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Method to assess the quality of casein used as superplasticizer in self-levelling compounds

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ABSTRACT

A fast and accurate method of assessing the quality of casein superplasticizer is presented. The method is based on analysis of the content of α-, β- and κ-casein proteins contained in whole casein by ion exchange fast protein liquid chromatography (FPLC). The chromatographic profiles of six commercial casein samples were determined, revealing that the amount of κ-casein present in the biopolymer is the main assessment criteria for the quality of casein. For high dispersing effectiveness, the content of κ-casein needs to be high. The reason is that at pH ~12, a high content of κ-casein results in submicelles possessing smaller size (diameter ~10 nm), as was proven by dynamic light scattering measurement (DLS). These smaller submicelles are supposed to adsorb on cement in higher amount than large submicelles. Using this FPLC method, the dispersing performance of any casein sample can be determined very quickly without physical testing of mortar.

1. Introduction

Superplasticizers (SPs) are widely used in cementitious materials such as grouts, mortars and concrete mixes. Their function is to reduce the water-to-cement ratio and to provide enhanced fluidity [1]. A variety of SPs has been developed in recent years, e.g. sulfonated naphthalene formaldehyde condensates (BNS) and sulfonated melamine formaldehyde condensates (PMS). Since the 1980s, application of a new generation of polycarboxylate (PC) superplasticizers has been studied [2]. Although PCs are not as tolerant to different cements like polycondensates, they allow to achieve high fluidity at very low water to cement ratios (as low as 0.15) and provide slump retention over a prolonged period of time. Finally, in the 1990s superplasticizers based on "small molecules" were introduced [3]. They consist of one or two anionic anchor groups which allow the molecule to adsorb onto the surface of cement hydrates, and a non-anionic, non-adsorbing side chain, typically made of polyethylene oxide units. A typical example is the methoxy polyethylene glycol ester of phthalic acid.

Casein is a biopolymer which is obtained by acid precipitation from milk. It has been known as a cement dispersant for a long time. It shows excellent plasticizing effect at low dosage and good compatibility with retarders, particularly citrate. In self-levelling underlayments (SLUs), casein stands out above all other superplasticizers because it not only provides excellent flow properties, but also shows a self-healing effect on the surface of the grout. Thus, any disturbance of the surface corrects itself to a planar spread. Casein contains a group of different phosphoproteins which account for approximately 80% of the total protein content of milk. The three major protein fractions are α-, β- and κ-casein. The relative amounts of these proteins can vary considerably, depending on the species of cattle, the seasonal food, and whether the cow is in the lactation period or not.

To understand the difference between the three major protein fractions, it is important to point out that the casein proteins contain different amounts of phosphate groups which are linked to serine through esterification. This way, the casein proteins become anionic polyelectrolytes. α-casein possesses 8 to 13 phosphoseryl residues and carries the highest negative charge of all casein proteins (−24 at pH 6.7). β-casein contains 5 phosphoseryl residues and the net charge of this molecule is −13 at pH 6.7. Compared to α- and β-casein, the net charge of κ-casein derives from two parts, one carrying the positive and one possessing the negative charge. κ-casein is the only protein which is soluble in the presence of Ca^{2+}, because it contains only one phosphate group.

Casein exists in milk in the form of colloidal particles, so called "micelles". Their diameter was found to range between 90 and 140 nm. They are composed of the protein molecules and calcium phosphate clusters, $Ca_9(PO_4)_6$. In 1984, WALSTRA proposed a commonly accepted model for the structure of casein micelles [4]. The model suggests that casein micelles are built of roughly spherical subunits or sub-micelles (see Fig. 1). The composition of the submicelles varies, and their size is in the range between 12 and 15 nm in diameter. The submicelles are kept together by hydrophobic interactions between the proteins and calcium phosphate clusters. The stability of the micelles depends on the environmental conditions. For example, in the presence of a calcium

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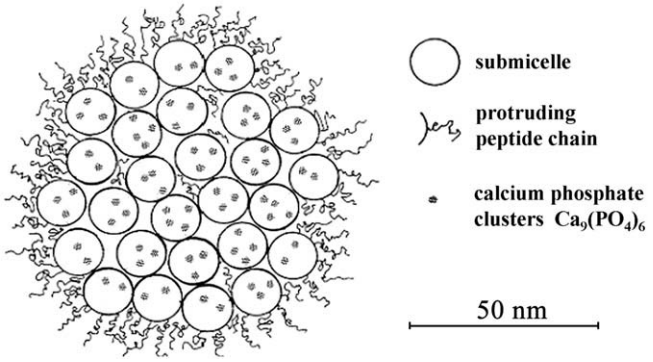

Fig. 1. Model of casein micelle as proposed by WALSTRA [4].

chelating agent such as EDTA [5], the micelles dissociate. Also, solvent-mediated disintegration of casein micelles by heating milk in the presence of ethanol or by introducing urea to the aqueous solution as a chaotrophic agent has been reported [6,7]. In addition, alkaline induced dissociation of casein micelles has also been reported. There, a reduction in the turbidity of skimmed bovine milk was observed when the pH was raised above 9. Obviously, in highly alkaline environment the casein micelle shows a decreased intramicellar stability [8,9].

Although the application of casein as admixture can be tracked back to ancient Rome, investigation on its working mechanism and functional components only occurred very recently. In 2008, we isolated pure α-, β- and κ-casein from whole casein and characterized the single proteins with respect to their specific anionic charge amount in cement pore solution, their adsorption behavior on cement and their zeta potentials [10]. The study revealed that α-casein is the main fraction responsible for the outstanding plasticizing effect of casein. It adsorbs on cement in high amount whereas β-casein shows medium and κ-casein the lowest adsorption. Most recently, intercalation of α- and β-casein into a Ca-Al-LDH host structure which yields a biopolymer-inorganic hybrid material has been proven [11]. This result strongly suggests that dissociation of the casein micelles occurs in highly alkaline solutions (pH ~12).

However, some disadvantages of casein based SPs have been reported over the past decades of application. Among them are a smell of ammonia, caused by decomposition of the proteins at high pH, and enhanced mould formation because casein is a perfect nutrition for *Aspergillus niger* and other moulds. For formulators, a major problem is that the quality of casein varies widely, depending on a serial of factors, such as species of animals, sampling season and manufacturing method, etc. Accordingly, widely fluctuant plasticizing effects are observed for caseins of different batches. Therefore, the industry routinely tests every casein sample extensively and assesses its performance before application. Unfortunately, this testing process is very time consuming and a significant amount of mortar specimens have to be prepared which is a considerable time and cost factor.

To resolve this problem, we studied several commercial casein samples and developed a simple, fast and inexpensive method for assessing the quality of casein by fast protein liquid chromatography (FPLC). FPLC is a widely used technique which allows rapid separation of protein mixtures [12]. Due to their different anionic charge, α-, β- and κ-casein adsorb differently on the column of a cationic media and elute under different buffer conditions. In a previous study, we have shown that this method also allows a large scale fractionation of casein, yielding the pure α-, β- and κ-casein proteins at gram level [13]. In the present work, we first assessed the dispersing effectiveness of the six commercial casein samples over a period of 60 min using a mini slump test. Next, we characterized the casein samples by FPLC and determined the content of each protein present in the casein sample. Furthermore, DLS measurements of the casein samples were carried out in alkaline solution as a model for the SLU pore solution. The goal was to study a potential dissociation of the casein micelles and the effect of micelle disintegration on the plasticizing effect. Based on these findings, the criteria for the quality of casein to be used as superplasticizer in SLUs is proposed.

2. Materials and methods

2.1. Casein samples

Six commercial casein samples provided by Ardex GmbH, Witten/Germany, were used directly without any treatment and purification. The samples were designated as casein #1, casein #2, casein #3, and so on. The colour of the samples varied between white, yellowish and grey white.

2.2. SLU formulation

For performance testing of the casein samples, a simplified SLU paste formulation was used in this study (see Table 1). The formulation is based on a ternary binder system with a water to binder ratio of 0.25. Flowability was measured versus time according to EN 12706. A slump cone with an internal diameter of 30 mm and a height of 50 mm was used.

2.3. FPLC

2.3.1. Instrument

An ÄKTA Explorer (GE Healthcare, Germany) was used as FPLC system in this study. 1 mL Resource Q column (GE Healthcare, Germany) was used as ion exchange media. Absorption at 280 nm was monitored by a UV detector. Solutions of the casein samples were injected into a 100 µL loop on the injection valve.

2.3.2. Preparation of buffers

For the chromatographic analysis of caseins, an imidazole/HCl buffer containing 3.3 M urea was used. To obtain eluent buffer A, 1.362 g imidazole (Merck) and 198 g urea (urea pellets, biochemical grade from Merck) were dissolved in 900 mL ultra pure water under stirring. The pH of the solution was adjusted to 7.0 by carefully adding diluted hydrochloric acid. After that, 2 mL 3-mercapto-1,2-propanediol (Merck) and ultra pure water were added until the total volume of the solution reached 1000 mL. This way, an eluent buffer A containing 20 mM imidazole, 3.3 M urea and 0.2% (v/v) 3-mercapto-1,2-propanediol was obtained. Preparation of the eluent buffer B was done in the same manner. The only difference was that additionally it contained 0.5 M NaCl (Merck).

2.3.3. Pretreatment of casein sample for FPLC

100 mg of casein, 720 mg of urea, 5 mg of EDTA and 2 mg of dithiothreitol (Merck) were dissolved in 2 mL buffer A. 0.1 mL 3-mercapto-1,2-propanediol was added to this solution and the pH was adjusted to 7 by addition of diluted ammonia. After 10 min of ultrasonication, the solution was filtered through a 0.2 µm Millipore membrane (Macherey–Nagel, Düren/Germany) to remove insoluble particles which disturb the following chromatography process.

Table 1
Simplified SLU formulation used in the study.

Component	Function	wt.%
Ordinary Portland cement (CEM I 42.5 R)	Binder	47.3
Calcium aluminate cement (approximately 40 wt.% Al_2O_3)	Binder	32.8
$CaSO_4$ (synthetic anhydrite)	Binder	19.1
Li_2CO_3 (particle size < 40 µm)	Accelerator	0.3
Sodium potassium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$)	Retarder	0.4
Casein	Superplasticizer	0.1
Water (for 100 wt.% of dry blend)		25.0

Table 2
Chromatographic protocol for the FPLC separation of casein proteins.

Volume (mL)	Procedures/parameter
0	Injection of sample to 100 µL loop Sample loading
0–5	Wash out the unbound sample Flow rate 0.5 mL/min
6–51	Linear gradient 0–40% (v/v) buffer B Flow rate = 2 mL/min
52–61	Platform 40% buffer B (v/v) Flow rate = 2 mL/min
62–107	Linear gradient 40%–100% (v/v) buffer B Flow rate = 2 mL/min

2.3.4. Chromatography

To separate and detect the single protein fractions in casein, it is necessary to assure that the peaks from different fractions are clearly separated and no cross contamination occurs between each other. To meet this requirement, a chromatographic protocol was designed. The different steps are listed in Table 2.

2.4. Dynamic light scattering (DLS)

DLS measurements were conducted on a Malvern Zetasizer Nano ZS instrument where a He–Ne laser with a wavelength of 632.8 nm is used as light source. The back scattering angle is set to 173º and the temperature is stabilized at 25 °C. The particle size distribution was obtained by CONTIN analysis.

For each measurement, 10 mg of the casein sample was dissolved in 10 g of 40 mM aqueous NaOH. After filtration through a 0.2 µm Millipore membrane (Macherey–Nagel, Düren/Germany), the solution was ready for DLS measurement.

3. Results and discussion

3.1. Dispersing effectiveness of casein samples

Flowability of SLU pastes containing the different casein samples was determined by measuring the spread flow according to EN 12706. The results are shown in Fig. 2. Casein #1 performed best of all the samples. Tested over a time span of 1 h, the flow remained constant at ~16 cm for 40 min before it dropped as a result of accelerating cement hydration. Casein samples #2 and #3 also performed quite well whereas samples #4–6 showed inadequate dispersing power. This was particularly true for casein #6 which hardly gave a plasticizing effect. According to the supplier of the casein samples, the products tested here were randomly selected from different batches shipped by a manufacturer. The huge variation in quality which becomes evident here and ranges between excellent and no effect at all confirms the huge problem of formulators who have to screen these materials and select the suitable ones.

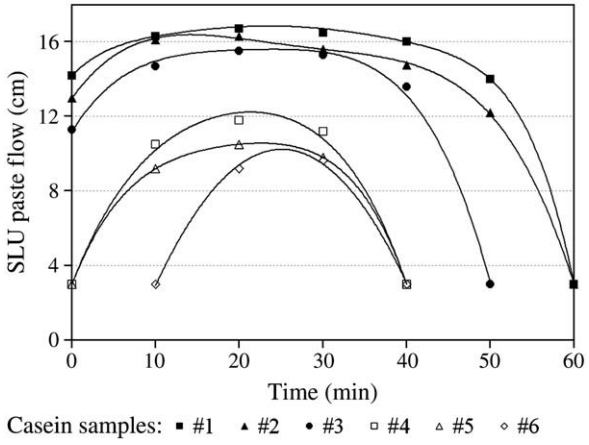

Fig. 2. Flowability versus time for self-levelling grout containing 0.1 wt.% of different commercial casein samples.

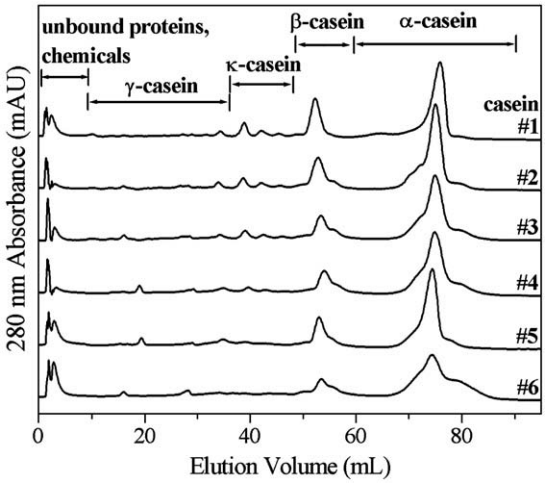

Fig. 3. FPLC profiles of six commercial casein samples, separated on Resource Q column.

3.2. Protein separation by FPLC

Next, the six casein samples were characterized by anion exchange FPLC. Their elution profiles are shown in Fig. 3. The first peaks of substances eluted at 0–10 mL were ascribed to unbound proteins and some chemicals added during sample preparation. The elution profiles occurring at 11–35 mL corresponded to a group of protein species called γ-casein, which is present in trace amounts and does not contribute to the plasticizing effect. In good quality casein samples such as caseins #1–3, the intensity of these peaks was very low, indicating that the active material (α-, β- and κ-casein) were not diluted by these inactive components. The following peaks can be ascribed to the proteins with plasticizing effect. The amount of κ-casein fraction was identified as the group of peaks from elution volume ~36 mL to ~47 mL. The family of β-casein proteins eluted from ~48 mL to ~60 mL elution volume whereas the α-casein protein appeared from ~61 mL to ~85 mL elution volume. The peaks for α- and β-casein were very well resolved, indicating that no cross contamination occurred and that the proteins were completely separated. Sufficient separation between β- and κ-casein was achieved as well.

Earlier we have reported that relative to β- and κ-casein, pure α-casein performed the best plasticizing effect in SLUs [10]. Therefore, we expected that casein samples containing a higher amount of α-casein will exhibit a better plasticizing effect. Based upon this assumption, casein #1 was supposed to contain the largest and casein #6 the lowest amount of α-casein. However, integration of the elution profiles of α-casein shown in Fig. 3 revealed a peak area of 327 mAU mL for casein #1 and of 359 mAU mL for casein #6 (see Table 3). Clearly, the peak areas are not consistent with our theory. Surprisingly, the content of α-casein is not the key parameter which controls the plasticizing effectiveness of casein.

Table 3
Peak area integrals for α-casein and relative percentages of α-, β- and κ-casein contained in the commercial casein samples, calculated on peak areas.

	Casein #1	Casein #2	Casein #3	Casein #4	Casein #5	Casein #6
Peak area for α-casein (mAU mL)	327	371	346	353	357	359
α-casein %	68.9	70.6	73.6	75.0	72.4	80.0
β-casein %	22.9	22.5	20.3	20.3	23.4	18.3
κ-casein %	8.2	6.9	6.2	4.7	4.2	1.6

Former publications have already reported that the composition of casein affects certain properties such as size of micelle, colloidal stability, coagulation properties etc. [14,15]. Therefore, the question arises as to whether the composition of casein, i.e. the relative amounts of α-, β- and κ-casein, represents the factor influencing casein's performance as a superplasticizer. To investigate, the percentage of peak area attributed to each protein fraction and the ratios between peak areas of α- and κ-casein (α/κ) and β- and κ-casein (β/κ) were calculated by integration of the chromatograms. The results are listed in Table 3 and Fig. 4, respectively. Note that in this calculation, the different absorbance coefficients of the casein proteins were not taken into account. Consequently, the results do not represent the actual content of the proteins in the casein samples.

According to Table 3, no clear trend was observed for the α- and β-casein content. However, the percentage of peak area representing the κ-casein protein decreased steadily from casein #1 to casein #6. This is exactly in line with the performance of the casein samples found in the flowability tests. It can be concluded that the more κ-casein is contained in a sample, the better is its plasticizing effect.

In Fig. 4, a clear trend was shown in terms of peak area ratios. There, the ratios of the peak areas α/κ- and β/κ-casein increased distinctly from casein sample #1 to casein sample #6. Namely, for the α/κ-casein ratio, values of 8.39 for casein #1, 10.20 for casein #2, 11.93 for casein #3, 16.01 for casein #4, 17.20 for casein #5 and 49.50 for casein #6 were found, indicating that huge differences existed between samples. This confirmed that the lower are the α/κ- and β/κ-casein ratios, the better is the plasticizing effect of a casein sample. From this data, it was concluded that not only the absolute content of κ-casein, but also the relative amount of α- to κ- and β- to κ-casein could be applied as a quality criterion for the dispersing power of casein. The difference expressed by the α/κ- and β/κ-casein ratios is more significant than the % of κ-casein only. Therefore, it allows a more detailed classification and assessment of the quality of casein samples. Based on our method, a κ-casein content of >6% and an α/κ-casein ratio of <12 appear to be the reference levels for high quality casein. When a casein sample shows a κ-casein content of below 6% or an α/κ-casein ratio of more than 12, it can be expected to perform poorly as superplasticizer or even fail entirely.

3.3. Dissociation of casein micelles

The analysis of the relative amount of κ-casein confirmed that this protein plays the key role in controlling the quality of different casein samples. As pointed out before, this conclusion seems to be in conflict with results published earlier [10], where α-casein was found to show superior plasticizing effect in comparison with the pure β- and κ-casein proteins. To understand this phenomenon, the behavior of casein micelles in highly alkaline environment needs to be looked at. In previous studies, casein micelles were found to be unstable in alkaline solution (pH 8–11). There, they dissociate into a subunit made of α-, β- and κ-casein which is much smaller in size than the original micelles [8,9]. In SLUs, casein is exposed to a pH value of ~12. Thus, one can expect that when casein is used in SLUs, substantial disintegration of the casein micelles will occur, and much smaller submicelles will be present.

To investigate this effect quantitatively, we dissolved casein sample #1 in 40 mM aqueous NaOH (pH ~12) and performed DLS measurements to gain information about the particle size of casein in this strongly alkaline medium. The results are shown in Fig. 5.

The particle size distribution calculated by scattering intensity of this casein sample (see Fig. 5a) showed a typical bimodal distribution profile, where one population exhibited a mean diameter between ~80 and ~200 nm, presumably representing casein micelles. Between ~8 and ~13 nm, a second population appeared, supposedly representing submicelles. Although the larger particles (micelles) showed a much higher scattering intensity, based on the number distribution the smaller ones (submicelles) proved to be by far the predominant species existing in solution (see Fig. 5b). They accounted for a weight fraction of 98.9%. Thus, the DLS data reveals that in highly alkaline environment, the casein micelles practically completely dissociate into submicelles. This suggests that in SLUs not the large casein micelles, but instead very small submicelles adsorb onto the surface of the binder and perform the superplasticizing effect. From this data, we propose a model for the behavior and working mechanism of casein superplasticizer in cement paste (see Fig. 6).

It is now concluded that in a SLU formulation, casein dissociates into submicelles due to the alkaline pH. Other researchers have found that the size of casein micellar structure is especially closely related to the content of κ-casein [15]. Due to its more pronounced hydrophilic domains, κ-casein is located preferentially on the surface of the micelles. This protein forms an outer layer which covers the core of the submicelle. Through this mechanism, the amount of κ-casein present in a casein sample determines the final size of the submicelles [16]. Therefore, a casein containing a high proportion of κ-casein tends to form a large number of micelles possessing smaller size. Concerning the six casein samples tested here, the relative amount of κ-casein detected by FPLC decreased in the order from casein #1 to casein #6. Accordingly, their particle sizes in alkaline solution are supposed to increase by the same order. To verify, average particle sizes of the submicelles released from the six casein samples were

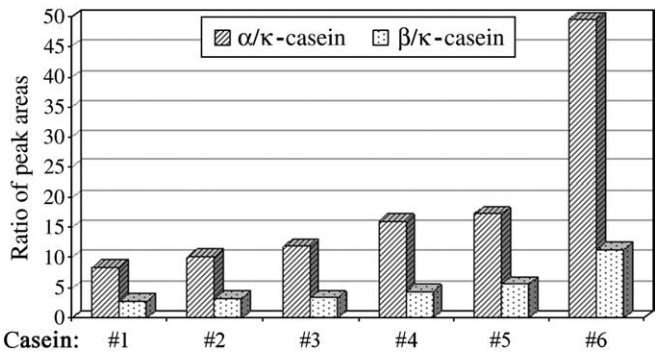

Fig. 4. Ratios of peak areas for α/κ- and β/κ-casein proteins in six commercial casein samples, determined by FPLC.

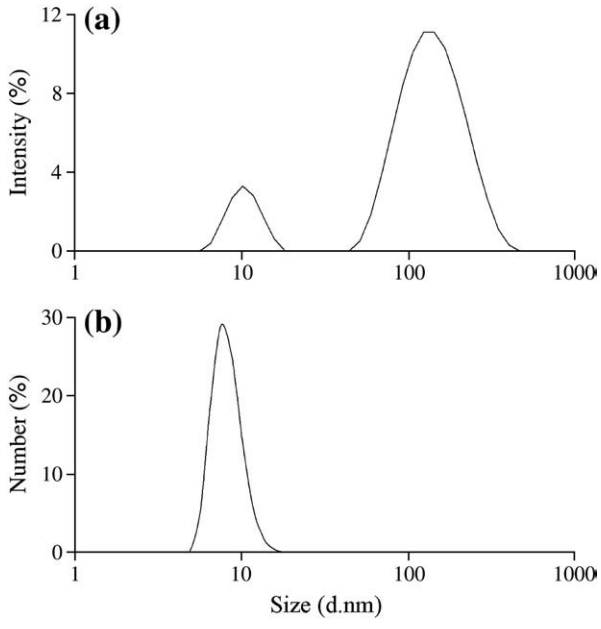

Fig. 5. Particle size distribution (diameter) for casein sample #1 dissolved in aqueous NaOH (pH ~12): (a) size distribution by intensity; (b) size distribution by number.

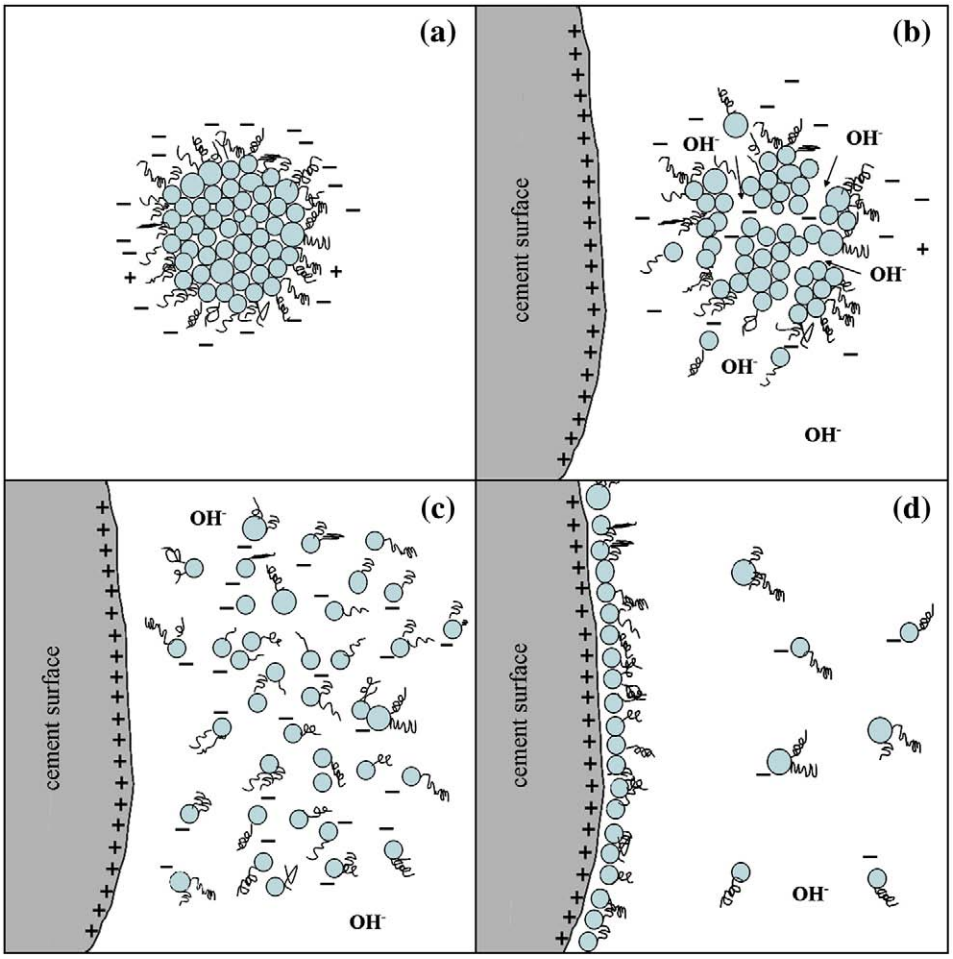

Fig. 6. Proposed model for the interaction between casein biopolymer and cement: (a) casein micelle exhibiting a negative surface charge from phosphate groups; (b) in the alkaline environment of cement pore solution, the micelle starts to disintegrate; (c) micelle dissociates into numerous submicelles; (d) some negatively charged submicelles adsorb onto positively charged cement surface, resulting in an adsorption equilibrium.

measured in alkaline solution by DLS. The results are presented in Fig. 7.

For all the samples, the mean diameters of the submicelles were of the order of ~10–20 nm. Casein #1, for example, exhibited the lowest mean diameter of 9 nm whereas casein #6 showed the highest mean diameter of 16 nm. Consequently, the particle size showed a clear tendency to decrease with increased amount of κ-casein. The DLS data confirms that the particle size of the casein submicelles directly correlates with the respective amount of κ-casein. This result seems to contradict our previous finding that from all casein proteins, pure α-casein provides the best dispersing effect [10]. The difference may be explained by the fact that *pure* proteins adsorb as such onto the surface of cement whereas here, casein submicelles interact with the binder. Additional experiments, for example measurement of the thickness of the adsorbed protein layer, can provide more insight into their different behaviours.

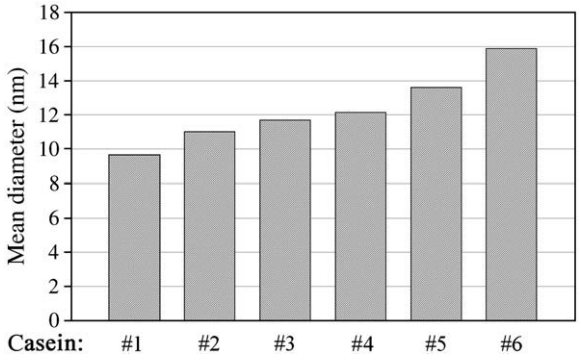

Fig. 7. Mean diameter of submicelles of different casein samples, measured by DLS in alkaline solution (pH ~12, $c_{casein} = 1$ mg/mL).

It can be concluded that when added to a SLU formulation, casein dissociates into submicelles of different sizes which depend on the content of κ-casein. Smaller submicelles can adsorb in larger numbers onto the cement surface and thus will provide a more densely packed polymer layer. Through this mechanism, stronger electrostatic repulsion between the cement particles is instigated and higher flowability of the SLU paste is achieved.

4. Conclusion

Six commercial casein samples were tested with respect to their dispersing power in a SLU formulation. Their quality was found to show huge variations. Ion exchange FPLC was used to examine the relative content of the different casein proteins, namely α-, β- and κ-casein. It revealed that the amount of κ-casein plays the key role in controlling the superplasticizing effectiveness of casein in SLUs. The percentage of κ-casein relative to total protein content by peak area and the relative ratio of α-casein to κ-casein can be applied as criteria for the quality of caseins. It was found that the ratio of α- to κ-casein allows the most accurate assessment of casein quality. The working mechanism of casein as superplasticizer for cement was analyzed by measuring DLS profiles of the casein particles in alkaline solution. It

suggested that the casein micelles dissociate into submicelles of 10–20 nm in diameter. The size of the submicelles decreases with increased content of κ-casein. The smaller submicelles are supposed to show higher adsorption on the binder and consequently produce a better superplasticizing effect.

Our study indicates that FPLC measurement may provide a fast and accurate method for assessing the quality of casein with respect to its dispersing effectiveness. First, it allows to check the purity (effective protein content) of a casein sample. Second, the dispersing effectiveness of a casein sample can be derived from the content of κ-casein and the relationship between the content of α- and κ-casein. Compared to conventional quality control tests common in industry which are based on physical testing of SLU mortar specimens, the FPLC method is less labor intensive.

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