Production of Alginate Beads by Emulsification/Internal Gelation

D. PONCELET

École Nationale d'Ingénieurs des Techniques des Industries Agricoles et Alimentaires (Enitiaa), BP 82225, 44322 Nantes, France

ABSTRACT: **Alginate microspheres were produced by emulsification/internal gelation of an alginate sol dispersed within vegetable oil, followed by a reduction in pH to release calcium from an insoluble salt. Microspheres with mean diameters ranging from 50 to 1,000 μm were obtained with standard deviations ranging from 35 to 45% of their mean value. Smooth, spherical beads were obtained with the narrowest size dispersion when using low guluronic and low viscosity alginate and a carbonate complex as calcium vector. The calcium salt must also be included within the alginate sol as a very fine powder to promote homogeneous gelation. Internal gelation was also tested with the dropping method. Observation of the beads produced revealed that the structure of the beads is more homogeneous than observed with external gelation. Shrinking is more important, although the diffusion of large molecules is faster with internal versus external gelation.**

KEYWORDS: **alginate bead production, emulsification, internal gelation, microspheres**

INTRODUCTION

Encapsulation in hydrogel beads remains one of the most usual methods of cell immobilization. Dropping an alginate solution into a calcium-gelifying bath permits one to produce relatively large quantity of beads although the productivity is inversely proportional to the bead volume.[1] Therefore, producing small beads in large quantity remains a challenge particularly under sterile conditions.

An emulsification/internal gelation method is proposed for producing small diameter alginate beads in large quantity. 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 of the dispersed alginate droplets may be initiated by releasing Ca^{2+} from an insoluble complex (calcium salt) through pH reduction.[2] By controlling the conditions under which the water-in-oil dispersion is produced, the bead size can be controlled from a few microns to millimeters in diameter. The purpose of this paper is to report latest developments in obtaining the narrowest size distribution.

Address for correspondence: D. Poncelet, Enitiaa, BP 82225, 44322 Nantes cedex 3, France. Voice: 33 2 51 78 54 25; fax; 33 2 51 78 54 67.
poncelet@enitiaa-nantes.fr

MATERIALS AND METHODS

Reagents

Grinsted and SKW alginates were obtained, respectively, from Grinsted Products, Brabrand, Denmark, and SKW Bio Industries, Paris, France and used as received. Canola oil was provided by Canada Packers, Montreal, Canada. Calcium carbonate (Setacarb) was obtained from Omya Paris. Other products, Span 80, calcium citrate, calcium chloride, sodium bicarbonate and acetic acid were purchased from Sigma.

Preparation of Alginate Solution

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

Bead Production

Alginate (20 ml) was mixed with 1 ml of a suspension of insoluble calcium salt (500 mM Ca^{2+} equivalent). The carbonate, citrate, monohydrogenophosphate, oxalate, and tartrate salts of calcium were tested. The alginate–calcium salt mixture (21 ml) was dispersed in 100 ml canola oil in a turbine reactor by stirring at 200 to 500 rpm for 15 minutes. With continued agitation, 20 ml of canola oil containing 80 µl glacial acetic acid were then added to the emulsion, liberating divalent calcium for gelation of the alginate polyanions. After five minutes, the oil–bead suspension was added with gentle mixing to 150 ml of a 50 mM calcium chloride solution. After complete partitioning of beads to the aqueous phase, the oil was discarded, and the beads filtered on a 30 µm sieve and washed with 1% Tween solution.

While testing the internal gelation with extrusion techniques, the solution mentioned above was dropped in an acidic bath at different pH. Beads were left to stand for 10 min and then washed with water and kept in saline solution.

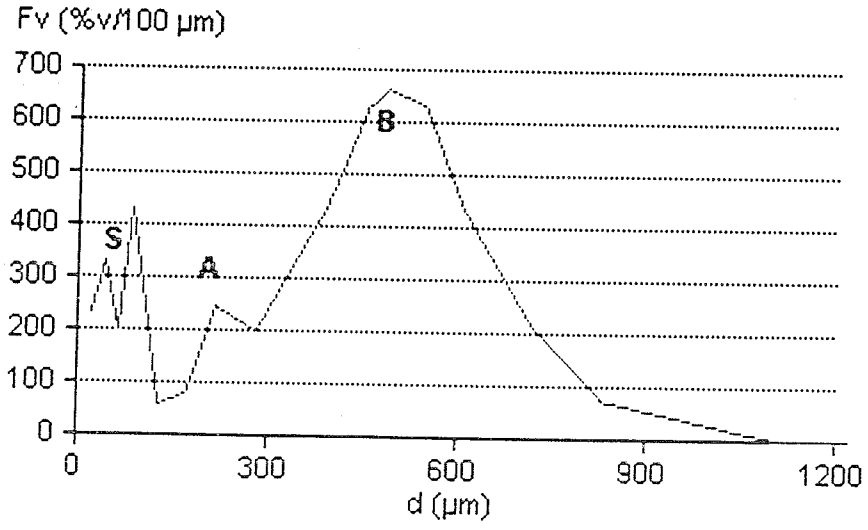

FIGURE 1. Typical size distribution of alginate bead batch.

Microbead Characterization

The volume distributions of beads were estimated using a Malvern 2605-Lc particle size analyzer. A typical size distribution (see FIGURE 1) was composed of two main peaks (A and B) and several small satellite peaks (S). The main peak (A or B) was characterized by its mean diameter and standard deviation values. The mean diameter of the main peak was evaluated by the mode (maximum frequency) and the standard deviation was obtained from the peak width at half height (FIG. 1). Reproducibility tests for the mean and standard deviation were evaluated to 15 and 8% of the mode, respectively. Mechanical resistance was evaluated qualitatively by viewing the rupture of beads under pressure microscopically.

Blue Dextran Release

Beads were suspended in a blue dextran solution (Sigma) for two hours and then transferred into distilled water. The blue dextran concentration was estimated by optical density. The ratio of the concentration in the solution, at a given time, to the concentration observed at complete release, was taken as the metric.

RESULTS

The size distribution of alginate beads prepared by emulsification/internal gelation (FIG. 1) was polymodal with two main peaks (A and B) and several small peaks (S). The distribution of the main peaks is a function of the type of alginate and calcium vector (see TABLE 1). Calcium oxalate, tartrate, phosphate, carbonate, and citrate were evaluated. In the working pH range (greater than pH 5), only citrate and carbonate salts permit spherical bead formation with moderate size polydispersities. More complex size distribution is formed with calcium citrate, whereas calcium carbonate often results in a single main peak. Beads prepared with calcium carbonate were more spherical than those prepared with calcium citrate. The lack of sphericity was attributed to some incomplete coalescence of the drops during the gelation period.

A combination of low guluronic and low or medium viscosity alginate and calcium carbonate gave the narrowest size distribution, whereas combining high guluronic and high viscosity alginate with calcium citrate provided the most complex multimodal distribution. Under optimum 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 peak A. The use of an alginate with a high guluronic content favors dominance of peak A.

The amount of calcium introduced into the alginate sol required to ensure complete gelation was 100 mM. 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 using calcium carbonate. 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 using

TABLE 1. Effect of calcium vector and alginate type on size distribution

Calcium Vector (50 mM Ca^{2+})	Alginate Viscosity (cp at 1%)	Guluronic Content[a]	Mode (μm)	Standard Deviation (%)	Peak A (%)	Peak B (%)	Peak S (%)
Citrate	100	low	720	34	78	16	6
	158	low	660	35	78	18	4
	480	low	690	35	78	20	2
	1,300	low	720	36	84	10	6
	1,464	high	610	34	85	5	10
	1,694	high	620	34	87	4	9
	>2,000	low	670	29	77	16	7
$CaCO_3$	100	low	423	36	—	88	12
	158	low	346	41	—	76	14
	480	low	423	36	—	88	12
	1,300	low	308	53	—	87	13
	1,464	high	390	56	35	58	7
	1,694	high	385	51	37	51	18
	>2,000	low	318	65	17	73	10

[a]Low, about 40% to high, about 70%, from providers sources.

larger amounts of acetic acid while limiting the pH reduction 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 microbead formulation (50 to 300 μm), the calcium salt particle size was reduced to ensure a more homogeneous dispersion within the alginate. Prior to 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 (from 30 μm to 2.5 μm). Microbeads obtained with ground or sonicated calcium powder resulted in gels with a higher mechanical resistance, improved sphericity, and the absence of residual calcium grains.

The gelation time was estimated, qualitatively, by measuring the stickiness or adhesion of alginate sol after mixing with the calcium salt (see FIGURE 2). At pH 8, gelation occurred within a few minutes using citrate, whereas the alginate–calcium carbonate mixture was still in a liquid state after 48 hours. Following acidification to pH 6.5, gelation occurred within a few seconds, was quasi instantaneous with calcium carbonate, and within 20 sec for the calcium citrate.

Modeling Internal Gelation

The release of calcium may be written as follows

$$Ca_xA_z \Leftrightarrow Ca^{2+} + zA^{(2x/z)-}$$

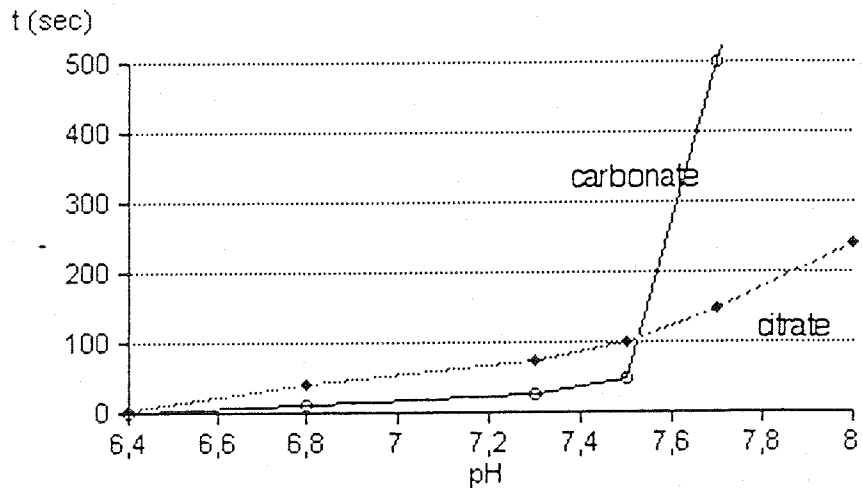

FIGURE 2. Gelification time of alginate solutions containing calcium vector at various pH values.

where A represents the anion. The free calcium concentration, $[Ca^{2+}]$, and the total free anion concentration, C_A (sum of the basic and acid forms), are related, at equilibrium, by

$$\frac{C_A}{z} = \frac{[Ca^{2+}]}{x}. \tag{1}$$

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

$$[A] = \varphi_0 C_A, \tag{2}$$

where φ_0 is the partition coefficient calculated from the acidity constants of the chemical species A and the pH.[3] Finally the solubility product may be written as follows

$$L = [Ca^{2+}]^x [A]^z. \tag{3}$$

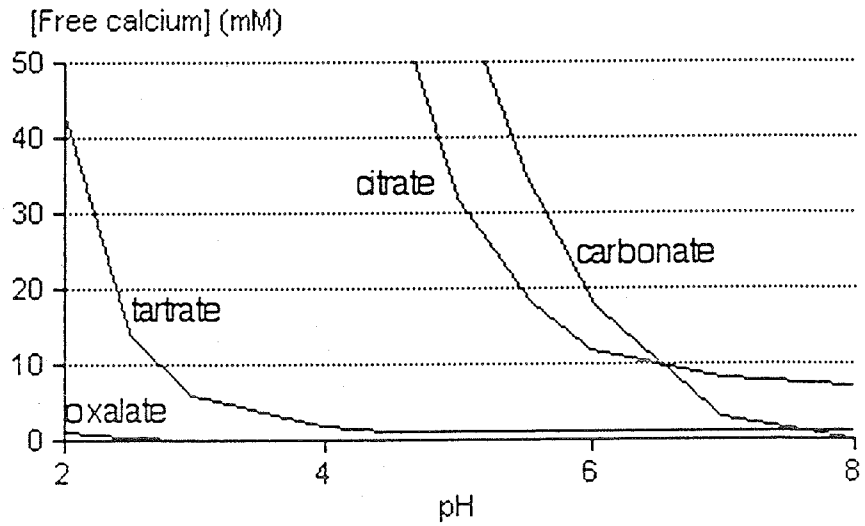

FIGURE 3. Free calcium concentration function of the calcium vector and pH.

Combining Equations (1) to (3) leads to:

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

FIGURE 3 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). The release of calcium occurs over a narrower pH range with calcium carbonate than with calcium citrate. At pH 7–8, the calcium concentration with calcium citrate remains higher than 8 mM, and initiate gelation of the alginate.

Physicochemical Parameter Influences

The Alginate viscosity had little influence on the size distribution (TABLE 1). Increasing the viscosity by a factor of 100 reduces 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%.

Extrusion-based Technology

Internal gelation could be applied to an extrusion-based technology. The alginate/calcium carbonate suspension was dropped in an acidic solution at different pH. TABLE 2 shows the influence of the bath pH on the droplet size and the shrinking factor (defined as the fraction of volume reduction during gelation). Correct beads could only be obtained in the gelation bath at a pH of six or below. The beads produced by internal gelation have lower mean sizes, and higher shrinking than those produced by external gelation. The shrinking factor increases with a reduction of the pH. Under optical microscopy, the internal gelation based beads shown a more homogenous structure (see TABLE 3). Large molecules (blue dextran at 2,000,000 daltons) escape faster from the internal gelation based beads than external gelation based beads (TABLE 3), indicating a higher egress permeability.

TABLE 2. Effect of the gelifying bath composition and pH on size and shrinking factor of beads done by extrusion

Bath	pH	*d* (mm)	Shrinking (%)
$CaCl_2$ 40mM	6.6	3	0.6
HAc/NaAc 3%	3.0	2	0.92
	4.0	2.2	0.88
	5.0	2.4	0.86
	6.0	2.8	0.8
	6.5	2.9 (?)	0.7
	7.0	no gelation	—

TABLE 3. Effect of the gelifying bath composition and pH on size and shrinking factor of beads done by extrusion

Bath	pH	Time	Release	Pore Structure[a]
$CaCl_2$	6.6	15 days	11%	inhomogeneous[b]
HAc/NaAc	5	15 days	60%	homogeneous

[a]Optical observation under a microscope.
[b]Inhomogeneity refers to the presence of pores, channels, and so forth, not to an alginate concentration profile.

DISCUSSION

The physicochemical conditions of alginate bead formation via internal gelation with a calcium vector primarily influences of the size distribution of the resulting microbeads. Physicochemical 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.

Alginate solutions at 2%w correspond to 103 mM of guluronic or manuronic monomers. The alginate was crosslinked with 25 mM divalent calcium (50 mM positive charge). Only the guluronic groups link sufficiently to calcium to produce strong gels even though all of the monomeric units have not been cross-linked. A calcium/alginate monomer ratio of 1/4 (25mM/100mM) seems to be 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. This assumes that the number of calcium vector grains is rather larger than the alginate droplets. If this is not the case, it will result in aggregation of the beads. However, spherical beads smaller than 50 μm may be formed using a higher calcium concentration and/or a smaller grain size.

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). The selection of a suitable calcium vector for internal gelation is, therefore, quite important. Over the pH range of interest, the concentration of free calcium must be very low initially with rapid release of calcium while reducing pH. A pKa value of the anions in the working range (6.5 to 7.5) is optimal for cell immobilization. Oxalate and tartrate were unacceptable, since they did not released calcium within a suitable pH range. Calcium phosphate was also rejected because of the large grain size, resulting in poor gelation.

A citrate complex resulted in large beads with a high variability in the size distribution. At pH 8, gelation was completed within four minutes whereas at a lower pH (6 to 6.5) gelation was not particularly fast (20 sec), although more rapid than under basic conditions. In contrast, calcium carbonate–alginate sol remained stable for more than 48 hours at pH 7.5. By reducing the pH to 6 or 6.5, essentially instantaneous gelation resulted. The beads were smaller and spherical and the size distribution was typically unimodal. The difference in behavior between the citrate and

carbonate complexes relates to the stoichiometry that defines the slope of the calcium release as a function of pH. A molecular calcium to anion ratio of 1 into the calcium vector ensures the maximum slope, permitting work within a smaller pH range.

The alginate composition is also an important parameter in alginate bead formulation. A high guluronic content and homopolymer blocks lead to higher interaction alginate/calcium and hence stronger gels. Moreover, in the emulsification method, premature gelation occurs faster with high guluronic alginate.

Microbead Size Distribution

The 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. With citrate complex as the calcium vector, the alginate bead size distribution was independent of the surfactant concentration and the alginate viscosity. It was expected that the pregelation of the alginate was masking the other effects. When using carbonate complex, the alginate viscosity had no effect on the mean size but the bead diameter was dependent on the surfactant concentration and, thus, the interfacial tension. Pregelation is, therefore, no longer a dominant factor in determining the size distribution.

In summary, the narrowest size distribution alginate bead produced by emulsification and internal gelation was obtained by using low guluronic 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.

Effect of pH To obtain good quality beads by extrusion/internal gelation, the gelifying bath must have a pH lower than 6. This does not imply that one decrease the pH in the beads since the release of calcium from calcium carbonate releases carbonate ions that act as a base. However, the need for low pH in the bath may be necessary to insure fast reaction at the droplet interface. In the case of external gelation method, the reaction between alginate and calcium at the droplet interface is very fast and a skin is formed very quickly, preventing the alginate from diffusing in the aqueous phase. This is a typical phenomenon in a symmetric membrane formation. Such fast reaction at the interface is most probably necessary in the case of the internal gelation.

Thermodynamic of Shrinking and Swelling

A larger shrinking of the beads formed by internal gelation is most probably due to the fact that the reaction takes place simultaneously throughout the volume of the beads. In the case of external gelation, when the gelled external layer becomes sufficiently rigid, gelation does not provoke additional shrinking. However, the external layer of beads formed by external gelation may be more compact than internally gelled core. This external layer then plays a role similar to a membrane. This is why the diffusion of large molecules is faster with beads formed by internal gelation, where gelation does not lead to such skin but most probably to large free space between compact alginate zones.

CONCLUSION

Internal gelation seems to be a very promising method to form beads in a large scale, under sterile conditions with a relatively low mean size. However, it is difficult to expect size dispersion (standard deviation) lower than 30% of the mean size. Using internal gelation in combination with dropping techniques may also offer interesting alternatives, reducing the polydispersity to a 5–10% range. Additional work is, however, required to optimize the methods and to define the final properties of the beads and then the scope of applications.

REFERENCES

1. PONCELET, D., B. PONCELET DE SMET, C. BEAULIEU & R.J. NEUFELD. 1992. Scale-up of gel bead and microcapsule production in cell immobilization. *In* Fundamentals of Animal Cell Encapsulation and Immobilization. M.F.A. Goosen, Ed.: 113–142. CRC Press, Boca Raton.
2. LENCKI, R.W.J., R.J. NEUFELD & T. SPINNEY. 1989. Microspheres and Method of Producing Same. U.S. Patent 4.822.534:Apr. 18, 1989.
3. PONCELET, D., A. PAUSS, H. NAVEAU, *et al.* 1985. Computation of physicochemical parameters, i.a. pH, in complex (bio)chemical system. Anal. Biochem. **150:** 421–428.