Casein glycomacropeptide pH-driven self-assembly and gelation upon heating

Martinez, María J.¹, Farías, María E.¹² and Pilosof, Ana M. R.¹

¹ Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria (1428), Buenos Aires, Argentina.
² Departamento de Tecnología, Universidad Nacional de Luján, Ruta 5 y 7, Luján (6700), Buenos Aires, Argentina.
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\textsuperscript{1} Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria (1428), Buenos Aires, Argentina.

\textsuperscript{2} Departamento de Tecnología, Universidad Nacional de Luján, Ruta 5 y 7, Luján (6700), Buenos Aires, Argentina.

*Corresponding author: Tel.: +54 11 45763377; fax: +54 11 45763366

E-mail address: apilosof@di.fcen.uba.ar
ABSTRACT

Casein glycomacropeptide (CMP) found in cheese whey is a C-terminal hydrophilic glycopeptide released from κ-casein by the action of chymosin during cheese making. In a previous work a self-assembly model for CMP at room temperature was proposed, involving a first step of hydrophobic assembly followed by a second step of electrostatic interactions which occurs below pH 4.5. The objective of the present work was to study, by dynamic light scattering (DLS), the effect of heating (35 – 85 ºC) on the pH driven CMP self-assembly and its impact on the dynamics of CMP gelation. The concentration of CMP was 3% w/w for DLS and 12% w/w for rheological measurements. The solutions at pH 4.5 and 6.5 did not show any change in the particle size distributions upon heating. In contrast the solutions at pH lower than 4.5 that showed electrostatic self assembly at room temperature were affected by heating. The mean diameter of assembled CMP increased by decreasing pH. For all solutions with pH lower than 4.5, the particle size did not change on cooling, suggesting that the assembled CMP forms formed during heating were stable. The gel point determined as G’–G’’ crossover, occurred in all systems at 70 ºC, but at different times. The rate of self-assembly determined by DLS as well as the rate of gelation increased with increasing temperature and decreasing pH from 4 to 2. Increasing temperature and decreasing pH, the first step of CMP self-assembly by hydrophobic interactions is speed out. All the self assembled structures and the gels formed at different temperatures were pH-reversible but did not revert to the initial size (monomer) but to associated forms that correspond mainly to CMP dimers.

Keywords: Casein glycomacropeptide, heat-induced self-assembly, heat gelation, pH.
1. Introduction

Casein glycomacropeptide (CMP) comprises the 64 amino acids of the hydrophilic C-terminal portion of κ-casein released after its specific cleavage by chymosin or pepsin. CMP contains all the posttranslational modifications (glycosylation and phosphorylation) present in κ-casein that contribute to its marked heterogeneity (Mikkelsen et al., 2005). Glycosylated forms of CMP (gCMP) represent about 50% of the total CMP (Mollé & Leonil, 2005) and it contains all carbohydrates originally present in κ-casein. The A and B forms of non-glycosylated CMP (aCMP) have molecular masses of 6787 and 6755 Da, respectively and the highest molecular mass up to 9631 corresponds to highly glycosylated CMP (Mollé et al., 2005). The most predominant carbohydrate is N-acetylneuraminic acid (sialic acid) (Coolbear, Elgar & Ayers, 1996). Sialic acid was found to be particularly important for biological and pharmacological activity. It is an acidic sugar with pKa value of 2.2. A higher concentration of sialic acid in CMP gives lower pI of this glycopeptides. The pI of aCMP is close to 4.1, which is related to the high amount of acidic amino acid side chains (Kreuβ, Strixner & Kulozik, 2009b). The positive charge of aCMP at pH levels lower than the pI originates from the three Lys residues as well as from the positively charged N-terminus, while all Glu and Asp residues are protonated. The pI of gCMP, in contrast, is at 3.15, as the negative charge of the sialic acid residues reduces the net charge of the amino acid backbone (Kreuβ et al., 2009b).

In the last years, CMP has been the subject of interest due to its beneficial biological and physiological properties (Maubois, 2008; Thomä-Worring, Sørensen & López Fandiño, 2006). Nevertheless, fewer studies exist on the functional performance of CMP. Recently, growing amount of studies revealed that CMP has outstanding surface properties (Martinez,
Carrera Sanchez, Rodríguez Patino & Pilosof, 2009) and can perform as a good foaming and emulsifying agent (Kreuβ, Krause & Kulozik, 2009a; Kreuβ et al., 2009b; Thöma Worringer, Siegert & Kulozik, 2007). CMP has an amphyphilic nature, which results from a partial glycosylation. The sugar residues are highly hydrophilic, whereas the peptide chain is more hydrophobic (Tolkach & Kulozik, 2005).

Studies on CMP gelation are scarce and sometimes contradictory (Thomä-Worringer et al., 2006). Burton and Skudder (1987) reported the gelation of a 9.3% (w/w) CMP solution at pH 4.5 and 20 ºC. However, Wang (2007) reported that CMP was also able to form a gel only at pH < 4.0.

In a previous work we have shown by dynamic light scattering (DLS) studies that CMP undergoes a pH-dependent self-assembly at room temperature (Farías, Martinez & Pilosof, 2010). Different self-assembled structures form over time at pH values less than 4.5 and as a result the solutions gel with time forming opaque gels. CMP concentration for gelation was pH-dependent. At pH 3.0 or 3.5 CMP gelled even at concentrations as low as 3% (w/w) but the times needed for gelation were as long as 6–15 days. When CMP concentration was higher than 7–8% (w/w), the gelation time was shorter (<50 h) than for lower concentrations and kept almost constant.

Self assembled structures formed at room temperature are partially pH-reversible, but dimers appear to be resistant to pH changes once formed (Farías et al., 2010).

A model to explain the pH driven self assembly and cold gelation of CMP has been proposed (Farías et al., 2010). The first stage of CMP self-assembly to form dimers would occur via pH-driven strong interactions of hydrophobic domains at values below 6.5. Once formed, these dimers are stable to pH changes.
A second stage of self-assembly by electrostatic interactions would occur below pH 4.5 between aCMP dimers with a net positive charge and negatively charged gCMP. One of the genetic variants of aCMP is less acidic than the other, being uncharged at around pH 4.3–4.6. Only at pH 4.0–4.1 all aCMP is uncharged, whereas gCMP retains a net negative charge due to sialic acid residues. Decreasing pH below 4, less acidic gCMP isoforms reach their isoelectric state, and at pH 3.15 all gCMP is in the isoelectric state (Kreuß et al., 2009b). Nevertheless, because of the low pK of sialic acid residues (2.2) local negative charges would be located on the glycan down to pH 2.2, allowing it to interact with positive charges.

Thus, at room temperature, in the pH range 2–4.5, the self-assembly via electrostatic bonds can proceed to form gel structures over time.

In the proposed model, it is evident that the second stage of self-assembly that leads to gelation is only possible in the presence of glycan side chains, mainly sialic acid, which retains negative charges down to pH 2.2.

In a more recent work (Martinez, Farías & Pilosof, 2010) it was observed that the increase of temperature from 25 to 50 ºC dramatically decreased the time for CMP gelation. A further increase of temperature slightly reduced this time.

The objective of the present work was to study, by DLS, the effect of heating on the pH driven CMP self-assembly and its impact on the dynamics of CMP gelation.

2. Materials and methods

2.1. Materials
BioPURE-GMP® casein glycomacropeptide (CMP) was provided by DAVISCO Foods International, Inc. (Le Sueur, MN, USA). Composition of CMP was: protein (dry basis) 83.0% (w/w) (N x 6.47) being CMP 90.0% (w/w) (N x 7.07) of total proteins, 0.6% (w/w) fat, 6.3% (w/w) ash and 6.0% (w/w) moisture. The degree of glycosylation is about 50%.

Powder sample of CMP was dissolved in Milli-Q ultrapure water at room temperature under agitation. Bulk concentration of the CMP solutions were 3% (w/w) for dynamic light scattering (DLS) measurements and 12% (w/w) for dynamics rheological measurements. The solutions were prepared freshly and kept 24 h at 4 °C. After that, the pH was adjusted from 2.0 to 6.5, immediately before the measurement, by using 1 N HCl or NaOH. To prevent bacterial growth 0.02% (w/w) NaN₃ was added to each sample.

2.2 Particle size determination

Dynamic light scattering (DLS) measurements were carried out in a Zetasizer Nano-Zs (Malvern Instruments, Malvern, UK) (measurements range of 0.6 nm to 6 µm), provided with a He-Ne laser (633 nm) and a digital correlator, Model ZEN3600. Measurements were carried out at a fixed scattering angle of 173°. Samples were contained in a disposable polystyrene cell.

Samples were heated inside the DLS equipment from 25 to 85 °C in steps of 5 °C and holding them at each temperature for 5 min. The autocorrelation function at each temperature was obtained in this equipment and then the size of particles. Two approaches were used to obtain size information, Cumulants and Contin algorithm (Farías et al., 2010). The z-average is useful when citing a single average value for the purpose of comparison, but clearly
inadequate for giving a complete description of the distribution results in polydisperse systems.

The samples for DLS measurements were filtered through a 0.45, 0.22 and 0.02 µm microfilter Whatman International Ltd. (Maidstone, England) before measurements. The assay was performed by triplicate on three individual samples.

2.3. Sol-gel transition

The sol-gel transition time was determined by a modified tilting-test (Relkin, Meylheuc, Launay & Raynal, 1998). Hermetically closed tubes containing 2 ml of 12% (w/w) CMP solutions were observed over time (min) at constant temperature (25 ºC and 70 ºC) and the gelation time ($t_{\text{gel}}$) was assumed to be reached when there was no deformation of the meniscus upon tilting. The gelation rate ($V_{\text{gel}}$) was calculated as $1/t_{\text{gel}}$. The average value of three individual samples is reported, with an experimental error < 10%.

2.4. Dynamic oscillation measurements

Dynamic oscillation measurements were performed in a Paar Physica controlled stress Rheometer (MCR 300) (Graz, Austria). The pH of CMP solutions was adjusted and immediately 700 µl solution were placed on the bottom plate of a parallel plate measuring systems, with a gap of 1 mm. The temperature of the bottom plate was controlled with a Peltier system (Viscotherm VT2, Paar Physica). Liquid paraffin was added around the plate edges to prevent dehydration of the samples. The frequency was 1 Hz and the strain was kept at 0.01 %, a value found to be in the linear viscoelastic region in preliminary experimentation.
The samples were rapidly heated from 25 ºC to the final temperature (70 ºC) in 1.5 minutes to simulate the heating conditions of the tilting test, kept at constant temperature for 30 minutes which was sufficient time to allow G’ reached the equilibrium, and then cooled to 25 ºC in 10 minutes. Dynamic oscillation values quoted are the means of the measurements on three different CMP samples.

3. Results and discussion

3.1. Effect of heating on CMP self assembly

Fig. 1 shows the effect of heating temperature from 35 to 85 ºC on the intensity size distribution of 3% (w/w) CMP at different pH values. It is also included the initial size distribution at room temperature (25 ºC), immediately after pH adjustment.

The predominant lower size peak of the initial size distributions shows that the state of association of CMP is pH dependent as the corresponding diameter increased to higher sizes (2.5 to 6.5 nm) when decreasing the pH from 6.5 to 3.0. The maximum of the predominant lower size peak, at pH 6.0 or above, was almost constant at around 2.5 nm and, in accordance with the molecular mass estimation by the Zetasizer Nano-Zs software, corresponds to the monomeric form of CMP (average Mw about 7500 Da). Above pH 6.5, CMP self association would be prevented due to the shielding by the strong negative charge, which is more pronounced for gCMP, keeping the monomeric form of CMP predominant (Farías et al., 2010). The increased hydrodynamic diameters of the predominant lower size peak of CMP size distributions at pH below 6.5, correspond to CMP self assembled forms like dimers, tetramers, hexamers and other oligomers (Farías et al., 2010). Decreasing the pH below 6.5,
there is a strong increase of the zeta potential up to the pI (Kreuß et al., 2009b) because of increasing protonation of acidic AA side chains. Therefore the shielding by the negative charges starts to decrease, allowing the N-terminal hydrophobic domain (AA 1–5) of CMP which is not covered by the negative charge, to interact first, followed by the hydrophobic domains located in the centre of the peptide chain. Thus, the first stage of CMP self-assembly to form dimers would occur via pH-driven strong interactions of hydrophobic domains at pH values below 6.5.

A second stage of self-assembly by electrostatic interactions would occur below pH 4.5 between aCMP dimers with a net positive charge and negatively charged gCMP, leading to more assembled CMP forms.

When heating the solutions from 35 to 85 °C inside the DLS equipment, the intensity size distributions changed, for systems with pH 3.0, 3.5 and 4.0 (Fig. 1). In contrast, the solutions at pH 4.5 (not shown) and 6.5 did not show any change in the size distributions upon heating. In a previous work (Farias et al., 2010), it was shown that at pH between 4.5 and 6.5, CMP associate through hydrophobic interactions but do not form self assembled structures through electrostatic interactions at room temperature. Thus, results in Fig. 1 point out that temperature does not promote CMP self assembly at these pH values. In contrast the solutions at pH lower than 4.5 that showed electrostatic self assembly at room temperature were affected by heating.

The intensity size distribution for the solutions at pH 3.0 and 3.5 (Fig. 1) indicated that an important decrease in the intensity of the lower size peak of CMP occurred by increasing temperature with a concomitant increase in the higher size peak of the distributions that moved to higher sizes, indicating the formation of more assembled structures by increasing temperature. Nevertheless, the population corresponding to the higher sizes formed below 55
°C was negligible as it can be seen from the volume size distributions (Fig. 1). By increasing
the temperature in the range 55-85 °C, the lower size peak almost disappeared from both the
intensity and volume size distributions, and more assembled structures predominated with
sizes above 1000 nm.

At pH 4.0 (Fig. 1), the intensity of the lower size peak of CMP was also reduced by
increasing temperature with a concomitant increase in the higher size peak of the distributions
that moved to higher sizes. However the population corresponding to the higher size was very
small at all temperatures, as shown from the volume size distribution where this peak is not
apparent.

Fig. 2 shows the evolution of z-average (a measure of the mean hydrodynamic
diameter) of CMP with heating time in comparison with the evolution at room temperature
(25 °C). The rate of assembly was about 5-10 times lower at room temperature, as determined
from the slope of the curves (R² > 0.95).

Fig. 3 shows the change of the hydrodynamic mean diameter (z-average) upon heating
and cooling CMP solutions at pH 3.5, 4.0, 4.5 and 6.5. Although the size distributions of
CMP solutions at pH 4.5 and 6.5 (Fig. 1) did not significantly change with heating time (or
increasing temperature), the z-average values slightly increased on heating. It could be due to
small changes in the positions of the higher size peaks. Nevertheless, the z-average value of
these solutions decreased upon cooling, being the final z-average values slightly higher than
the initial ones.

In contrast, CMP solutions at pH below 4.5 showed an important increase of the z-
average value with increasing heating time, related to the marked changes in the size
distributions in Fig. 1. The increment on the z-average of CMP with heating time was higher
as pH decreased. For all solutions with pH lower than 4.5, the z-average did not change on cooling, suggesting that the assembled CMP forms formed during heating were stable.

3.2. Heat-induced gelation of CMP

Fig. 4 shows the dynamics of gelation of 12% (w/w) CMP solutions at pH between 2.0 and 3.5 upon heating them up to 70 ºC, holding the sample for 30 min at this temperature and then cooling down to 25 ºC for 10 min. As the heating was very fast (from 25 ºC to 70 ºC in 1.5 min) the gelation can be considered to occur in the holding period at 70 ºC. The gelation was performed at 70 ºC because at temperatures above 55 ºC a maximum effect of temperature is reached, as shown in Fig. 1 and in a previous work (Martinez et al., 2010).

The gel point \( t_{gel} \), determined as G'–G'' crossover, occurred in all systems at 70 ºC, but at different times. After the gel point, G' continued to develop and then levelled off. G' did not further increase upon cooling as it was reported by Martinez et al. (2010). This would indicate that hydrogen bonds would not have an important role in the stabilization of CMP gels. The G' values strongly decreased by decreasing of pH, but tan \( \delta = G''/G' \) was constant about 0.03, revealing that gel behaviour is primarily elastic. The tan \( \delta \) is considered to represent the relative viscoelasticity within the network (Aguilera, Xiong & Kinsella, 1993).

Interestingly, in the system at pH 3.0 and more marked at pH 3.5 two gel points were apparent (Fig. 4 C and D). This unusual behaviour may be ascribed to the existence, within this pH range, of two different self assembled structures that gel at different times. The scheme in Fig. 5 shows the electric states of dimers that could form in mixtures of aCMP and gCMP as a function of pH. Between 6.5 and 4.5 all kind of dimers have a net negative charge, which increases with the degree of glycosylation. Below pH 4.5 aCMP starts to be protonated
and below pH 4.0 where all aCMP is protonated, aCMP dimers would exhibit a positive 
charge. Therefore, between pH 4.0 and pH 3.15 where all gCMP isoforms become also 
protonated, the dimers positively charged of aCMP could electrostatically interact with 
negatively charged dimers containing gCMP. This kind of assembly would involve strong 
electric forces. Below pH 3.15 dimers containing gCMP start to exhibit a positive charge 
like the dimers containing only aCMP. As previously shown (Farías et al., 2010), even when 
all dimers carry a positive charge (i.e. below pH 3.15) electrostatic assembly still occurs 
leading to gelation. Because of the low pK of sialic acid residues (2.2) local negative charges 
located on the glycan would interact with positive charges. Thus, in the pH range 2.0–4.5, the 
self-assembly via electrostatic bonds can proceed to form gel structures over time. 
Nevertheless, the electrostatic interactions involved in the self assembly of dimers at pH 
below 3.15 should be weaker.

Therefore, the first gel point in Fig. 4 C and D would be due to the rapid assembly of 
 oppositely charged dimers and the second one to the assembly of dimers with similar positive 
charge by means of weaker electrostatic interactions. Gels formed at pH 2.0 and 2.5 would 
only involve weak electrostatic interactions between CMP dimers. These weaker electrostatic 
interactions also impacted on the elastic character of gels that was lower at pH below 3.5

The rates of formation of the primary gel structure (V_{gel}) at the gel point were 
evaluated as 1/t_{gel} and plotted as a function of pH (Fig. 6) together with the gelling rates (V_{til}) 
obtained by tilting test (1/t_{til}). The gelling rates obtained by the tilting test (V_{til}) were lower 
than those obtained from small strain measurements (V_{gel}), because a more developed gel 
structure is needed to resist the deformation imposed by tilting, especially at pH 3 and 3.5 
where V_{til} including the second increment of G′. The first gel point (V_{gel1} in Fig. 6) seemed to 
be not affected by pH. Instead V_{til} at 70 °C increased with decreasing pH from 4 to 2 which
reveals the strong influence of pH on the first stage of self assembly involving hydrophobic interactions between CMP monomers to form stable dimers. At pH below 6.5 the shielding by the negative charges starts to decrease, allowing the hydrophobic domains of CMP to interact (Farías et al., 2010; Kreuβ et al., 2009b).

Fig. 6 also includes de gelation rates by the tilting test at room temperature (25 ºC) which were much lower than those obtained at 70 ºC. This trend is similar to that observed in Fig. 2, for DLS measurements of the initial steps of CMP self assembly.

3.3. Testing pH reversibility of heat assembled CMP and gels

As was shown above, CMP assembled structures formed upon heating below pH 4.5 were stable on cooling, as well as the gels. Thus the question we address here is if these assembled structures are pH reversible (i.e by adjusting pH to 7.0).

To this end CMP assembled structures obtained at pH 3.5 upon heating from 35 to 70 ºC, were tested for pH-reversibility. In Fig. 7 the z-average following pH adjustments, from 7.0 to 3.5 and back to 7.0 is plotted during the heating and the cooling period. The starting solution of CMP at pH 7.0 and 25 ºC showed a maximum diameter of the lower size peak about 2.3 nm (similar to the value for CMP at pH 6.5 showed in Fig. 1), corresponding to the monomeric form of CMP (Farías et al., 2010). The z-average value at 25 ºC was 6-7 nm (Fig. 7) which was higher than the maximum value of the lower size peak due to the presence of particles with higher size at this pH (Fig. 1). Following pH adjustment to 3.5 the heat treatment started and z-average increased rapidly with increasing temperature up to values about 2000 nm. During cooling from 70 to 25 ºC, the z-average kept almost constant, indicating the stability of the assembled structures formed. At this point (at 25 ºC) the pH was
returned to 7.0 and z-average determined over time. It can be observed a rapid and marked
decrease of z-average, being the final value 9 nm, which is slightly higher than the initial one
(6-7 nm). This behavior was similar to that observed when testing pH-reversibility of CMP
assembled at room temperature (Farías et al., 2010).

Fig. 8 shows the comparison of the intensity and volume size distributions of the
initial CMP solution at pH 7.0 and that obtained at the end of the test in Fig. 7. It can be seen
that the size distributions before and after the experiment did not coincide. The maximum
value of the lower size peak was 2.3 nm before the experiment and 4.25 nm after it (Fig. 8 A),
corresponding to the monomeric and dimeric forms of CMP, respectively. The size
displacement of the predominant lower size peak was also observed in the volume size
distribution (Fig. 8 B). These results indicate that, although the heating at pH < 4.5 promoted
the formation of CMP assembled structures stable to cooling, these structures were almost
reversible at neutral pH, to stable dimeric forms.

Similar results were obtained when the pH-reversibility of CMP gels formed at 70 ºC
(Fig. 4) was tested. The gels (12% w/w, formed at pH from 2.0 to 3.5) were diluted 1:5 in
Milli-Q ultrapure water and then the pH was adjusted to 7.0. The samples were totally
transparent and no precipitates were observed. The intensity size distributions at pH 7.0 (Fig.
9 A) for the CMP pre-gelled samples showed two populations, being the predominant first
peak 3.6 nm. The populations of higher size and intensity were negligible as it can be seen in
the volume size distributions (Fig. 9 B). In the same figure it is included the size distribution
of the initial solution at pH 7.0 (obtained by dilution 1:5 of a 12% (w/w) CMP solution). The
first peak was also predominant for this solution (maximum d(H) at 2.1 nm), as indicated the
volume size distribution (Fig. 9B).
From the comparison of initial solutions and those from gelled samples, it can be concluded that the gelled samples did not revert to the initial size but to associated forms that correspond mainly to CMP dimers (3.6 nm).

4. Conclusions

The results obtained from DLS studies on heating CMP solutions and on the dynamics of CMP gelation indicate that the mechanism of CMP self assembly upon heating is the same we proposed for CMP self assembly at room temperature (Farías et al., 2010).

CMP self-assembly would include a first stage of hydrophobic self-assembly to form dimers (which are stable to further pH changes) which then interact through electrostatic bonds to form pH reversible gels over time (Fig. 10). However, increasing temperature, the first step of CMP self-assembly by hydrophobic interactions is speed out, because high temperatures increase the potential for these interactions (Bryant & McClements, 1998). This suggests that stage I (Fig. 10) would be the rate determining step of CMP gelation.

The present results point out that pH is also very important in controlling CMP assembly and gelation. The pH modulates de rate of self assembly and gelation by favoring hydrophobic interactions as pH decreases below 4.5 (Fig. 10).

Also pH modulates the elastic properties of gels because it affects the electric state of the different CMP fractions (A and B variant of aCMP and the different glycosylated isoforms) in a different way, leading them to assemble by strong or weak electrostatic interactions depending on the pH, thus determining the characteristics of the gels.

The high thermal stability of CMP in comparison to other whey proteins was described by Brody (2000), Martin Diana, Fraga & Fontecha (2002) and Moreno, López
Fandiño & Olano (2002). However, the results obtained in this study show that this peptide is stable to heat only at pH ≥ 4.5. These results agree with Lieske, Konrad & Kleinschmidt (2004) who studied the influence of pH and heating on the isolation of CMP by ultrafiltration of whey and verified that CMP became increasingly heat-sensitive at acidic pH (from 3.8 to 5.2), whereas they did not observe changes at neutral pH.

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References


Legends for figures.

**Figure 1.** Intensity (left plots) and volume size (right plots) distribution for CMP solutions at 3% (w/w) and pH: 3.0, 3.5, 4.0 and 6.5, at different steps of temperature during the heating (°C): 25 (□), 35 (▲), 45 (◇), 55 (►), 65 (○), 75 (■) and 85 (★).

**Figure 2.** Evolution of z-average as a function of time for CMP solution at 3% (w/w). 25 °C (■) and heat treatment (up to 85 °C) (○) at pH: (A) 3.0, (B) 3.5 y (C) 4.0.

**Figure 3.** Z-average during the heating and cooling of solutions of CMP 3% (w/w) at pH: 3.5 (◇), 4.0 (▲), 4.5 () y 6.5 (■). The (——) temperature profile is also depicted.

**Figure 4.** G' (■) and G’’ (○) evolution during the heat-induced gelation of 12% (w/w) CMP solution at pH: (A) 2.0; (B) 2.5; (C) 3.0 and (D) 3.5. The (——) temperature profile is also depicted.

**Figure 5.** Scheme of the possible electric states of dimers in mixtures of aCMP and gCMP as function of pH. The arrows indicate the pH range where dimers can assume a positive charge.

**Figure 6.** $V_{ui}$ at 25 ºC (○) and 70 ºC (●) and $V_{gel1}$ at 70 ºC (□) for 12% (w/w) CMP as a function of pH. Error bars: standard error (n = 2 or 3).
Figure 7. Evolution of z-average (■) as a function of temperature for CMP solution at 3% (w/w) with change of pH from 7.0 (at 25 °C) to 3.5 (from 25 to 85 °C and during the cooling until 25 °C) and then returning to 7.0 (at 25 °C).

Figure 8. Intensity (A) and volume (B) size distribution of CMP solutions at 3% (w/w) and pH 7.0, before (□) and after (●) the test of heat-induced reversibility (at pH 3.5) brought about by pH adjustment. Temperature 25 °C.

Figure 9. Intensity (A) and volume (B) size distribution at 25 °C for diluted (1:5) 12% (w/w) CMP gels at pH 7. Gelation was performed at 70 °C at different pH: 2.0 (▲); 2.5 (◇); 3.0 (▷) y 3.5 (●). CMP solution at pH 7 is also depicted (□).

Figure 1
Figure 2

(A) pH 3.0
Slope = 0.149
Slope = 0.033

(B) pH 3.5
Slope = 0.142
Slope = 0.015

(C) pH 4.0
Slope = 0.0513
Slope = 0.0058
Figure 4

- A: $t_{gel} = 1.5$ min
- B: $t_{gel} = 2.0$ min
- C: $t_{gel} = 2.6$ min
- D: $t_{gel} = 2.6$ min, $t_{gel2} = 9.8$ min
Figure 5

Self-assembly by hydrophobic interactions

Electrostatic interactions to form gel structures over time

 Isoelectric state of sialic acid

All aCMP is in the isoelectric state

All gCMP is in the isoelectric state

2.2

pH

3.0

3.5

3.15

4.0

4.5

5.5
Figure 6

![Graph showing the pH dependence of Vg and Vt at 25°C and 70°C.](image_url)
Figure 8

(A) Graph showing intensity (%) vs. particle diameter (nm) with peaks at 2.3 nm and 4.25 nm.

(B) Graph showing volume (%) vs. particle diameter (nm) with peaks at the same particle diameters as in (A).
Figure 9

(A) Intensity (%)

(B) Volume (%)

Particle diameter (nm)

3.6 nm

2.1 nm

ACCEPTED MANUSCRIPT
Figure 10

Stage I

$\text{(CMP)}_M \xrightarrow{H > T; < pH} \text{(CMP)}_D$

Stage II

$n\text{(CMP)}_D \xrightarrow{E} \left[\text{(CMP)}_D\right]_n$