Controlled Calcium Release in the Absence and Presence of an Ion-Binding Polymer

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The calcium-induced structuring of pectin solutions has been exploited in many diverse areas of science and technology for many decades, as indeed has controlled calcium release as a methodology for obtaining homogeneous gels in such systems. However, many physical chemistry facets of the release, such as the determination of the rate-limiting step and the explicit investigation of the effects of the ion-binding polymer on the ion release process, have received scant attention. In this work, it is demonstrated that the release of calcium via the controlled dissolution of $CaCO_3$ by GDL can be rate limited *either* by GDL hydrolysis or by $CaCO_3$ dissolution, dependent on the particle size. The influence of the ion release rate on the gelation of pectin has also been studied, using a methodology to ensure that the resultant mechanical properties of the different systems were all measured at the same pH. Furthermore, for the first time, explicit evidence is provided to support the hypothesis that the presence of the pectin does not significantly influence the dissolution rate of $CaCO_3$.

Introduction

It has long been appreciated that the interaction between ionic polysaccharides and specific metal ions plays a crucial role in fulfilling the structural physiological requirements of both land and marine plants.[1−3] These adapted structuring solutions have been exploited in vitro for many decades using extracted biopolymers such as pectins, alginates, and carrageenans, mixed with relevant cations.[4,5] Applications of this technology are diverse[6] and include systems used as cell immobilization matrixes,[7−9] ion-exchange media,[10,11] controlled drug delivery vehicles,[12,13] and rheology modifiers for the textural design of food products.[14−16] With increasing current interest in the study of soft condensed matter, ion-induced ordering transitions such as these also offer excellent model systems in which to investigate the isothermal assembly of biopolymeric networks.

The binding of such anionic polysaccharides to their adapted ion of choice has been shown previously to take place on the order of milliseconds to seconds.[17,18] This poses considerable practical difficulties in controlling the introduction of the polymer and ion in order to avoid heterogeneous large-scale precipitation before distribution of the ions throughout the system can be achieved through mixing. In nature, this control is achieved by post-placement enzymatic modification. Polymeric material that has a fine structure that elicits particularly strong ion sensitivity is not biosynthesized directly in this form; rather, it is first transported to the required site as a less sensitive mother polymer and subsequently modified enzymatically to generate the required functionality. Practically, in applications such as food gels, the contrary approach has been taken to circumvent the mixing problem. Rather than what might be thought of as a controlled release of ion-binding polymer segments, it is typically the ions that are liberated by using controlled release systems, such as employing the hydrolysis of GDL to solubilize otherwise poorly soluble salts.[19]

In such cases, it is important to understanding the evolution of system properties that changes are viewed within the context of the time-course of ion release. Conventionally, such release has been monitored by using ion-specific electrodes to measure the increasing free ion concentration generated by the designed release system. However, it should be noted that, owing to the difficulty of using such devices in gelling systems, these experiments have routinely been carried out in stirred systems that lack the ion-binding polymer itself. It has become largely accepted that the release profile ascertained in such a manner is a good approximation to that which would be obtained in the presence of ion-binding material. However, it seems conceivable that the presence of calcium binding polymers might influence the dissolution of, for example, $CaCO_3$, by sequestering the free calcium and driving the reaction or by modifying the mass transport of the calcium ions. It seems unclear, therefore, whether the grounds for this assumption have been adequately investigated, and it is the purpose of the work reported herein to explicitly test this hypothesis.

Materials and Methods

Pectin. The anionic polysaccharide pectin was utilized for this study, epitomizing, as it does, a commonly exploited calcium binding biopolymer. The sample used was derived from lemon peel and was obtained from CP Kelco ApS, DK 4623 Lille Skensved, Denmark. It had a galacturonic acid content of around 90%, of which approximately 35% was methyl-esterified. The distribution of the methyl-esterified residues along the backbone is likely to be random, owing to the nature of the commercial de-esterification process.

Calcium Carbonate. Samples of $CaCO_3$ powders were both purchased from Fisher Scientific, Bishop Meadow Rd, Loughbourough, LE11 5RG, UK and kindly provided by Provencale s.a., Avenue Frédéric Mistral, 83172 Brignoles Cedex, France. The latter samples were produced from the same quarried material through crushing, sieving, and milling to give a series of samples with common particulate properties and mean sizes of 1.7, 5, and 10 μm, respectively. The Fisher sample had a

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mean diameter of 20 μm. The particle size distributions were approximately log-normal with 60% or greater of the particles having diameters falling within a factor of 2 from the mean.

Calcium Release. All release matrixes described were initially titrated to pH 6.0 using 1 and 0.1 M NaOH. The calcium release from the carbonate salt was triggered by the release of protons from the hydrolysis of glucono-δ-lactone (GDL), which was obtained from Fisher. The release of free calcium was followed using a calcium-selective electrode (model 93−20) obtained from Thermo Orion Europe, 12−16 Sedgeway Buisness Park, Cambridgeshire, UK, CB6 2HY, with a single junction reference electrode (filling solution Orion Cat No. 900011). Calibration was performed daily using fresh $CaCl_2$ solutions. All water used had a resistivity of at least 18.2 MΩ.

Free calcium concentrations were also assessed by performing colorimetric titrations with the disodium salt of ethylenediaminetetraacetic acid (EDTA).[20] Aliquots were taken from a beaker in which the calcium release was initiated, each corresponding to a time-point in the calcium release profile. The solution pH of each aliquot was increased prior to titration to 10 by the addition of ammonia/ammonium chloride buffer (1.20 M NH_4Cl/21% NH_3 solution). The color indicator used was Eriochrome black T (EBT). The titration of solutions filtered through 0.45-μm Millipore filters was compared to unfiltered solutions in order to investigate any influence on titrate volume from remaining $CaCO_3$ particles. Identical results were obtained in both cases.

The total calcium concentration of solutions was also analyzed using a PerkinElmer OPTIMA 3000DV Inductively Coupled Plasma-Atomic Emission Spectrometer (ICP-AES) to asses the importance of any impurities. Quantification was performed by comparison of the emission intensity at 422.6 nm with that of known standards prepared from a 10 000 ppm calcium standard for ICP; BDH "ARISTAR" grade, product number 455172T.

Rheology. The rheological consequences of the calcium release in systems containing pectin were monitored by measuring the storage and loss moduli at a strain of 0.5%, a frequency of 1 Hz, and a temperature of 293 K, using a Physica UDS 200 stress-controlled rheometer (Paar Physica, Stuttgart, Germany) with a cuvette geometry. These measurements were carried out using 2% w/w pectin solutions, with the $CaCO_3$ concentration such that the complete dissolution yielded 12.4 mM calcium ions. This is equivalent to a reactivity ratio for the final gel of $R = 2*[Ca2+]/[COO^-] = 0.4$, the value of which was selected based on previous studies.[21] Such a ratio can be considered as the fractional saturation of de-esterified galacturonate by calcium ions. A stoichiometric amount of GDL was always used (24.8 mM) to ensure that the pH was held as near constant as possible. It is important to stress that using this methodology means that the comparison of final gel strengths of samples in which the calcium has been released at different rates is carried out at the same pH. This has frequently not been controlled in previously reported studies, although it is known that the pH of such gels can significantly alter the materials properties. The salt and GDL were added to the pectin solution immediately prior to sample loading.

Image Analysis. The decreases in the $CaCO_3$ particle sizes accompanying dissolution were also monitored in these pectin containing systems, in real time, using light microscopy and image analysis. Images were acquired using a Leica microscope with 10× or 20× magnification and recorded using a CCD camera. Binary images were thresholded and analyzed using Zeiss KSRUN 3.0. The total amount of undissolved material was estimated by first taking the pixel area of each counted particle and then deriving an equivalent radius and hence an equivalent spherical volume from this. Subsequently, these volumes were summed to give a total undissolved volume of $CaCO_3$ that was finally represented as a fraction of the initial volume.

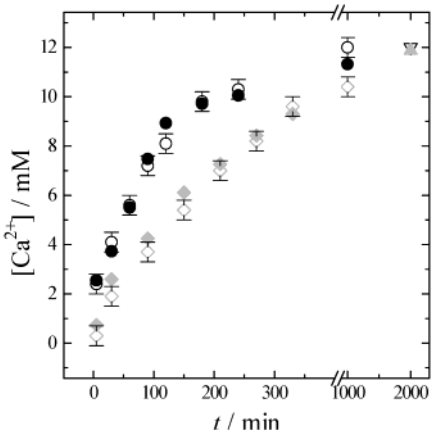

Figure 1. Calcium release from carbonate particles with 1.7 (circles) and 20 μm (diamonds) mean diameters, generated from 12.4 mM of the carbonate salt in water (at pH 6.0) by adding the stoichiometric equivalent of GDL (24.8 mM). Filled symbols, calcium electrode measurements; open symbols, results from EDTA titrations. The error bars give an indication of the uncertainty in the measurement as assessed from a number of repeats. The triangles are the final calcium concentrations monitored by ICP-AES (down, 1.7 and up, 20 μm).

Results and Discussion

Figure 1 shows the time-course of calcium release from two powders with mean diameters of 1.7 and 20 μm, resulting from the addition of 12.4 mM carbonate salt to deionized water (at pH 6.0) and subsequently adding the stoichiometric equivalent of GDL (24.8 mM). The results are obtained from measurements carried out on aliquots using both a calcium electrode and an EDTA titration. It can be seen that there is a good agreement between the two methodologies. Furthermore, the calcium content of the solutions after extended time periods was measured using ICP-AES and was also found to be in good agreement with the electrode and EDTA results.

It is worth mentioning, however, that when the calcium electrode was left in the reaction mixture throughout the time-course of the experiment, in an attempt to measure the evolving free ion concentration in-situ, very different results were obtained, so that the dissolution appeared considerably faster than the results of the EDTA titrations indicated. Following the experiments using the electrode with aliquots, in which the electrode was well cleaned between measurements and subsequent stop-clean-remeasure experiments, it was determined that despite the stirring of the solutions this seeming discrepancy originated from the binding of calcium to electrode membrane throughout the course of the experiment. Subsequently, great care was taken to ensure that such artifacts did not affect the results.

To determine the rate-limiting step in the calcium release process, and in particular assess the relative importance of the GDL hydrolysis and the dissolution of the $CaCO_3$ particles, the changes in proton concentration were also measured simultaneously with the calcium ion release. It has previously been shown that the rate constant for GDL hydrolysis varies significantly with pH.[22] This complicates matters significantly, as the pH of our reaction mixtures over the first minutes is a complicated function of the initial rates of particulate dissolution

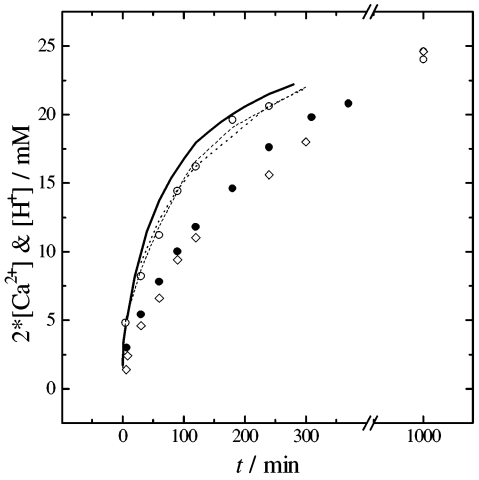

Figure 2. Calcium (symbols) and proton (lines) release profiles measured in aqueous solution (initially pH 6.0) following the addition of 24.8 mM GDL to 12.4 mM carbonate particles with 1.7 (open circles, solid), 5 (filled circles, dashed), and 10 (diamonds, dots) μm mean diameters.

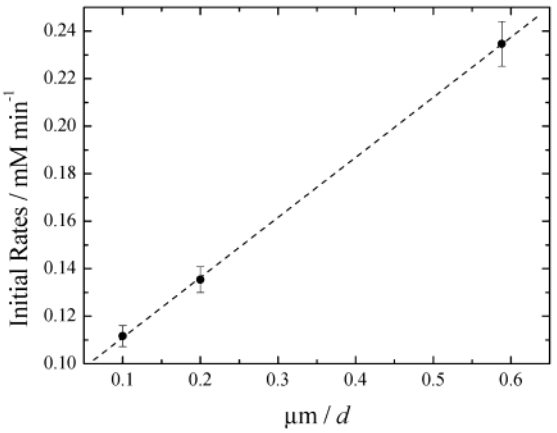

Figure 3. Initial rates of calcium release plotted against the reciprocal of the mean diameter for the 1.7, 5, and 10-μm particles from Provencale.

(and thereby particle size and porosity) and GDL hydrolysis. However, the proton release for all systems was calculated from the experimental pH using an incremental first order reaction scheme in which the rate constant used in each time increment was determined according to the pH, from relations given previously.[22] These data are shown in Figure 2, and it can be seen that for the smallest particle size (1.7 μm), the proton and calcium release rates are very similar, especially considering that the experimental uncertainties in the proton release measurement are around 5−10% (primarily because of the sensitive dependence of the rate constant on the pH). This suggests that for this powder the hydrolysis of GDL may well be the rate-limiting step. However, for larger powders from the same manufacturer (5 and 10 μm) it can be seen that the calcium release is substantially slower than the proton release, suggesting that, for these powders, the dissolution of the particles is rate limiting. This clearly agrees with the observation that while the proton release rates for the 1.7, 5, and 10-μm particles are very similar, the calcium release rates differ and are dependent on $CaCO_3$ particle size.

Furthermore, by fitting the increase in calcium concentration with time, an initial release rate is obtained, which is found, as shown in Figure 3, to scale linearly with the reciprocal of the particle size. This demonstrates that the rate is proportional to the total surface area of the particles (for the same total volume, the total surface area decreases as the reciprocal of any increase in radius), as would be expected for a process rate limited by $CaCO_3$ dissolution. The data-point for the 1.7 μm particles appears to fit well on this plot despite the fact that the calcium release rate is very similar to that of the GDL hydrolysis. This suggests that for these powders this size approximately marks the transition between the rate-limiting steps. Above 1.7 μm, it can be expected that increasing the particle size will slow the calcium release in a predictable way, based on the reciprocal of the mean size. Below 1.7 μm however, reductions in size would be expected to have little further effect on the rate, owing to the calcium release rate already being close to that of the GDL hydrolysis. (It is, however, worthy of comment that, in such highly coupled systems, changes in particle size may have indirect consequences for the release rate, by for example, modifying the *initial* pH obtained on the introduction of the powder and therefore the subsequent proton release rate.)

It is noteworthy that when the same study was carried out with the larger $CaCO_3$ particles obtained from Fisher, the proton release rate was substantially slower than that obtained with the powders from Provencale, so that in this case, the process was still rate limited by the GDL hydrolysis. This was true despite the particles being substantially larger than those in the powders provided by Provencale that were clearly limited by particle dissolution. This can be explained if the particle size at which the dissolution process becomes rate limiting is dependent on the physicochemical properties of the carbonate particles. Indeed, the dependence of the dissolution rate on the chemistry of the surface and on the presence of surface active inhibitors or coatings has been examined in systems where carbonate surfaces have been treated directly with acid.[23−25] Furthermore, it has been reported that different carbonate sources and production methods can produce particles with quite different topologies and porosities.[26,27]

Figure 4(a) shows the corresponding rheological consequences of using the 1.7, 5 and 20 μm systems investigated above to release calcium into 2% w/w pectin solutions. It appears that particles that dissolve somewhat faster do yield an increased gelation rate, although the final gel strengths are comparable (it should be noted, as described in Materials and Methods, that these final gels have identical pH values). Similar findings have been made with alginate solutions gelled with unadulterated and subsequently sonicated calcium carbonate particles.[28]

The calcium concentration required for gelation can be assessed by taking the crossover of the storage and loss moduli (G' and G'', respectively) as a criteria for gelation[29] and comparing the time at which this occurs with data showing the calcium release. Such an assessment of gelation times for the different particle sizes is shown in Figure 4(b). (This is essentially a zoom into the early time region of Figure 4(a).) It can be seen that this rheologically determined gelpoint occurs almost immediately for the 1.7-μm particles and after approximately 15 and 47 min for the 5 and 20-μm mean particle sizes, respectively. It is interesting to note that the observed crossovers all occur at an elastic shear modulus of around 2 Pa. This is consistent with the idea that the elastic modulus is directly proportional to the number of cross-links in the network,[30,31] and suggests that there are a critical number of junction zones required for percolation and the evolution of gel properties. In this picture, the varying rates of calcium release only alter the time taken to achieve this critical fraction. Upon consultation of the calcium release data, it is found that these times all correspond to an approximate calcium concentration

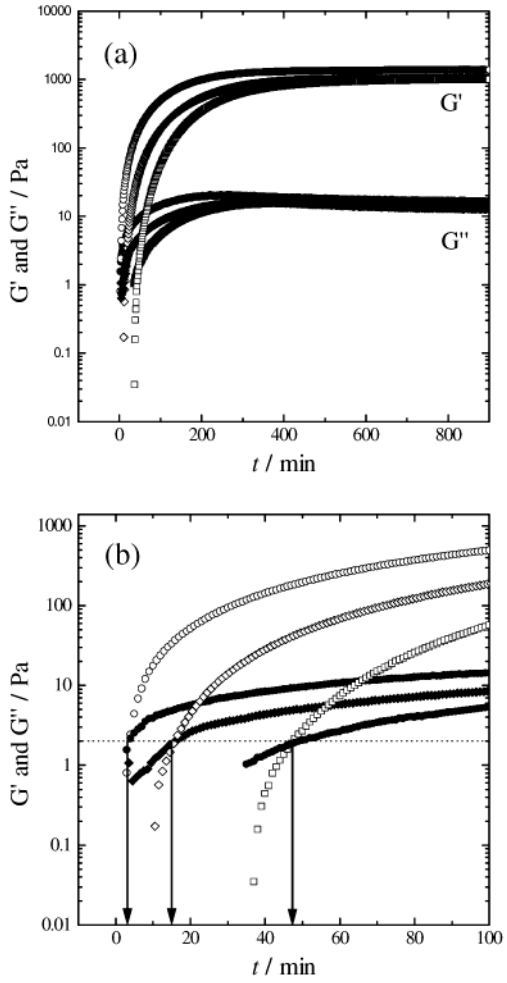

Figure 4. The rheological consequences of using the 1.7 (circles), 5 (diamonds), and 20 (squares) μm systems to release calcium into 2% w/w pectin solutions (a) over 900 min, showing the final gel strength, (b) over the first 100 minutes showing the crossover of G′ and G″, taken as the gelpoint.

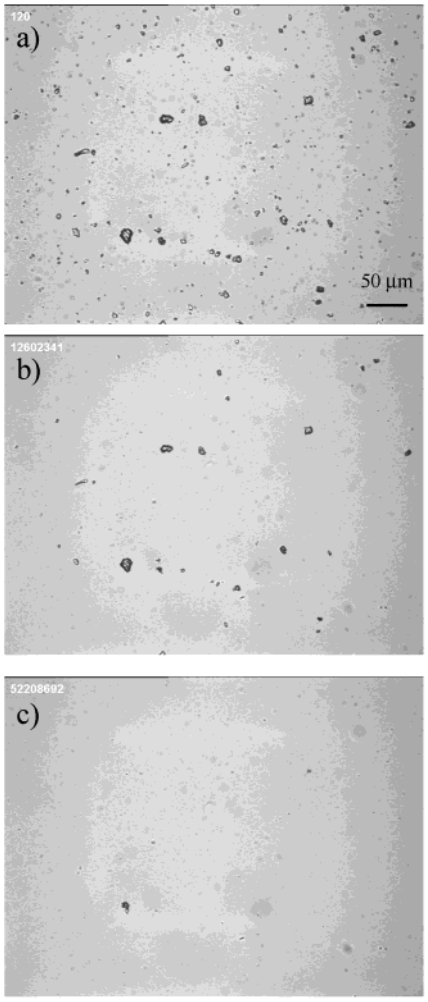

Figure 5. A representative time sequence of images for the 5-μm particles undergoing dissolution as described in the text (a) 5 min, (b) 215 min, (c) 20 h.

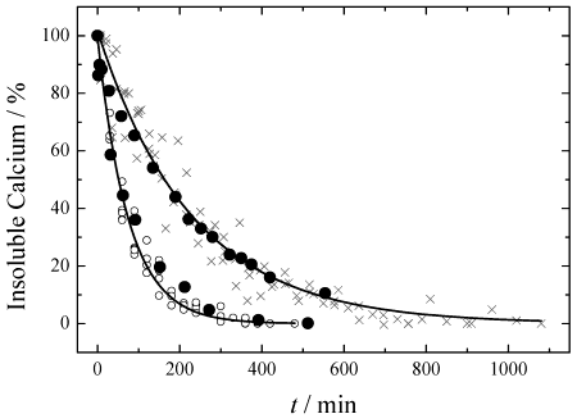

Figure 6. Image analysis data-sets obtained for the dissolution of the 1.7 (open circles) and 5 (crosses) μm particles in the presence of 2% pectin solution. These data were fitted to exponential decays, (shown in the figure, as solid lines) to facilitate the comparison of the image analysis data with the results of the measurements carried out in the absence of polymer using an EDTA titration (filled circles).

of between ~2.0 and 3 mM (corresponding to a required R value of between 0.07 and 0.10). It is interesting to note that similar critical concentrations have been observed in studies of alginate gelation.[32] Ongoing work involves examining how this R value depends on polymeric fine structure.

However, as described previously, such a comparison of the rheological data with calcium release profiles is not obviously justified. In the gelation experiments, the released calcium ions will react with the pectin as they are released, whereas in the absence of pectin, the calcium in the release experiments will remain free in solution. In an attempt to study the influence of pectins on the GDL-induced dissolution of $CaCO_3$, the $CaCO_3$ particles themselves were visualized as a function of time and the decrease in their size observed by image analysis. Figure 5 shows representative images recorded during the dissolution of the 5-μm particles. The change in the total volume of undissolved particles was monitored by analyzing a time-series of such pictures. Large concatenated data-sets (from five repeat experiments) representing the dissolution of the 1.7 and 5 μm particles were then produced and can be seen in Figure 6. These image analysis data sets were then fitted to exponential decays (again shown in the figure) to facilitate the comparison of the image analysis data with the results of the measurements carried out in the absence of polymer by conventional techniques. The increase of free calcium in the equivalent pectin-absent systems, as measured by the EDTA titrations, has been transformed into a percent decrease of undissolved $CaCO_3$, and hence can be compared to the image analysis data obtained in the presence of pectins. It appears from the results shown in Figure 6 that within experimental uncertainty the dissolution rate of the calcium carbonate is unaffected by the presence of the ion-binding polymer.

Conclusions

It has been demonstrated that the release of calcium via the controlled dissolution of initially poorly soluble salts by GDL can be rate limited *either* by GDL hydrolysis or by $CaCO_3$ dissolution. For a particular powder, the pertinent rate-limiting step is dependent on particle size. However, we have also shown that the relevant size at which such a transition occurs depends crucially on the physiochemical properties of the particles. While practically this is of great interest and provides many routes to manipulating the release kinetics, it also highlights the need for care in the comparison of published work.

We have also examined the influence of release rate on the gelation of the ion-binding anionic polysaccharide pectin, using a methodology to ensure that the resultant mechanical properties of the different systems were all measured at the same pH. Thus, it has been shown, by using smaller particle sizes of $CaCO_3$ to increase the calcium release rate, that an increase in release rate does correspond to a reduction in gel-time, but that (at least for variation investigated here) the resultant gels have similar final gel strength.

Most importantly, for the first time, we have provided explicit evidence that the presence of pectins does not significantly influence the dissolution rate of $CaCO_3$. Hence, the dissolution rates of $CaCO_3$ generated by the hydrolysis of GDL, measured in absence of pectins, can indeed be used as a reliable indication of the actual calcium concentration with time in a gelling system.

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