temperature and pH variations or additions of salts and chelating agents. These modifications also affect the casein micelles properties, especially their mineral balance. The links between the environmental modifications and the different properties of the casein micelles, including their mineral balance, their colloidal and functional properties, are more or less understood and depend on the functionality studied. In addition, only the effects of single modifications were reported in the literature.

The emphasis of the present Ph.D. project was put on filling this knowledge gap with the general objective to understand and establish the relationships that exist between the environmental modifications, the mineral balance of casein micelles, their colloidal and functional properties (with special attentions paid on emulsion and rennet coagulation functionalities).

Briefly, the strategy was to obtain suspensions of casein micelles in water and to modify their environments:

- in adding different concentrations of Na_3Cit (from 0 to 40 mmol kg^{-1}) (Part A),

Or,

- in simultaneously varying five (Part B, chapter 1) or three (Part B, chapter 2) of the following factors: pH (from 5.7 to 7.7); Na_3Cit (from 0 to 30 mmol kg^{-1}); NaCl (from 0 to 100 mmol kg^{-1}); $CaCl_2$ and $MgCl_2$ (from 0 to 15 mmol kg^{-1}). The suspensions of modified casein micelles were then characterized in terms of mineral balance, colloidal properties, emulsion (Part A) or rennet coagulation functionalities (Part B).

2 Answering the Ph. D. objective

In total, five environmental factors were studied throughout this project, all having different consequences on the mineral balance of the casein micelles. Both individual and multifactorial approaches enable to highlight several casein micelles behaviors regarding their colloidal and functional properties. The choice was made to deeply characterize the casein micelles in terms of mineral balance, colloidal and functional properties. Common biophysical (DLS, LLS, turbidimetry) and biochemical analysis techniques (atomic absorption, ionic chromatography, nitrogen content determination) were combined to advanced ones (AsFlFFF, NTA, cryo-TEM and SAXS). The complexity of the multifactorial approach implemented in Part B, chapters 1 & 2, was managed with the help of statistical tools (experimental design, PCA and multiple regression).

The results presented in Parts A and B of this manuscript contribute to answer the four following questions:

I – What are the consequences of environmental modifications of the casein micelles on their mineral balance?

How the mineral balance was impacted, in an individual manner, by each of the environmental modifications studied in this project was already well understood (see literature review section).

The individual effect of Na_3Cit addition on the mineral balance, reported in Part A, was in agreement with the literature (Table 1). However, the work carried out in Part B brought original knowledge because it focused on cases where such modifications were applied simultaneously to casein micelles in suspensions.

Decrease in pH and Na_3Cit addition dominantly affected the mineral content of the casein micelles in solubilizing Ca and Pi (Tables 1 and 2). On the contrary, addition of $CaCl_2$ and $MgCl_2$ were the only factors that showed mineralizing effects in increasing micellar Ca and Mg contents (Table 3). These effects were minor compared to the demineralization induced by variations in pH and Na_3Cit addition. However, no correlations were found between $CaCl_2$ and $MgCl_2$ with colloidal Pi or Cit concentrations, which suggested that these factors did not form insoluble salts and only associated with negatively charged caseins. NaCl addition had no effect on colloidal ions concentrations; however this salt was mainly responsible for both the increases in diffusible Na and Cl concentrations (Table 4).

II – How do these modifications influence the colloidal properties of casein micelles?

The most important changes were related to the partial or total disruption of the casein micelles. The main consequence was the release of smaller particles (~ 10 to 20 nm in radius) detected by AsFlFFF and qualified of small CAs in Part A and detected by SAXS and cryo-TEM and qualified of dense regions in Part B (chapter 2). This phenomenon was already known to be caused by the individual addition of chelating agent such as Na_3Cit, which was confirmed in Part A (Table 1, Fig. 1A). However, the multifactorial studies performed in Part B demonstrated that casein micelles disruption also occurred under multifactorial conditions. The micellar disruption strongly depended on the addition of Na_3Cit (Part B, chapter 1 - Table 1 and Fig 2) or NaCl (Part B, chapter 2 – Table 4 and Fig 3). In addition, pH decrease only caused the swelling of the micelles (increase in size) and did not led to their disruption (Table 2, Figs. 2 and 3).

III – What effects do these modifications have on the functionalities of casein micelles?

<u>Emulsion functionality.</u> Regarding this functionality, addition of Na_3Cit caused a decrease in the emulsion droplets size and a decrease in bridging flocculation, which corresponded to an increase in emulsifying capacity of the casein micelles. However, their emulsion-stabilizing

capacities decreased: none of the emulsions was stable against creaming and flocculation over storage. All of the emulsions were stable against coalescence (Fig. 1).

Rennet coagulation functionality. RCT and firmness were mainly impacted by variations in pH, addition of Na_3Cit and NaCl. Addition of Na_3Cit and increase in pH (Part B, chapter 1 – Tables 1 and 2 and Fig 2) increased the RCT and decreased the gel firmness up to the complete inhibition of the coagulation. Thresholds differentiating coagulating and non-coagulating suspensions could not be defined given that those factors strongly interacted together (Tables 1 and 2). NaCl had significant effects in the three-factors study, in which it caused an increase in RCT and a decrease in gel firmness while decreasing the pH had opposite effects (Tables 4 and 2, Fig. 3).

IV – Which relationships link the mineral balance, the colloidal and the functional properties of casein micelles?

Two casein micelles properties are of major importance on their functional properties: their mineral content and their disruption that led to the release of small particles (named CAs or dense regions, Parts A and B, respectively). The latter can be a consequence of the former, which depended on the environmental modifications applied.

Emulsion functionality

The release of small CAs from casein micelles was a consequence of their CaP depletion, itself induced by the addition of Na_3Cit. Briefly, the disruption of casein micelles into smaller aggregates generated more “protein surface” able to stabilize larger amount of milkfat / water interface and led to the formation of smaller emulsion droplets (increased emulsifying capacity). Regarding the emulsion-stabilizing capacity, the presence of non-adsorbed small aggregates that induced depletion-flocculation enhanced the flocculation of the emulsified droplets.

Rennet coagulation properties

The disruption of casein micelles under multifactorial modifications of their environment was caused by two different phenomena. In the five-factor study (Part B, chapter 1), the disruption was a consequence of CaP solubilization induced by the addition of a Ca chelating salt (Fig. 2). Indeed, CaP links α_{s1}, α_{s2} and β-caseins together in forming nanoclusters that entrapped their phosphoseryl residues. However, pH decrease down to 5.7 only caused the swelling of the micelles (increase in size) which demonstrated that the solubilization of micellar CaP do not

necessarily led to their disruption. In the three-factor study (Part B, chapter 2), pH decrease had the same effect while NaCl caused the casein micelles disruption most probably in breaking up some electrostatic interactions linking the casein molecules. Indeed NaCl had no impact on micellar CaP.

The presence of such aggregates revealed to have a large impact on the gel firmness as indicated by the significant contribution of n_b (number of dense regions determined by SAXS experiments) to the firmness model (Part B, chapter 2). In addition, the casein micelles size (r_a, also determined by SAXS experiments) and their mineral content also affected the gel firmness. Indeed, n_{cCaP} (number of smallest X-ray scatterers, determined by SAXS experiments) strongly correlated with the Ca and Pi contents of the casein micelles (Part B, chapter 2), whether it was interpreted as CaP nanoclusters or protein inhomogeneities (Fig 3).

Table 1: Consequences of Na_3Cit addition on the casein micelles properties highlighted by the work carried out during the Ph.D. project.

Environmental modification		Properties impacted		Variations
Addition of Na_3Cit	Individual modification (Part A)	Mineral balance	Diffusible minerals	Increase in Ca and Pi concentrations Decrease in Na concentration
		Colloidal properties	Soluble casein	Increase
			Charge	Constant zeta potential
			Hydration	Constant followed by a slight decrease
			Micellar size	Decrease (dissociation)
		Emulsion functionality	**Emulsifying capacity:** Droplet size Bridging flocculation Interfacial tension	**Increase** Increase Decrease Constant
			Emulsion stability: → Creaming Thickness of the creamed layer → Flocculation droplet size → Coalescence Droplet size in presence of SDS	**Not stable** → Not stable Decreased → Not stable (increase) Increase → Stable Constant
	Multifactorial modifications (Part B – chapter 1 : five-factors)	Mineral balance	Diffusible minerals	Increase in Ca and Pi, Cit, and Na concentrations
		Colloidal properties	Micellar Size	Decrease
			Turbidity	Decrease
		Coagulation functionality	**Rennet Clotting Time**	**Five-factor study** General tendency: increase Interactions: - strong with pH
			Firmness	**Five-factor study** General tendency: decrease Interactions: - strong with pH - less important with NaCl

Table 2: Consequences of pH decrease on the casein micelles properties highlighted by the work carried out during the Ph.D. project.

Environmental modification	Properties impacted		Variations
pH decrease Multifactorial modifications (Part B – chapter 1: five factors and chapter 2: three factors)	Mineral balance	Diffusible minerals	Increase in Ca and Pi concentrations
	Colloidal properties	Number of CaP nanoclusters or potein inhomogeneities n_{cCaP} or n_{cPI} (SAXS)	Decrease
		Micellar size d_{mean} (DLS) r_{NTA} (NTA) r_s (SAXS)	No correlation Increase Increase
		Turbidity	No correlation (five-factors) Increase (three-factors study)
		Micellar electron density $\Delta\rho_s$ (SAXS)	Decrease
		Number of dense regions n_b (SAXS)	No correlation
		Size of the dense regions r_b (SAXS)	No correlation
	Coagulation functionality	**Rennet Coagulation Time**	**Five-factor study** General tendency: decrease Quadratic effect Interactions: - strong with Na_3Cit - less important with $CaCl_2$ **Three-factor study** Increase
		Firmness	**Five-factor study** General tendency: decrease quadratic effect Interactions: - strong with Na_3Cit - less important with $CaCl_2$ **Three-factor study** Indirectly implicated in an interaction through its effect on CaP solubilization

Table 3: Consequences of $CaCl_2$ and / or $MgCl_2$ additions on the casein micelles properties highlighted by the work carried out during the Ph.D. project.

Environmental modification	Properties impacted		Variations
Addition of $CaCl_2$ or $MgCl_2$ Multifactorial modifications (Part B – chapter 1: five factors → $CaCl_2$ and $MgCl_2$ and chapter 2: three factors → $CaCl_2$ only)	Mineral balance	Colloidal minerals	Increase in Ca concentration ($CaCl_2$) or in Mg concentrations ($MgCl_2$)
		Diffusible minerals	Increase in Ca concentration ($CaCl_2$) or in Mg concentration ($MgCl_2$); but no solubilization of micellar cations
	Colloidal properties	Number of CaP nanoclusters or potein inhomogeneities n_{cCaP} or n_{cPI} (SAXS)	No correlation ($CaCl_2$)
		Micellar size d_{mean} (DLS) r_{NTA} (NTA) r_s (SAXS)	No correlation No correlation ($CaCl_2$) No correlation ($CaCl_2$)
		Turbidity	No correlation
		Micellar electron density $\Delta\rho_s$ (SAXS)	No correlation ($CaCl_2$)
		Number of dense region n_b (SAXS)	Decrease ($CaCl_2$)
		Size of the dense regions r_b (SAXS)	No correlation ($CaCl_2$)
	Coagulation functionality	**RCT**	**Five-factors study** General tendency: no impact Weak interaction of $MgCl_2$ with Na_{Cl} Weak interaction of $CaCl_2$ with pH **Three-factors study** No impact
		Firmness	**Five-factors study** General tendency: no impact Weak interaction of $CaCl_2$ with pH **Three-factors study** Increase (Indirectly implicated: release of dense regions, that decrease the gel firmness, limited by $CaCl_2$ addition)

Table 4: Consequences of NaCl addition on the casein micelles properties highlighted by the work carried out during the Ph.D. project.

Environmental modification	Properties impacted		Variations
Addition of NaCl Multifactorial modifications (Part B – chapter 1: five factors and chapter 2: three factors)	Mineral balance	Diffusible minerals	Increase in Na and Cl concentrations (dominant effect on diffusible Cl concentration) No effect on Ca and Pi concentration
	Colloidal properties	Number of CaP nanoclusters or potein inhomogeneities n_{cCaP} or n_{cPI} (SAXS)	No correlation
		Micellar size d_{mean} (DLS) r_{NTA} (NTA) r_a (SAXS)	No correlation (five-factors) No correlation Decrease
		Turbidity	No correlation
		Micellar electron density $\Delta\rho_a$ (SAXS)	No correlation
		Number of dense region n_b (SAXS)	Increase
		Size of the dense regions r_b (SAXS)	Decrease
	Coagulation functionality	**RCT**	**Five-factors study** General tendency: slight impact Weak interaction with $MgCl_2$ **Three-factors study** Increase
		Firmness	**Five-factors study** General tendency: slight impact Weak interaction with Na_3Cit **Three-factors study** Decrease (Indirectly implicated: release of dense regions favored by NaCl addition decrease the gel firmness)

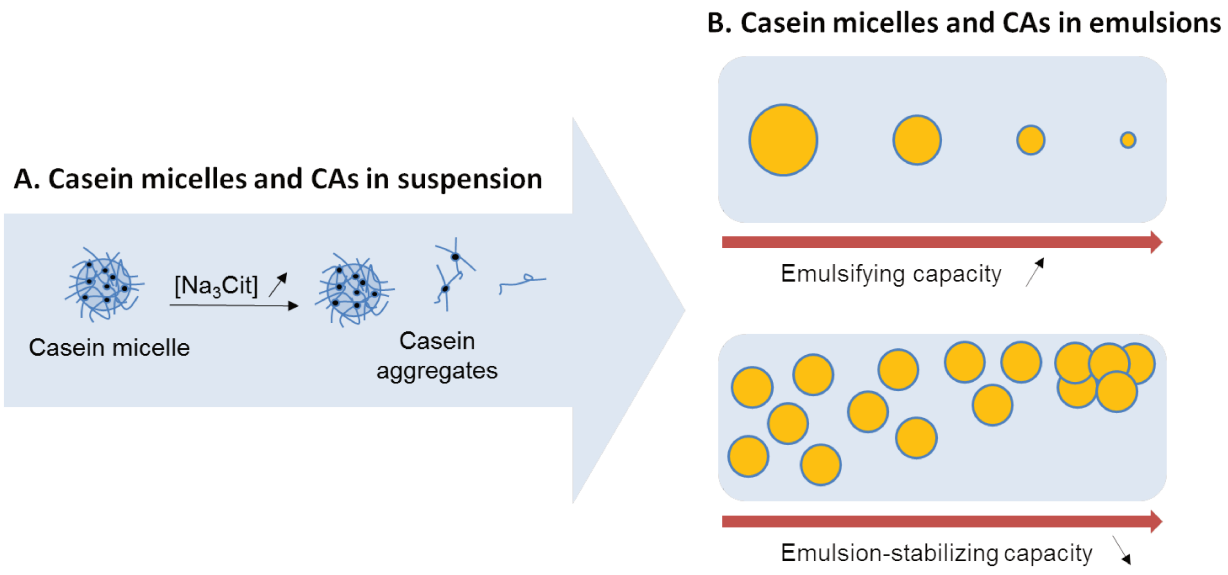

Figure 1. Graphical abstract of the main relationships highlighted in Part A. Emulsion functionality. A. Disruption caused by the solubilisation of micellar CaP, induced by the addition of Na_3Cit (from 0 to 40 mmol kg^{-1}), is responsible for B. the increase in emulsifying capacities and the decrease in emulsion stabilizing capacities of casein micelles and agregates

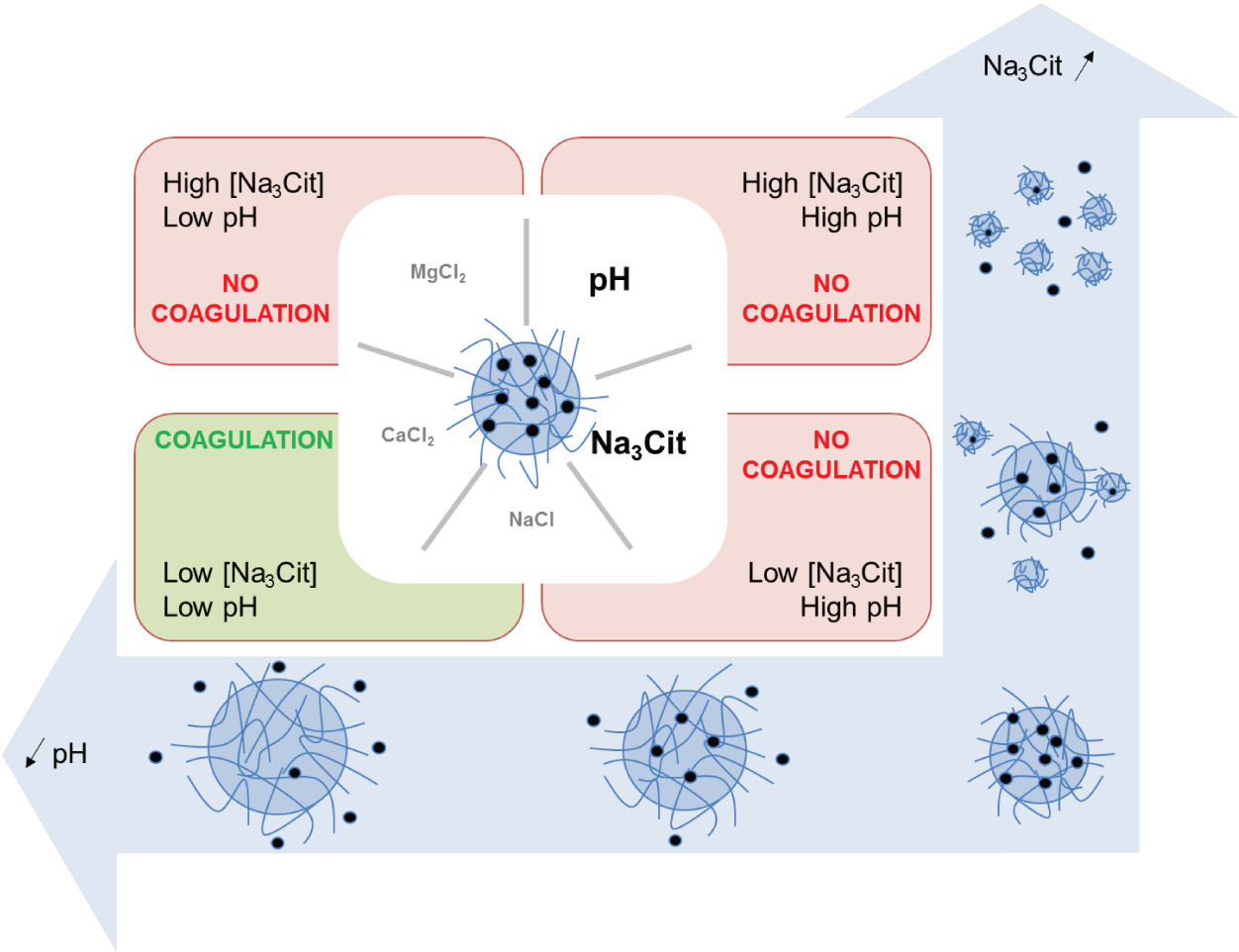

Figure 2. Graphical abstract of the main relationships highlighted in Part B, chapter 1. Rennet coagulation functionality (five-factor study). Variations in pH (from 5.7 to 7.7) and addition of Na_3Cit (concentrations between 0 and 30 mmol kg^{-1}) have major impacts on the rennet coagulation properties of the casein micelles suspensions. Increase in Na_3Cit concentration and decrease in pH are responsible for the micellar CaP solubilization. The former caused the disruption of the micelles while the latter only caused their swelling. Coagulation only occurs at low Na_3Cit concentrations and low pH.

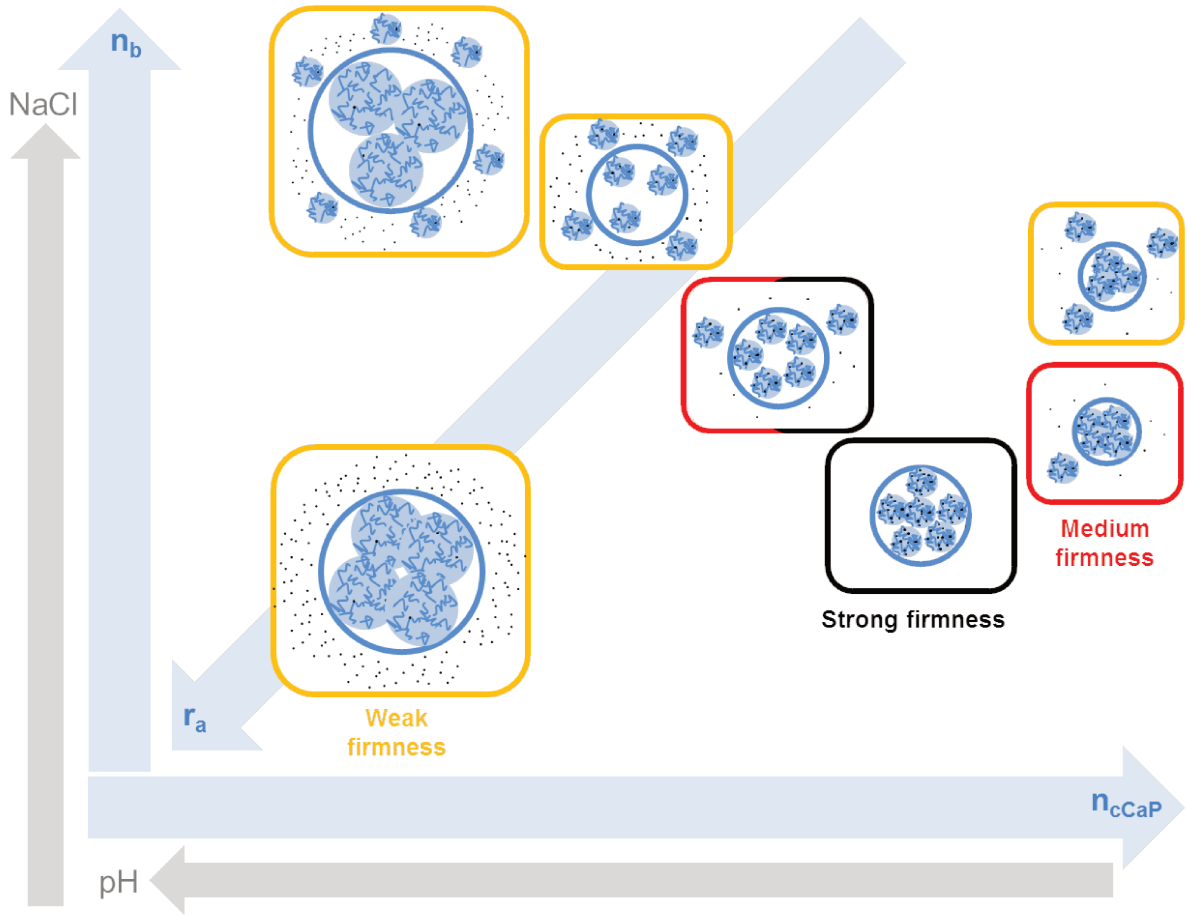

Figure 3. Graphical abstract of the main relationships highlighted in Part B, chapter 2. Rennet coagulation functionality (three-factor study). Gel firmness depended on the colloidal and mineral content of the casein micelles. Suspensions that led to the formation of gels of weak firmness are framed in orange, medium firmness in red and strong firmness in black. Increasing the number of dense regions (n_b) decreased the gel firmness while the size of the casein micelles (r_a) and their mineral content (n_{cCaP}) contribute to the gel firmness through an interacting effect. NaCl addition increased the number of dense regions (n_b increase) while pH decrease caused the solubilization of micellar CaP (n_{cCaP} decrease). Both factors affected the micellar size (r_a).

3 The limits of the study and potential applications of the results

All the results were obtained on model systems composed of purified casein micelles suspended in milli-Q water. Such systems were chosen for understanding reasons, as they reduced milk complexity in limiting the study to caseins and minerals only (absence of lactose, milk fat and whey proteins). The validity of the results on more realistic, but different systems, such as fresh or reconstituted milks, is unsure. It would be interesting to reproduce the same approach on interest systems (e.g. milk or milk concentrates) to verify the reproducibility of the results.

During the P.h. D., this approach on more complex sytem was carried out on raw skimmed milk for the study of the emulsion functionality (data not shown). Besides the presence of lactose and whey proteins also known to have emulsifying properties, the tendencies were the same, i.e. enhancement of the emulsifying properties and decrease of the emulsion-stabilizing properties following the addition of Na_3Cit. These results suggest that differences in the mineral content of the diffusible phase and the presence of whey proteins in milk had no major influences on the observed properties.

The full experimental design carried out in the three-factor study (Part B, chapter 2) can be considered as a sub-design of the five-factor study (Part B, chapter 1). Indeed, pH, NaCl and $CaCl_2$ and their ranged of variations were included in the experimental area covered by the first experimental design. In the three-factor study, limiting the perimeter of the study to suspensions that coagulate only, in eliminating Na_3Cit and tightening the pH range, emphasized the role of NaCl. This demonstrated that each factor depended on others and that the relationships established were valid on the area defined by the experimental design only (system, factors and ranges of pH and concentrations). Caution must be applied in regards to their generalization.

The study of the emulsion functionality was carried out on large droplet size emulsions (~ 12 µm in diameter). It brought several advantages as it facilitated the visualization of the droplets using confocal microscopy, facilitated the determination of interfacial protein contents and enhanced the emulsions destabilization. However, such droplet size range was not conventional compared to the type of emulsions produced in the industry (~ 1 µm in diameter) and can be considered as a limitation for the transfer of the results on emulsified systems containing smaller droplets.

The study of the rennet coagulation properties (Part B, chapters 1 and 2) focused on the secondary phase (aggregation of the casein micelles) through the determination of RCT, and on the finality of the coagulation mechanism through the determination of the gels firmness. Important casein micelles properties were highlighted and hypotheses were discussed

concerning their respective roles in the gel formation. However, the knowledge provided by the experiments was not enough to suggest any complete mechanism. Indeed, little information was brought in regards to the first (κ-casein hydrolysis) and the third (cross-linking and reorganization of the gel) phases of the mechanism. The approach applied in this project could be further developed using other analytical methods, such as characterization of the enzymatic phase in determining CMP content to evaluated the peptide release (first phase), and characterization of the gels structure thanks to rheological and microscopical measurements during coagulation (second and third phases).

In Part B, chapter 2, the firmness differences were explained thanks to SAXS parameters that represented the colloidal and mineral state of the modified casein micelles. Such model is not usable for routine prediction of sample coagulation – unless performing SAXS experiments – which is not realistic to apply in an industrial context. The prime aim of this model was to highlight the mineral and colloidal properties of the casein micelles that were responsible for their gel firmness properties. The extent of casein micelles disruption can be assessed by other more accessible analytical techniques, e.g. in determining soluble proteins content or the size-distribution profile of the casein micelles in suspension. Regarding the mineral state, atomic absorption spactrometry and ionic chromatography or inductively coupled plasma measurements are already employed some industrial research laboratories.

4 Further outlooks

4.1 Benefits of the multifactorial and statistical approaches

One of the main originality of the present project resides in the establishment of relationships between the mineral balance, the colloidal and functional properties that brought better understandings of the casein micelles behaviors in model dairy products. The establishment of such relationships was reached by using both individual and multifactorial approaches, the latter being more powerful in terms of time and financial savings and in providing substantial information compared to the individual approach. The multifactorial approach present the advantage of being easily adaptable to the study of other functionalities, such as thermal stability, acid coagulation or foaming, just to name a few. Environmental modifications could also be extended to the assessment of the effects of temperature, pressure or concentration variations, or other chemical agent additions.

4.2 The question of nanoscale particles in suspension

One of the results of the present Ph.D. project is that the casein micelles disruption into small aggregates induced by Na_3Cit and NaCl additions significantly impacted their functional properties. Therefore, it seems important to successfully characterize the small particles (~ 10 – 20 nm in radius) in terms of size and proportion compared to the larger casein micelle aggregates (~ 50 - 100 nm in radius). The stake is a better understanding, and by extension a better monitoring, of the dairy products properties.

Determining the size of particles, such as macromolecules or colloids, in suspension is a big challenge in numerous biological fields (Beliciu & Moraru, 2009; Gaumet, Vargas, Gurny, & Delie, 2008; Kestens et al., 2016; Provder, 1997; Scherer, Leung, Owyang, & Shire, 2012). The main difficulty is the determination of size distributions, especially in the case of mixtures of several populations of particles; which is the case of disrupted casein micelles. Many techniques are available: they are based on different physical principles and more or less complex methods of data processing. During the entire project, a large range of techniques was covered:

- AsFlFFF was used to characterize the micellar dissociation induced by the addition of Na_3Cit (Part A). The results obtained were consolidated by τ and soluble proteins measurements;

- DLS was applied in the case of the five-factor study (Part B, chapter 1), coupled to τ measurements;

- Cryo-TEM, NTA and SAXS analyses were used for the size characterization of the suspensions produced in the framework of the three-factor study (Part B, chapter 2), also in addition with τ measurements. Although cryo-TEM micrographs were only used to prove the presence of released dense regions ($\Gamma_{s/l}$ measurements), the establishment of particle size distributions would have been feasible.

Table 5 summarizes the main characteristics of each technique and further details can be found in the cited articles. Direct comparisons of each technique reveal discrepancies between the results. Indeed, they depend on the manner the samples were prepared, the physical principle on which the characterizations were based and how the data were analyzed. Nevertheless, the fact that small aggregates were observed using different size-measurement methods strengthen the hypothesis of casein micelles disruption.

Each of these techniques were assessed in terms of benefits and limitations (Table 6). The major source of uncertainties on the adequation of the experimental measurements and the real state of the samples is linked to behavior differences established, or suspected, between the small aggregates and the large casein micelles. As an example, some of the light scattering techniques (DLS, NTA) were not as successful for the detection of small aggregates compared to the others. Indeed small particles considerably less scattered than large ones. AsFlFFF technique was more resolvent thanks to the separation of different population of particles prior their characterization. Uncertainties remained regarding the impact of sample preparation, especially with the level of dilution (NTA, DLS, AsFlFFF) or adsorption and freezing (cryo-TEM) of the samples. SAXS analysis was the only technique that did not needed any transformation of the samples and brought substantial knowledge on internal organization of the casein micelles. However, the high level of expertise required for the instrument handling and the data treatment, the high cost of the technique and the lack of accessibility to the facilities clearly eliminated this technique for the performing of routine experiments.

As a recommendation, couple at least two methods, based on different physical principles, could strengthen any size-measurements. The choice of the techniques should consider the level of information required (mean diameter, whole size distribution, internal structure characterization), the final aim of the measurement (routine measurements, deeper fundamental study) and the expertise and financial means available.

From all the techniques employed during the project, AsFlFFF seemed to be the best compromise for routine measurement, in terms of resolution, level of expertise required and financial cost. It also present the advantage to provide MW characterization of the different aggregates. Another possibility would be to improve the DSL measurements in partitioning the samples prior analysis. This could be done by successive centrifugal sedimentations at different rates as performed by Marchin, Putaux, Pignon, & Léonil (2007). Other methods, which were not used during this Ph.D. project, could be of interest for the characterization of casein micelles disruption. It includes techniques based on chromatography (Griffin, Lyster, & Price, 1988), ultrasound (Povey, 2013) or diffusible wave spectroscopy (Alexander & Dalgleish, 2006; Moitzi, Menzel, Schurtenberger, & Stradner, 2011) principles.

Table 5: Main characteristics of the different analytical techniques used for the determination of casein micelles size. * The size distribution profiles of different casein micelles in suspensions determined by cryo-TEM measurements were not presented in the manuscript. However, it seemed important to mention this technique given that it was another possibility for particle size measurement. The results presented in Part B – chapter 2 corresponds to $\Gamma_{s/l}$ determined by particle detection on the cryo-TEM images.

Technique - detection type	Physical principle	Sample preparation	Type of particle detection	Data analysis	Type of radius	Type of weighting & averaging type	Reference in the manuscript	Principle and casein micelles examples in
AFIFFF-MALLS	Rate of diffusion by Brownian motion against a cross-flow	Suspension diluted with a CaP saturated solution (eluent) – fractionation due to parabolic profile laminar flow	Multi angle light scattering: intensity of light scattered by ensemble of particles co-eluting at a given time	AFIFFF theory	Sphere equivalent hydrodynamic radius	Scattered light intensity	Part A (entire size distribution profiles)	(Guyomarc'h, Violleau, Surel, & Famelart, 2010)
DLS	Rate of diffusion due to Brownian motion	Suspensions diluted 10 times in their own diffusible phase	Fluctuation of the intensity of light scattered by the ensemble of all particles in the measurement volume	Numerical deconvolution of autocorrelation function	Sphere equivalent hydrodynamic diameter	Scattered light intensity & arithmetic mean	Part B – chapter 1 (d_{mean})	(Alexander & Dalgleish, 2006; Beliciu & Moraru, 2009)
Cryo-TEM*	Electron beam imaging based on transmitted electrons	Thin vitrified films formed in loading the suspensions (not diluted) on metallic mesh grids and rapidly frozen in liquid ethane	Individual detection of particles on 2D images.	The detection and size measurement were automated through the use of macros	Numerous possibilities: area-equivalent circular, Feret's, maximum or minimum linear diameters…	Number & arithmetic mean	Part B – chapter 2 ($\Gamma_{s/l}$)	(Danino, 2012; McMahon & McManus, 1998)
NTA	Rate of diffusion due to Brownian motion	Highly diluted (40 000 times) suspensions in their own diffusible phase	Identification of individual particle trajectories in video image	Video analysis of tracked particles' mean-square displacements and velocity in 2D through length scale and time calibration	Sphere equivalent hydrodynamic radius	Number & arithmetic mean (Shulz equation modelling)	Part B – chapter 2 (r_{NTA})	(Tran Le, Saveyn, Hoa, & Van der Meeren, 2008)
SAXS	Angular distribution of X-rays scattered at surface of suspended particles	Direct measurement of not diluted suspensions	Fluctuation of the intensity of X-rays scattered by the ensemble of all particles in the measurement volume	Numerical deconvolution of angular scattering pattern and modelling according to Bouchoux et al. (2010).	Sphere equivalent hydrodynamic radius	Scattered X-ray intensity & arithmetic mean	Part B – chapter 2 (r_a and other variables : $\Delta\rho a$, r_b, r_c, n_b, n_c)	(de Kruif, 2014)

Table 6: Benefits and limitations of the different analytical techniques used for the determination of distributions of casein micelles size.

Technique - detection type	Benefits	Limitations
AFIFFF-MALLS	- high resolution → separation of the constituent of polydisperse suspension - no stationary phase → no interactions of the particles with any materials, separation according to the size and density of the particles - wide size range available (from nm to µm) - low shear force applied → no deformation, compatible with fragile particles	- high dilution of the sample prior and during the separation step - large amount of eluent needed → litre scale - not easy to use, needs quite an expertise to be correctly manipulated - particle must be spherical for accurate analysis - little use on casein micelle reported in the literature
DLS	- wide size range available (from 3 nm to 3µm) - not expensive - fast technique and automated - widely use → easy to access and use - can be coupled to other separation techniques (centrifugation, flow-field fractionation)	- poor resolution on polydisperse suspensions → biased to large entities, scattered intensity proportional to r^6 - dilution required → highly transparent samples - particles must be spherical for accurate analysis
Cryo-TEM*	- high resolution → individual particle detection - wide size range available (> 5 nm) - direct visualization of the samples - no dilution required at normal milk concentration - give access to other characteristics such as particle shape	- expensive technique - high level of expertise required → to operate → for the images treatment - time consuming → numerous step for the sample preparation → needs tens of images - uncertainties on the adequacy between the prepared samples and the original samples → involve a loading on a grid and a rapid freezing steps of the samples
NTA	- higher resolution than DLS → individual tracking of particles - wide size range available (from nm to µm) - compatible with fluorescent dyes - not expensive - quite easy to access and use	- extreme dilution of the samples → 40 000 times in our case - unsure about the resolution → based also on light scattering → focus in favor of large particles - little use on casein micelle reported in the literature
SAXS	- no sample manipulation needed (no dilution, no loading, no freezing, no coating etc) - give access to other structural characteristics of the casein micelle	- size range limited in the upper limit to 800 nm - expensive - high level of expertise required → to operate → for the data treatment and modelling - not easily accessible → peer review application → only a few synchrotron in the world → lab SAXS instrument available, but size range is even narrower - no size distribution, only average value per population - no consensus on the mathemical model to use for the scattering patter treatment

4.3 Internal organization of the casein micelle:

How the casein micelles are formed, how they respond to the various treatments applied in dairy product manufacture are controlled by the interactions between their constituents, i.e. caseins (α_{s1}, α_{s2}, β and κ-caseins), minerals (mainly Ca and Pi) and water. These interactions are of different nature and are intimately linked to the specificities of the casein molecules (Horne, 2017; McMahon & Oommen, 2013):

- Electrostatic interactions mainly intervene in the formation of CaP nanoclusters bridging α_{s1}, α_{s2} and β-caseins containing phosphate centers. These interactions would also be responsible for the Ca bridging of two negatively charged casein sites and different ionic parings between charged amino acids or with mineral ions.

- Hydrophobic interactions would also be a source of “attraction” for the hydrophobic regions of the caseins in limiting their exposure to water.

- Hydrogen bonds and Van der Wall forces are other common protein interactions that would be implicated in the formation of casein micelles and / or casein structures of derived dairy products.

Each of the main model that intends to define the internal organization of the casein micelles (presented in the literature review section of this manuscript) takes into account at least one of these interaction types. To date, diverging points of view remain on the role and importance these interactions, especially in regard to the ones that involved the casein chains only (Farrell, Brown, & Malin, 2013; Holt, 2016; Holt, Carver, Ecroyd, & Thorn, 2013; Horne, 2017; Huppertz, 2013; Thorn, Ecroyd, Carver, & Holt, 2015). The argumentations of the different authors mainly relied on the chemical nature of the caseins and on the impacts of different environmental modifications, always in an individual manner.

A multifactorial approach seems relevant to address the thorny issue of the internal organization of the casein micelles. Indeed, it would enable to simultaneously variate each of the above-mentioned interactions in judiciously selecting several environmental factors. The experimental designs used in the present study (Part B, chapters 1 and 2) were not constructed to meet this challenge of the micellar internal organization as it was not the aim of the project. However, the choice of the factors, pH, Na_3Cit, NaCl, $CaCl_2$ and $MgCl_2$, put emphasis on the importance of the electrostatic interactions that are at the basis of the mineral balance, which was the target of the present project.

pH decrease up to 5.7, one of the predominant factors of our study, unequivocally led to the solubilization of micellar Ca and Pi and caused swelling of the micelles without leading to their disruption (Part B, chapters 1 and 2). This result confirmed that other interactions than CaP-casein interactions would be responsible for the aggregation of the caseins. However, Ca chelation using Na_3Cit, that had a similar impact than acidification on the solubilization of micellar Ca and Pi, significantly correlated with the progressive disruption of the casein micelles, both in controlled (Part A – when dialysis step was applied) or not controlled (Part B – when several factors, comprising salts additions, varied) ionic environments. In the case of acidification, H^+ that had higher association constants, are more efficient than other ions in screening the negatively charged phosphoseryl residues released by the solubilization of Ca and Pi. Therefore, electrostatic repulsions between casein chains would be limited which contributed to the casein micelle stability. In the multifactorial study, confronting pH, NaCl and $CaCl_2$ variations at the same time (Part B, chapter 2), NaCl revealed to be of major influence on the release of dense regions without having any effect on the mineral content of the casein micelles. Again, this result supported the argument that not only mineral-casein but also other interactions are responsible for the micellar stability. These interactions would most probably be of electrostatic nature as ionic strength is reported to enhance hydrophobic interactions (Rajamani, Truskett, & Garde, 2005).

Such discussion demonstrated the potential of multifactorial studies. As suggestion, a similar multifactorial study could be easily performed with the selection of relevant factors for the specific study of all kinds of interactions mentioned at the beginning of this paragraph. Variations in pH, addition of Ca chelating agent and NaCl (ionic strength) would enable to vary the electrostatic interactions (protein-minerals and protein-protein) while temperature variations and / or addition of detergent such as SDS could help in the study of hydrophobic effects. In addition, not only the factors but also the analysis methods should be selected in relevance with this interaction question.

DISCUSSION GENERALE, CONCLUSION ET PERSPECTIVES

1 Revue du contexte et de la stratégie

Les micelles de caséines sont au cœur du ce projet de thèse. Elles sont l'un des composants majeurs du lait et représente 80 % des protéines laitières. Les caséines sont à la base de nombreuses fonctionnalités des produits laitiers et sont largement utilisées en technologie alimentaire, spécifiquement laitières, pour leurs propriétés remarquables. Les caséines peuvent être utilisées en tant que micelles de caséines lors de la transformation du lait en produits laitiers mais elles existent également sous la forme d'ingrédient en poudre, tels que des concentrés ou isolats micellaires, des caséines acides ou présure ou des caséinates, qui sont par la suite ajoutés à des formulations alimentaires. Dans chaque cas, les procédés technologiques employés affectent les propriétés des caséines de façon à former les produits désirés. A titre d'exemple, la précipitation acide suivit de la neutralisation par NaOH sont appliquées au lait pour former du NaCas à partir de micelles de caséines. Cet ingrédient est reconnu pour ces propriétés émulsifiantes améliorées. La présure est ajoutée au lait pour déstabiliser les micelles de caséines et former des gels, ce qui correspond à la première étape de la fabrication de fromages.

Souvent, les propriétés des produits finaux sont ajusté par la modification de facteurs environnementaux, tel que des variations de température et de pH ou l'addition de sels et d'agents chélatants. Ces modifications affectent aussi directement les propriétés des micelles de caséine, en particulier leur équilibre minéral. Les liens entre les modifications environnementales et les différentes propriétés des micelles de caséines, en incluant leur équilibre minéral, leur propriétés colloïdales et fonctionnelles, sont plus ou moins compris et dépendent de la fonctionnalité étudiée. De plus, seuls les effets d'un facteur à la fois ont été reportés dans la littérature.

Ce projet de thèse s'inscrit dans le but de combler ce manque de connaissance avec pour objectif général de comprendre et d'établir les relations qui existent entre les modifications environnementales, l'équilibre minéral des micelles de caséine, leurs propriétés colloïdales et fonctionnelles (avec une attention spéciale portée sur les fonctionnalités émulsion et coagulation présure).

Brièvement, la stratégie à consistée en l'obtention de suspensions de micelles de caséine dans l'eau et dans la modification de leurs environnements :

- en ajoutant des concentrations différentes de Na_3Cit (de 0 à 40 mmol kg^{-1}) (Partie A)

Ou,

- en faisant varier de manière simultanée cinq (partie B, chapitre 1) ou trois (partie B, chapitre 2) de facteurs suivants : pH (de 5.7 à 7.7) ; Na_3Cit (de 0 à 30 mmol kg^{-1}) ; NaCl (de 0 à 100 mmol kg^{-1}) ; $CaCl_2$ et $MgCl_2$ (de 0 à 15 mmol kg^{-1}). Les suspensions de micelles de caséine modifiées ont été caractérisées en termes d'équilibre minéral, de propriétés colloïdales, de fonctionnalités émulsion (partie A) et coagulation présure (partie B).

2 Répondre aux objectifs du projet de thèse

Au total, cinq facteurs environnementaux ont été étudiés lors de ce projet, chacun ayant des conséquences différentes sur l'équilibre minéral des micelles de caséine. Des approches à la fois mono et multifactorielles ont permis de mettre en évidence certain comportement des micelles de caséine en termes d'équilibre minéral, de propriétés colloïdales et fonctionnelles. Des techniques courante d'analyse biophysique (DLS, LLS, turbidimétrie) et biochimiques (spectrométrie d'absorption atomique, chromatographie ionique, détermination de l'azote) ont été combinées à des techniques plus avancées (AsFlFFF, NTA, cryo-TEM et SAXS). La complexité de l'approche multifactorielle utilisée dans la partie B, chapitres 1 et 2 a été gérée avec l'aide d'outils statistiques (plans d'expérience, PCA et régression multiple).

Les résultats présentés dans les parties A et B de ce manuscrit contribuent à répondre aux quatre questions de recherche suivantes :

I – Quelles sont les conséquences des modifications environnementales des micelles de caséine sur leur équilibre minéral ?

La façon dont l'équilibre minéral des micelles a été impacté, d'une manière individuelle, par chacune des modifications étudiées dans ce projet était déjà bien compris et décris dans la littérature (voir section revue de la littérature). L'effet individuel de l'ajout de Na_3Cit sur l'équilibre minéral, reporté dans la partie A, était en accord avec les précédents travaux publiés (Table 1). Cependant, les travaux réalisés dans la partie B ont apportés des connaissances originales

parce qu'ils se sont focalisés sur des cas ou de telles modifications d'environnement ont été appliquées de façon simultanées aux micelles de caséines en suspension.

La diminution de pH et l'ajout de Na_3Cit sont les effets dominants affectant le contenu minéral des micelles de caséine, en solubilisant le Ca et Pi (Tables 1 et 2). A l'inverse, l'addition de $CaCl_2$ et de $MgCl_2$ ont été les seul facteurs montrant des effets minéralisant en augmentant les contenus micellaires en Ca et Mg (Table 3). Ces effets ont été mineures comparés à la déminéralisation induite par les variations de pH et de Na_3Cit ajouté. Cependant, aucune corrélation n'a été trouvée entre $CaCl_2$ et $MgCl_2$ avec les concentrations colloïdales de Pi ou de Cit, ce qui suggère que l'ajout de ces sels n'ont pas permis de former des sels insolubles et que les ions ajoutés ce sont seulement associés aux résidus caséiniques négativement chargés. L'ajout de NaCl n'a eu aucun effet sur les concentrations en ions colloïdaux bien que ce sel eut été responsable de l'augmentation des concentrations de Na et Cl diffusibles (Table 4).

II. – Comment ces modifications influencent-elles les propriétés colloïdales des micelles de caséine ?

Les changements les plus importants ont été liés à la dissociation partielle ou totale des micelles de caséine. La conséquence principale a été la libération de plus petites particules (~ 10 à 20 nm de rayon) détectées par AsFlFFF et qualifié de « petits CAs » dans la partie A et détectées par SAXS et cryo-TEM et qualifiées de « régions denses » dans la partie B (chapitre 2). Ce phénomène était déjà connu pour être causé par l'addition individuelle d'agent chélatant comme le Na_3Cit, ce qui a été confirmé dans la partie A (Table 1, Fig. 1A). Cependant, les études multifactorielles réalisées dans la partie B ont démontrées que la dissociation des micelles de caséine se produisait aussi dans des conditions multifactorielles. La dissociation micellaire a été fortement dépendante de l'addition de Na_3Cit (partie B, chapitre 1 – Table 1 et Fig. 2) ou de NaCl (partie B, chapitre 2 – Table 4 et Fig. 3). De plus, la diminution de pH a seulement causé le gonflement des micelles (augmentation de taille) et n'a pas mené à leur dissociation (Table 2, Figes. 2 et 3).

III – Quels sont les effets de ces modifications sur les fonctionnalités des micelles de caséine ?

<u>Fonctionnalité émulsion :</u> En ce qui concerne cette fonctionnalité, l'ajout de Na_3Cit a été responsable de la diminution de la taille des gouttelettes des émulsions et une diminution de leur floculation par pontage, ce qui correspond à une augmentation des capacités émulsifiantes des micelles de caséine. Cependant, leurs capacités de stabilisation des émulsions à diminué et

aucune des émulsions n'était stable face au crémage et la floculation au cours du stockage. Toutes les émulsions ont été stables face à la coalescence (Fig. 1).

Fonctionnalité coagulation présure : Le RCT et la fermeté des gels ont été principalement affectés par les variations de pH, les ajouts de Na_3Cit et de NaCl. L'ajout de Na_3Cit et l'augmentation de pH (partie B, chapitre 1 – Tables 1 et 2 et Fig. 2) ont augmenté le RCT et diminué la fermeté des gels jusqu'à l'inhibition complète du phénomène de coagulation. Les seuils différenciant les suspensions coagulantes et non-coagulantes n'ont pu être établis étant donné que ces facteurs interagissent fortement ensemble (Tables 1 et 2). NaCl a montré des effets significatifs dans l'étude trois-facteurs, dans laquelle il a été responsable de l'augmentation du RCT et de la diminution de la fermeté des gels tandis que la diminution de pH possédait des effets opposés (Tables 4 et 2, Fig. 3).

IV – Quelles rel*ations lient l'équilibre minéral, les propriétés colloïdales et fonctionnelles des* micelles de caséines ?

Deux propriétés des micelles de caséines sont d'une importance majeures pour leur fonctionnalités : leur contenu minéral et leur dissociation qui mènent à la libération de petites particules (appelées CAs ou régions denses, parties A et B, respectivement). Ce dernier phénomène pouvant être une conséquence de la modification du contenu minéral, qui elle même dépend des modifications environnementales appliquées.

Fonctionnalité émulsion

La libération de petits CAs des micelles de caséine a été une conséquence de leur déplétion en CaP, elle-même induite par l'addition de Na_3Cit. Brièvement, la dissociation des micelles de caséine en plus petits agrégats a générée plus de « surface protéique » capable de stabiliser une plus grande quantité d'interface matière grasse laitière / eau et menant à la formation de plus petites gouttelettes d'émulsion (augmentation de la capacité émulsifiante). En ce qui concerne les capacités de stabilisation des émulsions, la présence de petits agrégats non adsorbés ont causé le phénomène de déplétion-floculation et ont augmenté la floculation des gouttelettes des émulsions.

Fonctionnalité coagulation présure :

La dissociation des micelles de caséine sous l'effet de modifications multifactorielles de leur environnement a été causée par deux phénomènes différents. Dans l'étude à cinq-facteurs

(partie B, chapitre 1), la dissociation a été une conséquence de la solubilisation du CaP induite par l'ajout d'un agent chélatant du Ca (Fig. 2). En effet, le CaP lie les caséines αs_1, αs_2 et β ensemble en formant des nanoclusters qui prennent au piège les résidus phosphoséryls. Cependant, une diminution du pH jusqu'à 5.7 a seulement causé le gonflement des micelles (augmentation de taille) ce qui a démontré que la solubilisation du CaP micellaire ne mène pas nécessairement à la dissociation des micelles de caséine. Dans l'étude trois-facteurs (partie B, chapitre 2), la diminution de pH a montré un effet identique tandis que l'addition de NaCl a été responsable de la dissociation des micelles de caséine, très probablement en diminuant les interactions électrostatiques liant les molécules de caséine entre elles. En effet, NaCl n'a pas montré d'influence sur le CaP micellaire.

La présence de petits agrégats dissociés s'est révélé avoir un impact majeur sur la fermeté des gels comme indiqué par la contribution significative de n_b (le nombre de régions denses déterminé par les analyses SAXS) dans le modèle de la fermeté (partie B, chapitre 2). De plus, la taille des micelles de caséine (r_a, également déterminée grâce aux analyses SAXS) et leur contenu minéral affectent aussi la fermeté des gels. En effet, n_{cCaP} (le nombre des plus petits diffuseurs de rayons-X, déterminé par SAXS) était fortement corrélé aux contenus en Ca et Pi des micelles de caséine (partie B, chapitre 2), que ce nombre soit interprété en tant que nanoclusters de CaP ou d'inhomogénéité protéiques (Fig. 3).

3 Les limites de l'étude et les applications potentielles des résultats

L'ensemble des résultats ont été obtenus sur des systèmes modèles composés de micelles de caséines purifiées et resuspendues dans l'eau milli-Q. De tels systèmes ont été choisis pour des questions de compréhension, étant donné qu'ils réduisent la complexité du lait en limitant l'étude aux caséines et aux minéraux seulement (absence de lactose, de matière grasse laitière et de protéines sériques). La validité des résultats sur des systèmes plus réalistes, mais différents, comme des laits frais ou reconstitués, n'est pas garantie. Il serait intéressant de reproduire les mêmes approches sur des systèmes d'intérêts (ex : lait, ou lait concentrés) afin de vérifier la reproductibilité des résultats.

Au cours de la thèse, cette approche sur des systèmes plus complexes à été effectuée sur du lait frais écrémé pour l'étude de la fonctionnalité émulsion (données non présentées). Malgré la présence de lactose et de protéines sériques également connues pour leur propriétés émulsifiantes, les tendances observées ont été identiques, c'est-à-dire l'augmentation des

capacités émulsifiantes et la diminution des capacités stabilisante d'émulsions suite à l'ajout de Na_3Cit. Ces résultats suggèrent que les différences dans les contenus des phases diffusibles et la présence de protéines sériques dans le lait n'ont pas d'influences majeures sur les propriétés observées.

Le plan d'expérience complet réalisé dans l'étude trois-facteurs (partie B, chapitre 2) peut être considéré comme un sous plan de l'étude cinq-facteurs (partie B, chapitre 1). En effet, le pH, NaCl, et $CaCl_2$ ainsi que leurs gammes de variations étaient inclus dans l'aire expérimentale couverte par le premier plan d'expérience. Dans l'étude trois-facteurs, limiter le périmètre de l'étude aux suspensions qui coagulent seulement, en éliminant le facteur Na_3Cit et en resserrant la gamme de pH, a permis de souligner le rôle du NaCl. Ceci démontre que chaque facteur dépend des autres et que les relations qui ont été établies sont valides dans les conditions définies par les plan expérimentaux seulement (système, facteurs et gammes de pH et concentrations). Une éventuelle généralisation de ces résultants devra être effectuée avec précaution.

L'étude de la fonctionnalité émulsion a été réalisée sur des gouttelettes d'émulsions de grande taille (~ 12 µm de diamètre). Ce choix comprend plusieurs avantages étant donné qu'il facilite la visualisation des gouttelettes par microscopie confocale, qu'il facilite la détermination des concentrations protéiques aux interfaces et accélère la déstabilisation des émulsions. Cependant, une telle gamme de taille de gouttelettes n'est pas conventionnelle si l'on se réfère aux types d'émulsions produites dans l'industrie (~ 1 µm de diamètre) et peut être considérée comme une limite pour le transfert de nos résultats à des systèmes émulsifiés contenant de plus petites gouttelettes.

L'étude de la fonctionnalité coagulation présure (partie B, chapitre 1 et 2) se concentre sur la phase secondaire (agrégation des micelles de caséines) au travers de la détermination du RCT, et sur la finalité du mécanisme de coagulation par la détermination de la fermeté des gels. Les propriétés importantes des micelles de caséines ont été soulignées et des hypothèses concernant leurs rôles respectifs dans la formation de gels ont été discuté. Cependant, les connaissances produites par les expériences n'ont pas été suffisante pour permettre de suggérer un mécanisme complet. En effet, peu d'information a été apporté concernant la première (hydrolyse de la caséine κ) et de la troisième étape (réticulation et réorganisation du gel) du mécanisme. L'approche utilisée dans ce projet pourrait être développé en utilisant d'autres techniques analytiques, telles que la caractérisation de la phase enzymatique par le dosage du CMP afin d'évaluer la libération peptidique (première étape), et la caractérisation de

la structure des gels grâce à des méthodes rhéologiques et microscopiques pendant la coagulation (deuxième et troisième étapes).

Dans la partie B, chapitre 2, les différences de fermeté sont expliquées par des paramètres SAXS qui représentent les états colloïdal et minéral des micelles de caséines modifiées. Ce modèle n'est pas directement utilisable pour des prédictions de routine de la coagulation d'échantillon – sauf en réalisant des expérimentations SAXS – ce qui ne semble pas réaliste pour une application industrielle. Le but premier de ce modèle était donc de mettre en évidence les propriétés minérales et colloïdales des micelles de caséines qui sont responsables de la fermeté des gels présure. L'étendue de la dissociation des micelles de caséine peut être évaluée par d'autres techniques analytiques plus accessibles, par exemple en déterminant les protéines solubles ou les profils de distribution de taille des micelles de caséine en suspension. En ce qui concerne l'état minéral, la spectrométrie d'absorption atomique et la chromatographie ionique ou la spectrométrie à plasma à couplage inductif sont des techniques déjà employées dans certains laboratoires de recherche.

4 Perspectives

4.1 Bénéfices des approches multifactorielles et statistiques

Une des originalités principales de ce projet de thèse est l'établissement de relations entre l'équilibre minéral, les propriétés colloïdales et fonctionnelles qui apportent une meilleure compréhension des comportements des micelles de caséine dans les produits laitiers. L'établissement de telles relations a été atteint grâce à l'utilisation d'approches mono et multifactorielles, la dernière étant plus efficiente en termes de gain de temps et financier et en fournissant des informations conséquentes comparé à l'approche mono factorielle. L'approche multifactorielle présente l'avantage d'être facilement adaptable à l'étude d'autres fonctionnalités, telles que la stabilité thermique, la coagulation acide ou les capacités moussantes, pour n'en citer que quelques unes. Les modifications environnementales pourraient aussi être étendues à l'évaluation des effets de la température, de la pression ou de variation de concentration, ou d'ajout d'autres agents chimiques.

4.2 La question des nanoparticules en suspension

L'un des résultats de ce projet de thèse est que la dissociation des micelles de caséine en de petits agrégats induite par des ajouts de Na_3Cit et NaCl impacte de manière significative leurs propriétés fonctionnelles. Il parait alors important de caractériser avec succès ces petites particules (~ 10 – 20 nm de rayon) en termes de taille et de proportion comparé aux plus grandes micelles de caséine (~ 50 – 100 nm de rayon). L'enjeu est une meilleure compréhension, et par extension un meilleur contrôle des propriétés des produits laitiers.

Déterminer la taille de telles particules, comme des macromolécules ou des colloïdes, en suspension est un grand challenge dans de nombreux domaines biologiques (Beliciu & Moraru, 2009; Gaumet, Vargas, Gurny, & Delie, 2008; Kestens et al., 2016; Provder, 1997; Scherer, Leung, Owyang, & Shire, 2012). La plus grande difficulté réside dans la détermination des distributions de taille, spécifiquement dans le cas de mélanges de plusieurs populations de particules, comme dans le cas des micelles de caséines dissociées. De nombreuses techniques sont disponibles : elles sont basées sur des principes physiques différents et des méthodes de traitement des données plus ou moins complexes. Au cours de l'ensemble du projet de thèse, une large gamme de ces techniques a été couverte :

- l'AsFIFFF a été utilisée pour caractériser la dissociation micellaire induite par l'addition de Na_3Cit (partie A). Les résultats obtenus ont été renforcés par des mesures de τ et de contenu en protéines solubles.

- La DLS a été appliquée dans le cas de l'étude cinq-facteurs (partie B, chapitre 1), couplée à des mesures de τ.

- Des analyses en cryo-TEM, NTA et SAXS ont été utilisée pour la détermination de la taille des particules en suspensions produites dans le cadre de l'étude trois-facteurs (partie B, chapitre 2), également avec la réalisation de mesure de τ. Bien que les images de cryo-TEM aient été utilisées uniquement pour prouver la présence de régions denses dissociées des micelles de caséine (mesures de $\Gamma_{s/l}$), les distributions de taille de particules auraient également pu être déterminées.

Le Tableau 5 résume les principales caractéristiques de chaque technique et des détails complémentaires peuvent être trouvés dans les articles cités. Des comparaisons entre chaque technique révèlent les divergences entre les résultats. En effet, elles dépendent de la manière dont les échantillons ont été préparés, le principe physique sur lequel les caractérisations sont basées et comment les données ont été analysées. Néanmoins, le fait que de petits agrégats aient été observés en utilisant différentes techniques de mesure de taille renforce l'hypothèse de la dissociation des micelles de caséine.

Chacune de ces techniques a été évaluée en termes de bénéfices et limites (Table 6). La source majeure d'incertitude sur l'adéquation entre les mesures expérimentales et l'état réel des échantillons est liée aux différences de comportements établies, ou suspectées, entre les petits agrégats et les grandes micelles de caséine. A titre d'exemple, certaine des techniques de diffusion de la lumière (DLS, NTA) n'ont pas été aussi efficace dans la détection des petits agrégats comparés aux autres techniques. En effet, les petites particules diffusent considérablement moins que les plus grosses. L'AsFIFFF a été plus résolutive grâce à la séparation préalable des différentes populations de particules avant leur caractérisation.

Des incertitudes demeurent au sujet de l'impact de la préparation des échantillons, spécifiquement en ce qui concerne le niveau de dilution (NTA, DLS, AsFIFFF) ou l'adsorption et la congélation (cryo-TEM) des échantillons. Le SAXS est la seule technique qui ne nécessite aucune transformation des échantillons et qui apporte des connaissances supplémentaires au sujet de l'organisation interne des micelles de caséine. Cependant, le haut niveau d'expertise nécessaire au maniement des appareils ainsi que pour le traitement des données, le coût

financier élevé et le manque d'accessibilité des équipements éliminent cette technique pour la réalisation d'expériences de routine.

A titre de recommandation, le couplage d'au moins deux méthodes d'analyses, basées sur des principes physiques différents, pourrait renforcer les mesures de tailles. Le choix des techniques devrait considérer le niveau d'information requis (diamètre moyen, distribution de taille, caractérisation de la structure interne), le but final de la mesure (mesure de routine, étude fondamentale plus approfondie) et le niveau d'expertise et les moyens financiers disponibles.

Parmi toutes les techniques employées lors de ce projet de thèse, l'AsFlFFF semblait être le meilleur compromis pour des mesures de routine, en termes de résolution, de niveau d'expertise requis et de coût financier. Cette technique présente également l'avantage de fournir la caractérisation des poids moléculaire des différents agrégats. Une autre possibilité serait d'améliorer les mesures par DLS en partitionnant les échantillons avant analyses. Ce pourrait être fait par des étapes successives de centrifugation à différentes vitesses comme réalisé par Marchin, Putaux, Pignon, & Léonil (2007). D'autres méthodes, qui n'ont pas été utilisées durant ce projet de thèse, pourrait présenter un intérêt dans la caractérisation de la dissociation des micelles de caséine : des techniques basées sur des principes chromatographiques (Griffin, Lyster, & Price, 1988), d'ultrasons (Povey, 2013) ou de spectroscopie d'ondes diffusibles (Alexander & Dalgleish, 2006; Moitzi, Menzel, Schurtenberger, & Stradner, 2011).

4.3 L'organisation interne des micelles de caséine

La manière dont les micelles de caséine se forment, la façon dont elles répondent aux différents traitements appliqués lors de la fabrication des produits laitiers sont contrôlées par les interactions enter leurs constituants, c'est-à-dire les caséines (α_{s1}, α_{s2}, β et κ), les minéraux (principalement Ca et Pi) et l'eau. Ces interactions sont de différentes natures et sont intimement liées aux spécificités des molécules de caséine (Horne, 2017; McMahon & Oommen, 2013) :

- les interactions électrostatiques interviennent principalement dans la formation de nanoclusters de CaP en pontant les caséines α_{s1}, α_{s2} et β qui contiennent des centres phosphate. Ces interactions pourraient également être responsable du pontage par le Ca de deux sites caséiniques négativement chargés et de différents appariement entre des acides aminés chargés et / ou des ions minéraux.

- les interactions hydrophobes pourraient aussi être une source « d'attraction » pour les régions hydrophobes des caséines en limitant leur exposition à l'eau.

- les liaisons hydrogènes et les forces de Van der Wall sont d'autre interactions protéiques courantes qui pourraient également être impliquées dans la formation des micelles de caséine et / ou de structures caséiniques de divers produits laitiers.

Chacun des principaux modèles qui tentent de définir l'organisation interne des micelles de caséine (présentés dans la section revue de la littérature de ce manuscrit) prennent en compte au moins un de ces types d'interaction. A ce jour, des points de vue divergents demeurent au sujet du rôle et de l'importance de ces interactions, plus spécialement vis-à-vis de ceux qui concernent la formation d'intéractions entre les chaînes caséiniques seulement (Farrell, Brown, & Malin, 2013; Holt, 2016; Holt, Carver, Ecroyd, & Thorn, 2013; Horne, 2017; Huppertz, 2013; Thorn, Ecroyd, Carver, & Holt, 2015). Les argumentaires des différents auteurs reposent principalement sur la nature chimique des caséines et sur l'impact des différentes modifications environnementales, toujours de manières individuelles.

L'approche multifactorielle semble pertinente pour s'attaquer à cette question épineuse de l'organisation interne des micelles de caséines. En effet, elle permettrait de faire varier de manière simultanée chacune des interactions mentionnées ci-dessus en sélectionnant de façon judicieuse plusieurs facteurs environnementaux. Les plans d'expériences utilisés lors de cette étude (partie B, chapitres 1 et 2) n'ont pas été construits de manière à répondre à ce challenge de l'organisation interne des micelles de caséine étant donné que ce n'était pas le but de ce projet. Cependant, le choix des facteurs pH, Na_3Cit, NaCl, $CaCl_2$ et $MgCl_2$ souligne l'importance des interactions électrostatiques qui sont à la base de l'équilibre minéral, la cible de ce projet de recherche.

La diminution du pH jusqu'à 5.7, l'un des facteurs prédominant de l'étude, provoque de manière inéquivoque la solubilisation des Ca et Pi micellaires et conduit au gonflement des micelles sans induire leur dissociation (partie B, chapitres 1 et 2). Ces résultats confirment que d'autres interactions que celles entre le CaP et les caséines sont responsables de l'agrégation des caséines. Cependant, la chelation du Ca en utilisant le Na_3Cit, qui a un effet similaire à la l'acidification sur la solubilisation des Ca et Pi micellaires, corrèle de manière significative à la dissociation progressive des micelles de caséine, que ce soit dans un environnement ionique contrôlé (partie A – lorsqu'une étape de dialyse fut réalisée) ou non (partie B – lorsque plusieurs facteurs, dont des ajouts de sels, variaient). Dans le cas de l'acidification, les H^+ qui possèdent

des constantes d'associations les plus élevées, sont plus efficaces que les autres ions pour l'écrantage des charges négatives des résidus phosphoséryles libérés lors de la solubilisation du Ca et du Pi. De ce fait, les répulsions électrostatiques entre les chaines caséiniques seraient limitées, ce qui contribuerait à la stabilité des micelles de caséines. Dans l'étude multifactorielle qui confronte les variations simultanées de pH, d'ajout de NaCl et de $CaCl_2$ (partie B, chapitre 2), le NaCl s'est avéré avoir une influence majeure sur la libération de régions denses sans avoir aucun effet sur le contenu minéral des micelles de caséine. A nouveau, ce résultat soutient l'argument que les interactions minéraux-caséines ne sont pas les seules interactions responsables de la stabilité micellaire. Ces autres interactions seraient probablement de nature électrostatiques étant donné que la force ionique est connue pour accroître les interactions hydrophobes (Rajamani, Truskett, & Garde, 2005).

De telles discussions montrent tout le potentiel des études multifactorielles. A titre de suggestion, une étude multifactorielle similaire pourrait être facilement réalisée en sélectionnant des facteurs pertinents pour l'étude de tous les types d'interactions mentionnées au début de ce paragraphe. Des variations de pH, l'ajout d'un agent chélatant et de NaCl (force ionique) pourraient permettre de faire varier les interactions électrostatiques (protéines-minéraux et protéines-protéines) tandis que des variations de température et / ou l'addition de détergent tel que le SDS pourraient aider dans l'étude des effets hydrophobes. De plus, pas seulement les facteurs mais aussi les méthodes d'analyses devraient être sélectionnées avec pertinence en regard de cette question des interactions.

Thesis output

THESIS OUTPUT

1 Articles

Lazzaro, F., Saint-Jalmes, A., Violleau, F., Lopez, C., Gaucher-Delmas, M., Madec, M.N., … Gaucheron, F. (2017). Gradual disaggregation of the casein micelle improves its emulsifying capacity and decreases the stability of dairy emulsions. Food Hydrocolloids, 63, 189–200. https://doi.org/10.1016/j.foodhyd.2016.08.037

Lazzaro, F., Pezennec, S., Lechevalier, V., Gaucheron, F. Rennet coagulation properties of casein micelles are affected by the interdependence of environmental factors upon multifactorial modification of their mineral balance. To be submitted to Food Hydrocolloids

Lazzaro, F., Bouchoux, A., Raynes, J., Williams, R., Ong, L., Hanssen, E., Lechavallier, V., Pezennec, S., Hyun-Jung, Cho., Logan, A., Gras, S., Gaucheron, F. Tailoring the structure of casein micelles through a multifactorial approach to manipulate and understand their rennet coagulation properties. To be submitted to Food Hydrocolloids.

2 Oral communications

Lazzaro, F., Saint-Jalmes, A., Violleau, F., Lopez, C., Gaucher-Delmas, M., Madec, MN., Beaucher, E., Gaucheron, F. The gradual disaggregation of casein micelles: emulsifying capacities and stability of dairy emulsions. The international hydrocolloids conference, May 16-20 2016, Guelph, Canada.

Lazzaro, F., Pezennec, S., Lechevalier, V., Gaucheron, F. Predictive models of the firmness and clotting time of rennet gels obtained from casein micelles in different mineral environments: a multifactorial approach. Third international symposium on minerals & dairy products, September 20-22 2017, Wuxi, China.

3 Poster

Lazzaro, F., Saint-Jalmes, A., Beaucher, E., Lopez, C., & Gaucheron, F. Formation and stability of dairy emulsions prepared with gradually demineralized casein micelles. IDF World Dairy Summit, September 20-24 2015, Vilnius, Lithuania.

4 Supervision

Supervision of C. Provault, from the University of Bordeaux (master 1), from April to August 2017. She worked on an internship project titled “preparation of a protein based ingredient (liquid or powder) from minerally modified casein micelles keeping cheese making properties: proof of concept” that was defined according to the results obtain during this Ph. D. project.

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ABSTRACT

Caseins micelles, composed of caseins, minerals and water, are under dynamic equilibria, they constantly exchange materials with their surrounding environments. In addition, casein micelles possess valuable functionalities in regards to the formation of dairy products, such as the ability to stabilize emulsions or to form rennet gels. Environmental changes, such as variations in pH, additions of salts or chelating agents, affect the casein micelles equilibria and lead to modifications in their compositional and colloidal properties. Such changes also modify their functional properties, although this aspect is poorly described in the literature. This project aimed to understand the relationships that link the environmental modifications, the mineral balance, the colloidal and functional properties of the casein micelles. The impact of five modifying factors (pH, Na_3Cit, NaCl, $CaCl_2$, $MgCl_2$) of the casein micelles were studied and the focus was placed on emulsion and rennet coagulation functionalities. pH decrease and addition of chelating agent were responsible for the main mineral modifications in solubilizing the micellar calcium phosphate. The former only induce the swelling of casein micelles while the latter led to their disruption into smaller aggregates. NaCl had no impact on the micellar mineral content but also caused the release of small aggregates, as revealed by electronic microscopy and small angle X-ray scattering analyses. The decisive role of micellar calcium phosphate on the functionalities was confirmed and this study highlighted the importance of monitoring the release of small aggregates, as they strongly affected emulsions stability and gels firmness. $CaCl_2$ and $MgCl_2$ additions slightly increased the mineral content of casein micelles and had minor impacts on their rennet coagulation.

Keywords: casein micelle, environment, pH, Na_3Cit, NaCl, $CaCl_2$, $MgCl_2$, mineral balance, colloidal properties, functionalities, emulsion, rennet coagulation, experimental design, multifactorial approach, predictive models

Les micelles de caséines, composées de caséines, minéraux et eau, sont en équilibres dynamiques, elles échangent en permanence de leur matière avec leur environnement. Les micelles de caséines possèdent d'intéressantes fonctionnalités pour la fabrication de produits laitiers, telles que leur capacité à stabiliser des émulsions et à former des gels sous l'action de la présure. Des changements environnementaux, variations de pH, additions de sels ou d'agents chélatants, affectent les équilibres des micelles et induisent des modifications de leurs compositions et propriétés colloïdales. Ces changements modifient également leurs propriétés fonctionnelles, bien que cet aspect soit peu décri. Le but de ce projet était de comprendre les relations liant l'environnement, l'équilibre minéral, les propriétés colloïdales et fonctionnelles des micelles de caséines. L'impact de cinq facteurs (pH, Na_3Cit, NaCl, $CaCl_2$, $MgCl_2$) modifiant les micelles fut étudié en focalisant sur leurs propriétés émulsifiantes et coagulantes par la présure. L'acidification et l'addition de Na_3Cit ont causé les modifications minérales les plus importantes en solubilisant le phosphate de calcium micellaire. Le premier conduisit au « gonflement » des micelles alors que l'agent chélatant causa leur dissociation en petits agrégats. L'ajout de NaCl n'eut aucun impact sur le contenu minéral des micelles mais provoqua aussi la libération d'agrégats, révélée par cryo microscopie électronique et diffusion de rayons-X aux petits angles. Le rôle du phosphate de calcium sur les fonctionnalités fut confirmé et l'étude révéla l'importance de contrôler la libération des agrégats compte tenu de leurs impacts sur la stabilité des émulsions et la fermeté des gels. $CaCl_2$ and $MgCl_2$ augmentaient légèrement le contenu minéral des micelles et eurent des impacts mineurs sur leur coagulation.

Mots clefs : micelle de caséines, environement, pH, Na_3Cit, NaCl, $CaCl_2$, $MgCl_2$, équilibre mineral, propriétés colloidales, fonctionalités, émulsion, coagulation presure, approche multifactorielle, plan d'expérience, models prédictifs

RÉSUMÉ *ABSTRACT*

Comprendre les comportements des micelles de caséines dans des environnements variés, de leur équilibre minéral à leurs propriétés colloïdales et fonctionnelles : émulsion et coagulation présure

Les micelles de caséines, composées de caséines, minéraux et eau, sont en équilibres dynamiques, elles échangent en permanence de leur matière avec leur environnement. Les micelles de caséines possèdent d'intéressantes fonctionnalités pour la fabrication de produits laitiers, telles que leur capacité à stabiliser des émulsions et à former des gels sous l'action de la présure. Des changements environnementaux, variations de pH, additions de sels ou d'agents chélatants, affectent les équilibres des micelles et induisent des modifications de leurs compositions et propriétés colloïdales. Ces changements modifient également leurs propriétés fonctionnelles, bien que cet aspect soit peu décri. Le but de ce projet était de comprendre les relations liant l'environnement, l'équilibre minéral, les propriétés colloïdales et fonctionnelles des micelles de caséines. L'impact de cinq facteurs (pH, Na_3Cit, NaCl, $CaCl_2$, $MgCl_2$) modifiant les micelles fut étudié en focalisant sur leurs propriétés émulsifiantes et coagulantes par la présure. L'acidification et l'addition de Na_3Cit ont causé les modifications minérales les plus importantes en solubilisant le phosphate de calcium micellaire. Le premier conduisit au « gonflement » des micelles alors que l'agent chélatant causa leur dissociation en petits agrégats. L'ajout de NaCl n'eut aucun impact sur le contenu minéral des micelles mais provoqua aussi la libération d'agrégats, révélée par cryo-microscopie électronique et diffusion de rayons-X aux petits angles. Le rôle du phosphate de calcium sur les fonctionnalités fut confirmé et l'étude révéla l'importance de contrôler la libération des agrégats compte tenu de leurs impacts sur la stabilité des émulsions et la fermeté des gels. $CaCl_2$ and $MgCl_2$ augmentaient légèrement le contenu minéral des micelles et eurent des impacts mineurs sur leur coagulation.

Mots-clefs : micelle de caséines, environement, pH, Na_3Cit, NaCl, $CaCl_2$, $MgCl_2$, équilibre minéral, propriétés colloidales, fonctionalités, émulsion, coagulation presure, approche multifactorielle, plan d'expérience, modèles prédictifs

A comprehensive investigation of the behaviors of casein micelles in multiple environments, from their mineral balance to their colloidal and functional properties: focus on emulsion and rennet coagulation functionalities

Caseins micelles, composed of caseins, minerals and water, are under dynamic equilibria, they constantly exchange materials with their surrounding environments. In addition, casein micelles possess valuable functionalities in regards to the formation of dairy products, such as the ability to stabilize emulsions or to form rennet gels. Environmental changes, such as variations in pH, additions of salts or chelating agents, affect the casein micelles equilibria and lead to modifications in their compositional and colloidal properties. Such changes also modify their functional properties, although this aspect is poorly described in the literature. This project aimed to understand the relationships that link the environmental modifications, the mineral balance, the colloidal and functional properties of the casein micelles. The impact of five modifying factors (pH, Na_3Cit, NaCl, $CaCl_2$, $MgCl_2$) of the casein micelles were studied and the focus was placed on emulsion and rennet coagulation functionalities. pH decrease and addition of chelating agent were responsible for the main mineral modifications in solubilizing the micellar calcium phosphate. The former only induce the swelling of casein micelles while the latter led to their disruption into smaller aggregates. NaCl had no impact on the micellar mineral content but also caused the release of small aggregates, as revealed by electronic microscopy and small angle X-ray scattering analyses. The decisive role of micellar calcium phosphate on the functionalities was confirmed and this study highlighted the importance of monitoring the release of small aggregates, as they strongly affected emulsions stability and gels firmness. $CaCl_2$ and $MgCl_2$ additions slightly increased the mineral content of casein micelles and had minor impacts on their rennet coagulation.

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