Drug Development and Industrial Pharmacy, 27(8), 825–829 (2001)

RESEARCH PAPER

Calcium Alginate Microspheres of *Bacillus subtilis*

M. C. Lamas,[1,2] C. Bregni,[1,*] M. D'Aquino,[1] J. Degrossi,[1] and R. Firenstein[2]

[1]*Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Junin 956, Piso 6°, (1113) Buenos Aires, Argentina*
[2]*Faculty of Biochemistry and Pharmaceutical Sciences, University of Rosario, Rosario, Argentina*

ABSTRACT

Microspheres of Bacillus subtilis *were prepared using sodium alginate. Some typical properties of microencapsulated systems, such as microorganism content, particle size, and germination time, were studied. Calcium alginate microspheres were obtained by the emulsification method, dripping into a solution of calcium salt. The conditions of the preparation steps were very soft to produce calcium alginate microspheres containing cells with no apparent changes in general biological properties. The hydrogel matrix provides protection without preventing communication with the surrounding medium.*

Key Words: *Alginate;* Bacillus subtilis*; Emulsification; Microspheres*

INTRODUCTION

Calcium alginate microspheres represent one of the most common methods for immobilization of enzymes, proteins, and living cells as well as for the controlled release of drugs. The success of this simple encapsulation method is based on the mild conditions needed and the low cost of the process (1–4).

The material to be encapsulated is usually mixed with an alginate solution, and the mixture is dripped into a solution containing Ca^{2+} ions, resulting in the instantaneous formation of microspheres that entrap cells or drugs within a three-dimensional

*Corresponding author. E-mail: cbregni@ciudad.com.ar

lattice. However, to the best of our knowledge, there are a limited number of reports describing the microencapsulation of microbial cells. Alginates are a family of polysaccharides composed of α-L-guluronic acid (G) and β-D-mannuronic acid (M) residues, arranged in homopolymeric blocks of each type (MM, GG) and in heteropolymeric blocks of each composition; these are reported to have a major impact on the properties of the different systems.

Some hydrophilic polymers have ion-binding properties. Among these are alginates, which belong to a family of unbranched binary copolymers of linked acid residues. Alginates have the ability to bind multivalent cations, which is the basis of their gelling properties and leads to the formation of covalent bonds, yielding insoluble hydrogels (5).

Alginates form strong gels with divalent cations like Ca^{2+}, giving both strength and flexibility. Such a cross-linking process stiffens and roughens the polymer and reduces the swelling in solvents. The soluble sodium alginate was cross-linked with calcium chloride, resulting in the formation of the insoluble calcium alginate. This strong thermostable gel has properties that largely depend on the characteristics of the polymer and the preparation method.

Natural polymers are used both as carriers and as determinants of the release rate in controlled-release systems. The main advantages of natural polymers lie in their biocompatibility and biodegradability without producing systemic toxicity on administration. In spite of the increasing popularity of this method of encapsulation, demonstrated by a large amount of research, the majority of reports mainly describe the controlled-release application of the microspheres, while few contributions have reported about the microencapsulation of microbial cells (6–8).

The objectives of the present study were to investigate the possibility of microencapsulation of a model (nonpathogenic) microorganism (*Bacillus subtilis*) with sodium alginate and to study the viability of the microorganism after the preparation step.

EXPERIMENTAL

Materials

Alginate sodium salt, low viscosity, was purchased from Sigma. Ether sulfuric high-performance liquid chromatography (HPLC) grade was purchased from Merck. The nonionic surfactants used were sorbitan monooleate (Span 80) and polyoxyethylene sorbitan monooleate (Tween 80), manufactured by Atlas Company. Bacterial spores and vegetative viable cells (*Bacillus subtilis*, strain ATCC 6633) were used as biological materials. Microbial counts were performed in plate dishes using tryptic soy agar (Difco). Bacto-Peptone (Difco) was used to prepare suspensions and dilutions. Aldrich supplied the other chemicals used. All materials were used as received.

Methods

Preparation of Water-in-Oil Primary Emulsions

The primary emulsion was prepared by a one-stage emulsification procedure. The sodium alginate solution containing the suspended cells was emulsified in an equal volume of the oil external phase. The composition of the oil phase was 70 ml of liquid vaseline, 30 ml of ether sulfuric, and 3% w/w sorbitan monooleate (Span 80). The primary water-in-oil (w/o) emulsion was obtained by means of a small vortex mixer (Whirlimixer). After preparation, the formation of the emulsion was confirmed by microscopic examination (Fig. 1).

The emulsion was characterized by measuring the viscosity (Brookfield RVT viscometer) and conductivity (YSI 3418 conductivity cell, Yellow Spring Instruments, Yellow Spring, OH; cell constant K of 0.1/cm). Samples for conductivity measurement were prepared using 0.1 molar sodium chloride as

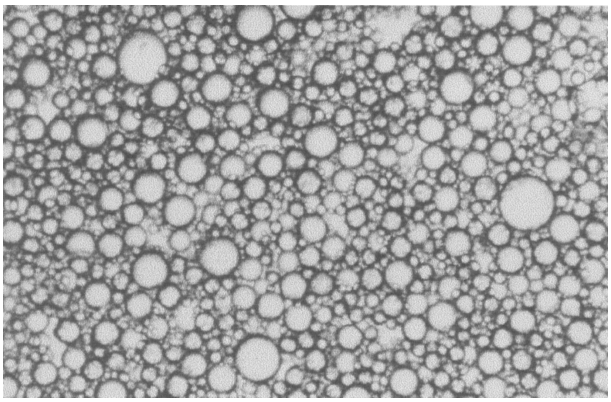

Figure 1. Light micrograph of water-in-oil emulsion (magnification 100×).

the aqueous phase. Viscosity and conductivity measurements were carried out in triplicate at 25°C.

Preparation of the Microspheres

Microspheres were prepared by ionic cross-linking gelation, mainly based on an intermediate stage of primary emulsion. Alginate gel microspheres were prepared by the cross-linking of sodium alginate, participating in the aqueous internal phase of the primary emulsion, with calcium ions. The material to be encapsulated is usually mixed with an alginate solution (2% w/v), and the mixture is dripped into a solution (40 ml) containing calcium ions (4% w/v), resulting in the instantaneous formation of microspheres. The ratio between sodium alginate and Ca^{2+} was 1:2. The supernatant was decanted; the sediment was washed with distilled water (200 ml); and the system was filtered and dried at 37°C until the residue reached a constant weight. Two types of microspheres were prepared: type I and type II, which contained spores and vegetative viable cells, respectively. Both types of microspheres were prepared in the same way.

Morphology

The formation of microspheres was monitored by optical microscopy (Optiphot, Nikon).

Particle Size Distribution

Standard U.S. sieves ranging from 50 to 700 μm were used to determine the particle size of 10-g microspheres. This procedure was repeated three times for different batches (Figs. 2 and 3).

Scanning Electron Microscopy

Scanning electron micrographs (SEMs) of the microspheres were obtained using a scanning electron microscope (Jeol JSM-35-C) at the required magnification at room temperature after coating with 200 Å gold under vacuum. The working distance of 4 cm was maintained, and the acceleration voltage used was 6 kV, with the secondary electron image (SEI) as a detector.

Microbial Counts

Microspheres (1.0 g) were suspended in 10 ml of peptone water (0.1% w/v) and stirred at room temperature in a vortex for 2 min. Decimal dilutions in peptone water (0.1% w/v) were made, and 0.1 ml samples of the appropriated dilutions were inoculated on petri dishes with tryptic soy agar. The plates were incubated 48 h at 37°C. The number of colonies was counted, and this corresponded to the number of the microencapsulated cells.

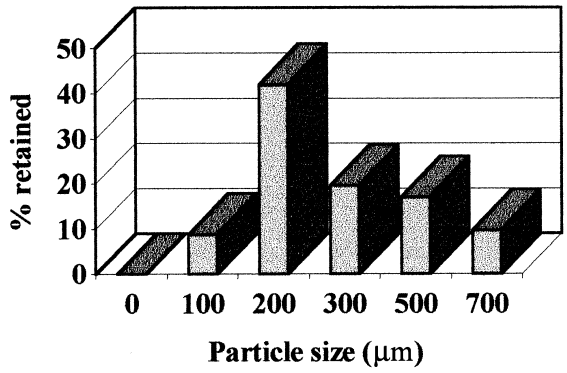

Figure 2. Particle size distribution of microspheres with vegetative viable cells (data are expressed as the mean for three to five experiments).

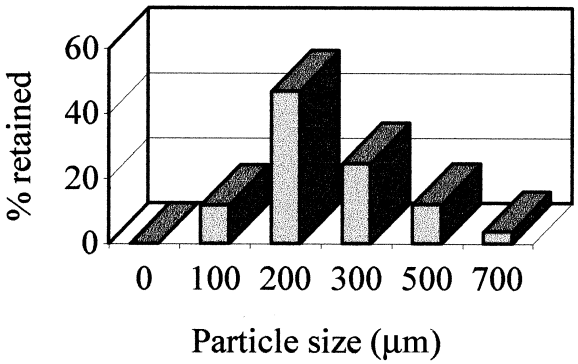

Figure 3. Particle size distribution of microspheres with spores (data are expressed as the mean for three to five experiments).

RESULTS AND DISCUSSION

Two types of microspheres were prepared: type I, spores (alginate concentration 2% w/v); and type II, vegetative viable cells (alginate concentration 2% w/v). The microspheres obtained were polydisperse systems with a particle size distribution conforming to a normal distribution and with mean diameters between 50 and 300 μm. The morphology

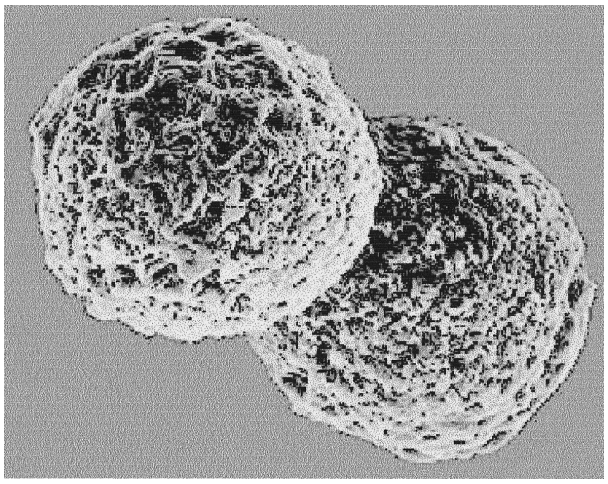

Figure 4. Scanning electron micrograph of alginate microspheres.

of the microspheres as examined by SEM (Fig. 4), which indicated the microspheres had surfaces with rugosity.

The microspheres were well formed, and in spite of their soft hydrogel nature, they resisted all the manipulations in liquid media during experimental work. However, an increase in microsphere concentration in aqueous media was obtained, but it was not possible to filter the microspherical product. The difference between microspheres prepared with microbial spores and with vegetative viable cells is evident from the data for the average number of cells per microsphere and the germination time (Table 1). The germination time of encapsulated cells was significantly higher than that of the cells not encapsulated (control), giving a relative indication of protection achieved by the process of microencapsulation (Table 2). After the lag time due to encapsulation, cell growth was uninhibited, and there was no difference between encapsulated and free cells. The viability of encapsulated cells was kept unchanged during the experiments, and since no difference was noticed, it can be presumed that it can be kept for much longer. Polymerization conditions allowed obtaining microencapsulated cells for 150 days.

Table 1

Alginate Microsphere Germination Properties Including Bacillus subtilis

Time (days)	Type I, Spores (CFU/g)	Type II, Viable Cells (CFU/g)
0	3.1×10^9	2.8×10^9
30	3.0×10^9	3.4×10^7
90	1.2×10^8	8.9×10^7
150	1.0×10^8	4.3×10^7

CFU/g, colony-forming units/g.

Table 2

Microorganism Without Encapsulation

Time (days)	*Bacillus subtilis* Spore Suspension at 7°C (CFU/g)	*Bacillus subtilis* Vegetative Cells Suspension at 7°C (CFU/g)
0	3.5×10^9	3.2×10^9
30	2.5×10^9	2.7×10^8
90	1.6×10^7	1.8×10^6
150	2.8×10^6	6.7×10^5

CFU/g, colony-forming units/g.

CONCLUSION

The results of this study showed that the encapsulation of microbial spores and vegetative viable cells of a model microorganism could be satisfactorily achieved using sodium alginate. Very mild conditions during the preparation step allowed the production of microspheres containing cells with no apparent changes in their general biological properties, but provided with protection by a soft hydrogel matrix; at the same time, communication with the surrounding medium was not prevented completely.

ACKNOWLEDGMENT

We wish to acknowledge the support of this work provided by UBACyT (01/TB12 and TB21).

REFERENCES

1. Pepeljnjak, S.; Filipovic-Grcic, J.; Jalsenjak, V. Pharmazie **1994**, *49*, 436–437.
2. Al Musa, S.; Abu Fara, D.; Badwan, A.A. J. Controlled Release **1999**, *57*, 223–232.

3. Takka, S.; Acarturk, F. J. Microencapsulation **1999**, *16* (3), 275–290.
4. Takka, S.; Acarturk, F. J. Microencapsulation **1999**, *16* (3), 291–301.
5. Vennat, B.; Lardy, F.; Arvouet-Grand, A.; Pourrat, A. Drug Dev. Ind. Pharm. **1998**, *24*, 27–35.
6. Watnasirichaikul, S.; Davies, N.; Rades, T.; Tucker, I. Pharm. Res. **2000**, *17*, 684–689.
7. Edwards-Levy, F.; Levy, M.C. Biomaterials **1999**, *20*, 2069–2084.
8. Funueanu, G.; Nastruzzi, C.; Carpov, A.; Desbrieres, J.; Rinaudo, M. Biomaterials **1999**, *20*, 1427–1435.