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Preparation and characterization of Ca-alginate microspheres by a new emulsification method

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Abstract

The preparation and characterization of Ca-alginate microspheres obtained by a new procedure from alginic acid, treated with an excess of NaOH and subsequently with a very concentrated solution of $CaCl_2$ are described. The produced particles have a spherical shape with an average diameter of 350 μm. The particles show a relative dense and homogeneous internal structure in comparison with previously reported alginate microcapsules (the bulk density being 0.47 g/cm^3), consequently the particle matrix is characterized by a high density of charged carboxylic groups. Particles produced with a contact time of 1 h showed a degree of crosslinking of 54%. Microspheres have a good stability, and despite of very low degree of swelling, they have a good solvents regain, and an excellent ionic binding capacity of cationic drugs. After preparation the beads were loaded by ionic complexation with an antitumor aromatic tetramidine. In addition, the in vitro release of drug has been studied. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Ca-alginate; Microspheres; Emulsification

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1. Introduction

The alginate microencapsulation procedure is, from a theoretical point of view, rather simple, nevertheless the production of microcapsule with optimal characterisics (e.g. spherical shape or swelling capacity) requires the strict control of a number of parameters. These include the type, source and concentration of the alginate used and the type (i.e. the alginate characteristics affect the capsule geometry), and molecular weight and con-

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centration of the cations used for the gelation process (Salib et al., 1978).

Ca-alginate microspheres are generally prepared by two alternative methods, (a) by dropping aqueous alginate into a solution of calcium salt (Salib et al., 1978; Matsumoto et al., 1986; Kazushi et al., 1995) or by (b) emulsification method under gentle stirring (Wan et al., 1990; Poncelet et al., 1995).

The particles produced by the first method are generally large (more than 1 mm), and the attempts to obtain smaller particles requires special extrusion device that can have the disadvantage of the high cost and possible clogging (Skjâk-Braek and Martinsen, 1991). The second method is less used and studied because of the drawbacks caused by the high viscosity of starting alginate solutions. In addition the resulting microspheres are generally non-spherical and with a high tendency to clumping.

Both methods use relatively low alginate concentrations (2–4%, w/v) and diluted calcium chloride solutions (Lin and Ayres, 1992; Pope et al., 1994; Sugawara et al., 1994; Velings and Mestdagh, 1994), this results in microspheres with a loose internal structure, representing good encapsulation devices for living cells but they are rather unsatisfactory for low molecular weight compounds. In fact in this type of particles the drug is encapsulated in a permeable polymeric shell, rather than in a homogeneous matrix. This results in a usually rapid release of the drug, not allowing long term release protocols.

Taking into account these considerations, a new strategy for alginate microspheres preparation using highly concentrated aqueous alginate solutions would be highly desirable, even if in the literature can be found alternative methods for the production of spherical particles which do not involve high cost (Rajaonarivony et al., 1993; Hsu et al., 1994; Willaert and Baron, 1996).

The aims of our work were to try solving the drawbacks of the methods currently available in literature and to test weather alginate microsphere can be used as delivery system for low molecular weight compounds. In this respect in this paper the production and characterization is described of alginate miscospheres containing the anticancer agent TAPP-Br.

2. Materials and methods

2.1. Chemicals

Alginic acid isolated from Laminaria digitata (Fluka, Switzerland) had the following characteristics as reported from the manufacturer: $F_G = 0.41$, $F_M = 0.59$, $F_{GG} = 0.25$, $F_{MM} = 0.43$, $F_{GM,\,MG} = 0.16$. The calculated intrinsic viscosity of sodium alginate, dissolved in 0.1 M NaCl, at 25°C was 477 ml/g, as measured by an Ubbelohde viscometer. Cellulose–acetate–butyrate, C.A.B-551-0.2 was purchased from Eastman, Kingsport, TN, USA. The 2′-bromo derivative of 1,3-di-

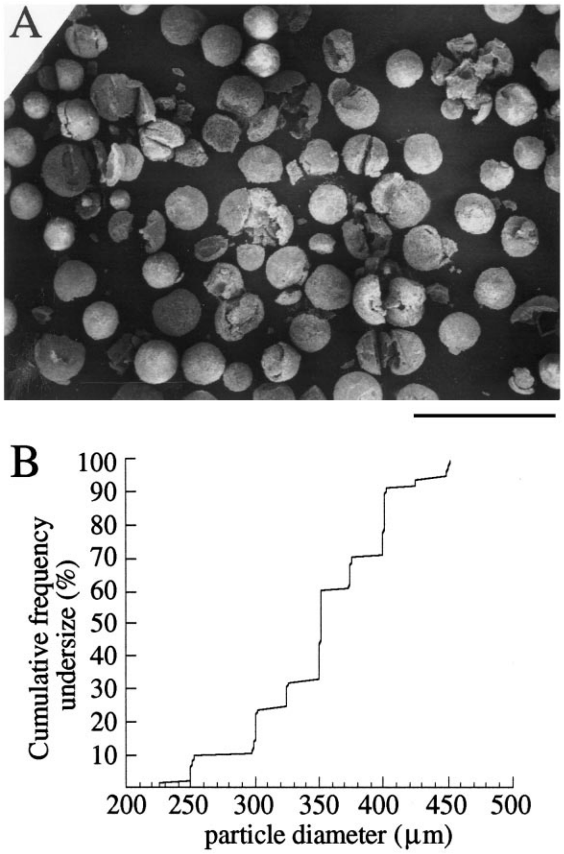

Fig. 1. Alginate microparticles. A: Scanning electron micrographs of microspheres, the bar corresponds to 1035 μm. In the picture some microspheres do not appear completely spherical since some particles were cut in order to study the internal morphology. B: Cumulative frequency distribution plot of the microspheres.

Table 1
Effect of temperature on the production of microspheres

Alginic acid (g)	NaOH (g)	$CaCl_2$, 5 N (ml)	Reaction time (min)	Temperature (°C)	Observations
1.5	0.5	1	15	60	Spherical microparticles
1.5	0.5	1	15	35	Amorphous polymer aggregate, only few particles
1.5	0.5	1	15	25	Amorphous polymer aggregate, only few particles
1.5	0.5	1	15	15	Amorphous polymer aggregate, only few particles

In all cases the following experimental parameters were employed: a 50 mm diameter vessel, a 35 mm diameter rotor, an oil phase constituted of 1,2-dichloroethane containing 1 g of Cellulose–acetate–butyrate as stabilizer, a volume ratio of the alginate solution to 1,2-dichloroethane of 0.25 and a stirring speed of 500 rpm.

Table 2
Effect of NaOH concentration on the production of microspheres

Alginic acid (g)	NaOH (g)	$CaCl_2$, 5 N (ml)	Reaction time (min)	Temperature (°C)	Observations
1.5	0.3	1	—	60	Rapid formation of amorphous aggregates
1.5	0.3 (+0.1 NaCl)	1	—	60	Rapid formation of amorphous aggregates
1.5	0.3 (+0.2 NaCl)	1	—	60	Rapid formation of amorphous aggregates
1.5	0.3 (+0.3 NaCl)	1	—	60	Amorphous polymer aggregate
1.5	0.4	1	20	60	Amorphous polymer aggregate, only few particles
1.5	0.5	1	15	60	Spherical microparticles

In all cases the following experimental parameters were employed: a 50 mm diameter vessel, a 35 mm diameter rotor, an oil phase constituted of 1,2-dichloroethane containing 1 g of Cellulose–acetate–butyrate as stabilizer, a volume ratio of the alginate solution to 1,2-dichloroethane of 0.25 and a stirring speed of 500 rpm.

(*p* - amidinophenoxy)bis - 2,2(*p* - amidinophenoxy-methyl)propane (TAPP-Br) was a generous gift of prof. Ferroni, Department of Pharmaceutical Sciences, University of Ferrara. Synthesis and analytical data of TAPP-Br have been reported elsewhere (Ferroni et al., 1984).

2.2. Preparation of microspheres

Ca-alginate microspheres were prepared as follows: 1.5 g alginic acid were dispersed in 6 ml bidistilled water. 0.5 g NaOH were dissolved in 4 ml water. The NaOH solution was instantaneously added to the alginic acid dispersion under powerful magnetic stirring and left to react for 15 min at 60°C to get a partial depolymerization. The resulting alginate solution was pre-heated at 60°C and then mixed with 40 ml of 1,2-dichloroethane (ratio aqueous phase/organic phase being 1:4) containing 2.5% w/v cellulose–acetate–butyrate as dispersing agent. After 15 min, 1 ml of 5 N $CaCl_2$ solution pre-heated at 60°C was added to the mixture. The formed emulsion was left under stirring at 60°C for different periods of time, comprised between 10 and 60 min. Afterwards, Ca-alginate microspheres were recovered by filtration through a sintered glass filter, under vacuum.

Table 3
Effect of Ca ions concentration on the production of microspheres

Alginic acid (g)	NaOH (g)	$CaCl_2$ (ml; N)	Reaction time (min)	Temperature (°C)	Observations
1.5	0.5	0.5; 5	15	60	Amorphous polymer aggregate
1.5	0.5	1; 5	15	60	Spherical microparticles
1.5	0.5	2; 5	—	60	Rapid formation of amorphous polymer aggregate
1.5	0.5	1; 5	—	60	Destabilization of the dropped emulsion system
1.5	0.5	5; 1	—	60	Destabilization of the emulsion system

In all cases the following experimental parameters were employed: a 50 mm diameter vessel, a 35 mm diameter rotor, an oil phase constituted of 1,2-dichloroethane containing 1 g of cellulose–acetate–butyrate as stabilizer, a volume ratio of the alginate solution to 1,2-dichloroethane of 0.25 and a stirring speed of 500 rpm.

Further hardening and removal of dispersing agent were performed by washing the microspheres with acetone. The particles were finally washed with a mixture of acetone:water 1:1 until no chloride ions were detectable in the effluents, as checked by titrimetic method with $AgNO_3$. The obtained particles were then completely dried at 40°C, under vacuum.

2.3. Morphological and dimensional analysis

The morphological and dimensional analyses of the microspheres were performed by optical and electron microscopy (SEM). Microspheres size and size distribution were determined by SEM photomicrographs analyzing about 500 microspheres.

2.4. Evaluation of cross-linking

The extent of microparticle ionic-crosslinking was indirectly determined by evaluating the amount of calcium ions ionically bound to the carboxylic groups of alginate in microparticles. This value was defined as degree of substitution (DS), indicating the percentage of Na ions substituted by Ca ions. 100 mg of microspheres were extensively washed with water for complete removal of the excess of unbound calcium ions. The calcium content in the washing solution was determined by complexometric method, using a 0.1 N EDTA solution, in the presence of murexide as indicator (Liteanu, 1959; Moe et al., 1991). Microspheres were then treated with a 0.1 N HCl solution, in order to remove all the ionically bound calcium ions, subsequently quantitated by the same method above described.

2.5. Determination of solvent regains

Solvents regains have been made both in water (representing a hydrophilic solvent) and in toluene (representing a hydrophobic solvent). 1 g dried microparticles were submerged in water or toluene for 24 h. The wet microspheres were centrifuged at 3000 rpm for 1 h. Afterward, particles were weighted and the solvent regain was calculated by difference with the dry weight.

2.6. Determination of swelling degree and bulk density

Swelling tests were performed using an optical microscope equipped with a micrometric device. The microspheres placed in aqueous solution were observed until the equilibrium was reached. At least 50 microspheres were considered for each batch and the mean value and S.D. were calculated.

The bulk density of microspheres was calculated by measuring the volume of a weighted amount of dried particles in measuring cylinders.

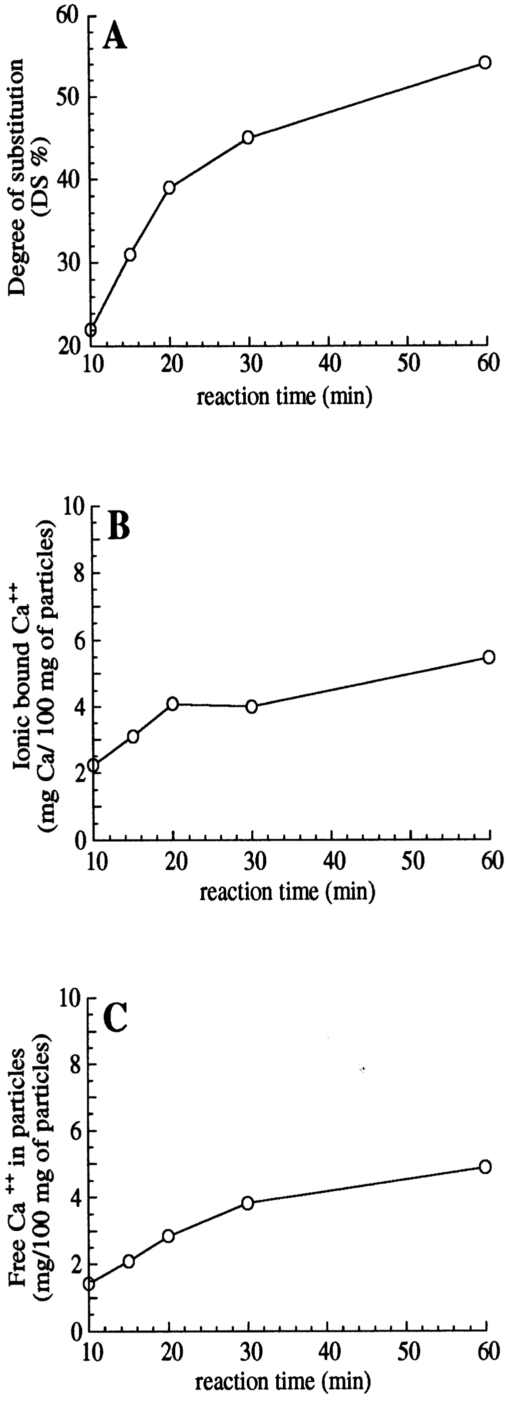

Fig. 2. Effect of reaction time on the alginate microspheres. Percentage of degree of substitution (DS) (panel A), ionic bound Ca ions (panel B) and free Ca ions (panel C) as function of the reaction time. All the reported parameters were calculated as reported in the experimental section.

2.7. Loading of TAPP-Br into Ca-alginate microspheres

100 mg of dried alginate microspheres were left for 2 h at 20°C in 2 ml of an aqueous solution containing different concentrations of TAPP-Br (2.5, 5, 10, 25 mg/ml), under gentle magnetic stirring. The amount of the ionic bound drug was determined by difference, evaluating the amount of drug in the filtrate (obtained after microspheres isolation) by UV spectrophotometry. Determinations were performed at 260 nm using a previously constructed calibration curve and a SPEKORD M42 C Spectrophotometer (Zeiss, Jena, Germany). In order to check a possible Ca/TAPP-Br exchange, the content of calcium ions in the filtrate was also performed.

2.8. Determination of in vitro release of TAPP-Br from microspheres

In vitro release of TAPP-Br was performed by dialysis method as previously reported (Nastruzzi et al., 1994). 100 mg of microspheres were introduced into a dialysis tube (molecular weight cut off 10000–12000 Medi Cell, UK), then placed in buffers at different pH under magnetic stirring. 1 ml samples were removed at various time and analysed spectrophotometrically for TAPP-Br content.

3. Results and discussions

3.1. Preparation of Ca-alginate microspheres

A method for the rapid preparation of alginate microparticles is presented here. The preparation procedure, based on a emulsification technique allows to obtain microparticles having a dense and homogeneous internal structure with small pores.

Microparticles were produced using as starting material a low viscosity sodium alginate (intrinsic viscosity 477 ml/g) which, before use, was further degraded by alkaline hydrolysis in NaOH solution (Haug et al., 1967). The use of the low molecular weight polymer allowed (a) to obtain a highly

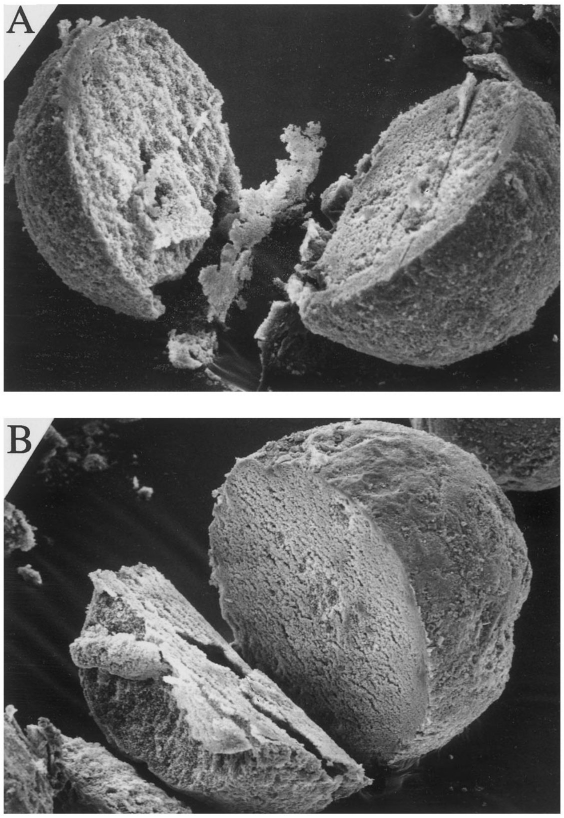

Fig. 3. Scanning electron micrographs showing the internal morphology (cross-sections) of alginate microparticles obtained by using a reaction time of 15 (panel A) or 60 min (panel B). The bar corresponds to 100 μm.

concentrated starting polymer solution and (b) to facilitate the diffusion of calcium ions into the gel network during the microparticle production process (Peters and Van Bommel, 1992).

Optimal results, both in term of particle yield and geometry were obtained by using the following experimental parameters. A 50 mm diameter vessel, a 35 mm diameter rotor, an organic phase constituted of 1,2-dichloroethane containing 1 g of Cellulose–acetate–butyrate as stabilizer, a volume ratio of the alginate solution to 1,2-dichloroethane of 0.25, a stirring speed of 500 rpm, a temperature of 60°C and a reaction time of 15 min. Employing these preparation parameters we obtained microspheres with an average diameter of 350 μm and good spherical geometry (see Fig. 1). In addition, our preparation procedure allows to obtain a high microsphere recovery (over 95%) with respect to the starting amount of alginate used. In order to evaluate the influence of the different preparation parameters on the microsphere characteristics a series of experiments was carried out focusing on temperature, Na and Ca ions concentration and reaction time.

3.2. Effect of temperature on the production of microspheres

To enhance the diffusion of calcium ions into alginate gel, the preparation of microsphere was conducted at the relative high temperature of 60°C, that causes the decrease of viscosity of the

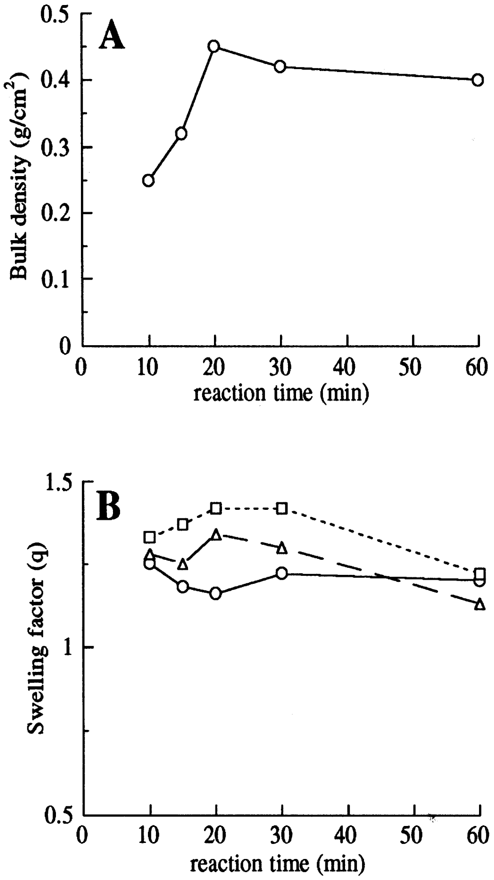

Fig. 4. Influence of reaction time on the microsphere bulk density (panel A) and microsphere swelling (panel B). Swelling determinations were performed in aqueous buffers at pH 1 (●), pH 7 (■) and pH 13 (▲).

Table 4
Effect of reaction time on the production of microspheres

Alginic acid (g)	NaOH (g)	$CaCl_2$, 5 N (ml)	Reaction time (min)	Temperature (°C)	Observations
1.5	0.5	1	10	60	Spherical microparticles
1.5	0.5	1	15	60	Spherical microparticles smooth surface
1.5	0.5	1	20	60	Spherical microparticles
1.5	0.5	1	30	60	Spherical microparticles
1.5	0.5	1	60	60	Spherical, rigid microparticles

In all cases the following experimental parameters were employed: a 50 mm diameter vessel, a 35 mm diameter rotor, an oil phase constituted of 1,2-dichloroethane containing 1 g of cellulose–acetate–butyrate as stabilizer, a volume ratio of the alginate solution to 1,2-dichloroethane of 0.25 and a stirring speed of 500 rpm.

solution (Cottrell and Kovacs, 1980). Particles with a good morphology (spherical shape) were effectively produced only at 60°C whilst working at lower temperature (i.e. 35, 25, and 15°C) resulted largely in the formation of an amorphous (non-spherical) polymer aggregate and only few non spherical and friable microspheres (see Table 1).

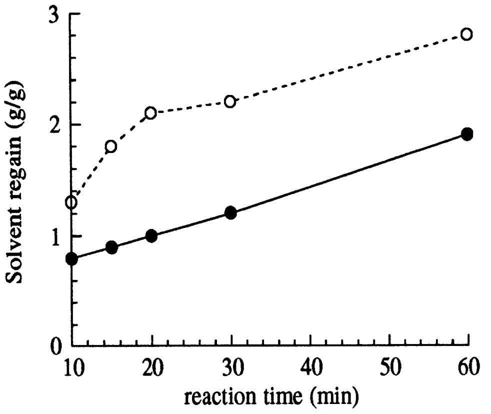

Fig. 5. Solvent regain of Ca-alginate microspheres in water (○) and in toluene (●).

Table 5
Swelling factors of Ca-alginate microspheres

pH 1	pH 7	pH 13	TAPP-Br 5 mg/ml
1.25	1.33	1.28	1.80
1.18	1.37	1.25	1.70
1.16	1.42	1.34	1.78
1.26	1.28	1.42	1.72
1.20	1.22	1.13	1.76

q = swollen volume/dried volume of particles, determined by optical microscopy.

3.3. *Effect of NaOH concentration on the production of microspheres*

The preliminary alkaline treatment of the polymer with an excess of sodium hydroxide plays two important roles. Firstly, it causes the hydrolysis of alginate, reducing the molecular weight of the polymer, thus allowing to obtain a concentrated polymer solution (up to 15%, w/v) characterized by a low viscosity. Secondly, it promotes the

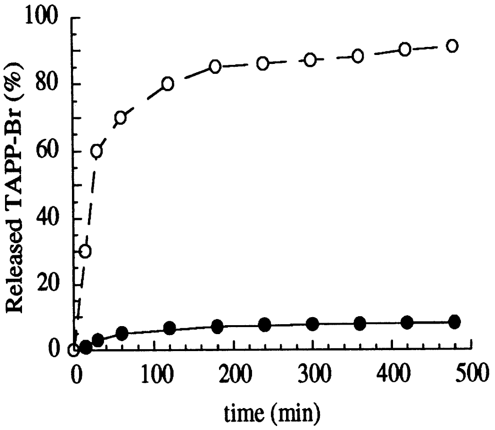

Fig. 6. TAPP-Br release profiles from Ca-alginate microspheres, determined by a dialysis method as previously published (Nastruzzi et al., 1994). The reported values represent the average of three independent experiments (bars = S.D.). Release experiments were performed at pH 1.2 (○) or pH 4.8 (●) buffers.

formation of $Ca(OH)_2$ competitive reaction with the formation of Ca-alginate complex. In this respect it is important to note that if an excess of NaOH is not used the formation of microspheres is totally hindered. This behaviour was demonstrated in a series of experiments where a lower amount of NaOH was employed, adjusting the total ionic strength by addition of different amounts of NaCl (see Table 2).

3.4. Effect of Ca ions concentration on the production of microspheres

Optimal results in term of microsphere recovery and morphology were obtained by adding in a single step a concentrated $CaCl_2$ solution (1 ml, 5 N). It is to be note that the amount of Ca ions appear to be a very critical parameter. When different amount of Ca ions were employed, for instance 0.5 or 2 ml, 5 N $CaCl_2$ solutions, a rapid destabilization of the emulsion system was observed, causing the formation of an irregular (non-spherical) polymer aggregate. Moreover, it is to be underlined that if the same amount of Ca ions was added in the form of a diluted solution ($CaCl_2$, 5 ml, 1 N) or the concentrated solution (1 ml, 5 M) was added dropwise, again only non spherical polymer aggregates were formed (see Table 3).

3.5. Effect of reaction time on the production of microspheres

In order to evaluate the effect of the reaction time on microparticle morphology, the microspheres were left, before isolation, in the presence of Ca ions for different periods of time, comprised between 10 and 60 min. The degree of substitution (DS), taken as cross-linking indicator, and the concentration of $Ca(OH)_2$, present in the washing solution after microparticles isolation, were calculated for each time as reported in the experimental section. From the analysis of the results reported in Fig. 2, it was found that both parameters increased in an approximately linear fashion during the first 30 min.

After isolation microparticles were in addition analysed both in term of morphology and cross-linking efficiency.

Particles with ideal size and shape were obtained with a reaction time of at least 15 min (100% yield, 31% D.S.), longer reaction times led to the formation of more rigid beads, still maintaining a spherical shape. Cross-sectional analysis performed by electron microscopy (reported in Fig. 3) shows that the particles have a relatively rough and uniform surface and a homogeneous internal structure, characterized by a microporosity reflecting the incubation time in the presence of Ca ions.

It is to be underlined that the $Ca(OH)_2$ formed in situ could play an important role on the microspheres morphology.

Its relatively low solubility in water (77 mg/100 ml at 75°C) could in fact lead to an additional crosslinking of alginate particles, whilst the removal of precipitated $Ca(OH)_2$ could account for the uniform microporosity observed within the particle matrix.

3.6. Effect of reaction time on microspheres density and swelling

The influence of reaction time on the microsphere bulk density is shown in Fig. 4 A. After 20 min the microspheres reach the highest bulk density owing to a volume contraction. The volume and the weight loss due to the syneresis phenomenon (Andresen and Smidsrød, 1977; Yotsuyanagi et al., 1987), so-called maturation step, takes rapidly place in our case.

Velings and Mestdagh (1995) clearly show that in other cases the maturation step is generally slow, resulting in a steady state equilibrium after about 15 h, depending to the cation concentration, the ionic strength, and pH.

The swelling of the alginate microsphere was in addition determined by measuring the volume increase of particles at the equilibrium in aqueous buffers at different pH (see Fig. 4B and Table 4). Because of the high degree of crosslinking the microspheres showed in all cases a relatively small degree of swelling, apparently looking hydrophobic. This latest feature could be convenient for biomedical applications, especially when prolonged drug release is requested.

3.7. Solvent regain

The profiles of water and toluene regain against time reaction of Ca-alginate microspheres are presented in Fig. 5. These data, in agreement with the microscopic analysis of the microparticle cross-section (see Fig. 3), indicated that the beads have a so-called microreticular structure (Kremmer and Boross, 1979) presenting a highly cross-linked structure with a high dry-matter content and a low specific solvent uptake in swelling, when compared to macroreticular gels. The prepared microparticles can be thus included in the class of aerogels in which the network of particles remains unchanged after the removal of the dispersive medium (Kremmer and Boross, 1979).

3.8. Loading of microparticles with the antitumor drug TAPP-Br

The high loading capacity of alginate microparticles was demonstrated using different concentrations of the antitumour drug TAPP-Br in aqueous solution; namely 2.5, 5, 10, and 25 mg/ml. The loading was found in all cases almost quantitative, this result was attributed to the strong ionic interactions occuring between the polycation TAPP-Br and the highly negatively charged alginate polymer. Indeed, the high density of carboxylic groups present on the microspheres strongly interacts with the four positively charged drug (Nastruzzi et al., 1993).

In order to better evaluate the loading process with TAPP-Br the microparticle swelling in the presence of 5 mg/ml TAPP-Br solution was determined. As it is clearly evident from the data reported in Table 5, microparticles swelling is increased in the presence of the positively charged drug. This evidence was tentatively attributed to the fact that the positively charged tetra-amidine displaces calcium ions when forming an electrostatic complex. It is to be considered that from a therapeutical and biological point of view, a drug delivery system with a high drug loading capacity is very advantageous, limiting the needing of administer high level of carrier.

3.9. Release of TAPP-Br from alginate microspheres

Preliminary studies of TAPP-Br release in a simulated vascular tumor (pH = 5.0, acetate buffer) (Al-Shamkhani and Duncan, 1995) for loaded microspheres (D.S. = 45%) with different drug content (5%, 10%, 33% w/w) have been performed.

The amount of drug released was very low for each sample (Fig. 6). Changing the acetate buffer with phosphate buffer (pH = 4.8), knowing its affinity for multiple cations and the fact that it is a human fluid body component, we could not improve our results. The unexpected reabsorbtion of the initially released drug is due probably to some rearrangements between calcium ions and TAPP-Br. After that no change takes place and an equilibrium seems to be settled. In acidic conditions (pH = 1.2) the drug was released almost entirely just after 30 min. All these results confirm our assumption that TAPP-Br forms a polyelecrolyte complex and a further investigation on these interactions must be performed.

3.10. Conclusions

First of all, we would like to underline that our results are highly reproducible, in fact all the experiments were conducted in triplicate and standard deviations were always within ± 8. The produced Ca-alginate microspheres have, in comparison with the previously reported alginate beads, a high content of carboxylic groups per volume unit, a dense and microporous internal structure.

Microparticles with a high degree of crosslinking can be rapidly produced using a very simple procedure. They are not friable, and with a low swelling tendency. The produced beads show a so-called microreticular structure (Kremmer and Boross, 1979), presenting a high degree of crosslinking, a high dry-matter content and a low specific solvent uptake when compared to macroreticular gels. Taking together our data indicate that the prepared microspheres can be inserted in a particular class of aerogels in which the particle network remains unchanged after the removal of the dispersive medium (Kremmer and Boross, 1979).

All these special characteristics make the Ca-alginate microspheres particularly interesting for biomedical applications. In this respect, TAPP-Br loaded microspheres could be proposed for the experimental therapy of tumors by following subcutaneous or chemoembolization administration procedures.

To this respect, it should be remembered that alginate itself having a high content of β-D-mannuronic acid residues display an antitumor and immunomodulating activity (Otterlei et al., 1991; Fujihara and Naguma, 1992; Espevik et al., 1993).

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