PGA-HSA coated microspheres : encapsulation and release of a bioactive peptide

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Introduction

Our laboratory develops for many years a new method, avoiding the use of crosslinking reagents, for the preparation of microparticles (Lévy and Edwards-Lévy, 1996). This method is 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, for example). Upon alkalization, covalent amide bonds are created between the two polymers. A preliminary study has shown the biocompatibility of the microparticles in osteoblast cultures (Hurteaux, 2005).
Our project is to evaluate these microspheres as a delivery system. We chose a product of biological interest, the KRFK peptide, involved in the cell bone proliferation mechanism thanks to the activation of the latent complex of transforming growth factor-β1 (Adams, 2001 ; Centrella, 1994).
The aim of this work was to understand the encapsulation process and to acquire a better knowledge on the nature of interactions between the microparticles and this product, as a function of microparticle preparation parameters and of the presence of different ions in the surrounding medium. Cytotoxicity of the microspheres was evaluated in monocyte cultures (THP-1).

Material and methods

Preparation of the microspheres.

- Microspheres (MS) consisting of an alginate gel core surrounded by a membrane made of PGA-HSA: 10 mL of an aqueous phase consisting of 2 % (w/v) PGA (Kelcoloid S, ISP), 1 % sodium alginate (AlgNa, Manugel, ISP) and 4 % HSA (Baxter) in saline was emulsified at a stirring speed of 1500 rpm or 4000 rpm in 50 mL of an oily phase (isopropyl myristate, SDF) containing 5 % Span 85 (Seppic). After 5 min stirring, 35 mL of a $CaCl_2$ solution (5 or 20 %) was added and agitation was continued for 15 min. After centrifugation, AlgCa microspheres were resuspended in 50 mL of a 2.5 % HSA aqueous solution and magnetically stirred. 10 mL of 0.5 M NaOH was added dropwise and the transacylation reaction was allowed to develop for 15 min. The reaction was stopped by dispersion of coated MS in 50 mL of imidazole buffer pH 7 for 15 min. They were then washed (water (Fresenius) + Tween 20 2 %, once) and rinsed (water, 3 times). The particles were then lyophilized.
- Microspheres of PGA-HSA without alginate gel: 6 mL of an aqueous phase consisting of 2 % PGA and 20 % HSA in water was emulsified in 40 mL of isopropyl myristate containing 5 % Span 85. After 5 min stirring, 2 mL of a 2 % NaOH solution in 95 % ethanol was added and agitation was continued for 15 min to allow the transacylation reaction to occur. Then, 2 mL of a 8.5 % (v/v) acetic acid solution in ethanol was added. After 15 more min, agitation was stopped and the MS were washed and rinsed as previously described, and finally lyophilized.

Microscopic observations and diameter determination

The MS were observed with a light microscope (Olympus, BH-2) equipped with a CCD camera (DP-50) coupled with an AnalySIS software (Soft Imaging System).
Diameter measurements were performed using a laser diffraction technique (Coulter Particle Sizer type LS200, Beckman-Coulter).

Evaluation of cytotoxicity in monocytes cultures

THP-1 monocytes were harvested with 1.5 mg of lyophilized MS for 24 h to 72 h in RPMI-1640 medium (Life Technologies, Inc) supplemented with 10 % fetal calf serum, 2 mM glutamine, penicillin (5000 U/mL) and streptomycin (25 µg/mL). Cells were then counted and the percentage of viability determined (trypan blue exclusion test). Observations of the morphology of the cells in contact with the MS were realised by SEM.

Saturation experiments

5 mg of lyophilized MS were rehydrated by 10 mL of water for 1 h at 37°C. 1 mL of the suspension was transferred in 5 mL of KRFK solutions of known concentrations in pure water. The resulting suspension was incubated at 37°C and magnetically agitated for 1 h. Aliquots of 600 µL were centrifuged and the supernatants were analyzed by HPLC (mobile phase water/acetonitrile 80/20 added by 0,5 % of TFA, UV detection at 210 nm). Experiments were made in triplicate.

Displacement experiments

Dilutions of saline (NaCl 9 g/L) were used instead of pure water to evaluate interactions between the KRFK peptide and the MS. The fraction of free peptide in the medium was determined by HPLC.

Results and discussion

Morphological characteristics of the microspheres

Figure 1 presents the morphological aspect of the MS prepared by the first method. Microspheres appeared homogeneous. The surfaces were not perfectly smooth and some fibrillar material could be observed at the surface of the microspheres. The presence of the membrane was confirmed by reliquification of the gel core by sodium citrate. This treatment dissolves AlgCa, allowing to visualize the membrane by itself. No significant difference was observed between the weakly (Figure 1b) and strongly (Figure 1a) gelified MS.

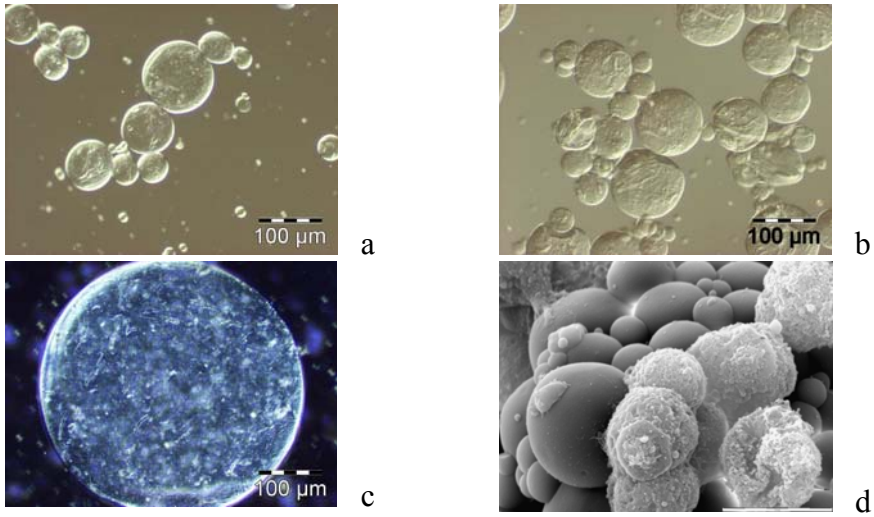

Figure 1. (a-c) Microphotographs of PGA-HSA coated microspheres. (a) small MS, gelation with $CaCl_2$ 20 %; (b) small MS, gelation with $CaCl_2$ 5 %; (c) large MS, gelation with $CaCl_2$ 20 %, **(d) SEM observation of THP-1 cells in contact with the MS (magnification x3500).**

MS produced by the second method (without AlgCa gel core) were of similar morphological aspect. The average diameters of the particles, which varied according to the stirring speed during the emulsification step, were about 100 µm ("small" MS) and 500 µm ("large" MS).

Evaluation of cytotoxicity in monocytes cultures

THP-1 cells were viable (> 95%) whatever the type of MS added. Cell morphology was not affected by the presence of MS, as presented in Figure 1d. Some pseudopodes were observed, indicating a good adhesion of the cells at the surface of the microspheres.

KRFK interactions with the microspheres

At the equilibrium, a fraction of the KRFK molecules was captured by the MS. The free fraction was measured and compared to the initial amount of peptide, allowing the determination of the bound fraction. According to Scatchard, a graph could be drawn representing the relation:

$$B/F = K (n-B)$$

where B is the amount of moles of KRFK bound to 1 mg of microspheres, F is the concentration of free peptide in solution, n the total number of mol sites for 1 mg of microspheres and K the equilibrium constant. Data are presented in Figure 3 for the different types of MS.

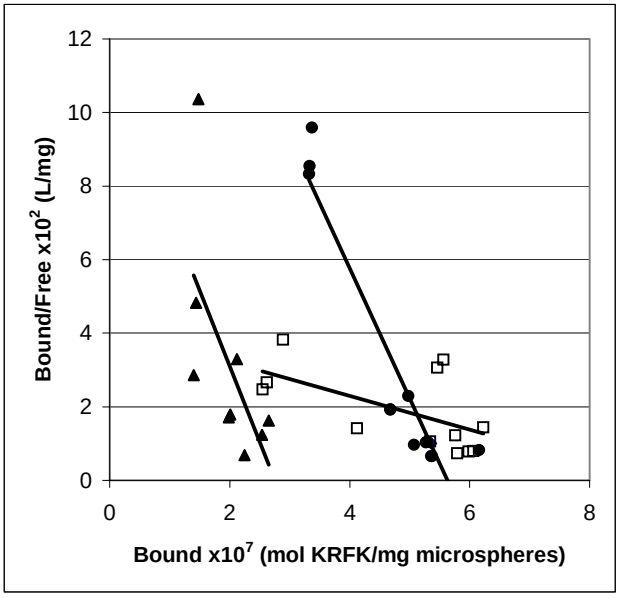

Figure 3. Scatchard plots of KRFK interactions with the microspheres. (●) small MS, gelation with CaCl2 20 %; (□) small MS, gelation with CaCl2 5 %; (▲) large MS, gelation with CaCl2 20 %.

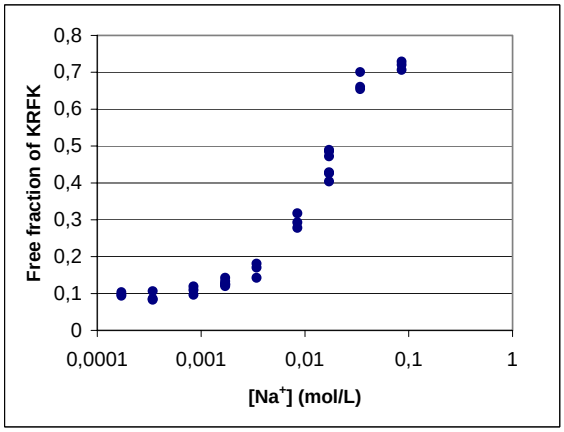

Figure 4. Effect of addition of Na^+ ions on the equilibrium between KRFK and the microspheres.

Results point out a variability, which can mainly be explained by the microsphere preparation process. For each MS type, one straight line can be drawn, indicating that there is only one class of binding sites.

The size of the microspheres (●, 100 µm vs. ▲, 500 µm) affected the total number of binding sites available for the peptide (intersection with the x axis), but not the affinity of the MS for KRFK (slope). Values are given in Table 1. This result, which could be related to the higher specific surface exposed to the peptide for the small particles suggests that the binding sites for KRFK would be located near the surface.

	Small MS, $CaCl_2$ 20% (●)	Small MS, $CaCl_2$ 5% (□)	Large MS, $CaCl_2$ 20% (▲)
K (L/mol)	$35.2.10^4 \pm 4.0.10^4$	$4.6.10^4 \pm 1.9.10^4$	$41.3.10^4 \pm 18.5.10^4$
n (mol/mg)	$5.6.10^{-7} \pm 0.6.10^{-7}$	$9.0.10^{-7} \pm 2.2.10^{-7}$	$2.7.10^{-7} \pm 0.9.10^{-7}$

Table 1. Values determined from the Scatchard representation: equilibrium constant (K) and total number of binding sites (n).

The gel strength (●, strongly gelified vs. □, weakly gelified) mainly affects the affinity for the peptide, and seems to influence the total number of sites of interactions between the KRFK and the microspheres. Ionic interactions between the positive KRFK molecules and the negative charges of alginate may explain these differences: a weakly gelified microsphere presents more accessible carboxylate groups than a strongly gelified one, in which more Ca^{2+} ions interact with alginate charges. In a similar way, Sawaya *et al.* (1987) showed that the reticulation rate of crosslinked albumin microcapsules had an influence on the total number of binding sites available for ions.
There was almost no interaction between microspheres produced by the second method and the peptide, enhancing the idea that the binding sites are located in the alginate gel core of the coated microspheres.
Hypothesis of ionic interactions was tested by adding NaCl, in the KRFK bound to microspheres (small and strongly gelified). The equilibrium fraction of free peptide is presented as a function of the Na^+ concentration in Figure 4. The results indicate that the presence of Na^+ ions induces a release of the peptide from the particles and give a new argument in favor of an ionic interaction model.

Conclusion

The method used in this work allowed to produce coated microspheres of 100 μm or 500 μm in diameter, with different gel strengths. Those particles were able to interact with a peptide sequence involved in cell bone proliferation thanks to ionic interactions between the positive charges of KRFK and the negative charges remaining available in the alginate gel. Positive ions could easily displace the peptide through ion exchange. The smaller the microspheres, the larger the total number of binding sites, indicating a possible location of the sites at the surface. Particles were shown to be biocompatible in monocyte culture, allowing us to investigate further the peptide loaded-microspheres as biomaterial for bone regeneration.

Bibliography

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