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Production of alginate beads by emulsification/internal gelation. II. Physicochemistry

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Abstract Alginate microspheres were produced by emulsification/internal gelation of alginate sol dispersed within vegetable oil. Gelification was initiated within the alginate sol by a reduction in pH (7.5 to 6.5), releasing calcium from an insoluble complex. Smooth, spherical beads with the narrowest size dispersion were obtained when using low-guluronic-acid and low-viscosity alginate and a carbonate complex as the calcium vector. A more finely dispersed form of the complexed calcium within the alginate sol promotes a more homogeneous gelification. Microsphere mean diameters ranging from 50 µm to 1000 µm were obtained with standard deviations ranging from 35% to 45% of the mean.

Introduction

The immobilization or isolation of chemicals and biologicals in small spherical particles has proven to be of great utility in a variety of industrial applications. Since the mid 1970s (Kierstan and Bucke 1977), alginate has been the most commonly used material for cell entrapment (Lim and Sun 1980; Prevost et al. 1985; Redenbaugh et al. 1986; Shiotani and Yamane 1981). Large-diameter alginate beads (typically 2–5 mm) are usually produced by the extrusion method and low production rates (ml/h). Smaller-diameter beads are often preferred because of enhanced rates of bioconversion (Neufeld et al. 1991) and to avoid bead rupture resulting from the accumulated fermentation gas.

Coaxial air-jet devices (Miyawaki et al. 1980), electrostatics (Hommel et al. 1988) and vibrating needles (Hulst et al. 1984) result in smaller beads (down to 0.5 mm). However, as the bead number produced per unit of time is somewhat independent of size, techniques for small-droplet production are not well suited to industrial scale-up. To make beads on a large scale, many needles would have to be run concurrently, a complex and awkward solution (Dabora 1967).

In a previous study (Poncelet et al. 1992a), an emulsification/internal gelation method was described for producing small-diameter alginate beads (200–1000 µm), potentially on a large scale (m^3/h). The difficulty in using dispersion/external gelation techniques with ionic polysaccharide is that the calcium source ($CaCl_2$) is insoluble in the oil phase. As an alternative, internal gelation (Lencki et al. 1989) of the dispersed alginate droplets may be initiated by liberating Ca^{2+} from an insoluble complex (calcium citrate) through pH reduction. By controlling the conditions under which the water-in-oil dispersion is produced, the bead size can be controlled from an few micrometers to millimetres in diameter. Alginate/calcium citrate dispersions can be produced under low-shear conditions thus protecting fragile encapsulants. Furthermore, since dispersions can be produced industrially on very large equipment, the scale-up potential of this process is almost unlimited. Since biocompatible materials are used, the technique is suitable for biological or food applications.

The principal drawback of the emulsification method is the resultant broad size distribution. Polymodal distributions were observed (Poncelet et al. 1992a) with peaks in the small-diameter range attributed to the

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formation of satellite beads during breakage of the main droplets (Grace 1982). The existence of two major peaks (primary and secondary) appears more specific to the alginate system as only one main peak was observed with carrageenan or agarose beads (Audet and Lacroix 1989; Neufeld et al. 1991). The principal difference is that alginate sol is gelified through a physicochemical process (release of calcium) while carrageenan and agarose beads are obtained by temperature reduction. Improved physicochemical control may reduce polydispersion during alginate bead formulation. This is the objective of the present study.

Materials and methods

Reagents

The characteristics of the sodium alginates used in this work have been summarized previously (Poncelet et al. 1992a). Grinsted and Sanofi alginates were offered respectively by Grinsted Products, Brabrand, Denmark, and Sanofi Bio Industries, Paris, France. Canola oil was provided by Canada Packers, Montreal, Canada. The surfactant Span 80 (sorbitan triolate, S-7135) was purchased from Sigma (St. Louis, Mo., USA), calcium carbonate from Baker (Montreal, Canada). Reagent-grade calcium citrate, calcium chloride, sodium bicarbonate and acetic acid were purchased from Anachemia (Montreal, Canada).

Bead production

Sodium alginate (1%–4% w/v) was dissolved by mixing in a Waring blender or some other high-shear device for 2 min. Solutions stood for at least 1 h to allow deaeration, and acid (or base) was generally added to adjust the pH to the desired value (typically 7.5).

Before use, the encapsulant was dispersed into the alginate at a ratio of up to 1:1. This alginate preparation (20 ml) was mixed with 1 ml suspension of insoluble calcium salt (500 mM Ca^{2+} equivalent) resulting in a suspension of the 25 mM calcium salt in alginate sol. The carbonate, citrate, monohydrogenphosphate, oxalate and tartrate salts of calcium were tested. The alginate sol was at times buffered with the sodium salt of the calcium counter-ion to allow easier control of pH.

The alginate/calcium-salt mixture (21 ml) was dispersed within 100 ml Canola oil by stirring at 200–500 rpm for 15 min. With continued agitation, 20 ml Canola oil containing 80 µl glacial acetic acid was then added to the emulsion, liberating divalent calcium for gelification of the alginate polyanions. After 5 min, the oil-bead suspension was added with gentle mixing to 150 ml 50 mM calcium chloride solution. After complete partitioning of beads to the aqueous phase, the oil was discarded, and the beads washed and filtered on a 30-µm sieve (Tetko) with 1% Tween solution.

Reactors

The emulsification step was performed in round-bottom cylindrical reactors agitated by a marine impeller. The configuration and characteristic dimensions of the impeller and reactor are given in Fig. 1.

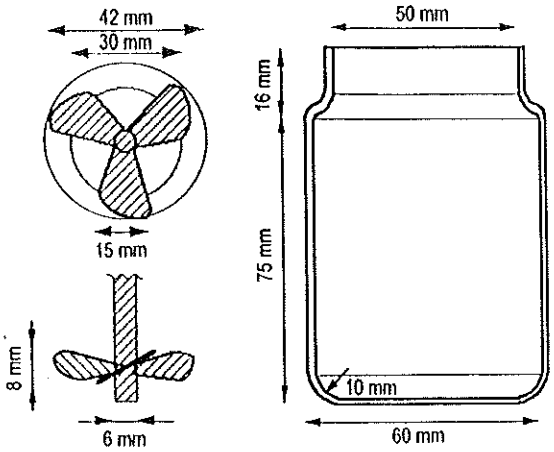

Fig. 1 Reactor and impeller configuration

Bead size distributions

Volume distributions of beads were obtained using a Malvern 2605-Lc particle-size analyser. A typical size distribution (Fig. 2) was composed of two main peaks (A and B) and several small satellite peaks. The main peak (A or B), was assumed to follow the normal law, typically representing more than 75% of the total bead volume, and was characterized by a mean diameter with a standard deviation value (σ). The extent of divergence from this simple model was estimated by the percentage of bead volume included in the other peak (A or B) plus the percentage of bead volume in the combined satellite peaks of particles with diameters under 150 µm.

The mean value of the main peak was evaluated by the mode (maximum frequency) and the standard deviation (σ) was obtained from the peak width at half height (Fig. 2). Reproducibility tests for the mean and standard deviation were evaluated to 15% and 8% of the mode respectively (Poncelet et al. 1992a).

Alginate viscosity

The viscosity of the alginate solution was measured using a Brookfield viscosimeter LVT with an adapter UL for small volumes (16 ml).

Results

Figure 2 presents a typical size distribution for alginate beads formed by emulsification/internal gelation. The size distribution was polymodal with two main peaks A ($\geq$ 500 µm) and B ($<$ 500 µm), together representing 85%–95% total bead volume and the balance consisting of several small peaks (S) under 150 µm diameter. Dominance of peak A or B depended on the type of alginate and calcium vector utilized (Fig. 3) and, in certain cases, the secondary peak (A or B) was eliminated. Five calcium sources were tested: oxalate, tartrate, phosphate, carbonate and citrate. Table 1 and Fig. 3 summarize results obtained for beads formed with either calcium citrate or calcium carbonate under similar conditions. Larger beads with a more complex size distribution, characterized by two main peaks,

were formed with calcium citrate, in comparison with calcium carbonate which resulted in one main peak in most cases. Beads were not formed with calcium oxalate nor with calcium tartrate. With phosphate as the calcium source, the salt formed agglomerates at the centre of the beads and incomplete gelification resulted in bead clumping.

Beads prepared with calcium carbonate were more spherical than those prepared with calcium citrate. In the latter case, a significant number of capsules had either an ellipsoïde form (diameter ratio lower than 1.1 : 1) or an irregular undulating surface (with a maximum amplitude of 10% of the diameter). The different effects (see below) that reduce the size dispersion led also to an improvement in the sphericity both in terms of number of affected capsules and extend of the divergence. The lack of sphericity was attributed to some incomplete coalescence of the drops during the gelation period.

Fig. 3 and Table 1 show that a high-guluronic-acid alginate complex with citrate resulted in a large peak A (85% bead volume). With calcium carbonate, peak A decreased to 35%. The use of a low-guluronic-acid alginate with calcium citrate favoured peak B up to 20% of the total bead volume. Combining calcium carbonate and low-guluronic-acid alginate results in a dominance of peak B, representing up to 90% of the total bead volume. Under these conditions, peak A was eliminated except for very-high-viscosity alginate. In summary, the use of calcium carbonate in place of citrate strongly increases peak B to the detriment of

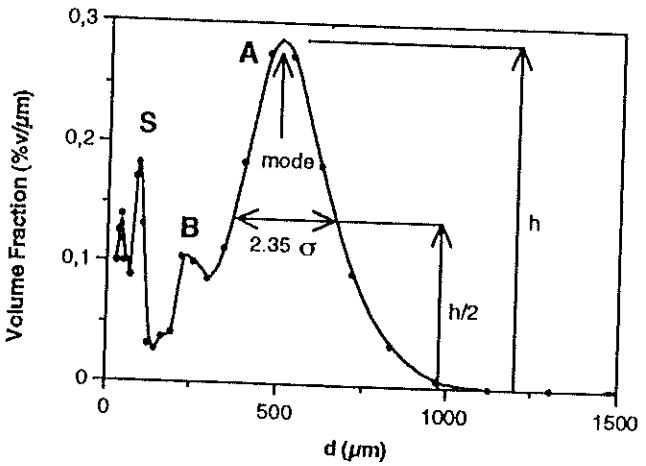

Fig. 2 Estimation of the size distribution parameters (alginate Aldrich 1.8%, Span 80 2%, calcium citrate 50 mM Ca^{2+}, 400 rpm)

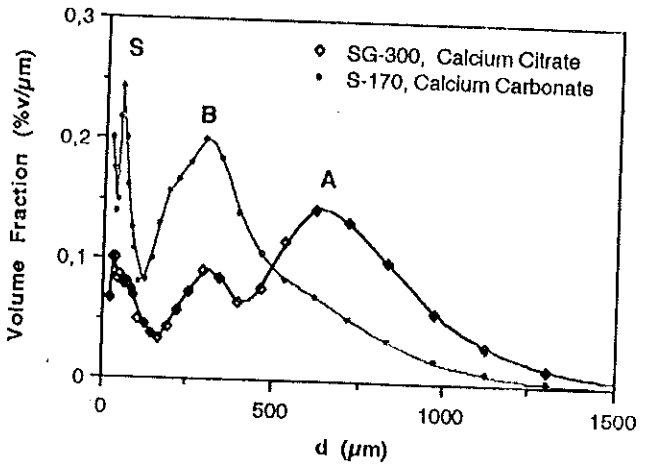

Fig. 3 Impact of the type of alginate and calcium source on the size distribution (alginate 1.8%, cross-linker 50 mM Ca^{2+}, Span 80 2%, 400 rpm; d bead diamter, σ standard deviation

Table 1 Selection of calcium vector and type of alginate (400 rpm, round-bottom reactor, 2% Span 80, alginate 1.8%)

Alginate	Viscosity (cP)	Guluronic acid content	Mode[a] (µm)	σ[a] (%)	Distribution of material (% by vol.)		
					Peak A	Peak B	Peak S
Calcium citrate 50 mM Ca^{2+}							
FD-170	100	Low	720	34	78	16	6
FD-120	158	Low	660	35	78	18	4
S-170	480	Low	690	35	78	20	2
FD-124	1300	Low	720	36	84	10	6
SG-300	1464	High	610	34	85	5	10
SG-500	1694	High	620	34	87	4	9
S-550	> 2000	Low	670	29	77	16	7
Calcium carbonate 50 mM Ca^{2+}							
FD-170	100	Low	423	36	—	88	12
FD-120	158	Low	346	41	—	76	14
S-170	480	Low	423	36	—	88	12
FD-124	1300	Low	308	53	—	87	13
SG-300	1464	High	390	56	35	58	7
SG-500	1694	High	385	51	37	51	18
S-550	> 2000	Low	318	65	17	73	10

[a] For the main peak (largest percentage)

peak A. The use of an alginate with a high guluronic content favours the dominance of peak A (Table 1).

Calcium, when released from the carbonate complex, favoured the formation of satellite peaks in the size distribution. The volume of the satellite beads increased slightly from an average of 6% by vol. ($\pm$ 3%) to 11% by vol. ($\pm$ 5%) when carbonate was used in place of citrate (Table 1). No clear trend was evident between alginate type and the volume of the satellite bead.

The standard deviation (σ) of the main peak was larger when calcium carbonate was combined with alginate of high viscosity (> 1300 cP) ($\sigma = 56 \pm 6\%$) than with calcium citrate ($\sigma = 34 \pm 3\%$) or alginate of low viscosity (< 500 cP) ($\sigma = 38 \pm 3\%$). Peak B predominated with calcium carbonate; however, the residual peak A formed a tail (Fig. 3) that interfered in the measurement of the peak B width, giving rise to larger standard deviation values. In addition, peak A was symmetric (normal law) while peak B was asymmetric, typically represented by a log/normal law.

The amount of calcium introduced into the alginate sol was 100 mM to ensure complete gelification. However, a number of insoluble calcium complex grains were apparent in the resulting beads. The calcium concentration was then reduced to 25 mM without observing any change in bead size, shape or mechanical strength.

The initial pH of the alginate sol was set to 8 but was later reduced to 7.5 when calcium carbonate was used. The final pH was varied by changing the amount of acetic acid introduced to the dispersion system and/or by use of carbonate buffer. For final pH values lower than or equal to 6.5, strong and spherical beads were obtained with calcium carbonate complex. Beads were stronger and more spherical when larger amounts of acetic acid were used, while the pH reduction was limited by enhancing the buffer capacity of the alginate solution.

Commercial calcium citrate or carbonate powders consist of grains with diameters of 30 µm. For small-bead formulation (50–300 µm) it was necessary to reduce the calcium salt particle size to ensure a more homogeneous dispersion within the alginate. Microscopic examination revealed that the calcium complex consisted of aggregates with salt grains ranging from 2 µm to 2.5 µm. Before use, the calcium carbonate suspension was thus sonicated with an Ultrasonic homogenizer or the dry powder was ground with a mortar and pestle to disrupt the aggregates; however, the treatment did not reduce the grain size itself. Beads obtained with ground or sonicated calcium powder resulted in beads with a higher mechanical resistance, improved sphericity and without residual calcium grains. From the respective volumes of calcium carbonate (density = 1.8 g/cm^3) and alginate introduced in the system, and assuming grains of 2.5 µm diameter, the number of calcium carbonate grains was computed as a function of the alginate droplet size (Fig. 4). The number of grains per drop decreased from 300 for 150 µm droplet diameter to 11 grains for 50 µm.

Gelification time was estimated qualitatively by measuring the stickiness or adhesion of alginate sol after mixing with the calcium salt (Fig. 5). At pH 8, gelification occurred within a few minutes when citrate was used, while the alginate/calcium-carbonate mixture was still in a liquid state after 48 h. Following acidification to pH 6.5, gelification occurred within a few seconds being quasi-instantaneous with calcium carbonate and within 20 s for the calcium citrate.

The release of calcium may be written as:

$$Ca_xA_z \downarrow \leftrightarrow x\ Ca^{2+} + z\ A$$

where A represents the anion. The free calcium concentration, [Ca^{2+}], and the total free anion concentration,

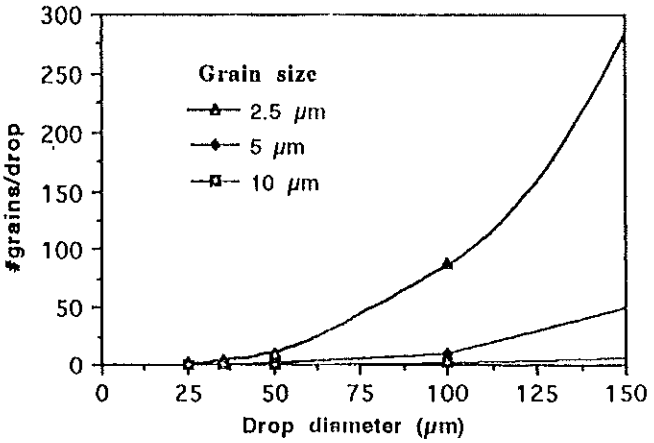

Fig. 4 Number of clacium carbonate grains per alginate droplet (alginate 20 ml, calcium carbonate 25 mM, grain density 1.8 g/ml)

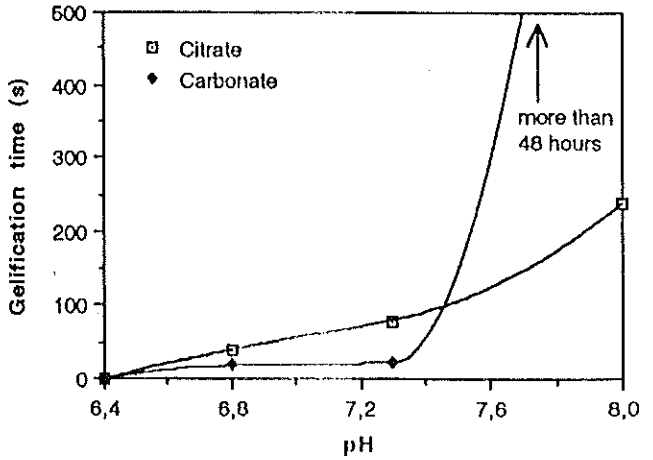

Fig. 5 Gelification time of alginate solution with varying pH (alginate FD-124, calcium citrate and carbonate 50 mM Ca^{2+})

c_A (sum of the basic and acid forms), are related by:

$$\frac{c_A}{z} = \frac{[Ca^{2+}]}{x} \quad (1)$$

The free basic anion concentration, [A], is given by:

$$[A] = \varphi_0 c_A \quad (2)$$

where φ_0 is the partition coefficient, which may be calculated from the acidity constants of the chemical species A and the pH (Poncelet et al. 1985). Finally the solubility product may be written as:

$$L = [Ca^{2+}]^x [A]^z \quad (3)$$

Combining Eqs. 1–3 leads to:

$$[Ca^{2+}] = \sqrt[x+z]{L\left(\frac{x}{z\ \varphi_0}\right)^z} \quad (4)$$

Figure 6 gives the free calcium concentration as a function of the pH for various calcium vectors. Calcium is not released from oxalate or tartrate complex in the pH range of interest (pH > 5). Release of calcium occurs in a narrower pH range with calcium carbonate than with calcium citrate. Moreover, at pH 7–8, the calcium concentration with calcium citrate remains higher than 8 mM, sufficient to initiate gelification of the alginate.

Alginate viscosity had little influence on the size distribution (Fig. 7 and Table 1). Increasing the viscosity by a factor of 100 reduced the peak mode by less than 10%. However, the size dispersion is smallest for low-viscosity alginate. In contrast, increasing the surfactant concentration decreased the mean bead size asymptotically as the surfactant concentration approached 1% (Fig. 8).

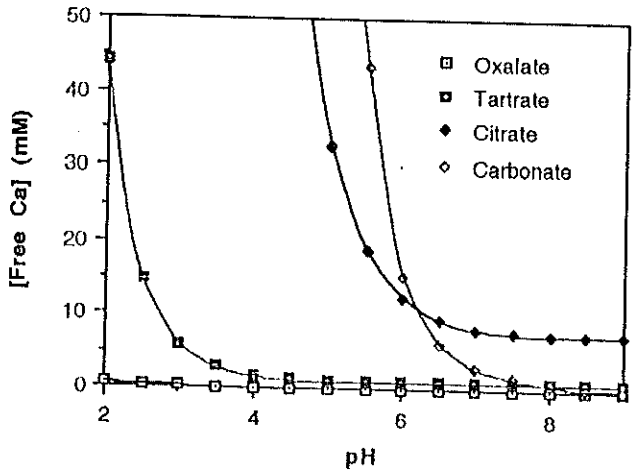

Fig. 6 Free calcium concentration versus pH for different calcium compounds

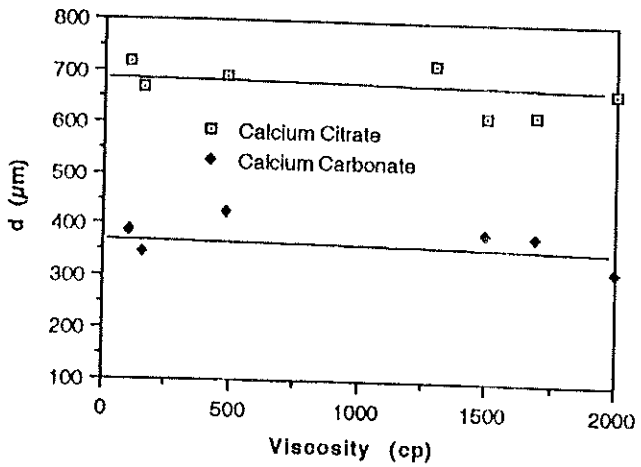

Fig. 7 Influence of the alginate viscosity on the peak mode (Span 80 2%, cross-linker 50 mM Ca^{2+}, 400 rpm)

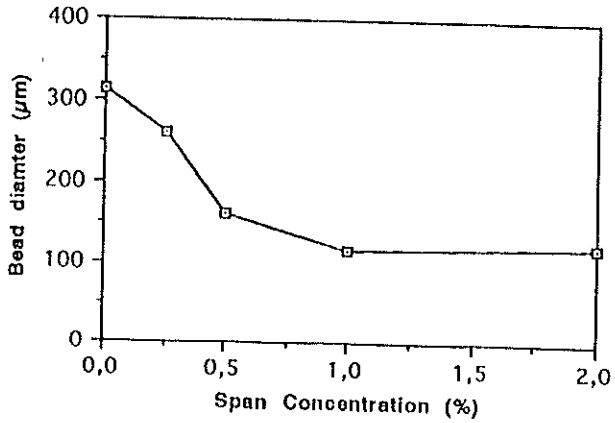

Fig. 8 Bead size versus surfactant concentration (calcium carbonate 25 mM Ca^{2+}, 400 rpm)

Discussion

The methodology for the formation of alginate microspheres (less than 1 mm in diameter) using a technique of emulsification/internal gelation was described in a previous report (Poncelet et al. 1992a). While alginate remains the preferred entrapment medium for immobilization of biological materials, techniques for large-scale formulation in industrial applications have not been developed (Poncelet et al. 1992b), and the high viscosity of alginate sols impedes small bead formation. The ability to formulate alginate beads with controlled diameter for large-scale applications is the key to industrial use.

In the present study, the physicochemistry of alginate bead formation via internal gelation with a calcium vector was studied. From all the parameters involved in bead formation, the physicochemical conditions are of primary importance for the control of the size distribution. Important factors include the type and form of the

calcium vector, its concentration, the initial and final pH values and the selection of alginate composition and structure.

A 2% (by weight) alginate solution corresponds to 103 mM (corresponding to 103 mM negative charges) in guluronic or manuronic acid residues (M_r = 194). The alginate was cross-linked with 25 mM divalent calcium (50 mM positive charge). According to Martinsen et al. (1989), only guluronic acid groups link to calcium and strong gels are obtained even though not all of the monomer has been cross-linked. A calcium/alginate monomer ratio of 1/4 (25 mM 100 mM) appears sufficient to ensure strong bead formation. Higher ratios lead to residual insoluble calcium in the beads.

Calcium availability within the alginate gel is assured by achieving a homogeneous distribution within the alginate sol and from droplet to droplet. Assuming that the number of dispersed calcium grains per droplet must be larger than 10, with 25 mM calcium and a grain size equal to 2.5 μm, droplets smaller than 50 μm will not gel homogeneously (Fig. 4) resulting in aggregation of the beads. Beads smaller than 50 μm may be formed by using a higher calcium concentration and/or a smaller grain size. Replacing the insoluble calcium salt with a soluble calcium complex (EDTA) may permit the formation of beads a few micrometres in diameter.

Selection of a suitable calcium vector for internal gelation depends on the range of initial to final pH values desired. Typically, neutral pH values are appropriate for live cell immobilization. Acid-tolerant cultures such as lactic acid bacteria may be immobilized at a lower pH range (7 initial to 5 final). Over the pH range of interest, the concentration of free calcium must be low initially with rapid release of calcium upon reduction in pH. A pK_a value of the anions in the working range (6.5–7.5) is optimal for cell immobilization. Oxalate and tartrate were unacceptable, as calcium was not released within a suitable pH range. Calcium phosphate was also rejected because of the large grain size resulting in poor gelification. Citrate complex resulted in large beads with a high variability in the size distribution. Even at pH 8, gelification was completed within 4 min (Fig. 5) and thus largely advanced before emulsion equilibrium was achieved. At a lower pH (6–6.5), gelification was not particularly fast (20 s).

In contrast, the calcium-carbonate/alginate sol remained stable for over 48 h at pH 7.5 without gelification. The drop in pH to 6 or 6.5 resulted in instantaneous gelification. Consequently the beads were smaller, and the size distribution was typically unimodal (neglecting satellite peaks).

The difference in behaviour between the citrate and carbonate complexes relates to the stoichiometry that defines the slope of the calcium release as a function of pH (Fig. 6). A molecular calcium-to-anion ratio of 1 in the salt ensured the maximum slope, permitting work within a smaller pH range.

The alginate composition is also an important parameter in alginate bead formation. Martinsen et al. (1989) have shown that calcium primarily cross-links the guluronic residues. A high guluronic acid content and homopolymer blocks lead to stronger gels. But, in the emulsification method, these alginates result in premature gelification yielding larger beads with a larger dispersion. Since commercial alginate is usually characterized by its viscosity, the work of Smidsrod (Smidsrod and Skjak-Braek 1990) and that of the present study shows that a structural characterization of alginate is needed to control bead properties and formation.

Generally droplet size distributions in emulsions, and resulting bead diameter distributions, are determined by the relation between the dispersive forces and either the surface tension or the viscosity of the discontinuous phase (Calabrese et al. 1986; Hinze 1955). With citrate complex as the calcium vector, the alginate bead size distribution was independent of the surfactant concentration and the alginate viscosity (Poncelet et al. 1992a). It was expected that the pregelification of the alginate was masking the other effects. When carbonate complex was used, the alginate viscosity (in the tested range) had no effect on the mean size but the bead diameter was dependent on the surfactant concentration and thus the interfacial tension. Pregelification was then no longer a dominant factor in determining the size distribution.

In summary, the narrowest size distribution for alginate beads produced by emulsification/internal gelation was obtained by using low-guluronic-acid and low-viscosity alginate, small and dispersed grains of calcium carbonate complex, within the pH range of 7.5 (initial) to 6.5 (final). The distribution was characterized by one main peak with a standard deviation of 36% representing 90% of the total bead volume. Emulsification/internal gelation appears promising for large-scale immobilization within alginate gels.

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