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Thermal behavior of water in micro-particles based on alginate gel

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Abstract Alginate has been established as a very versatile material in the preparation of hydrogel capsules for trapping therapeutic biomolecules and cells. The physicochemical properties, the mechanism and the processing of gel formation are now well established. In the frame of a project aiming at the exploitation of encapsulation of therapeutic proteins in alginate gel particles, the procedure of preparation, characterization, gel-drying and re-hydrating has been explored for the shelf-life of the encapsulated biomolecules. Here, the results of a calorimetric study on the freezing and dehydration process of alginate microcapsules is presented. The work aims at the description of water state(s) and its removal under "controlled conditions" in the presence of bioprotectant sugars.

Keywords Alginate gel beads · DSC · Gel dehydration · Water freezing and melting · Frozen and un-frozen water · Trehalose

Introduction

Alginates and pectins are well known for their gelling properties under conditions in which ordered chain aggregation (crystallite-like) is favored upon interaction of charged groups with divalent cations [1]. The results of equilibrium studies of the binding of calcium ions discriminate the higher affinity of calcium with pectin with respect to that with alginate. However, the different behavior is ascribed, at least as far as the extent of binding is concerned, to the different composition of the binding sites in polygalacturonate with respect to alginate, in which homooligomeric glucuronate sequences have been shown to be similarly effective [2], although binding affinity has been demonstrated also for long blocks of strictly alternate copolymeric sequences [3]. Indeed, alginates are a family of copolymers which contain varying amounts of 1,4′-linked β-D-mannuronic acid (M) and α-L-glucuronic acid (G) residues [4]. The M/G ratio and their distribution along the chains are strongly dependent on the particular species of algae from which it is extracted [5].

From the general thermodynamic point of view, the overall distribution of binding modes of ions by polyelectrolytes (i.e. non-localized, localized and site-biding) satisfies the electrostatic requirements given by the charge density of the polymer and depends on the very polyelectrolyte nature of the chain [6 and references therein]. Due to their polyelectrolyte character, the 'binding' of counter ions is a peculiarity stemming from the intrinsic charge density of the polymers and of the structures induced, making the final state the result of several contributions that can be thermodynamically modeled [6]. It is not uncommon, however, that the free-energy term alone suffers from some heuristic simplicity, in the absence of knowledge of other thermodynamic functions, such as enthalpy or volume. Indeed, previous experimental results on the interaction of several divalent cations with alginates and pectins show a complex (positive or negative) enthalpy change, while a positive volume change is always measured upon mixing polyelectrolyte solutions with salt solutions [7]. The latter result is in agreement with the

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more general observation that ion–ion interaction occurs with desolvation of the interacting groups and contributes, therefore, to the entropy term of mixing.

From the mere macromolecular point of view, the process of polyuronate gelation occurs with an extensive demixing heterogeneity of chain distribution in solution. Local aggregation of chains is counterbalanced by large domains of low (or null) concentration of polymer. Thus, the physico-chemical properties of the gel system are dominated by the presence of an excess of water "trapped" within the porous three-dimensional polymer matrix. Water molecules in polysaccharide/water systems are categorized into three kinds of water, i.e. non-freezing water (NFW), freezing bound water (FBW) and "freely"-freezing water (FW) [8, 9]. Non-freezing water belongs to the hydration shell and is tightly bound to the polysaccharide molecules and therefore cannot crystallize. Freezing bound water is weakly bound to polysaccharide molecules; consequently, it can crystallize, but the melting temperature is lower than that of bulk water. Freely freezing water is not influenced by polysaccharide molecules, hence the physical properties of this type of water are the same as those of bulk water. Bound water plays an important role in the formation and stability of the junction zones in gel forming polysaccharides [9], as well as for the survival of microorganisms [10].

In the technological applications, these gel systems are of significant interest for pharmaceutical application (e.g. for drug and vaccine delivery) and for the food industry [11], by using processing techniques such as spray-drying, fluidized bed drying and melt extrusion [12, 13]. In the more general preparation process, microspheres are produced in non-dry conditions and, therefore, removal of the excess free-water molecules from gel matrices is necessary to solve stability problems that arise during storage. Formulation and procedures to drying hydrogels are described in literature, without offering, however, a detailed thermodynamic characterization of complex gel beads containing therapeutic components and bioprotectants.

The aim of this work is to study the thermal behavior of water in alginate gel in the presence of a model protein (bovine serum albumin, BSA) and of trehalose added as a protein stabilizer. Trehalose, a naturally occurring osmolyte, is known to be an exceptional stabilizer of proteins and helps retain the activity of enzymes in solution as well as in the freeze-dried state [14]. Its natural activity and its approval in food and pharmaceutical use make this molecule an extremely suitable mean for controlling water mobility and dry food stability under several conditions [15]. Besides the morphological characterization of gel beads, both the freezing–melting behavior and the evaporation process have been studied and discussed within the known thermodynamics of hydrated biopolymer systems. For this purpose, DSC is a suitable mean that is commonly used to detect the different water fractions and in very small quantity. A slightly different formulation is studied in another paper from the viewpoint of rheological behavior in order to rationalize the contributions of the several components to the gelling structure formation [16].

The reported hydrogel matrices have been largely used to incorporate proteins for controlled release applications [17]. The stability of the gel structure and the tailored properties toward protein loading and release depend on the characteristics of the polymer and the preparation method [18]. In gel particle technology, three methods are commonly described in literature to prepare alginate microspheres: (a) mixing a solution of calcium ions with sodium alginate solution, (b) via a w/o emulsification technique and (c) complexation of oppositely charged polyelectrolytes [19]. However, the effectiveness of pharmaceutical, nutraceutical and food products depends also on the ability of preserving the active ingredient bioavailability, especially along the gastro-intestinal tract following oral administration [20, 21]. It should be also remarked that due to the safety of alginate-based delivery systems (GRAS) such compounds have been extensively developed for biomedical, food and pharmaceutical applications [20, 22, 23].

It is generally accepted that protein release from alginate matrices occurs following two mechanisms, that is: (a) diffusion of the protein through the pores of the polymer network and (b) degradation of the polymer network [19]. Water diffusion through a polymeric hydrogel layer has also been considered as one of the major factors affecting drug release rate [24]. The rates of diffusion can be minimized by lowering the water activity of the carbohydrate matrix, that is by controlling the material properties [12]. In addition, the porosity of an alginate gel can be significantly reduced by partially drying the beads. However, complete dehydration of alginate beads can result in surface cracking which can facilitate the surface erosion of the beads upon rehydration [19].

Materials and methods

Materials

Sodium alginate (Alginic acid sodium salt from brown algae $[\eta] = 5.6$ dL/g, $F_G = 0.4$), Albumin from bovine serum (BSA, minimum 98%) and D(+)Trehalose (dihydrate) were purchased from Sigma (Sigma-Aldrich Inc., St. Louis, MO, USA); calcium chloride dihydrate was a RP product from Carlo Erba Reagenti, Rodano (Italy). All other chemicals were of analytical grade.

Preparation of beads

Preparation of alginate spheres/microspheres was carried out starting from solutions containing alginate or alginate in combination with bovine serum albumin (BSA) and/or trehalose. BSA was chosen as a model protein and trehalose as a natural bioprotector [14]. The concentration of active solutes in the starting solutions was: alginate 2% w/v, BSA 10% w/v and trehalose 10% w/v. According to the common procedure, the gel beads were prepared by adding drop-wise each starting solution to a gelling solution, containing $CaCl_2$ 50 mM, under gentle stirring, at room temperature. The spherical gel beads were maintained in the gelling solution for about 10 min, in order to allow ion diffusion and hydrogel hardening. After curing, the beads were rinsed with deionized water and used directly in the experiments. The diameter of the beads depends on the size of the needle used and the viscosity of the alginate solution.

Scanning electron microscopy

The 3D-structure of alginate beads has been characterized by SEM microscopy. Alginate beads have been cut for the cross section observation, freeze-dried and deposited on the specific holder. Samples were sputter-coated with Au/Pd using a vacuum evaporator (Edwards, Milano, Italy) and examined using a scanning electron microscope (model 500, Philips, Eindhoven, The Netherlands) at 10 KV accelerating voltage using the secondary electron technique.

Calorimetric analysis

Freezing and melting experiments were performed by using a power compensation calorimeter Pyris DSC1 (Perkin Elmer) in the temperature range between 223 and 283 K; scanning rate was 1 K min^{-1} during cooling and 1, 5 or 10 K min^{-1} during heating. The final experimental conditions were chosen to optimize both resolution and kinetic control [25]. Analysis was carried out under a nitrogen flux of 20 mL/min, using sealed pans. Evaporation curves were recorded employing a heat flux calorimeter DSC6 (Perkin Elmer), in the range 283–403 (or 423 in the presence of trehalose), at a scanning rate of 10 K min^{-1} under a nitrogen flux of 20 mL/min in open pans. Some experiments were also carried out in partially sealed (vented) pans in order to provide a controlled evaporation rate.

In all experiments only one sphere ranging from 5 to 15 mg was analyzed; the sphere was weighed before and after heating in order to calculate the water loss and hence the dry matter. Parallel experiments were carried out on a large number of spheres (more than 50), by calculating the percentage of dry matter from the mass loss of the spheres held at 90 °C in an oven for 1 day.

Results

Characterization of the gelling system and morphology of beads

Alginate beads appeared as spheres of about 2 mm (Fig. 1a). SEM images revealed that the internal structure of the sphere was characterized by a sponge-like aspect (Fig. 1b). In particular, the smallest pores were near the surface of the sphere, as it is known that the bead preparation method produces an excess of calcium–alginate structured networks on the surface of the beads [26]. The preparation method of spherical gel beads has been found reproducible and beads size and shape were sufficiently constant to allow a comparison of the thermal results. A fundamental quantity of interest in the experiments is the amount of water per particle dry weight. In principle, given the preparation method, the solid matter weight could be calculated from the composition of the gelling system. In the absence of large osmotic diffusion effects (other than calcium ions), a constant volume assumption would predict a final dry mass in the beads equal to that contained in the starting droplets with the relatively small change of the calcium ions substituting the sodium ions. As a matter of facts, independent analysis with several proteins, including BSA, showed that the actual content of protein (and consequently of trehalose) slowly decreases with time (release)

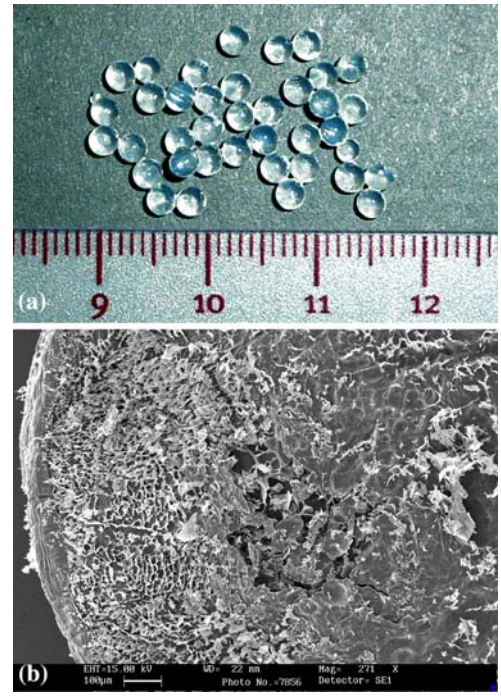

Fig. 1 **a** View of alginate beads; **b** SEM images of the a section of alginate beads

after an initial burst of the loaded material. Therefore, the amount of water per particle dry weight was evaluated by weighing the bead before and after dehydration.

Calorimetric analysis: melting

According to the standard procedure, all samples were subjected to a slow cooling scan (1 K min^{-1}) in the calorimeter down to 223 K, in order to freeze the water fraction. Thereafter, a heating scan was carried out and the melting was registered. A second heating scan was always recorded, after cooling at the same rate of 1 K min^{-1}.

The alginate spheres were initially studied on heating at three different heating rates that is 1, 5 and 10 K min^{-1}. Apart from the obvious influence of the scanning rate on the peak shape, the difference in the first part of the melting event is mainly due to a change (although small) in the onset temperature, while the rest of the peak becomes broader at higher scanning rate (Fig. 2). Indeed, variations in the thermal behavior were better emphasized at a scan rate of 5 K min^{-1}, especially in the presence of other components in the formulation. This observation could be related to the slow heating of the system and therefore to the possibility of a reorganization of the water molecules during melting. In fact, heating rate plays an important role in the DSC experiments because changes in shape of heat-flow versus temperature plots are clearly due to water migration inside the porous structure [25]. Consequently, all the experiments have been carried out at 5 K min^{-1}.

Heating traces of all samples of spheres in Fig. 3 showed only a broad unresolved curve, without a clear evidence of a separate peak for the fraction of water known as freezable bound water (FBW). The small shoulder in the curve of alginate solution becomes larger and more evident in all other sphere samples, producing an unusual broadening of the higher temperature melting profile. The thermogram of the alginate sphere with trehalose is similar to that of alginate solution. Occasionally (and randomly) the presence of undulations in the top part of the band revealed some small relative changes. This effect has not been considered significant and, therefore, has not been taken into account in the fitting procedure described below.

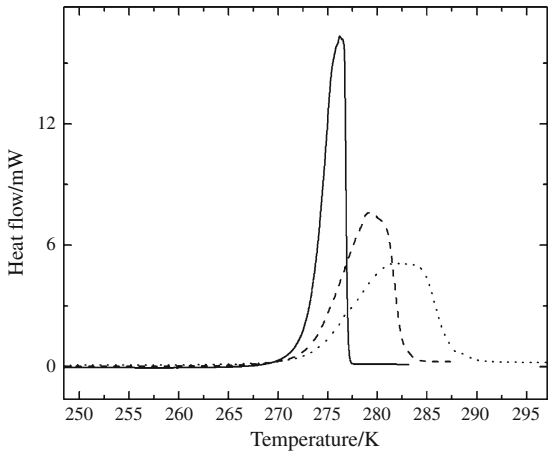

Fig. 2 Thermograms of an alginate sphere in sealed pan during heating scan at three different scanning rates: (—) SR 1 K min^{-1}, (——) SR 5 K min^{-1} and (·····) SR 10 K min^{-1}

Different parameters were evaluated in order to understand the complexity of the melting process. Calculation of T_{onset} and T_{end} was done with Pyris software (see data of Table 1). The difference between T_{end} and T_{onset} is mainly related to the mass of the samples, ranging between 5 and 6 K for samples with masses of 4–9 mg, with a linear dependence of the peak width on the sample mass.

Given for granted that the thermograms show a complexity that can be traced back to several melting processes, the analysis has been phenomenologically made without attributing this broadening to any specific series of events. The fitting of the melting curves, performed with Origin® software, revealed the possible presence of at least three Gaussian curves (Fig. 4). This result would suggest

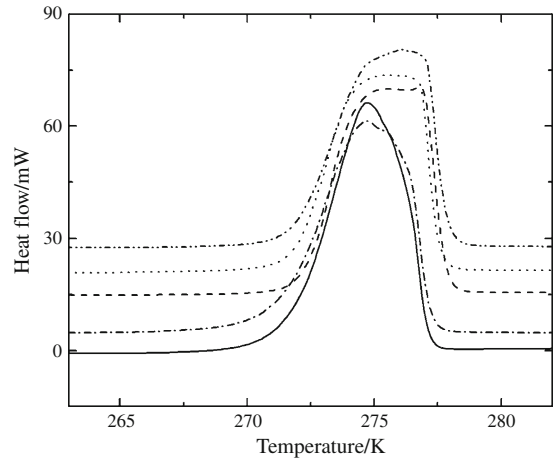

Fig. 3 Thermograms of alginate solution and alginate spheres in sealed pans during heating scan at SR 5 K min^{-1}: (—) alginate solution, (- - -) alginate sphere, (···) alginate sphere with BSA, (-·-) alginate sphere with trehalose and (-···-) alginate sphere with BSA and trehalose. Curves are displaced horizontally for clarity

Table 1 T_{onset} and T_{end} of alginate solution and alginate spheres calculated with Pyris software from curves recorded at SR 5 K min^{-1} during a heating scan

Sample	T_{onset}/K	T_{end}/K
Alginate solution	273.5	278.8
Alginate sphere	274.2	279.5
Alginate sphere + BSA	273.8	279.1
Alginate sphere + trehalose	273.7	279.4
Alginate sphere + BSA + trehalose	273.7	279.4

the presence of different "populations" of water. However, this does not mean that three different thermodynamic states of the water are present, but rather that the total melting process could be intended as the sum of at least three different steps of water fusion. According to this assumption, the three Gaussian curves were evaluated in terms of the position of the peaks and their relative area.

Among the different samples, position and area of the three peaks were comparable, suggesting an overall common melting behavior. The evaluation of the peak components revealed that the first peak is always far greater than the second and the third ones (Table 2). However, regarding all spheres samples, the difference between the first and the second peak is greater than the difference between the second and the third peak. The observations made so far suggest that water melting profile is in some way influenced by the presence of the alginate gel network, but the ratio between the total heat and the total amount of water undergoing melting remain approximately constant.

The total heat involved in the melting process, calculated with Pyris software, was compared to the theoretical heat that would have been expected on the basis of the total amount of water present. From the difference between the theoretical and experimental heats of melting, the amount of non-freezing water (NFW) was determined. In fact, assuming that the melting enthalpy of ice is temperature independent in the small temperature range and equal to 334 J g^{-1} (6.018 kJ mol^{-1}) [27], the difference in the heat of melting was attributed to the amount of non-freezing water [24]. The results revealed that, in all sphere samples, the amount of NFW was around 10% of the total water and a little bit higher for the alginate solution.

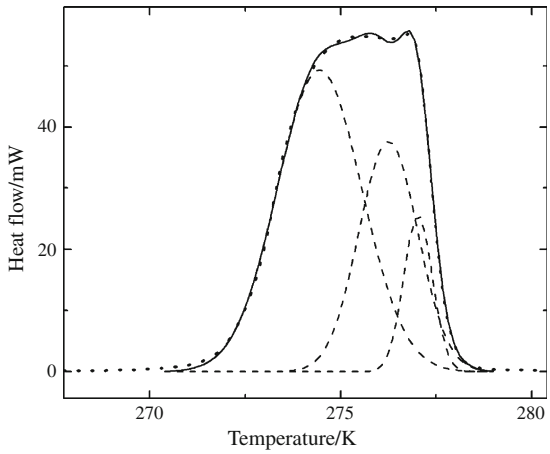

Fig. 4 Example of Gaussian fitting of a thermogram relative to alginate sphere in sealed pan during heating at SR 5 K min^{-1}; (····) heat flow measured, (—) Gaussian fit, (− −) Gaussian components

Table 2 Heat associated to the three curves deconvoluted with Gaussian fitting (see Fig. 4)

Sample	Peak 1/J	Peak 2/J	Peak 3/J
Alginate solution	1.044	0.675	0.334
Alginate sphere	1.556	0.688	0.224
Alginate sphere + BSA	0.973	0.452	0.161
Alginate sphere + trehalose	1.204	0.684	0.218
Alginate sphere + BSA + trehalose	1.800	0.768	0.289

The non-freezing water content and the total water content referred to 1 g of dry matter (W_{nf} and W_t, respectively) were calculated according to Hatakeyama [8] and plotted as W_{nf} versus W_t in Fig. 5. A correlation between the two water contents was evident, that is non-freezing water content increases with the increasing value of the total water content. This observation is in agreement also with data presented by Nakamura et al. [28]; the author showed that increasing the amount of water adsorbed with respect to the dry weight, the fraction of non-freezing water tends to level off to a certain amount, which equals the mass of the polymer, that is for example a sphere contains 10% polymer, 10% NFW and the rest is freely freezing water, FW.

Finally, some slight differences of the melting curve were noticed between the first and the second heating, showing a difference around 0.2 K in the positions of the peaks. This could imply a possible influence of the previous thermal history on the bead polymeric network. Variations in the structural properties have to be studied in detail, since no other properties were measured on beads after repeated freeze-thawing scans.

The results of the experiments here reported clearly show the absence of the fraction of freezing bound water in gel beads with low alginate content (2%), while this

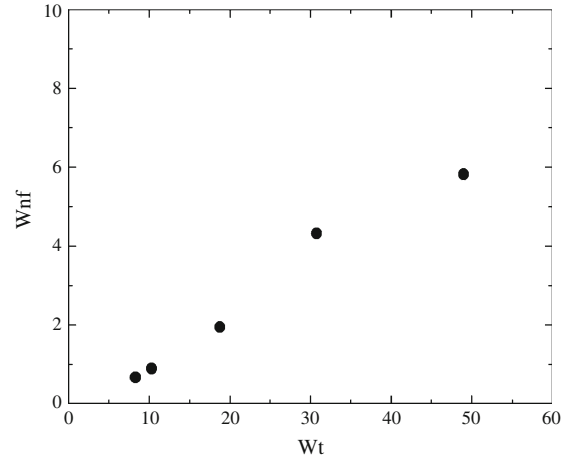

Fig. 5 Unfrozen water versus total water content. W_{nf} = unfrozen water (g)/dry matter (g), W_c = total water (g)/dry matter (g)

fraction has been reported in other literature works at higher alginate content (higher than ca. 4%) [24]. Whether the different results stem from the change in concentration or from the preparation process is an issue of interest and will be investigated.

Freezing

Crystallization of water, in all samples, appeared as a sharp peak. As expected, in addition to the depression of the freezing point due to the presence of a solute, the usual undercooling effect was shown, giving freezing phenomena in the range 254–267 K. The very small differences between first and second cooling suggest no major reorganization of the system, as already noticed in heating traces. Furthermore, the reproducibility of the two successive runs indicates that also the undercooling effects depend on the system properties and not from a random nucleation.

The thermal behavior of all the samples investigated was characterized by the presence of only one peak of crystallization, suggesting again that the fraction of freezing bound water (FBW) is not present as already found in the melting curves. The question of the presence of freezing bound water in alginate systems has been debated in literature. On one hand, Nakamura et al. [28] in a study of an alginate–water system containing gelling cations did not have evidence of a second melting peak, on the other hand Faroongsarng [24] more recently described water adsorbed on sodium alginate as bulk water and as freezing bound water, reporting two peaks both in the heating and in the cooling scan. Thus, giving for granted that all experiments have been carried out in similar conditions, it surprisingly appears that some differences in the state of polymer aggregation (gelling films versus hydrocolloid solutions) may change the topology of the polymer surface and give rise to the FBW peak.

The addition of trehalose to the polymeric system follows the trend already described in several literature works [29, 30]. In the present freezing experiments, it is evident that the addition of trehalose drifts the beginning of water crystallization to temperatures higher than those found in absence of the sugar.

Evaporation

Although evaporation experiments have been originally reported in the past, the literature does not give attention to this phenomenon in liquid-like systems, while other dehydration processes are amply studied. In view of the previous investigation on trehalose dehydration under several experimental conditions [31, 32], the study of water evaporation from alginate beads containing several additional components was considered. The complexity of the systems is evident from the thermograms obtained during heating in the range 283–403 K (or 283–423 K for systems with trehalose).

As a reference system, dehydration thermogram of bulk pure water in open pan is characterized by a continuous exponential increase of the heat flow up to the sharp peak with an abrupt decrease of the signal to the baseline. A similar behavior was observed also for the alginate solution, however in this case the decrease was not as rapid as for bulk water. Independently of the solute content, the thermograms of the sphere samples are characterized by a different shape, with a broader decrease after the maximum (Fig. 6a). A precise peak temperature was not always evident because of the presence of a wide plateau, suggesting continuously delayed water evaporation because of the interaction within the alginate matrix. After the plateau, other small evaporation processes occur before the signal gets completely off at temperature well above 373 K. The last part of the curve clearly reflects the influence of the other components (BSA and trehalose) on water evaporation. Still, the total heat involved in the evaporation process does not significantly differ within the samples.

Under controlled evaporation rate a quasi-stationary state can be reached in a DSC cell. The heat flow approximately measures the increase in vapor pressure with increasing temperature (that is an exponential Clausius–Clapeyron-like equation). Therefore, the total heat involved in the process can be analyzed in a logarithmic form (Fig. 6b). The results show a range of linearity in the first part of the heat flow versus temperature curves. The experimental curves can be grouped in two families, water and alginate solution on one side and all the gel beads on the other. However, this does not mean that the two different families present different heat of evaporation, but rather that the evaporation rates are different. Indeed, the figure would resemble a Clausius–Clapeyron plot only if the system could be in a thermodynamic equilibrium, while the present data are modulated by the water evaporation rates that must be different in the two families of experiments.

Thus, evaporation rates of free-water from the alginate beads is delayed by the calcium–alginate polymeric network which is known to be more densely packed at the bead surface. This hampering effect can be used to quantify the surface porosity in order to model the diffusion process and release of other molecules entrapped in the gel bead.

Finally, some calorimetric analysis was also performed in closed (vented) pans. This pan geometry generated a water pressure that is only slowly released. The thermograms obtained for spheres containing trehalose and trehalose with BSA were characterized by a very different behavior in the first part of the evaporation process, as

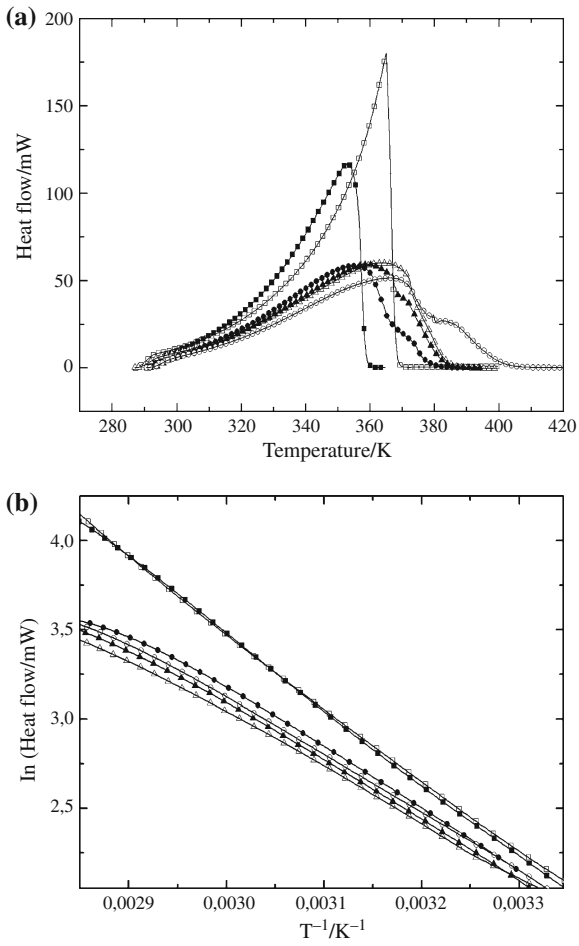

Fig. 6 **a** Thermograms of water evaporation from alginate solution and alginate spheres in open pans during heating scan at SR 10 K min^{-1}: (-■-) alginate solution, (-Δ-) alginate sphere, (-●-) alginate sphere with BSA, (-▲-) alginate sphere with trehalose and (-○-) sphere with BSA and trehalose; as a reference, the thermogram of a water drop is also reported (-□-). **b** Linearization of heat flow curves versus temperature in the exponential Clausius–Clapeyron-like equation: (-□-) water, (-■-) alginate solution, (-Δ-) alginate sphere, (-●-) alginate sphere with BSA, (-▲-) alginate sphere with trehalose and (-○-) sphere with BSA and trehalose

already reported in the technical bulletins. In addition to the evaporation process, a series of events related to the state of water in the hydrated components appeared in the thermograms, at least in part due to the trehalose polymorphic transitions [31]. The quite complicated interpretation of these curves has not yet fully worked out, thus further investigations are required.

Discussion and conclusions

The discussion of the data here presented would necessarily involve some issues about the current knowledge on the state(s) of water, as commonly analyzed and debated in the recent literature. As a matter of fact, the variability of molecular systems and the range of concentration studied often prevent a common understanding of the structural features under study. In addition, only if equilibrium processes are considered, then the schematic categories of the three state of water are reasonably useful. In other words, the continuously dynamical exchange can be fictively grouped in three distinct thermodynamic states, that of bulk water (freely to freeze, $\Delta G = 0$ at 273.15 K), that of bound water molecules (unable to freeze, $\Delta G >> 0$) and that topologically close enough to a molecular surface and therefore substantially perturbed ($\Delta G > 0$). However, the two normally expected calorimetric peaks of FBW and FW have already been reported to merge progressively into a single broad band at high swelling degree [33], as it has been found in the present study.

In addition, our systems present a common structural feature as far as the calcium–alginate network is concerned, which cannot be defined homogeneous as it is often assumed. It has been established that the method of gel bead production induces a calcium concentration gradient which decreases from the outward layer going to the inward part of the bead and adds up to the statistical heterogeneity of polymer distribution (fractal-like) containing some macroscopic clusters. As a consequence, also the polysaccharide distribution follows the same trend and the fraction of bound/unbound water as well. Under these circumstances, the calorimetric setup can still introduce a noticeable source of process modulation, in particular for the heating scan rates and the thermal geometries of the samples with the current size and mass. These compositional gradients will have to be taken into account in developing a correct mathematical understanding of heat conduction in a calorimetric experiment [34]. Although a specific model is not analyzed here, let only underline that slow scanning rates will uniformly transfer heat from the surface to the core, while high scanning rates will increase the role of the contact of the spherical bead with the aluminum surface.

Once the topological features of the matrix are assessed, the characterization of the water thermodynamics and morphology is necessary to devise a structural and dynamical understanding of the gel bead as a "thermodynamic" system which may exchange with the environment both energy (heat, elastic work) and matter (water and other released solutes). Among all nanostructured gels those based on natural polymers, like polysaccharides, appear to offer less outstanding material features, counterbalanced however by the high compatibility and sustainability that make them unvaluable in biopharmaceutics. In addition, alginate gels appear to possess a number of appealing thermodynamic properties such as hydrophilicity and thermostability. The paramount importance of the

enthalpimetric and volumetric contributions of the several constituents to the understanding the thermodynamics of the gelling phase formation resides in some original work of several laboratories published in the last decades. It clearly comes out the role of water displaced during the chain–chain interaction mediated by divalent cations, discriminating the entropic-driven from the enthalpic-driven association processes.

Future investigation within the present research work is therefore to fully analyze the water thermal behavior, including smaller alginate systems, i.e. micro- and nanocapsules, and to explore the water dynamic properties at fast timescales to be compared with the present thermodynamic properties.

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