Galactomannan-Alginate Synergism Applied in Albumin Encapsulation

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Summary: In this work, the rheological properties of the partial replacement of sodium alginate (AL) by galactomannan extracted from the seeds of *L. leucocephala* (GML), with or without the presence of calcium ions, were evaluated. The viscoelastic behaviour of polysaccharide blends at 15 $g \cdot L^{-1}$ showed an increase in viscosity when AL was 50% (w/w) replaced by GML. The addition of calcium ions (45 mmol) increased the apparent viscosity of the blend more than 17 times, at a shear rate of 4 s^{-1}. Micrographs of [GML/AL/ALB] Ca^{2+} crosslinked particles obtained by a spraying process were analysed by AFM and revealed spherical forms with diameters in the range of 518 nm, instead of 58.4 nm in the system without albumin. The results of ALB encapsulation (97% w/w) showed that the synergistic mixture GML/AL could be used, in the future, as a new carrier system.

Keywords: biopolymers; blends; gels; polysaccharides; rheology

Introduction

Alginate is a natural unbranched biopolymer composed of variable quantities and sequences, depending on the polysaccharide source, of 1-4-linked β-D-manuronic acid and α-L-guluronic acid residues.[1] It has been used in various industrial applications as a gelling, thickening and colloidal agent. Soluble sodium alginate can also be crosslinked by divalent cations to produce beads and microspheres, which can be used to encapsulate drugs, proteins and cells.[2–4] The most common technique for this purpose is the atomisation or spraying of the mixture of Na^+-alginate and protein into a divalent crosslinking solution, such as $CaCl_2$, using an extrusion device with a small orifice.[1,4]

Galactomannans are reserve carbohydrates found in the endosperm of some legume seeds. This biopolymer is composed of a linear chain of 1→4 linked β-D-mannopyranosil units with D-galactopyranosyl units α-(1→6) joined. The galactose distribution at the backbone varies from one botanical source to another and this difference controls properties such as viscosity, solubility, thickening and the stability of solutions.[5,6] For this reason, galactomannans are used as rheology modifiers in food,[7,8] cosmetics,[9] pharmaceutical products[10] and more recently in biotechnology processes e.g., culturing plant tissue,[11–14] among other applications. It can introduce gel properties to aqueous systems, where it is known to participate in synergistic interactions with other polysaccharides such as xanthan,[15,16] carragennan[17] and agar.[11] Synergistic interactions using galactomannans are of commercial interest because they offer the prospect of generating novel functionality, such as producing rheological or textural characteristics by using reduced levels of

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polysaccharides with lower costs.[5] Rheological measurement is considered as an analytical tool to provide fundamental insights into complex materials. The results are sensitive to temperature, concentration and physical state of dispersion. The linear visco-elastic material functions measured from oscillatory testing can be related to steady-shear behaviour,[18] and at low frequencies and shear rate, more reliable relationships can be established.

When dealing with complex systems, such as protein/polysaccharide, it is important to understand the efficiency of the mixture components since the behaviour of blend biopolymer solutions is the key to understanding the relationships between composition and bulk properties of systems (e.g., food).[19] The structure and texture of all biological materials depend on the ability of biopolymers to interact and to form organised structures. In this study, we examined the rheological behaviour of different mixtures of galactomannan and sodium alginate at a final concentration of $15\,g{\cdot}L^{-1}$ in the presence or absence of calcium ions. The system also included albumin ($5\,g{\cdot}L^{-1}$) and the nano-particles were obtained by spraying into $CaCl_2$ solution. The resulting morphology and size distribution was analysed by scanning electron microscopy (SEM) and atomic force microscopy (AFM).

Experimental Part

Materials

The isolation of galactomannan from *Leucaena leucocephala* seeds (50 g) collected in Curitiba, Parana, Brazil was performed as described by Lucyszyn et al.[11] In order to purify the polysaccharide, it was resolubilised in water at 25 °C, filtered under pressure through cellulose acetate membranes with 3.0, 0.8 and 0.22 μm pore diameter (Millipore®). Then, it was precipitated with excess ethanol to give the purified galactomannan fraction (GML), with a Mannose:Galactose (Man:Gal) ratio of 1.7, determined by alditol acetate composition. Sodium alginate (AL) was purchased from Proquimios, Rio de Janeiro/Brazil. Albumin from bovine serum (minimum 98%, A3803, batch 095K0708) and calcium chloride (minimum 96.0%, anhydrous powder, C4901, batch 077K0087) were from Sigma-Aldrich®, USA.

Chemical Analysis

Total carbohydrate was estimated by the phenol-sulfuric acid method[20] using glucose (Sigma Aldrich®) as the standard. The protein determination was performed with the Folin-Ciocalteau reagent (Merck®, Germany),[21] using bovine serum albumin (ALB) (Sigma Aldrich®) as the standard.

To determine the average molar mass, $\overline{M}_w$, the GML and AL solutions were first filtered through 0.45 and 0.22 μm cellulose acetate Millipore membranes. Then, they were tested by high-performance size exclusion chromatography (HPSEC), coupled with a differential refractometer and a multi-angle laser light scattering (MALLS) detector in a WYATT technology light scattering system. The eluent was 0.1 M $NaNO_2$-0.02% NaN_3 solution, with a controlled flow of $0.6\,mL{\cdot}min^{-1}$.

Rheological Measurements

To perform rheological experiments, the galactomannan and sodium alginate ($15\,g{\cdot}L^{-1}$) were solubilised in water and stirred overnight. Different mixtures of polysaccharides were prepared and then analysed. In order to observe the effect of calcium ions in the system, different concentrations of $CaCl_2$ (20 mmol, 30 mmol, 45 mmol or absence of Ca^{+2}) were added into polysaccharide mixtures, homogenised for more 60 min and then analysed in the rheometer. Experiments including ALB were prepared by mixing galactomannan and sodium alginate ($7.5/7.5\,g{\cdot}L^{-1}$ each) solution with albumin ($5\,g{\cdot}L^{-1}$); the final solution was then stirred overnight. After this, 45 mmol $CaCl_2$ aqueous solution was introduced and the system was homogenised for 30 more min.

The measurements were determined using a 35 mm diameter parallel plate and a 1.0 mm gap in a HAAKE Rheometer RS600, at 25 °C, accomplished with UTC unit. For steady flow measurements, the rheometer was programmed following a two-cycle of shear changing from 10^{-4} to $300\,s^{-1}$ in 5 min and back to $0\,s^{-1}$ in the next 5 min. The rheological parameters of shear stress, apparent viscosity and shear rate were obtained from the software. Frequency sweep measurements were performed between 5.10^{-1} and 1.10^{2} $rad{\cdot}s^{-1}$. In the viscoelastic region (3% of strain), the storage modulus (G', a measure of elastic property), loss modulus (G'', a measure of viscous property), dynamic complex viscosity (η^*) and phase angle (tan δ, ratio of loss modulus to storage modulus) were obtained directly from the software (HAAKE Rheowin Data Manager). Creep tests were performed using the same parallel plate configuration (gap 1.0 mm) as the dynamic rheological tests. Stress sweeps were performed on each sample to determine the appropriate applied shear stress for use in the creep experiments, in which a constant shear stress (3 Pa) was applied to the material for 120 s and the resulting strain was measured over a time of 650 s. The samples in the creep tests were also allowed to relax for 5 min prior to the start of each experiment. The experimental parameter is the creep compliance, J (t), determined by the Rheowin data software.

Preparation and Characterisation of the Particles Formed by [GML/AL/ALB] Ca^{2+}

Albumin (ALB, $5\,g{\cdot}L^{-1}$) was added into the mixture of galactomannan/sodium alginate, at final concentration of $7.5/7.5\,g{\cdot}L^{-1}$ each polysaccharide and stirred overnight. After this, the system was sprayed into a stirring $CaCl_2$ solution (45 mmol), utilizing a peristaltic pump from the Spray Drier, Labmac model LM-MSD 1.0. The particles produced were separated from the supernatant by centrifugation and the amount of protein encapsulated was determined as the difference between the initial amount and that remaining in supernatant.

The particle morphology, such as shape and size, was studied by atomic force microscopy (AFM) and scanning electron microscopy (SEM). For AFM, the beads were deposited on mica, adsorbed for 1 min, washed with water and left to dry for 24 h in controlled conditions. They was examined on a Shimadzu SPM-9500J3 using a 4.7 $N{\cdot}m^{-1}$ cantilever in tapping mode. For SEM samples, the beads were placed on the surface of an aluminum stub, coated with gold and observed using a Jeol JSM-6360 LV microscope.

Results and Discussion

Polysaccharides Analysis

The total carbohydrate content in the purified galactomannan sample (GML) was 83% (w/w) and the protein content was 5%. The average molar mass ($\overline{M}_w$) for GML and AL was 713,600 and 301,471 $g{\cdot}mol^{-1}$, respectively. The GML composition and the $\overline{M}_w$ results obtained were similar to other highly substituted varieties such as *Cyamopsis tetragonolobus*.[11] Finally, the alginate $\overline{M}_w$ experimental result confirmed the value furnished by the supplier.

Rheological Properties

To the best of our knowledge, this is the first study to have examined the rheological behaviour of blends of two natural polysaccharides (GML and AL), in order to evaluate a possible synergistic effect that is explained through an increase in the gel strength of the blends comparing with the behaviour of each isolated compound. The extension of synergism observed in the galactomannan mixtures is variable.[11] For example, the tendency to self-aggregate, presence of certain salts,[22] enhance the synergistic effects. As regards the galactomannans, the synergistic effects depend on both the degree and pattern of side chain substitution.[6]

Different mixtures were prepared, maintaining, in every situation, at final concentration of $15\,g{\cdot}L^{-1}$. The individual rheolo-

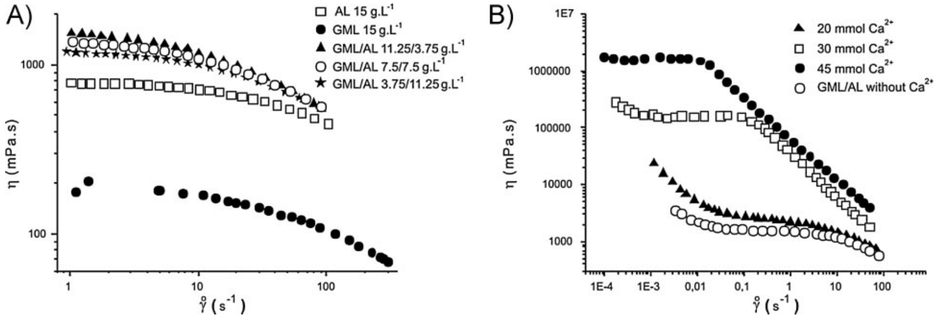

Figure 1.

Flow curves (apparent viscosity, η, *versus* shear rate, $\overset{o}{\gamma}$) in aqueous solutions at 25 °C: A. With different concentrations of GML and AL; B. With GML/AL mixture (7.5/7.5 $g \cdot L^{-1}$ each) and different concentrations of calcium ions.

gical behaviour of each biopolymer was compared with that of the mixtures and the data obtained are shown in Figure 1A. An increase in the viscosity by the flow curve was observed for all the mixture systems, each at least one order of magnitude higher in relation to the individual component (GML or AL in solution), suggesting an association between polysaccharides. These results may have been promoted by a good synergistic effect, due to a strong and efficient attraction between the biopolymers. Since the alginate is an anionic polysaccharide with a linear chain and galactomannan is a neutral one, probably the reason for the increase in viscosity observed for all systems is the intermolecular association effect occurring between polysaccharides, especially involving several hydrogen bonds. Similar results were obtained for other polysaccharide mixtures, such as glucomannan and galactomannan,[8] agar and galactomannan,[11] and xanthan and galactomannan.[15,16]

In order to observe the effect of calcium ions on the solution, the system selected was prepared with a mixture of GML/AL (7.5/7.5 $g \cdot L^{-1}$ each), into which different concentrations of calcium ions (20, 30 and 45 mmol) were added before rheological analyses were performed. Higher concentrations of calcium ions in solution, such as 50 and 100 mmol, were also tested, but the gels formed were brittle, so they could not be used for further analysis. It was observed from the flow curve (Figure 1B) that the addition of 45 mmol calcium ions contributed to an enhanced increase of apparent viscosity and pseudoplastic character. The values of viscosity increased more than 17 times for the mixture of GML/AL at a shear-rate of 4 s^{-1}. Therefore, a synergistic effect was exhibited for the blends GML/AL (7.5/7.5 $g \cdot L^{-1}$ each) containing 30 or 45 mmol of calcium ions, since the apparent viscosity of the mixture was much higher than the apparent viscosities of the individual components of the system at the same final concentration of 15 $mg \cdot mL^{-1}$. A more favourable crosslink between calcium and uronic residues of alginate was promoted by the interaction of GML with AL. It is known from the literature that sodium alginate, in the presence of divalent ions, can crosslink and form a gel matrix, called an "egg-box" structure.[23] At high ionic strengths, salts provide counter ions that reduce charge repulsion and therefore enhance aggregation and subsequent gelation.

Then, selecting a fixed quantity of each polysaccharide of 50% (w/w) and a 45 mmol calcium ions concentration, based on previous results, the viscoelastic behaviour of the mixtures was evaluated. All experiments were carried out in a region to which was applied a 3% deformation. Figure 2A shows the G'and G'' as a function of the angular frequency (ω). It was observed that GM/AL/Ca^{2+} behaves

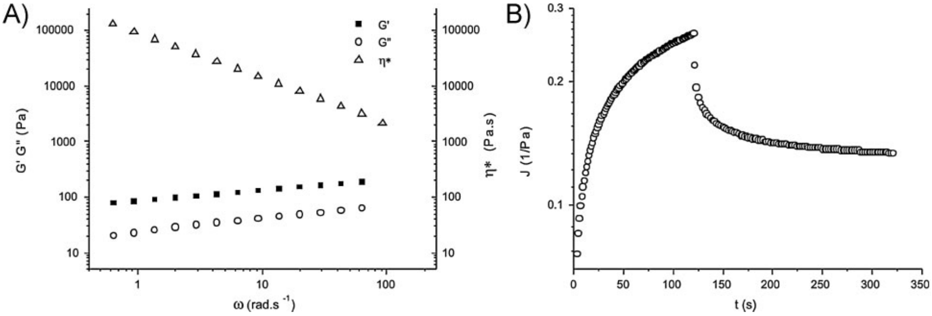

Figure 2.

A. Dynamic complex viscosity (η^*), elastic modulus (G') and viscous modulus (G'') versus angular frequency at 25 °C for a mixture of GML/AL (7.5/7.5 $g \cdot L^{-1}$ each), containing 45 mmol of calcium ions. B. Creep compliance (*J*) as a function of time (t) of GML/AL mixture (7.5/7.5 $g \cdot L\, g \cdot L^{-1}$ each) containing 45 mmol of calcium ions at 25 °C.

like a typical macromolecular entangled biopolymer in solution,[15] with the G' dominating over G'' in the interval of frequencies analysed. The modulus was slightly frequency dependent: the magnitudes of G' and G'' increased with the increase in the frequency and no evidence can be seen of a cross-over point. Since G' was greater than G'' for all frequencies analysed, this was an indication that the system has a dominant elastic nature or behaves as a gel. Tan δ (G''/G') values obtained were 0.27 to 0.33, within all frequency curves, which also showed an aspect of gel behaviour for the system. Furthermore, the η^* decreased linearly with increasing frequency of oscillations, which is also a typical result of a strong gel.[24]

Creep experiments were also performed in order to complete frequency sweeps analysis. Creep and recovery is a viscoelastic test that shows the differences between elastic and viscous responses. In this test, the other parameter evaluated was the time response, which depends on the stress applied. Figure 2B plots the creep compliance (*J*) for the mixture GML/AL/Ca^{2+}, showing the deformation of the system per unit stress. It can be observed that, initially, the creep grew rapidly, followed by a constant compliance. The recovery period was dependent on time and tends to a parallel abscise behaviour for both samples. The regeneration of the system theoretically extends to infinite time and in our experiments at 120 s, it was possible to see 88% of recovery. A viscoelastic response was observed, varying between stress and deformation. This is a typical elastic result, since 100% of recovery would be observed, theoretically, in the case of a pure elastic system. Therefore, the creep data confirmed the results obtained previously in the oscillatory analysis, which indicated gel behaviour for the polysaccharide mixtures.

It has been shown by different studies of alginate/protein[1,2,25] and galactomannan/protein[26–28] systems that the type of protein/polysaccharide interactions can vary due to the large variation in biopolymer structure and solvent conditions (pH, ionic strength, temperature). According to Tolstoguzov[29] the macromolecular interactions responsible for complex formation may be divided into three kinds:

1. interactions between the charged macromolecules;
2. interactions between oppositely charged (acidic and basic) side groups;
3. interactions between other available side groups of polyions.

These interactions can be classified more accurately as weak or strong, repulsive or attractive and specific or non-specific, in which the system reflects more the interactions between its components, then, the properties of these individuals components.[29,30]

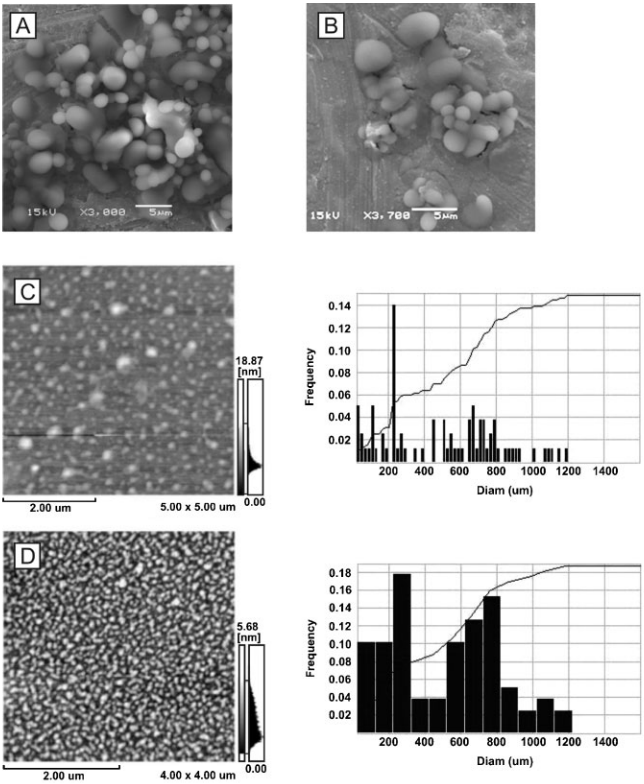

Figure 3.

A. SEM micrographs of GML/AL/Ca^{2+} particles. B. SEM micrographs of [GML/AL/ALB] Ca^{2+} particles. C. GML/AL/Ca^{2+} particles deposited on mica. D. [GML/AL/ALB] Ca^{2+} deposited on mica. Left: AFM topography image; right: diameter distribution histogram, in nm. Program SPIP v 4.

Development and Characterisation of [GML/AL/ALB] Ca^{2+} Systems

Currently, a large number of carrier systems are being developed for purposes such as the containment of drugs, proteins or genes in small vesicles or within polymeric matrices. These systems include nanoparticles, liposomes, microcapsules, polymer self-assembly and others.[1,3,4,10,31] In our work, particles were made by spraying the solution of polymer mixture (GML/AL, 7.5/7.5 $g{\cdot}L^{-1}$ each), without or with the ALB (5 $g{\cdot}L^{-1}$), into 45 mmol $CaCl_2$ aqueous solutions. In the system containing ALB, to confirm the encapsulation of the protein, the solution containing the particles was centrifuged and after the separation of the precipitate the protein in the supernatant was measured. The results showed a total of 3% of free protein in the solution, demonstrating that approximately 97% of ALB was included in the GML/AL/Ca^{2+} system.

SEM micrographs (Figure 3A, B) of the blends with and without the protein were made with the primary aim of observing the particle morphology. For both samples, spherical forms of different sizes were observed, whose surfaces appeared to be smooth, nonporous and with no evidence of fissures. However, no great difference could be observed. Therefore, a complementary AFM analysis was carried out and, in this microscopy analysis, it was possible to characterise the molecular size and shape of particles, improving the understanding of these biopolymers.[32]

Figures 3C and 3D (left) present AFM topographical images for the GML/AL/Ca^{2+} mixture deposited on a mica surface, with or without addition of albumin, respectively. Comparing the two, the differences were in the size and morphology of the particles formed. The microscopy images suggest that the particles have two main shape morphologies: spherical and rod-like. The average size of particles shown in the histogram of the system without albumin was 58.4 nm, instead of the 518 nm found for the system with protein (Figure 3C, D, right, respectively). This increase was probably due to the protein adsorption into the particles and also to a higher degree of hydration in the system containing the albumin, which is a consequence of the competition between the protein and alginate, both with negative charges, by calcium ions currently in the system.

Conclusion

This work demonstrated, by rheological analyses, the interaction between two natural biopolymers. The GML/AL mixtures at 15 g·L^{-1} showed a better synergism in the presence of 50% (w/w) of each polysaccharide in solution. The addition of Ca^{2+} (45 mmol) increased the values of viscosity more than 17 times, improved the interaction of polysaccharides and promoted the formation of a gel network. Albumin (5 g·L^{-1}) was included in the blend (with adsorption approximately of 97%) and then this system was sprayed into a calcium solution. The nano and micro particles obtained were characterised by AFM and SEM analysis and demonstrated promising features of GM/AL system as a bioactive compound carrier. And, specially, taking into account the cost of galactomannan compared to the alginate, this synergistic system may also have a cost potentially lower.

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[1] W. R. Gombotz, S. F. Wee, *Adv. Drug Delivery Rev.* **1998**, *31*, 267.

[2] S. Neiser, K. J. Draget, O. SmidsrØd, *Food Hydrocolloid* **1999**, *13*, 445.

[3] M. Leonard, M. R. de Boisseson, P. Hubert, F. Dalençon, E. Dellacherrie, *J. Control. Release* **2004**, *98*, 395.

[4] J. Zhang, X. Li, D. Zhang, Z. Xiu, *J. Appl. Polym. Sci.* **2008**, *110*, 2488.

[5] I. C. M. Dea, A. Morrinson, *Adv. Carbohydr. Chem. Biochem.* **1975**, *31*, 241.

[6] I. C. M. Dea, A. H. Clark, B. V. McCleary, *Food Hydrocolloid* **1986**, *1*, 129.

[7] N. Lucyszyn, F. Reicher, J. L. M. S. Ganter, in: "Prooceedings from IIIrd International Symposium on Natural Polymers and Composites – ISNAPol", 2000 (São Paulo - Brazil), p. 233.

[8] K. Yasar, T. Kahyaouglu, N. Sahan, *Food Hydrocolloid* **2009**, *23*, 1305.

[9] H. S. Koop, C. E. O. Praes, F. Reicher, C. L. O. Petkowicz, J. L. M. Silveira, *Mater. Sci. Eng. C* **2009**, *29*, 559.

[10] C. W. Vendruscolo, I. F. Andreazza, J. L. M. S. Ganter, C. Ferrero, T. M. B. Bresolin, *Int. J. Pharm.* **2005**, *296*, 1.

[11] N. Lucyszyn, M. Quoirin, H. S. Koehler, F. Reicher, M.-R. Sierakowski, *Sci. Hortic.* **2006**, *107*, 358.

[12] N. Lucyszyn, M. Quoirin, L. F. Ribas, M.-R. Sierakowski, *J. Hortic. Sci. Biotech.* **2006**, *206*, 310.

[13] N. Lucyszyn, M. Quoirin, L. F. Ribas, H. S. Koehler, M.-R. Sierakowski, *In Vitro Cell. Dev.-Pl.* **2006**, *42*, 287.

[14] N. Lucyszyn, M. Quoirin, M. M. Homma, M.-R. Sierakowski, *Biol. Plantarum* **2007**, *51*, 173.

[15] S. A. Khan, V. B. Pai, *Carbohyd. Polym.* **2002**, *49*, 207.

[16] M. Rinaudo, A. Moroni, *Food Hydrocolloid* **2009**, *23*, 1720.

[17] P. B. Fernandes, M. P. Gonçalves, J.-L. Doublier, *J. Texture Stud.* **1994**, *25*, 267.

[18] L. P. K. Lee, J. F. Steffe, *Food Eng. Prog.* **2002**, *6*, 195.

[19] S. Gaalloul, S. L. Turgeon, M. Corredig, *Food Hydrocoloid* **2009**, *23*, 1243.

[20] M. Dubois, K. Gilles, J. K. Hamilton, P. A. Rebers, F. Smith, *Anal. Chem.* **1956**, *28*, 350.

[21] E. F. Hartree, *Anal. Biochem.* **1972**, *48*, 422.

[22] E. R. Morris, in: "*Food Gels*", P. Harris, Ed., Elsevier Applied Science, London 1990, p. 291.

[23] C. M. Silva, A. J. Ribeiro, D. Ferreira, D. F. Veiga, *Eur. J. Pharm. Sc.* **2006**, *29*, 148.

[24] D. A. Rees, *Adv. Carbohyd. Chem. Biochem.* **1969**, *24*, 267.

[25] M. Panouillé, V. Larreta-Garde, *Food Hydrocolloid* **2009**, *23*, 1074.

[26] Y. A. Antonov, J. Lefebvre, J.-L. Doublier, *J. Appl. Polym. Sci.* **1999**, *71*, 471.

[27] G. E. Perissutti, T. M. B. Bresolin, J. L. M. S. Ganter, *Food Hydrocolloid* **2002**, *16*, 403.

[28] H. J. Kim, S. J. Choi, W. S. Shin, T. W. Moon, *J. Agr. Food Chem.* **2003**, *51*, 1049.
[29] V. B. Tolstoguzov, in: ‘‘*Food Proteins and Their Applications*’’, S., Damodaran, A. Paraf, Eds., Marcel Dekker, Inc., New York 1997, p. 171.
[30] K. K. T. Goh, A. Sarkar, H. Singh, in: ‘‘*Milk protein: Milk proteins from Expression to Food*’’, A. Thompson,, M. Boland,, H. Singh, Eds., Elsevier Inc., New York 2009, p. 347.
[31] C. Ribeiro, G. G. C. Arizaga, F. Wypych, M.-R. Sierakowski, *Int. J. Pharm.* **2009**, *367*, 204.
[32] V. J. Morris, A. R. Mackie, P. T. Wilde, A. R. Kirby, C. N. Mills, A. P. Gunning, *Lebensm. -Wiss. Technol.* **2000**, *34*, 3.