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Note

Albumin–alginate-coated microspheres: Resistance to steam sterilization and to lyophilization

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Abstract

The paper describes the effect of different thermal treatments on the morphology and binding properties of particles prepared using a transacylation reaction between two biocompatible polymers, namely propylene glycol alginate and human serum albumin.

Compared to control alginate gel microspheres, albumin–alginate covalent network offers a better resistance to the microspheres towards freezing, lyophilization and sterilization. The binding properties for methylene blue were not altered by the treatments. Moreover, stability in physiological environments opens interesting applications in biological and pharmaceutical fields.

Keywords: Alginate; Binding properties; Lyophilization; Microsphere; Transacylation; Sterilization

Alginate particles are commonly used as protective carriers for substances or cells (Reis et al., 2006; Uludag et al., 2000). For use as drug delivery systems, specific properties are required: mechanical resistance and stability in physiological environment, biocompatibility, sterility, and long-term storage capacity. Our laboratory develops a method leading to microspheres presenting improved stability and perfect biocompatibility in cell culture, which makes them particularly interesting for use as drug carriers (Edwards-Lévy and Lévy, 1999; Hurteaux et al., 2005). Stability is brought by the creation of stable membranes around alginate microparticles. The method, based on a transacylation reaction between ester groups of an alginate derivative (propylene glycol alginate, PGA) and amino groups of a protein (human serum albumin, HSA) is started upon a simple pH increase, and leads to the formation of covalent bonds between the two biopolymers.

We propose, in this note, to go further in the evaluation of these microspheres and determine if they could be sterilized or dried without major modifications of their properties. Steam sterilization, freezing and lyophilization were applied to the particles. Effects of these treatments were evaluated in terms of morphology, size, and binding properties of methylene blue, used as a model cationic drug, and compared to different control microspheres.

Coated microspheres consisting of an alginate gel core surrounded by a PGA–HSA membrane were prepared using a two-step method described elsewhere (Hurteaux et al., 2005). Briefly, the following parameters were used: *aqueous phase*: 2% PGA (Kelcoloïd S®, ISP), 1% sodium alginate (NaAlg, Manugel GHB®, ISP) and 4% HSA (Baxter) in saline; *emulsification*: 1500 rpm in 50 mL of isopropyl myristate (SDF) containing 5% (w/v) sorbitan trioleate (Seppic), 5 min; *gelification*: +35 mL of $CaCl_2$ (20%, w/v), 15 min; *membrane formation*: dispersion in 50 mL of a 2.5% HSA solution, magnetic stirring, +10 mL of 0.5 M NaOH, 15 min; *neutralization*: dispersion in 50 mL pH 7 buffer, 15 min; *washings*: polysorbate (Seppic) 2% (w/v) in water, then pure water.

Calcium alginate (CaAlg) microspheres were prepared using the following parameters: *aqueous phase*: 2% NaAlg in saline; *emulsification* and *gelification* as for coated microspheres; *washings* as previously described.

Microspheres consisting of calcium alginate gel containing unreacted PGA and HSA were prepared using the same parameters as for the coated microspheres, omitting the membrane formation step.

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Microparticles made of a PGA–HSA network without internal alginate gel were prepared as follows: 6 mL of 3% PGA and 20% HSA in water was emulsified (1500 rpm) in 40 mL isopropyl myristate + 5% sorbitan trioleate. After 5 min stirring, 2 mL of a 2% (w/v) NaOH solution in ethanol was added and agitation was continued for 15 min to allow the transacylation reaction to occur. Then, 2 mL of an 8.5% (v/v) acetic acid solution in ethanol was added. After 15 more minutes, the microspheres were washed as previously described.

Steam sterilization treatment consisted in a 15 min cycle at 121 °C (Mediclav 2000 AS) of microsphere suspensions in water, saline, or phosphate-buffered saline (PBS).

Observations of freezing effects (sample immersion in liquid nitrogen (−196 °C) for 24 h) were performed after thawing at room temperature.

Samples were freeze-dried in a Freezone 6 (LabConco, condenser temperature: −45 °C, pressure <0.5 mbar) after freezing aqueous suspensions of microspheres either in a freezer (−20 °C), or in liquid nitrogen. Samples were rehydrated in water for 3 h prior to observations.

Microparticle samples were observed by light microscopy (Olympus, BH-2) with interferential phase contrast. Photographs were selected from several observations (at least three samples per batch, and two batches).

Diameter measurements were performed using laser diffraction (Particle Sizer LS200, Beckman-Coulter).

The binding property for methylene blue was evaluated before and after sterilization of transacylated microspheres. A microsphere suspension in methylene blue solution (2.5 mg dry particles in 6 mL solution, dye concentration 30 μg/mL) was

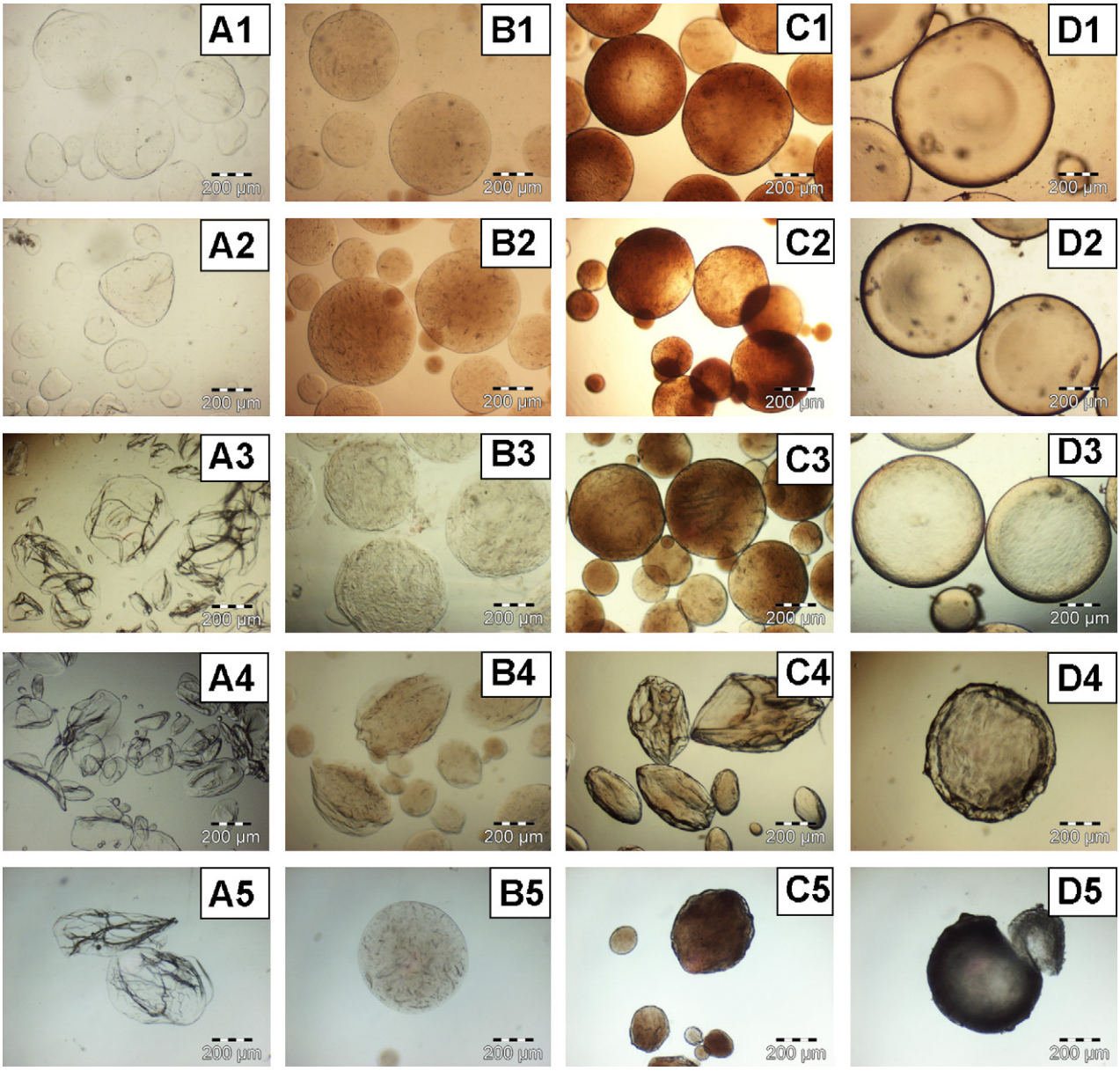

Fig. 1. Microphotographs of the four microparticle types before and after treatments. Calcium alginate microspheres (A), alginate microspheres containing unreacted PGA and HSA (B), PGA–HAS-coated microspheres (C) and PGA–HSA microparticles (D) without treatment (1), after sterilization in water (2), after freezing (−196 °C) and thawing (3), after freezing (−20 °C), lyophilization and rehydration (4), and after freezing (−196 °C), lyophilization and rehydration (5).

magnetically stirred for 3 h at 37 °C. The supernatant was measured by spectrophotometry at 664 nm, and the percentage of bound dye was determined by subtraction.

Microscopic observations revealed that calcium alginate microspheres presented an irregular shape, and appeared transparent (Fig. 1, line 1). The other particle types were less transparent, but the presence of HSA and PGA improved their sphericity, due to the interfacial properties of the two biopolymers (Hurteaux et al., 2005).

PGA–HSA microparticles appeared as shell-like structures, an external thick wall surrounding a distinct core. Particle breaking induced liquid material release from the core. A similar phenomenon had already been described, when larger beads (2 mm in diameter), constituted of calcium alginate added with PGA and HSA, were soaked in a diluted NaOH solution (Edwards-Lévy and Lévy, 1999). It was shown possible to restrict the pH increase to external layers of the beads by maintaining NaOH concentration below a threshold value, allowing, for example cell encapsulation in the neutral core of the beads (Shinya et al., 1999).

Calcium alginate microspheres were the most sensitive to freezing in liquid nitrogen and appeared shrunk after thawing (Fig. 1, line 3). The surface of microspheres containing unreacted PGA and HSA was rumpled, but the particles remained almost spherical. Microparticles prepared using the transacylation reaction were not affected by the freezing–thawing process. HSA is known to be cryoprotective for cells (Hreinsson et al., 2003), and either covalently bound to the particles or not, it appears to also preserve microparticle integrity after a freezing–thawing cycle.

The freezing rate is known to influence alginate gel structure (Shapiro and Cohen, 1997), and on the whole, lyophilized particles (Fig. 1, lines 4 and 5) rehydrated easier when freezing was performed in liquid nitrogen rather than in a freezer. Alginate gel microspheres, however, remained very crumpled after rehydration, whatever the freezing process. Although encapsulation in calcium alginate gel beads can be used as a means of preserving cells during freezing or freeze-drying (De Giulio et al., 2005), previous studies have also shown that the lyophilization of large beads may induce deformations on their surface (Zohar-Perez et al., 2004).

The presence of internal PGA and HSA improved sphericity of fast-frozen particles after rehydration. In addition to the aforementioned cryoprotective effect of HSA, it had already been shown (Gal and Nussinovitch, 2007) that the presence of filler molecules such as talc or starch inside alginate microspheres had a smoothing effect on the particle surface after freeze-drying, which was attributed to a resistance against collapse, brought by the filler (Zohar-Perez et al., 2004).

DeGroot and Neufeld (2001) reported that chitosan-coated alginate beads only partly recover their original diameter after lyophilization and rehydration, rigid membranes possessing less ability to be distorted than uncoated alginate particles. The thick PGA–HSA shell of the particles tested in the present study behaved in the same way and appeared mostly broken when frozen in liquid nitrogen. In this case the slow cooling procedure at −20 °C better preserved particle integrity.

All microparticle types survived sterilization in water (Fig. 1, line 2), but calcium alginate microspheres appeared partly shrunk. Alginate was previously shown to form stable gels over a 0–100 °C temperature range (Gombotz and Wee, 1998). However, to our knowledge, no literature exists concerning sterilization of alginate gel microspheres. Serp et al. (2002) have shown that large-sized alginate beads were sensitive to high temperature treatment. They observed a 23% decrease in bead diameter and shrinkage of the gel network after 20 min at 130 °C. In our study, the presence of PGA and HSA in the microparticles seemed to protect them from such structural modifications, since they kept their initial sizes. The internal network organization is currently being explored.

Aliquots of each microparticle suspension were transferred in saline or PBS. As expected, alginate microspheres, either containing unreacted polymers or not, redissolved in the ionic solutions, due to displacement of calcium ions from the gel. Microparticles of the two other types resisted to all tested solutions, thanks to the presence of the PGA–HSA network. They also survived sterilization in PBS and in saline, their mean diameters remaining unchanged after heat treatment (Fig. 2).

Binding properties of the microparticles towards methylene blue was determined before and after sterilization in water (Fig. 3). Coated microspheres were able to bind a higher amount of dye (∼80%) than PGA–HSA microparticles (∼15%). This cationic molecule is likely to bind to the microspheres thanks to ionic interactions with carboxylate groups of the inner alginate gel (Tomida et al., 1993; Gombotz and Wee, 1998). As the PGA possesses less binding sites (Sugawara et al., 1994), interactions with the dye were thus fewer with PGA–HSA microparticles, lacking calcium alginate gel core, than with coated microspheres. Sterilization did not change interactions between coated microspheres and dye molecules, which is indicative of gel structure preservation. The percentage of dye bound to PGA–HSA microparticles increased after sterilization (significant at the 5% level). An irreversible unfolding of HSA is known to occur when the protein is heated above 70 °C (Rezaei-Tavirani et al., 2006). Denaturation of the protein might have occurred during heating, exposing previously buried binding sites.

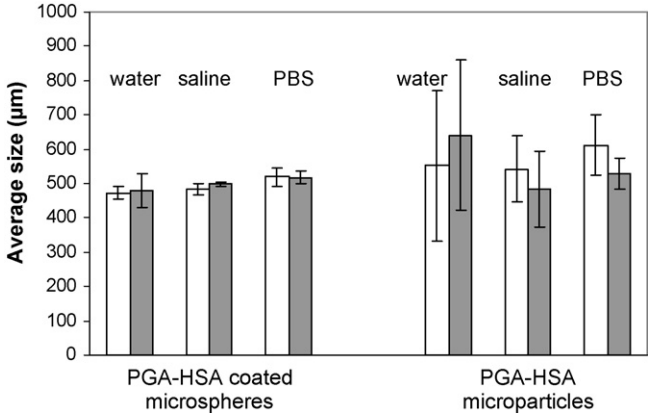

Fig. 2. Average size of transacylated microparticles before (white bars) and after (grey bars) sterilization (121 °C, 15 min) in water, saline and PBS. Data shown are the mean ± S.D. of six replicates (two batches, three determinations/batch).

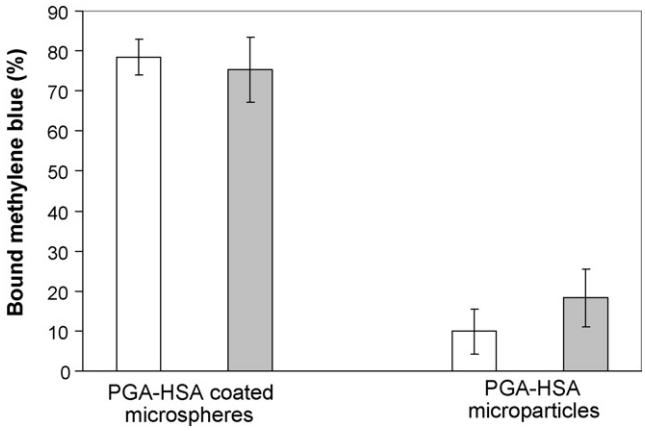

Fig. 3. Percentage of bound methylene blue onto transacylated microparticles before (white bars) and after (grey bars) sterilization (121 °C, 15 min) in water. Data shown are the mean ± S.D. of six replicates (two batches, three experiments/batch).

The results obtained in this study showed that the covalent PGA–HSA network brings to the microparticles good resistance towards some drastic treatments, as compared to control calcium alginate microspheres. In particular, the stability of these particles, only constituted of biocompatible and biodegradable polymers, to thermal treatments in saline or in PBS makes them particularly interesting for biomedical and pharmaceutical applications.

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