International Journal of Pharmaceutics 366 (2009) 103–110

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Serum albumin-alginate coated microspheres: Role of the inner gel in binding and release of the KRFK peptide

Maïté Callewaert[a,b], Jean-Marc Millot[c], Jacques Lesage[c], Dominique Laurent-Maquin[b], Florence Edwards-Lévy[a,*]

[a] CNRS UMR 6229, Institut de Chimie Moléculaire de Reims, Université de Reims-Champagne Ardenne, Faculté de Pharmacie, 51 rue Cognacq-Jay, 51100 Reims, France
[b] INSERM U926, Université de Reims-Champagne Ardenne, Faculté d'Odontologie, 51 rue Cognacq-Jay, 51100 Reims, France
[c] CNRS UMR 6237, MEDyC, Université de Reims-Champagne Ardenne, Faculté de Pharmacie, 51 rue Cognacq-Jay, 51100 Reims, France

ARTICLE INFO

Article history:
Received 23 April 2008
Received in revised form 4 September 2008
Accepted 4 September 2008
Available online 17 September 2008

Keywords:
Affinity
Binding properties
Membrane
Microsphere
Propylene glycol alginate
Transacylation

ABSTRACT

In continuation with our previous study using fluorescein-isothiocyanate (FITC)–Lys-Arg-Phe-Lys (KRFK) peptide, the aim of this work was to study the interaction of the unlabelled KRFK with calcium alginate gel microspheres coated with a serum albumin (HSA)-alginate membrane prepared using a transacylation method. Coated microspheres were prepared with two main sizes and two gel strengths. Control microspheres made of cross-linked alginate-HSA without calcium alginate gel were also prepared. A series of loading and release assays conducted with methylene blue showed the requirement of inner gel for binding the cationic molecule. Release experiments were performed in different media using unlabelled KRFK and coated microspheres. A plateau was reached within 1 h, in contrast with the slow release of the FITC-peptide observed in our previous work. This discrepancy was attributed to modified properties of the labelled peptide. Adsorption assays of KRFK on coated microspheres were performed in the presence of growing concentrations of NaCl or imidazole. The ions were able to displace the peptide from the particles, which demonstrated ionic interactions, probably involving carboxylate groups of alginate. Adsorption isotherms showed that gel strength influenced affinity (4×10^5 L/mol or 8×10^5 L/mol for gelation with 5% or 20% $CaCl_2$, respectively). Binding site number doubled (from 2.6×10^{-7} mol/mg to more than 5×10^{-7} mol/mg) when microsphere size decreased from 450 μm to 100 μm. Binding sites were assumed to be located in the gel underneath the membrane.

1. Introduction

Recently we reported on calcium alginate gel microspheres coated with a human serum albumin (HSA)-alginate membrane using a transacylation reaction (Hurteaux et al., 2005). Initially developed for large coated beads (Lévy and Edwards-Lévy, 1996; Edwards-Lévy and Lévy, 1999), the method was adapted to the preparation of microspheres by introducing an emulsification step of an aqueous solution of sodium alginate, propylene glycol alginate (PGA) and HSA in an oily phase. Addition of calcium chloride to the emulsion induced *in situ* gelation of the droplets. The resulting gel microspheres were transferred in a solution of HSA, and added with NaOH, which started the formation of amide bonds between ester groups of PGA and amino groups of the protein, giving a membrane around the particles. We showed that these particles were biocompatible, and able to release a fluorescein-isothiocyanate (FITC)-labelled biologically active peptide (lysine-arginine-phenylalanine-lysine, KRFK) during a prolonged period of time *in vitro* (Hurteaux et al., 2005). The peptide-loaded microparticles were intended to be fixed to a prosthetic biomaterial so as to slowly release KRFK locally, for an efficient stimulation of osteoblastic activity and of bone matrix production (Adams, 2001; Centrella, 1994), leading to a better osteointegration of the biomaterial (Tabata, 2000).

The first aim of the present work was to study the release kinetics of the unlabelled KRFK from the coated microparticles. After a preliminary study conducted with methylene blue chosen as a model of cationic molecule, several series of release experiments were performed with KRFK using different release media.

The second purpose of the work was to obtain information on the nature of the interaction of the peptide with the particles.

First, a series of loading experiments with KRFK was conducted in the presence of growing amounts of ions, namely NaCl or imidazole.

In the next step, KRFK adsorption assays were performed as a function of KRFK concentration. This study was conducted varying

* Corresponding author. Tel.: +33 326 91 80 53; fax: +33 326 91 37 44.
E-mail address: florence.edwards@univ-reims.fr (F. Edwards-Lévy).

0378-5173/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2008.09.011

the preparation parameters of the microspheres in order to investigate the influence of microsphere size (variation of stirring speed) and of gel strength of the core (variations of $CaCl_2$ concentration) on microsphere morphological characteristics on the one hand, and on interactions with the peptide on the other hand.

Three different types of coated microspheres were then prepared: small-sized microspheres (≈100 μm in diameter) prepared with $CaCl_2$ 20% (standard conditions), small-sized microspheres prepared with $CaCl_2$ 5%, and large-sized microspheres (≈450 μm in diameter) prepared with $CaCl_2$ 20%.

For comparison, control particles without calcium alginate gel were prepared by transacylation between PGA and HSA in emulsion. In this method, recently described (Callewaert et al., 2007), an aqueous solution of PGA and HSA is emulsified in an oily phase. Then an ethanolic solution of NaOH is added to the emulsion, in order to start the transacylation reaction, which resulted in microspheres made of a network of crosslinked polymers without calcium alginate gel.

2. Materials and methods

2.1. Materials

Sodium alginate (NaAlg, Manugel GHB®, supplier's specification: viscosity of a 1% (w/w) aqueous solution at 25 °C was 91 mPa s) and propylene glycol alginate (Kelcoloïd S®, supplier's specification: viscosity of a 2% (w/w) aqueous solution at 25 °C was 118 mPa s) were gifts from ISP. Human serum albumin was from Baxter. For the emulsion process, the oily phase was composed of isopropyl myristate (SDF) added with 5% (w/v) surfactant (sorbitan trioleate, Seppic). Ethanol (95%, v/v) was supplied by Charbonneaux-Brabant. Water (Fresenius Kabi) was sterile. For the washing step, water was added with polysorbate (Seppic) 2% (w/v). KRFK (95% grade) was from Neo-MPS. Other reagents were provided by Prolabo.

2.2. Preparation of the microparticles

2.2.1. Serum albumin-alginate coated microspheres

Microparticles 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, 10 mL of an aqueous phase consisting of 2% (w/v) PGA, 1% NaAlg and 4% HSA in saline was emulsified at a stirring speed of 4000 rpm (standard conditions, batch 1, Table 1) or 1500 rpm in 50 mL of the oily phase. After 5 min stirring, 35 mL of an aqueous $CaCl_2$ solution (20%) was added and agitation was continued for 15 min. After a centrifugation step, calcium alginate microspheres were resuspended in 50 mL of a 2.5% HSA aqueous solution and magnetically stirred. Ten milliliters of 0.5 M NaOH was added dropwise to the microsphere suspension and the transacylation reaction was allowed to develop for 15 min. The reaction was stopped by dispersing the coated microspheres in 50 mL of imidazole pH 7 buffer for 15 min. They were then washed (water + polysorbate, once) and rinsed (water, three times). Particles were either kept at 4 °C, or congealed and lyophilized (LabConco, Freezone 6).

Table 1
Membrane-coated alginate gel microspheres (batches 1–3) and control microparticles without calcium alginate gel (batch 4). Size of the microspheres as a function of the preparation parameters. Batch 1: standard conditions.

Batch	Stirring speed (rpm)	$CaCl_2$ concentration (%)	Diameter mean ± S.D. (μm)
1	4000	20	104 ± 112
2	1500	20	436 ± 278
3	4000	5	94 ± 86
4	2500	–	96 ± 84

2.2.2. Control PGA–HSA microparticles

Control microparticles without calcium alginate gel were prepared omitting the gelation step, as already described (Callewaert et al., 2007). Briefly, 6 mL of an aqueous phase consisting of 2% PGA and 20% HSA in water was emulsified in 40 mL of the oily phase at a stirring speed of 2500 rpm. After 5 min stirring, 2 mL of a 2% NaOH solution in 95% (v/v) 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 for the neutralization of the emulsion. After 15 more min, agitation was stopped and the microspheres were washed and rinsed as described for coated microspheres, and either kept at 4 °C, or congealed and lyophilized.

2.3. Morphological characterization

2.3.1. Optical microscopy

The microspheres were observed with a light microscope (Olympus, BH-2) equipped with interferential phase contrast and using a CCD camera (DP-50) coupled with the AnalySIS software (Soft Imaging System) for image recording. Photographs were selected from several observations.

In order to observe the internal structure of control PGA–HSA microparticles more accurately, microsphere samples were incubated 1 h at room temperature with tissue freezing medium (Jung, Leica Microsystems) before freezing at −20 °C. A series of transverse sections (thickness: 10 μm) was cut with a cryostat (Leica LM 1850, Leica Microsystems). Internal morphological observations were made using optical microscopy as described above, after staining with a 0.025% solution of Coomassie brilliant blue during 15 min, and rinsing with distilled water. This specific marker for proteins was used with the aim of locating the HSA in the microspheres. Such a study had already been performed with the coated microspheres in our previous work (Hurteaux et al., 2005).

2.3.2. Scanning electron microscopy

SEM observations (JSM-5400LV, JEOL) were made after alcohol dehydration of microsphere suspensions. The samples were then coated with a layer of Au/Pd (12–40 nm).

2.3.3. Size distribution

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

2.4. Interactions with methylene blue

This study was performed using small coated microspheres gelified with 20% $CaCl_2$ (batch 1) and control PGA–HSA microparticles without calcium alginate gel (batch 4, Table 1), respectively.

For loading experiments, 5 mg of freeze-dried microspheres were suspended in 10 mL of a 5-μg/mL methylene blue solution in water. The suspension was maintained at 37 °C and magnetically agitated. At intervals, 1.5 mL of the suspension was removed and centrifuged at 2000 rpm for 2 min. The methylene blue content of the supernatant was measured by reading the absorbance at 664 nm with a spectrophotometer (DU640B, Beckman).

For release experiments, 5 mg of freeze-dried microspheres were rehydrated with 20 μL of a 2.5-mg/mL methylene blue solution in water, according to the technique of Kawaï et al. (2000). After complete absorption of the liquid, the microspheres were suspended in 10 mL of water and magnetically agitated. The methylene

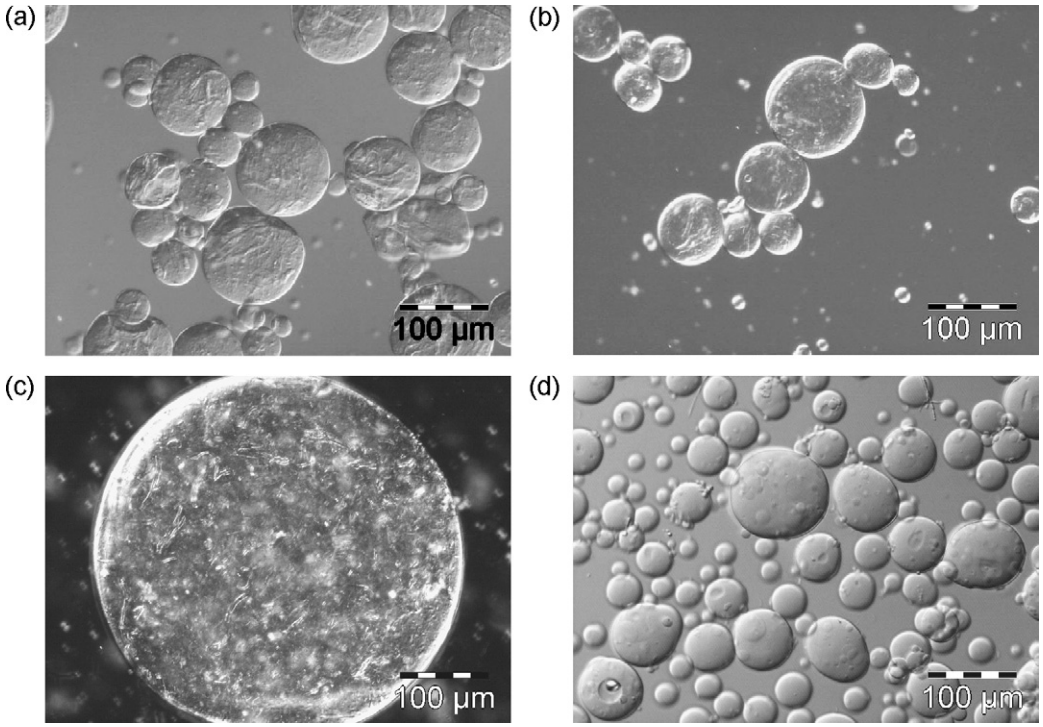

Fig. 1. Microphotographs of coated microspheres: (a) small, $CaCl_2$ 5%, batch 3; (b) small, $CaCl_2$ 20% batch 1; (c) large, $CaCl_2$ 20% batch 2; (d) of control PGA–HSA microparticles batch 4.

blue release as a function of time was followed as for the loading experiment.

For the two series of assays, three independent experiments were made and data are presented as mean ± standard deviation.

2.5. Peptide release kinetics from microparticles in various aqueous solutions

Five milligrams of freeze-dried microparticles of batch 1 were impregnated with 20 μL of a 26.3-mg/mL KRFK solution in water. The loaded particles (105.2 μg or 182 nmol KRFK/mg) were then immersed in 10 mL of release medium. Phosphate-buffered saline (PBS), imidazole pH 7 buffer and saline were tested. Release kinetics of KRFK from the particles was determined at 37 °C, under magnetic agitation in each release medium. At intervals, aliquots were centrifuged and the supernatants analyzed by HPLC.

The HPLC apparatus consisted of a binary HPLC pump (Waters 1525) and a UV absorbance detector (Waters 2487) set at 210 nm. Chromatography was performed at room temperature on a reverse-phase column (C18 Spherisorb ODS-2, 150 mm length, 4.6 mm diameter, Alltech). The mobile phase consisted of a mixture water/acetonitrile 80/20 containing 0.5% of TFA. The flow rate was 1 mL/min.

2.6. Interactions between KRFK and microspheres in the presence of growing concentrations of ions

The assays were performed at a constant concentration of KRFK (18.3 μg/mL, i.e., 31.7 μmol/L), using a 1-h incubation time. Five milligrams of freeze-dried microspheres of batch 1 were rehydrated in 10 mL of water for 1 h at 37 °C under magnetic agitation. One milliliter of the suspension was then transferred in 5 mL of KRFK solutions in NaCl solutions (NaCl concentration: 0–85 mmol/L) or pH 7 buffer dilutions (imidazole concentration: 0–50 mmol/L). After 1 h at 37 °C under magnetic agitation, 600 μL aliquots were centrifuged and the supernatants were analyzed by HPLC. Each point represents the mean of three measurements ± standard deviation.

2.7. Adsorption assays varying KRFK concentration

The assays were performed in pure water, at a concentration of KRFK varying from 5 μg/mL to 75 μg/mL, using a 1-h incubation time. Five milligrams of freeze-dried microspheres were rehydrated in 10 mL of water for 1 h at 37 °C under magnetic agitation. One milliliter of the suspension was then transferred in 5 mL of KRFK solutions of known concentrations in pure water (KRFK final concentration: 5–75 μg/mL, i.e., 8.65–129.8 μmol/L). After 1 h at 37 °C under magnetic agitation, 600 μL aliquots were centrifuged and the supernatants were analyzed by HPLC. Each point represents the mean of three measurements ± standard deviation.

This study was applied to the four different types of microspheres appearing in Table 1, namely three types of coated microspheres and control PGA–HSA microspheres without calcium alginate gel.

3. Results and discussion

3.1. Microparticle size

For this study, we set stirring speeds in order to prepare small microspheres (4000 rpm for particles of about 100 μm in diameter), and larger ones (1500 rpm for a mean diameter of about 450 μm). Combining the variations in the preparation parameters, we thus prepared small strongly gelified particles (batch 1), small weakly gelified particles and large strongly gelified particles, for which mean diameters and standard deviations are summarized in Table 1.

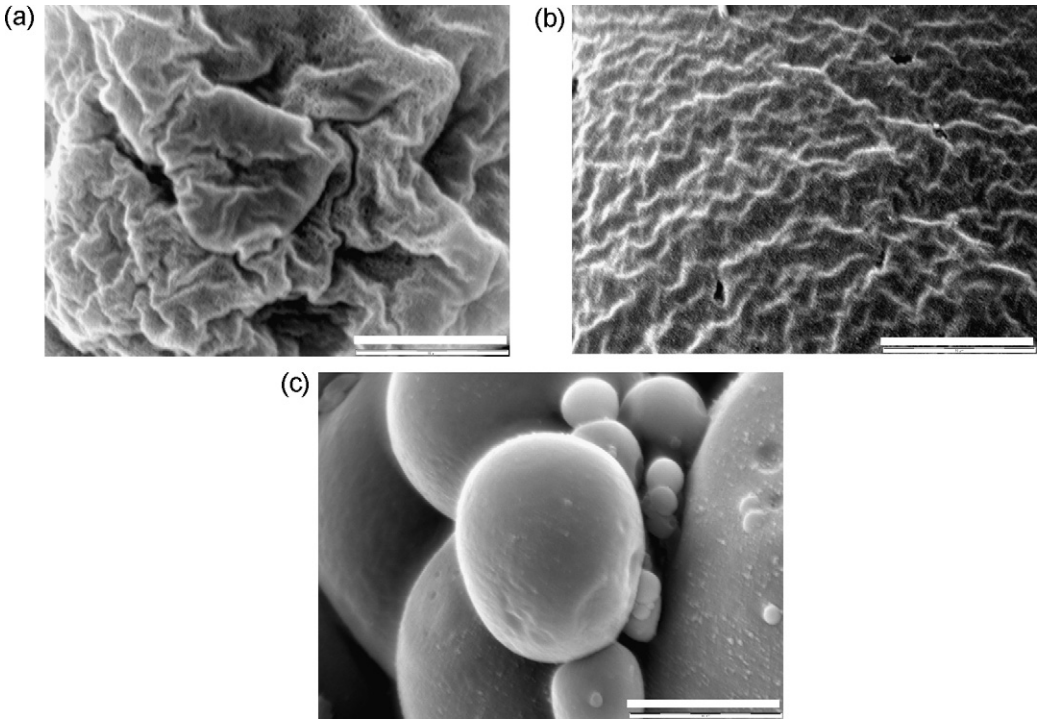

Fig. 2. SEM observations of coated microsphere surface: (a) small, $CaCl_2$ 5% batch 3; (b) small, $CaCl_2$ 20%, batch 1; (c) of control PGA–HSA microparticle surface, batch 4. Scale bar = 10 μm.

For the preparation of control microparticles lacking calcium alginate gel, the stirring speed had to be set at 2500 rpm to produce particles with a diameter close to the size of the small coated microspheres (Table 1).

3.2. Influence of preparation parameters on microparticle morphological characteristics

Using light microscopy, no significant difference could be observed between weakly ($CaCl_2$ 5%) and strongly ($CaCl_2$ 20%) small gelified microspheres (Fig. 1a and b). Raising the mean diameter of the microspheres did not alter the general morphological aspect (Fig. 1c). The control PGA–HSA microspheres looked very similar to the former ones (Fig. 1d).

SEM observations revealed that microsphere surface of coated microspheres presented different profiles depending on the $CaCl_2$ concentration used for the gelation step (Fig. 2). The surface of the weakly gelified microspheres was deeply rumpled (Fig. 2a) whereas that of strongly gelified microspheres was finely rugged (Fig. 2b). This observation can be related to the microsphere internal gel properties: a stronger gel prevents membrane deformation during sample dehydration for SEM examination. Furthermore, a denser cross-linked calcium alginate gel is expected to retain more HSA and PGA available for the transacylation step, leading to the formation of a stiffer and smoother membrane (Hurteaux et al., 2005).

In contrast, control microspheres revealed a very smooth and regular surface after dehydration (Fig. 2c). It seems to indicate that the PGA–HSA network constituting the spheres was rigid enough to withstand the dehydration step without deformation. This was confirmed by observation of the internal structure of the particles. In contrast with our previous study of slices of coated beads showing an intensely stained external layer (Hurteaux et al., 2005), slices of control microspheres revealed a more homogeneous inner morphology. They presented a blue color gradient up to the center, indicating that the protein was also present in deep regions of the microparticles (Fig. 3). The morphology of these PGA–HSA microparticles as a function of preparation parameters will be further investigated.

3.3. Interactions with methylene blue

Before evaluating the binding parameters of the microspheres with KRFK, preliminary studies were performed to examine the general behaviour of the microspheres towards a charged molecule. We chose methylene blue as a model, because it shares some similar properties with the peptide, i.e., a global positive charge and a close molecular weight.

Fig. 4 presents the results of interaction studies between methylene blue molecules and coated microspheres of batch 1, or control

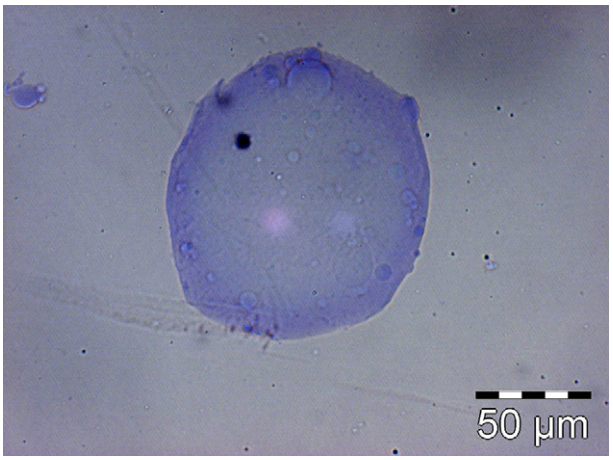

Fig. 3. Microphotograph of a section (thickness 10 μm) of a control PGA–HSA batch 4 microparticle stained with Coomassie blue. (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

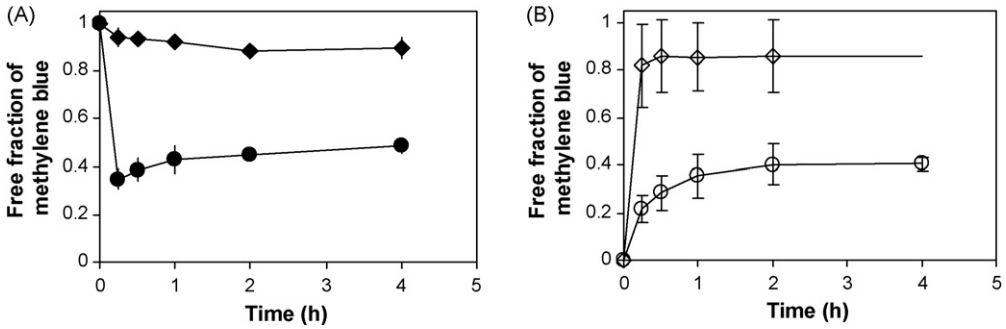

Fig. 4. Free fraction of methylene blue as a function of time, 5 mg microparticles in 10 mL aqueous medium, maximum dye concentration = 5 μg/mL: (A) loading experiment and (B) release experiment. Circles, small coated microspheres, batch 1 ($CaCl_2$ 20%); diamonds, control PGA–HSA microparticles, batch 4. Data are means of three independent experiments ± standard deviations.

PGA–HSA microparticles (batch 4). Two experiments were performed, i.e., loading (Fig. 4A) and release (Fig. 4B) assays.

In the two series of experiments, the curves reveal that an equilibrium situation was reached after about a 1-h contact.

The control PGA–HSA microparticles appeared to have no strong interaction with methylene blue. The loading experiment showed that the control microspheres absorbed only a small amount of methylene blue after 2 h (free fraction: 0.9), which did not increase after 4 h. In the release experiment, a burst effect was observed: after only 15 min, more than 90% of the methylene blue was released in the surrounding water, indicating a fast exchange between the microspheres and water. The fraction of molecules present in solution was close to 0.9 at the plateau.

The coated microspheres presented a different behaviour. During the first minutes of loading experiments, the microspheres captured a higher amount of molecules, a small part of which being released during the following hour until a plateau was reached (free peptide fraction: 0.49). This observation could be related to the swelling of freeze-dried microspheres during the first minutes of immersion in the solution, before the equilibrium situation. In the case of release, a plateau value was reached within about 1 h (free peptide fraction: 0.40).

These close plateau values show that both loading and release experiments led to comparable equilibrium situations. This observation is consistent with the existence of a maximal binding capacity for the molecule. The plateau values indicate that microspheres containing an alginate gel core presented a higher binding capacity for methylene blue than microspheres made of PGA and HSA only.

This preliminary study shows that interactions between a cationic small molecule and the microspheres results in an equilibrium situation. This makes loading and release experiments, as defined above, equivalent for the equilibrium characterization.

Moreover, comparing the methylene blue fraction fixed onto membrane-coated alginate gel microspheres with the small fraction fixed by particles without alginate gel (control particles), the binding of this cationic molecule was thought to concern the gel, and more precisely was hypothesized to occur via ionic interactions onto negatively charged carboxylate groups of alginate, as already observed (Martinsen et al., 1992; Gombotz and Wee, 1998; Chrétien et al., 2004).

3.4. KRFK release kinetics from microparticles in various aqueous solutions

When peptide-loaded microparticles of batch 1, were soaked in different ionic solutions, i.e., saline, PBS or imidazole pH 7 buffer, peptide release was surprisingly rapid in the 3 media and a plateau value was reached in less than 1 h (Fig. 5).

It should be pointed out that this result is very different from the release profile we previously observed with the FITC-labelled KRFK, where the release out of similar microspheres lasted more than 8 days in the imidazole pH 7 buffer. Recent studies indicate that fluorescent labels such as FITC appear to change the surface charge of proteins significantly, and thereby influence their behaviour in solution and their interactions with oppositely charged polyelectrolytes (Ramasamy et al., 2007). This phenomenon should be even more pronounced when the molecular weight of the fluorescent group used for the labelling is in the same range as the molecular weight of the labelled molecule, as it is the case for FITC (MW = 389.4 Da) and KRFK (MW = 577.7 Da). In addition, the FITC group is assumed to slow down diffusion of the peptide out of the microparticles due to steric hindrance reasons. This observation emphasizes the importance of using unlabelled molecules for release studies from drug delivery systems.

The fraction of KRFK released at the plateau was shown to depend on the release medium. When the experiment was performed in saline, 80% of the encapsulated peptide was released in 15 min. In PBS, the plateau value hardly reached 90% after 30 min. When using the imidazole buffer, 45% of the KRFK was released in 30 min. No further release of KRFK could be observed in the three different aqueous media after 20 days (480 h).

The plateau value depending on the aqueous solution composition, it was decided to study the nature of interactions between KRFK and the microspheres as a function of preparation parameters.

3.5. Interactions between KRFK and microspheres in the presence of growing concentrations of ions

KRFK adsorption experiments were performed in particle suspensions (0.5 mg microparticles in 6 mL of a 31.7-μmol/L KRFK solution) containing NaCl at different concentrations, in order to evaluate after 1 h a competitive effect of Na^+ ions towards KRFK. In the hypothesis of ionic interactions between KRFK and alginate, a displacement by other positive ions should be observed. The free peptide concentration was measured as a function of Na^+ concentration in the solution and the results are presented in Fig. 6.

In pure water, the free fraction of KRFK was 0.089 ± 0.003. For the most diluted saline solutions, the presence of Na^+ had no effect on free peptide fraction. For concentrations higher than 10^{-3} M NaCl, an increase in Na^+ concentration induced a progressive increase in free peptide fraction. As Na^+ and KRFK molecules are both positively charged, this observation can be explained by a displacement of KRFK by Na^+. This type of behaviour had already been described for

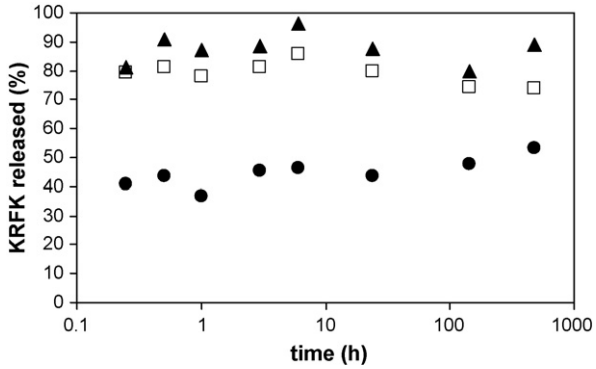

Fig. 5. Release kinetics of KRFK (910 nmol) from 5 mg of small coated microspheres, gelified with $CaCl_2$ 20%, batch 1, in 10 mL of saline (circles), PBS (triangles) or imidazole pH 7 buffer (squares).

calcium alginate microspheres loaded with a cationic drug (Tomida et al., 1993). It was also reported for hydrophilic albumin microspheres loaded with a salt of basic antitumour agent (Goldberg et al., 1984; Sawaya et al., 1987a, 1988). The coated microspheres then seemed to behave as ion-exchange resins. The most probable binding sites in the microspheres should be the carboxylate groups available in the alginate gel.

Fig. 6 shows that a Na^+ concentration of 85 mmol/L led to a KRFK free fraction of 0.72, corresponding to 0.023 mmol/L. An even higher Na + concentration would be required to release the whole amount of peptide. This observation could be indicative of a higher affinity of the microspheres for the peptide, as compared to Na^+ ions. Previous studies mentioned that the electrostatic interaction between divalent cations and their binding sites is stronger as compared with monovalent compounds, due to dual site binding (Sawaya et al., 1987a; Hänninen et al., 2007). The ε-amino groups of the two lysine residues of the KRFK, charged at neutral pH ($pK_a = 10.54$), probably bind two carboxylate groups of the particles leading to a stronger interaction.

This experiment was reproduced with imidazole buffer dilutions (Fig. 6). The values of free peptide compared for equivalent concentrations of Na^+ and imidazole showed similar trends, indicating that positively charged molecules with a higher molecular weight than sodium ion, like imidazole, can also exchange with peptide molecules

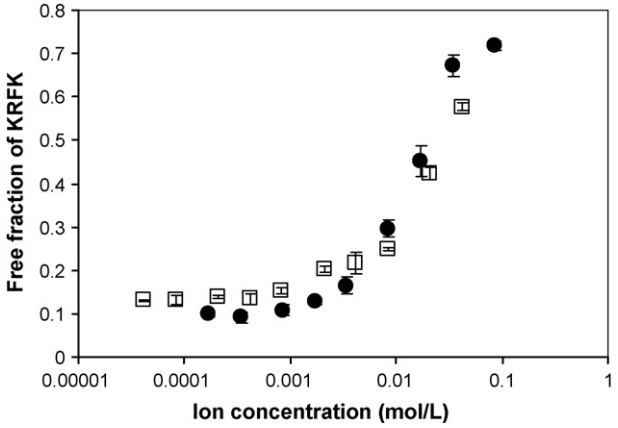

Fig. 6. Free fraction of peptide KRFK as a function of NaCl concentration (circles) or imidazole concentration (squares) after adsorption of KRFK on small coated microspheres, gelified with $CaCl_2$ 20%, batch 1, 0.5 mg microparticles in 6 mL of a 31.7-μmol/L KRFK solution. Data are means of three measurements ± standard deviations.

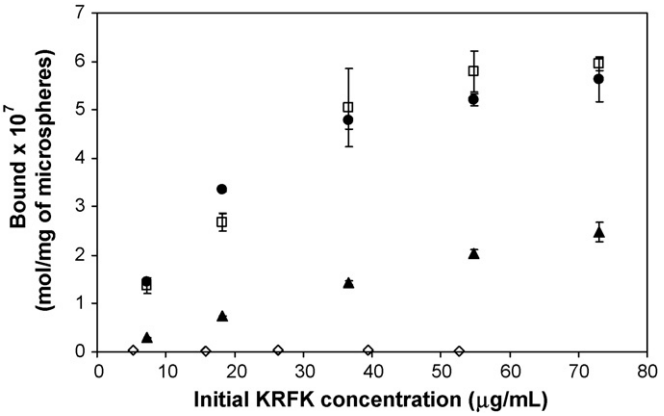

Fig. 7. Adsorption isotherms of KRFK on microparticles, 0.5 mg microparticles in 6 mL of KRFK solutions of various concentrations. Circles, small coated microspheres, gelation with $CaCl_2$ 20%, batch 1; squares, small coated microspheres, gelation with $CaCl_2$ 5%, batch 3; triangles, large coated microspheres, gelation with $CaCl_2$ 20%, batch 2; diamonds, PGA–HSA microparticles, batch 4. Data are means of three measurements ± standard deviations.

3.6. Adsorption as a function of KRFK concentration

The adsorption isotherms of KRFK onto the different microsphere types were determined using initial concentrations of KRFK in pure water in the 5–75 μg/mL range, i.e., 8.65–129.8 μmol/L. The results are presented in Fig. 7. The isotherm of KRFK adsorption onto control microparticles lacking calcium alginate gel showed that very low adsorption occurred in the concentration range used in this study. It confirms the results obtained with methylene blue and points out the requirement of an alginate gel for KRFK binding.

For the different coated microspheres, the amount of bound KRFK progressively increased with the initial peptide concentration. The gel strength influence was studied on small microspheres. The particles, strongly or weakly gelified, presented close adsorption isotherms. The maximal value was of about 5.5×10^{-7} mol/mg of microspheres. Adsorption of KRFK was significantly lower onto large microspheres ($p < 0.01$). For equal KRFK concentrations, the bound peptide amount was about twice smaller than for small microspheres. The maximal value was of about 2.5×10^{-7} mol/mg of microspheres.

The data obtained from isotherms of KRFK adsorption onto microspheres were processed according to the Scatchard plot's analysis in order to determine the binding site properties (Scatchard et al., 1950; Sawaya et al., 1987b). Scatchard described the equilibrium binding parameters by the equation:

$$\frac{B}{F} = K(n - B)$$

where B is the amount of KRFK moles bound to 1 mg of microspheres, F is the concentration of free peptide in solution, n is the maximum site number on 1 mg of microspheres and K the equilibrium constant of the system.

For control microparticles, adsorption was so low that no Scatchard plot could be drawn. Control microspheres were made of a network of PGA and HSA cross-linked through amide bonds. As compared to alginate, the PGA molecule presents much fewer binding sites (free carboxylates) able to link with a cation like KRFK, especially when linked to a protein. In this respect, the addition of PGA to alginate is known to decrease ionic interactions with divalent cations (Sugawara et al., 1994; Tateshita et al., 1993).

Values of K and n, obtained for the three types of coated microspheres and determined from the graph, are given in Table 2.

Table 2
Values determined from the Scatchard representation: equilibrium constant (K) and total binding sites number (n) as a function of size and gelation of coated microspheres

	Small MS, $CaCl_2$ 5% (□) (batch 2)	Small MS, $CaCl_2$ 20% (●) (batch 1)	Large MS, $CaCl_2$ 20% (▲) (batch 3)
K (L/mol)	$4.2 \pm 1.9.10^{5\#}$	$8.0 \pm 0.9.10^{5\#}$	$8.0 \pm 3.1.10^{5}$
n (mol/mg)	$5.8 \pm 2.0.10^{-7}$	$5.3 \pm 0.5.10^{-7*}$	$2.6 \pm 0.6.10^{-7*}$

#: $p < 0.01$; *: $p < 0.01$.

In contrast with the maximum number of binding sites, which was not modified, gel strength affected the affinity for the peptide. Strongly gelified microspheres presented higher affinity for the peptide ($K = 8.0 \times 10^5$ L/mol) than weakly gelified ones ($K = 4.2 \times 10^5$ L/mol) ($p < 0.01$). Previous studies had already shown an influence of gel strength on interactions between microspheres and some drugs or ions, a high calcium content in the alginate gel generally leading to a prolonged drug release (Tonnesen and Karlssen, 2002). In a recent study, Silva et al. (2006) reported that no systematic effect of Ca^{2+}/alginate mass ratio was observed on insulin encapsulation efficiency. The insulin encapsulation reached a maximum for a 7.3% (w/w) Ca^{2+}/alginate ratio and then decreased in a non-linear way when the Ca^{2+} concentration increased. It was explained by the ambiguous effect of an increase in gel strength. More molecules could be retained in the network but binding sites would become less accessible for insulin.

In the present study, gel strength did not significantly influence the binding site number, but influenced the affinity parameter. However, no simple interpretation could be drawn because variations in gel strength may have a lot of direct or indirect consequences like changes in Ca^{2+} and Na^+ local concentrations with impact on the available negative charges of alginate, modification of the network density (binding sites more or less accessible), changes in PGA and HSA internal concentrations (different nature of binding sites), etc.

The size of the microspheres (for 20% $CaCl_2$) did not significantly influence microsphere affinity for the peptide, but appeared to affect the maximum number of available binding sites. The small microspheres exhibited a number of binding sites of 5.3×10^{-7} mol KRFK per mg dry microspheres, and this binding site number fell to 2.6×10^{-7} mol/mg for the large ones. As small microspheres exposed larger specific area, this result suggests that the binding sites for KRFK would be located near the surface. As the covalent PGA–HSA network of control microspheres was shown to have very few binding sites for the peptide (see Fig. 7), it could mean that most of the binding sites would be located in the gelified part of the coated microspheres, underneath the PGA–HSA membrane.

In conclusion, the study demonstrates that the unlabelled KRFK interacts differently with the coated particles as compared with the FITC-labelled peptide. It is thought that the equilibrium situation was more rapidly reached due to changes in the properties of the unlabelled molecule, i.e., size, charge, and hydrophilicity. These changes were all the more pronounced as the small molecular size of the peptide was close to that of the FITC group of the labelled form. This resulted in a more rapid release of the unlabelled KRFK as compared with the FITC-labelled peptide.

In addition, this work brings information about the mechanisms involved in the interaction of charged molecules with microspheres consisting of a calcium alginate gel surrounded by a membrane of cross-linked PGA and HSA. Experiments performed with PGA–HSA microspheres without calcium alginate gel showed that the PGA–HSA network in these spheres did not ensure an efficient binding of KRFK, which confirmed the determining role of the alginate core in the coated microspheres for binding the peptide. Moreover, interactions between the peptide and the microparticles studied in this work were shown to be of ionic nature as demonstrated by the series of adsorption assays performed in the presence of growing amounts of ions. Negatively charged carboxylate groups of alginate are assumed to be concerned in binding. Lastly, the adsorption experiments conducted varying the preparation parameters of the microspheres showed that increasing the gel strength of alginate microspheres coated with a covalent membrane made of PGA and HSA enhanced the binding affinity, while the number of available binding sites remained unchanged. Moreover, an increase in microsphere size induced a decrease in binding site number.

As interactions occurred thanks to ionic bonding in the alginate gel, they were assumed to be located in the gel part, underneath the membrane.

Although our results could not show a release of KRFK over a prolonged period of time, these gel particles stabilized by an external membrane, easily lyophilized and easily loaded, might represent an interesting tool for the administration of peptides by alternative routes such as nasal route. In this respect, it is known that hydrogel microspheres can absorb water from the nasal mucosa and thus bring about a temporary dehydration of the epithelial membrane and opening of the tight junctions (Wang et al., 2006). Such microspheres thus can improve drug absorption.

Acknowledgements

The support of Région Champagne-Ardenne is gratefully acknowledged. The authors thank S. Bouthors for technical assistance with SEM.

References

Adams, J.C., 2001. Thrombospondins: multifunctional regulators of cell interactions. Annu. Rev. Cell Dev. Biol. 17, 25–51.
Callewaert, M., Laurent-Maquin, D., Edwards-Lévy, F., 2007. Albumin-alginate coated microspheres: resistance to steam sterilization and to lyophilization. Int. J. Pharm. 344 (1–2), 161–164.
Centrella, M., 1994. Transforming growth factor-β gene family members and bone. Endocr. Rev. 15, 27–39.
Chrétien, C., Boudy, V., Allain, P., Chaumeil, J.-C., 2004. Indomethacin release from ion exchange microspheres: impregnation with alginate reduces release rate. J. Control. Release 96, 369–378.
Edwards-Lévy, F., Lévy, M.-C., 1999. Serum albumin-coated beads: mechanical properties and stability. Biomaterials 20, 2069–2084.
Goldberg, E.P., Iwata, H., Longo, W., 1984. Hydrophilic albumin and dextran ion-exchange microspheres for localized chemotherapy. In: Davies, S.S., Illum, L., McVie, J.G., Tomlinson, E. (Eds.), Microspheres and Drug Therapy. Pharmaceutical, Immunological and Medical Aspects. Elsevier Science Publishers B.V., pp. 309–325.
Gombotz, W.R., Wee, S.F., 1998. Protein release from alginate matrices. Adv. Drug Deliv. Rev. 31, 267–285.
Hänninen, K.R., Murtomäki, L.S., Kaukonen, A.M., Hirvonen, J.T., 2007. The effect of valence on the ion-exchange process: theoretical and experimental aspects on compound binding/release. J. Pharm. Sci. 96, 117–131.
Hurteaux, R., Edwards-Lévy, F., Laurent-Maquin, D., Lévy, M.-C., 2005. Coating alginate microspheres with a serum albumin-alginate membrane: application to the encapsulation of a peptide. Eur. J. Pharm. Sci. 24, 187–197.
Kawaï, K., Suzuki, S., Tabata, Y., Ikada, Y., Nishimura, Y., 2000. Accelerated tissue regeneration through incorporation of basic fibroblast growth factor-impregnated gelatine microspheres into artificial dermis. Biomaterials 21, 489–499.
Lévy, M.-C., Edwards-Lévy, F., 1996. Coating alginate beads with cross-linked biopolymers: a novel method based on a transacylation reaction. J. Microencapsul. 13, 169–183.
Martinsen, A., Storro, I., Skjak-Braek, G., 1992. Alginate as immobilization material. II. Diffusional properties. Biotechnol. Bioeng. 39, 186–194.
Ramasamy, P., Raafat El-Maghrabi, M., Halada, G., Miller, L.M., Rafailovich, M., 2007. Examination of interactions of oppositely charged proteins in gels. Langmuir 23, 2021–2029.

Sawaya, A., Benoit, J.-P., Benita, S., 1987a. Binding mechanism of doxorubicin in ion-exchange albumin microspheres. J. Pharm. Sci. 76, 475–480.
Sawaya, A., Deyme, M., Benoit, J.-P., 1987b. Calcium and thiocyanate interactions with crosslinked albumin microcapsules: influence of the reticulation rate. Biomaterials 8, 211–216.
Sawaya, A., Fickat, R., Benoit, J.-P., Puisieux, F., Benita, S., 1988. Ion-exchange albumin microcapsules of doxorubicin: an in vitro release kinetic evaluation. J. Microencapsul. 5, 255–267.
Scatchard, G., Scheinberg, I.H., Armstrong Jr., S.H., 1950. Physical chemistry of protein solutions. V. The combination of human serum albumin with thiocyanate ion. J. Am. Chem. Soc. 72, 540–546.
Silva, C.M., Ribeiro, A.J., Figueiredo, I.V., Rocha Gonçalves, A., Veiga, F., 2006. Alginate microspheres prepared by internal gelation: development and effect on insulin stability. Int. J. Pharm. 311, 1–10.
Sugawara, S., Imai, T., Otagiri, M., 1994. The controlled release of prednisolone using alginate gel. Pharm. Res. 11, 272–277.
Tabata, Y., 2000. The importance of drug delivery systems in tissue engineering. PSTT 3, 80–89.
Tateshita, K., Sugawara, S., Imai, T., Otagiri, M., 1993. Preparation and evaluation of a controlled-release formulation of nifedipine using alginate gel beads. Biol. Pharm. Bull. 16, 420–424.
Tomida, H., Mizuo, C., Nakamura, C., Kiryu, S., 1993. Imipramine release from Ca-alginate gel beads. Chem. Pharm. Bull. 41, 1475–1477.
Tonnesen, H., Karlssen, J., 2002. Alginate in drug delivery systems. Drug Dev. Ind. Pharm. 28, 621–630.
Wang, J., Tabata, Y., Morimoto, K., 2006. Aminated gelatine microspheres as a nasal delivery system for peptide drugs: evaluation of in vitro release and in vivo insulin absorption in rats. J. Control. Release 113, 31–37.