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Removal of ciprofloxacin in simulated digestive media by activated charcoal entrapped within zinc-pectinate beads

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ABSTRACT

Beads made of a zinc-pectinate matrix containing activated charcoal were designed for the adsorption of colonic residual antibiotics responsible of the emergence of resistance. Bead stability was shown to correlate with bead zinc content, 0.08 mg/mg being the minimal amount of zinc that protects the egg-box structure against total disintegration. Moreover, the stability in simulated gastro-intestinal media was shown to be related to the composition of the incubation medium. Indeed, gastric medium was shown to extract a large amount of zinc inducing an early disintegration of the beads in the intestinal medium, making necessary their protection by gastro-resistant capsules. Simulated intestinal medium buffered by phosphate was not adapted for the disintegration studies since the formation of a zinc phosphate precipitate on beads surface enhances their resistance to further degradation by pectinases contained in colonic medium. On the other hand, beads incubated in HEPES were stable in intestinal medium and nicely degraded by pectinases contained in simulated colonic medium. Despite this stability, coating with Eudragit RS® was needed to prevent the early adsorption of antibiotics in intestinal medium. Adsorption studies in the simulated colonic medium show that the adsorption capacity of activated charcoal is not modified after its encapsulation within pectin beads making the elimination of ciprofloxacin reaching the colon clinically feasible.

1. Introduction

Bacterial resistance to antibiotics emerged shortly after their discovery and has risen continuously since. Nevertheless, as new antibiotics were being made available on a regular basis, it was not considered a major public health problem until the last decade (Okeke et al., 2005a,b). Indeed, as experience has shown, new antibiotics may be effective for a restricted period only and resistance appears sooner or later to each new drug mainly in relation with both their misuse and overuse in humans and animals (de Vries-Hospers et al., 1993). Emergence of resistance in bacteria can result from two different sequences of events, including either a direct one-step selection of resistant clones at the site of infection, or an indirect two-steps process in which commensal resistant bacteria are first selected in the natural ecosystems of humans. They can then transfer resistance mechanisms horizontally to pathogenic species. Such an horizontal transfer can occur in the same host in whom the resistant commensals have emerged, or in another host after inter-individual transmission of the resistant commensals (de Vries-Hospers et al., 1991; van der Waaij and Nord, 2000). It is now established that this second two-steps mechanism is the major route of evolution of bacterial resistance. Indeed, the diversity of bacterial species and their resistance genes present in the natural ecosystems become a quasi-unlimited source of new or variant resistance mechanisms (Salyers et al., 2004).

The intestinal tract is the site where most antibiotic resistance develops (O'Brien et al., 1980; Nikolich et al., 1994). Incomplete absorption of orally administered drugs, secretion of an antimicrobial agent by intestinal mucosa, salivary glands and bile can actually disturb the colonisation resistance of commensal colonic flora which give rise to the emergence of resistance to antibiotics by the indirect two-steps process described above (de Vries-Hospers et al., 1993). In this context, it is hypothesised that colonic inactivation of residual antibiotics can be a solution to maintain the stability of the intestinal microflora. Antibiotics inactivation can be achieved enzymatically as well as non-enzymatically (Welling et al., 1992; de Vries-Hospers et al., 1993). To deliver inactivating agent to the colon, it is possible to use bacteria responsive systems that allow site-specific targeting (Bourgeois et al., 2005a,b).

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For this purpose, we have designed beads made of pectin, a natural polysaccharide that can be enzymatically degraded by enzymes produced by colonic microflora (Sinha and Kumria, 2001; Vandamme et al., 2002). Pectin is composed of linear chains of (1 → 4)-linked α-D-galacturonic acid residues which are characterized by their degree of esterification (DE) and their degree of amidation (DA), both expressed as a percentage of carboxyl groups (esterified or amidated). DE and DA are important means to classify pectin (Liu et al., 2003). Amidated low methoxylated pectins (<50% DE) can react with divalent cations, such as calcium and zinc, to form insoluble egg-box networks. Through this mechanism, pectin has been used to prepare calcium or zinc-pectinate beads for the targeted delivery of drugs to the colon (Aydin and Akbuga, 1996; El-Gibaly, 2002; Bourgeois et al., 2005a,b). We have recently shown that the oral administration of beads containing β-lactamases in a mice model induced an increase of these β-lactams inactivating enzymes in the faeces which was not observed after administration of free β-lactamases (Bourgeois et al., 2005a,b). Moreover, the co-administration of β lactamases loaded beads and amoxicillin, a β-lactam antibiotic, did not modify the pharmacokinetics of this latest in a rat model (Bourgeois et al., 2008).

The goal of the present study is to develop a colonic delivery system of non-enzymatic inactivating agents such as therapeutic adsorbents that can be co-administrated with antibiotics. This system will have the advantage to be applied to a wide range of antibiotics since enzymatic inactivation is restricted to only few specific antibiotic classes (van der Waaij and Nord, 2000). The co-administration of adsorbents with an antibiotic requires that the latest can be protected from the adsorbent in the stomach and small intestine and perfectly adsorbed in the colon. This purpose can be achieved in co-administrating the adsorbents by an appropriate colonic delivery system. Activated charcoal (AC), a natural adsorbent, was encapsulated with success in several types of polymeric systems such as cross-linked agarose (Xu et al., 1981) and chitosan matrix (Chandy and Sharma, 1992) for haemoperfusion or alginate beads cross-linked with different types of metal ions for the removal of organic compounds (Lin et al., 2005). We here propose a system made of zinc-pectinate beads encapsulating activated charcoal in order to Inactivate residual antibiotics in the colon. Their *in vitro* behaviour in simulated intestinal media was clearly demonstrated.

2. Materials and methods

2.1. Materials

Amidated low methoxylated (LM) pectin (Unipectine™ OG 175C, degree of esterification from 22% to 28% and degree of amidation from 19% to 23%) as well as Eudragit® RS were gifts from Evonik Degussa Industries (Essen, Germany). Medicinal activated charcoal was obtained from Merck (Strasbourg, France). Zinc acetate dihydrate, ciprofloxacin and polyethylene glycol 300 (PEG) were obtained from Fluka Chemika (Buchs, Switzerland). 1-(2-pyridylazo)-2-naphthol (PAN), ammonia buffer (pH 10), DEAE dextran hydrochloride, poly-L-lysine, Pancreatine from porcine pancreas, Pectinex1 SP-L (26,000 PG/mL), a mixing of pectinases from Aspergillus aculeatus and other simulated fluids constituents (NaCl, HCl, KH_2PO_4, NaOH, HEPES) were all supplied by Sigma Aldrich (Saint-Quentin-Fallavier, France). Hydroxypropylmethylcellulose phthalate (HMPCP) was a gift from Seppic (Paris, France). HPLC grade solvents were purchased from Carlo Erba Reagenti (Milan, Italy).

Simulated digestive fluids were prepared according to the US Pharmacopeia XXVI Edition as followed:

- **Simulated gastric medium (SGM)** was prepared at a concentration of 2 g/L NaCl, adjusted to pH 1.2 using concentrated HCl (12 M).
- **Simulated intestinal medium (SIM)** contained 0.05 M KH_2PO_4, 77 mL NaOH 0.2 N, 10 g pancreatin (stock solution). The pH was finally adjusted to 6.8 with NaOH 1 M. In an optional SIM formulation, the phosphate buffer was replaced by HEPES (10 mM) buffer.
- **Simulated colonic medium (SCM)** was prepared in 10 mM HEPES, 145 mM NaCl solution. Different concentrations of pectinases were added to the solution and the pH was adjusted to 6 with NaOH 1 M.

2.2. Methods

2.2.1. Beads preparation

Pectin beads were prepared by ionotropic gelation using zinc acetate as gelling agent (El-Gibaly, 2002; Bourgeois et al., 2005a,b). Briefly, for unloaded beads, 3% (w/v) pectin solution was obtained by dissolving 1.5 g of pectin into 50 mL of distilled water. The bubble-free solution was then dropped, using a syringe pump and a 0.8 mm (inner diameter) needle into 50 mL of zinc acetate solution with gentle agitation at room temperature. By contact with zinc ions, pectin droplets formed gelled beads. Beads were allowed to stand in the zinc acetate solution for 30 min before being filtrated. Beads were then washed several times with distilled water. The procedure consisted of soakings of 1 min, in 50 mL ultra-pure water kept under magnetic stirring at 300 rpm. The effect of the number of soakings was evaluated. Beads were finally dried overnight at 37 °C.

Before being loaded into pectin beads, activated charcoal was hydrated into 50 mL of distilled water overnight. The AC suspension was then mixed with a pectin solution. The AC/pectin ratio were varied between 6/3 and 3/3 (w/w). Loaded beads were then prepared in the same manner as unloaded beads. The concentration of zinc acetate in the gelling bath was varied between 6 and 12% (w/w) and the number of washings between 0 and 7.

2.2.2. Bead coating by DEAE dextran

Beads coated with DEAE dextran (AC/pectin ratio = 5/3) were prepared as described above in a solution of zinc acetate (12%, w/v) containing 0.8, 1.2, 1.6 or 2% (w/v) DEAE dextran. A polyelectrolyte complex should form between the positively charged DEAE dextran and the negatively charged pectin (Huguet et al., 1995). The steps following bead preparation were similar to those described above.

Alternatively, the formation of the complex was performed by incubation of wet beads, before washing, during 20 min in a solution of DEAE dextran (0.8, 1.2, 1.6 or 2% (w/v)). Finally beads were washed only once using distilled water.

2.2.3. Bead coating by poly-L-lysine

Unloaded and loaded beads (AC/pectin ratio = 5/3) were prepared in a solution of zinc acetate (12%, w/v). After three washings, beads were incubated during 5 min in a solution of poly-L-lysine (0.05%, w/v) before being washed once more. A polyelectrolyte complex should form between the positively poly-L-lysine and the negatively charged pectin (Liu et al., 2003).

2.2.4. Bead coating by Eudragit® RS

The coating solution was prepared by dissolving 5 g of Eudragit® RS and 1 g of PEG 300 into 100 mL of acetone/ethanol (2/1, v/v). The coating process was carried out by the means of a conventional coating pan (inner diameter 10 cm). The coating equipment was specifically designed for this research to allow batch sizes of only 1 g beads (~750 beads). Beads were preheated in the coating pan

during 5 min. Pan speed was regulated at 20 rpm. The administration of the coating solution was performed manually, considering the small batch quantity, using a hand-held spray gun. Pan temperature was maintained at 37 °C by application of heated air from a heat gun at the outside wall of the pan. The end of the coating process was determined as a function of the increase of beads average weight.

2.2.5. Scanning electron microscopy

The typical external and internal structures of beads were investigated by scanning electron microscopy (SEM). SEM was performed using a LEO 1530 (LEO Electron Microscopy Inc, Thornwood, NY) operating between 1 and 3 kV with a filament current of about 0.5 mA. Dry beads, optionally sliced, were deposited on carbon conductive double-sided tape (Euromedex, France). They were coated with a palladium–platinum layer of ca. 4 nm using a Cressington sputter-coater 208HR with a rotary-planetary-tilt stage, equipped with a MTM-20 thickness controller.

2.2.6. Determination of zinc content in beads

In order to determine the amount of residual zinc contained in beads, 3 mg of beads were completely dissolved into 3 mL of diluted pectinases solution (1:25, v/v) to degrade them. Samples were then filtered on a 0.22 μm PVDF filter to remove AC and obtain a clear solution. Zinc was assayed as described (Arvand et al., 2007; Thanasarakhan et al., 2007). Briefly, one hundred microliters of sample containing Zn^{2+} ions and 1 mL of ammonia buffer solution (pH 10) were placed in 10 mL volumetric flasks. 3 mL of ethanolic PAN stock solution (5×10^{-4} M) was added and adjusted to 10 mL with ethanol. The sample was then shaken and left to stand in a thermostated bath for 10 min at 40 °C to allow complete formation of the PAN-Zn complex. Determination of PAN-Zn concentration was performed by measuring the absorbance at 550 nm. Measurements were performed in triplicate and expressed as zinc weight (mg/mg bead) ± standard deviation. Calibration was performed with known concentrations of zinc from 0.02 to 0.31 mg/mL.

To evaluate the possibility of ion-exchange between zinc contained in beads and cations present in the gastric and intestinal media, three nude beads loaded with activated charcoal (5/3 AC/pectin ratio) were placed into 4.5 mL of either SGM or SIM (prepared with HEPES buffer) and maintained at 37 °C under gentle tangential stirring. At specific times, beads were manually collected and disintegrated in a diluted solution of pectinases (1:25, v/v). Residual zinc in beads was then quantified as described above.

2.2.7. Determination of bead weight-loss during the drying process

Beads weight-loss during drying was determined by thermogravimetric analysis measuring bead weight before and after the drying process. Weight-loss was expressed as a percentage according to the equation:

$$\text{Weight loss}\,(\%) = \frac{\text{Wo} - \text{Weq}}{\text{Wo}} \times 100$$

where Wo and Weq represent the weight of wet and dry beads, respectively. Experiments were performed in triplicate as a function of the number of washing steps.

2.2.8. Stability of beads in simulated digestive media

Due to the relatively large amounts of beads required for standard disintegration methods described in pharmacopoeias and to the limitations of the laboratory-scale bead generator, an alternative disintegration method was employed. Briefly, three dry beads were introduced into 4.5 mL of test medium and maintained at 37 °C under gentle tangential stirring. Stability experiments were performed for 2 h in the gastric medium, 5 h in the intestinal medium and 5 h in the colonic medium.

In a first set of experiments, after 2 h of incubation in the SGM, the medium was replaced with SIM for 5 h. The same experiment was carried out after introduction of the beads in gastro-resistant coated capsules. Capsule coating was performed by dipping and drying capsules at least five times in an ethanol/acetone solution (50/50) containing 5% (w/w) HPMCP. In a second set of experiments, beads were placed directly in SIM for 5 h. In a third set of experiments, beads were placed in SCM until disintegration. In a last set of experiments, beads were first incubated for 5 h in SIM prepared with different buffers (phosphate or HEPES) before being transferred into SCM until disintegration.

Disintegration of beads was followed visually by AC release in the medium. Beads were considered stable if they were still intact after incubation time and if no AC release was observed in the medium. On the other hand, beads were considered completely disintegrated when they disappear from the medium and the medium become homogeneously black.

2.2.9. Adsorption kinetics

The efficiency of bare AC and AC-loaded beads to adsorb ciprofloxacin was tested under simulated digestive conditions, by determining the residual concentration of ciprofloxacin. Typically, 1 or 2 mg of beads corresponding to 0.625 or 1.25 mg AC were incubated with 1 mL of the medium SIM or SCM containing 100, 250 or 500 μg/mL ciprofloxacin, at 37 °C under gentle tangential stirring. The adsorption capacity of beads was also tested by incubating them first in SIM containing ciprofloxacin and then in SCM containing the same concentration of ciprofloxacin. In a control experiment, 1 or 2 mg unloaded zinc-pectinate beads were incubated with ciprofloxacin (100, 250 or 500 μg/mL) in 1 mL of either SIM or SCM prepared with HEPES. At desired time points, samples were centrifuged at 10,000 RPM using a microcentrifuge. The supernatant was filtered on syringe driven filter unit (Millex®-HV, 0.45 μm, PVDF, 4 mm) and analyzed using HPLC. A WaterTM 717 autosampler chromatographic system was employed, together with a WaterTM 486 absorbance detector (λ = 278 nm) and WaterTM 470 scanning fluorescence detector (λ_{exc} = 280 nm and λ_e = 460 nm). Chromatographic separations were performed on a C18 Symmetry® column (5 μm, 150 mm × 4.6 mm; Waters, France). Column temperature was maintained at room temperature (25 °C). The mobile phase consisted of 10% acetonitrile in 0.02 M NaH_2PO_4 solution acidified at pH 3 with orthophosphoric acid. The flow rate was set at 1 mL/min.

3. Results and discussion

The goal of the present experimental work was to design a formulation able to deliver activated charcoal to the colon and allowing it to exert its adsorption properties. It is believed that quick inactivation of residual antibiotics in the colon will protect the colonic flora against the development of antibiotic resistance in normal flora bacteria. The system is based on the entrapment of therapeutic particulate activated charcoal within pectin beads that will release their content like a Trojan horse in a site-specific manner. Such a system was never described before.

3.1. Preparation and characterization of pectin beads

Whereas pectin beads were developed for colonic delivery of low and high molecular weight compounds, they were never applied to the entrapment of particulate systems. When introduced drop by drop into a calcium chloride solution, the aqueous solution of LM pectin turns into gelled spherical beads that are formed by

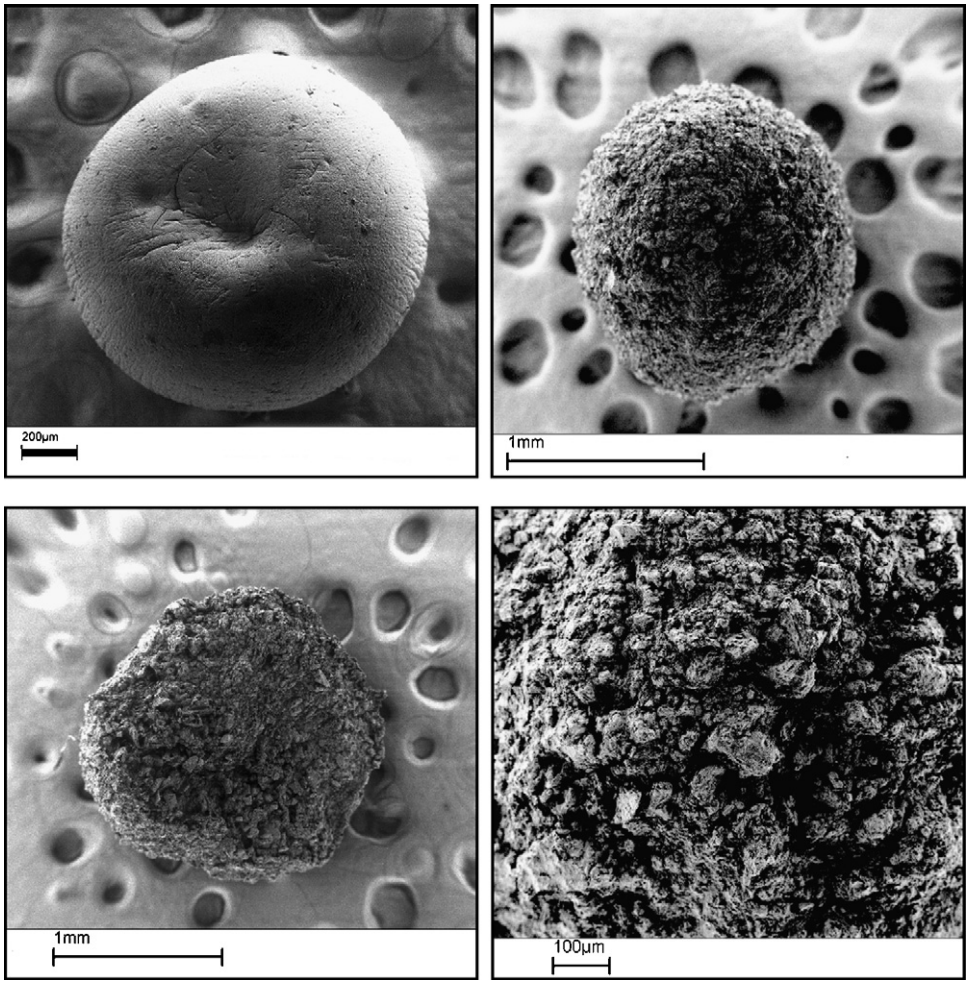

Fig. 1. SEM images showing typical unloaded (top-left, scale bar = 200 µm)) and loaded bead (top-right, scale bar = 1 mm), a sliced loaded bead (bottom-left, scalebar = 1 mm) and a zoom on a loaded bead surface (bottom-right, scale bar = 100 µm).

ionotropic gelation: intermolecular cross-links are formed between the negatively charged carboxyl groups of LM pectin and the positively charged calcium ions. Previous studies report the optimization of the amount of calcium necessary to obtain a stable network and it was demonstrated that a gelation time of 20 min is the minimum required for complete pectin cross-linking (Bourgeois et al., 2006; Sriamornsak et al., 2008). Moreover, increasing the concentration of divalent ions leads to a greater degree of cross-linking, resulting in a stronger gel (Sriamornsak, 1999). In addition, the use of zinc ions instead of calcium ions as alternative cross-linkers leads to a stronger pectinate gel whose stability in the upper gastro-intestinal tract is better (El-Gibaly, 2002; Chambin et al., 2006). Based on these results, we have prepared zinc-pectinate beads fixing the cross-linking time at 30 min which was considered optimal for beads formation. Nevertheless, it was necessary to optimize the process in order to obtain a homogenous incorporation of very hydrophobic activated charcoal particles within pectin aqueous solution and retain AC particles in pectin beads. Indeed, if activated charcoal is not sufficiently hydrated, a phase separation occurred when AC suspension was mixed with the pectin solution and an overnight hydration was necessary to ensure a complete incorporation in pectin solution. Finally, the incorporation of AC in pectin solution increases the viscosity markedly, causing the needle to clog. Therefore, the ratio of AC/pectin in the formulation must be established in such a way that it ensures an easy bead preparation and a satisfactory encapsulation efficacy. The ratio of (5/3) (w/w) was finely selected because it was the best to meet these conditions.

The resulting loaded beads were black and spherical with mean diameters of 1.56 ± 0.05 mm and mean weights of 1.21 ± 0.11 mg. Scanning electron microscopy observations of the loaded beads have shown a rather rough surface in comparison with nude beads (Fig. 1). The images of sliced loaded beads confirm the homogenous distribution of AC within the pectin matrix (Fig. 1). These results prove it was possible to homogeneously incorporate an important quantity of activated charcoal within the zinc-pectinate matrix. One can estimate that AC is completely entrapped and represents 62.5% of dry loaded bead since the loss of AC could not be observed macroscopically during either bead preparation or the washing steps.

3.2. Correlation between the amount of zinc retained by beads and their stability

The amount of zinc retained by beads may have critical consequences on their stability in the gastro-intestinal fluids. A minimal amount of zinc must be retained by beads after washings to ensure sufficient stability. In order to determine this amount, the stability of beads prepared with different concentrations of zinc was evaluated in NaCl–HEPES buffer as a function of the number of washings.

When the initial concentration of zinc acetate in the gelling bath doubles, the amount of zinc retained in beads increases significantly (Fig. 2). Beads prepared in 12% (w/v) zinc acetate always contain more zinc than those prepared in 6% (w/v) zinc acetate. However, zinc content considerably decreases after each washing. By determining bead weight-loss upon drying as a function of the

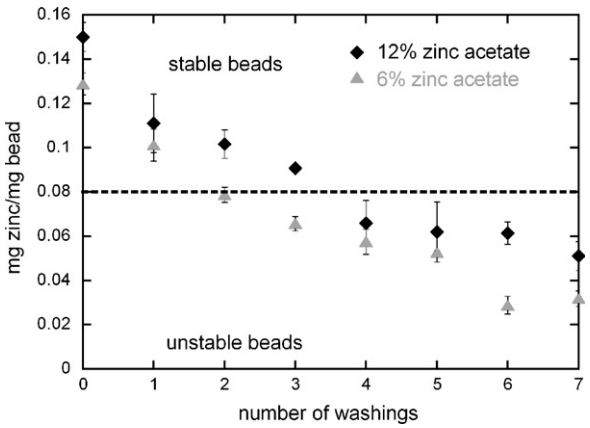

Fig. 2. Zinc content in beads (mg Zn/mg of beads) as a function of the number of washings and the concentration of zinc acetate in the gelation medium. Stability was evaluated in NaCl–HEPES for 5 h.

number of washings, one can further strengthen previous results: bead weight-loss reaches a plateau around 90% after 6 washings, meaning that the free zinc which is hygroscopic has been eliminated (Fig. 3).

As observed for calcium (Bourgeois et al., 2006), zinc retained in beads seems to exist under two different forms: cross-linked zinc, involved in the formation of Zn-pectinate network, and excess free zinc which can be eliminated by washing. Bourgeois et al. (2006) have demonstrated that only a small amount of retained calcium (~10%) is needed to form the Ca-pectinate network (Bourgeois et al., 2006).

One can notice that the stability of beads depends a lot on the amount of zinc retained in beads. Beads show a sufficient stability if they contain a minimal concentration of zinc (0.08 mg/mg bead). When beads are placed in the NaCl–HEPES buffer, the Na^+ ions present in the external solution exchange with the zinc retained in beads. In the initial phase of ion-exchange, the zinc ions present under free form are exchanged with sodium ions. Then, zinc ions participating to the egg-box structure start to exchange with sodium ions present in the buffer. Finally, beads begin to disintegrate when zinc ions in the egg-box structure diffuse out into the medium. This consideration is plausible because the egg-box structure has a strong auto-cooperative binding of Zn^{2+} ions and serves as a stable cross-linking structure within the gel. Therefore, to make beads stable enough, a minimal amount of zinc must be retained by beads to ensure that the ion-exchange with Na^+ takes place only

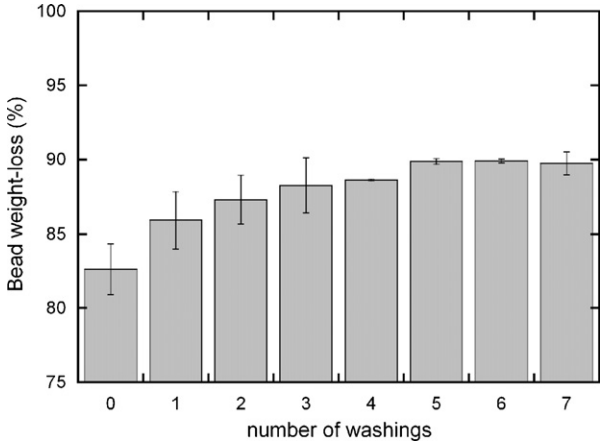

Fig. 3. Weight-loss of beads prepared in 12% (w/v) zinc acetate as a function of the number of washings.

Table 1
Disintegration times of free beads and beads encapsulated in gelatin capsules (uncoated or coated with HPMCP) in PBS-SIM with or without pre-incubation in SGM.

Medium	Free beads	Beads in uncoated Capsules	Beads in coated Capsules
SGM	>2 h	/	/
PBS-SIM after pre-incubation in SGM	<1 h	<1 h	>5 h
Directly in PBS-SIM	>5 h	/	/

with the free Zn^{2+} whereas the egg-box structure remains intact during the incubation in the SIM. We suppose that the concentration of 0.08 mg/mg of zinc in bead is the minimal concentration that provides sufficient free zinc and protects the network against the disintegration due to ion-exchange. For further experiments, the zinc concentration was fixed at 12% in the gelling bath and the number of washings to 3.

3.3. *Effect of pre-incubation in the gastric medium on further stability in simulated intestinal medium*

The stability of loaded beads in the intestinal medium was considered under different conditions. At first, the effect of pre-incubation in SGM was determined. When beads were incubated in this medium, they remained intact and no AC release was observed in the medium during 2 h of incubation. Direct incubation in the PBS-SIM medium induced a high preservation of charcoal retention inside the beads (Table 1). However, pre-incubation in SGM impacted on the stability in PBS-SIM where beads were completely disintegrated in less than an hour. Therefore, gastro-resistant capsules, coated with HPMCP, were needed to protect the beads from exposure to the harsh gastric environment and prevent their later disintegration in the intestinal medium (Table 1). When beads are placed in SGM and in SIM, an exchange process occurs between ions forming the Zinc-pectinate matrix and those present in the incubation medium (Ostberg et al., 1994; Sriamornsak and Kennedy, 2008). The exchange of Zn^{2+} retained by beads with H^+ or Na^+ from the gastric medium and with Na^+ from the intestinal medium occurs via diffusion through the polymer matrix. In SGM, zinc ions were severely extracted from beads as at least 95% of the retained zinc was released from beads after only 30 min incubation (Fig. 4), the total amount being released after one hour. As a result, the egg-box structure is depleted of cross-linkers. Beads keep apparently their structure intact since the pectinic acid, product of the ions exchange, is insoluble in the acidic environment (the pH of gastric

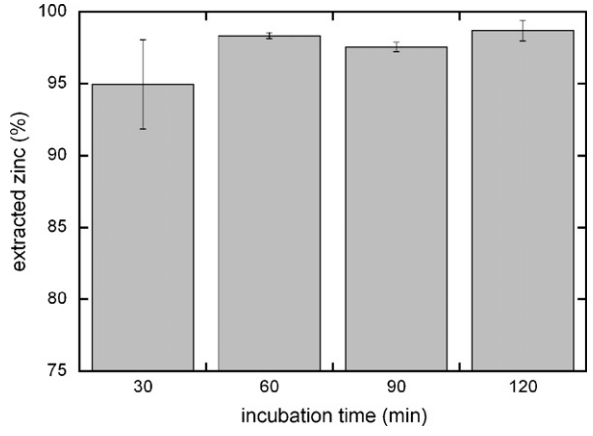

Fig. 4. Effect of incubation time in SGM on the amount of zinc extracted from beads.

Table 2
Disintegration times of beads incubated directly in SCM or after 5 h of pre-incubation in SIM prepared with different buffers as a function of the concentration of pectinases.

Incubation medium	Pectinases concentration in SCM (PG/mL)			
	0	650	1300	5200
Directly in SCM	>5 h	2 h.30	2 h	1 h.30
PBS-SIM + SCM	/	>20 h	>20 h	>20 h
Pancreatin-free PBS-SIM + SCM	/	>20 h	>20 h	>20 h
HEPES-SIM + SCM	>5 h	2 h.30	2 h	1 h.30

medium is below the pK_a of pectin 3.5). Afterwards, upon medium change and entrance into pH 6.8 of the SIM, the COOH groups of the pectinic acid turn into COO^-, which repel each other. Since beads are depleted of cross-linkers the structure is loose and a dramatic disintegration occurs (Atyabi et al., 2005; Sriamornsak and Kennedy, 2008).

3.4. Effect of pre-incubation in various intestinal media on further degradation in simulated colonic medium

Disintegration of beads in simulated colonic medium was achieved after pre-incubation in several simulated intestinal medium and compared to the disintegration obtained after direct incubation in SCM. The effect of pre-incubating beads in the PBS-SIM on their disintegration in SCM containing pectinolytic enzymes was considered. These beads were stable for more than 20 h (Table 2) whereas the ones directly incubated in the SCM containing pectinolytic enzymes were degraded rather fast (Table 2). Bead incubation in PBS-SIM induced the formation on their surface of a white layer that could be observed macroscopically. This layer does not arise from pancreatin since the same results were obtained using a pancreatin-free SIM. In fact, close examination of beads incubated in pancreatin-free PBS SIM using SEM shows that the white layer corresponds to a precipitate of small crystalline needles (Fig. 5). Since the formation of the precipitate on bead surface happens simultaneously with zinc release from beads in the intestinal medium, it has been hypothesized that the needles may arise from a reaction between zinc ions released from the beads and phosphate ions present in the medium, forming zinc phosphate (Fig. 5). This hypothesis is supported by the turbidity of the medium. Bajpai and Sharma (2004) and Ostberg et al. (1994) have also suggested the formation of a calcium phosphate precipitate, to interpret the increase of turbidity observed in the medium when calcium alginate beads were incubated in PBS. Since bead disintegration in SCM is due to the activity of pectinolytic enzymes, it is very likely that the zinc phosphate precipitate prevents pectinolytic enzymes from accessing and degrading the pectin network.

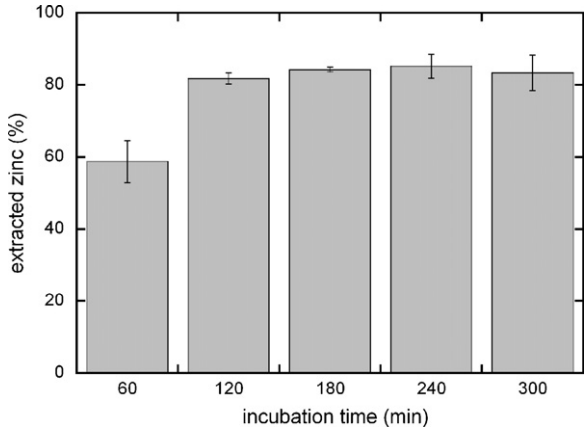

Fig. 6. Effect of incubation time in HEPES based SIM on the amount of zinc extracted from beads.

NaCl–HEPES was then proposed as an alternative to PBS for SIM buffering. Table 2 shows that beads are stable in SIM buffered by HEPES for more that 5 h. Moreover, pre-incubation of beads in this SIM does not modify their stability in SCM (Table 2). SEM images of beads incubated 5 h in HEPES-SIM do not show any formation of precipitate on the bead surface (Fig. 5). The determination of zinc release was carried out in a SIM buffered with HEPES. Fig. 6 shows that when beads were incubated in SIM there was also extensive displacement of zinc from beads. Nevertheless, 15% of the initial zinc remained retained by the beads even after 5 h of incubation (Fig. 6).

3.5. Ciprofloxacin adsorption kinetics

Adsorption kinetics studies were achieved both in simulated intestinal and colonic media. Gastric medium was excluded from these studies since ciprofloxacin would be protected from beads using gastro-resistant capsules.

In the first control experiment, ciprofloxacin was incubated separately in SIM and in SCM under the same conditions as the adsorption kinetics experiments. The concentration of ciprofloxacin remained stable during 5 h of incubation in both media (data not shown). This proves that ciprofloxacin does not undergo irreversible decomposition during the adsorption studies. Any decrease in ciprofloxacin concentration during adsorption kinetics studies would therefore arise only from adsorption.

In another control experiment, the adsorption capacity of bare AC and AC loaded in pectin beads was evaluated in SCM containing enzymes. Fig. 7 shows the adsorbed amount of ciprofloxacin versus the incubation time for two different initial concentra-

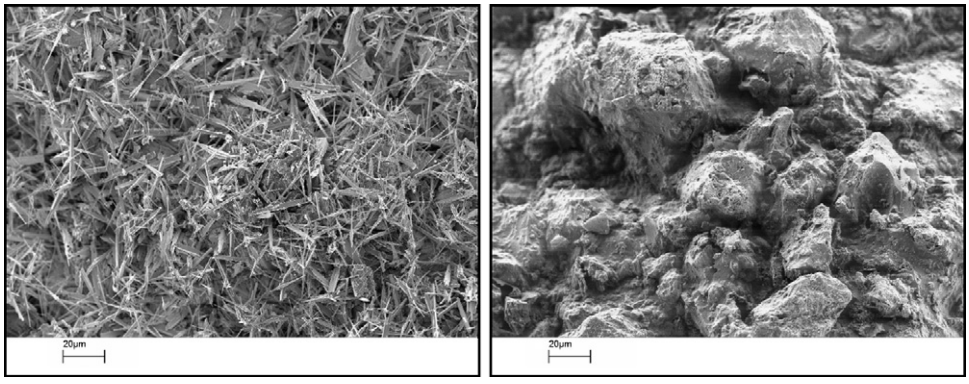

Fig. 5. SEM images of bead surface after 5 h of incubation in pancreatin-free PBS SIM (left) or HEPES SIM (right). Scale bars represent 20 µm.

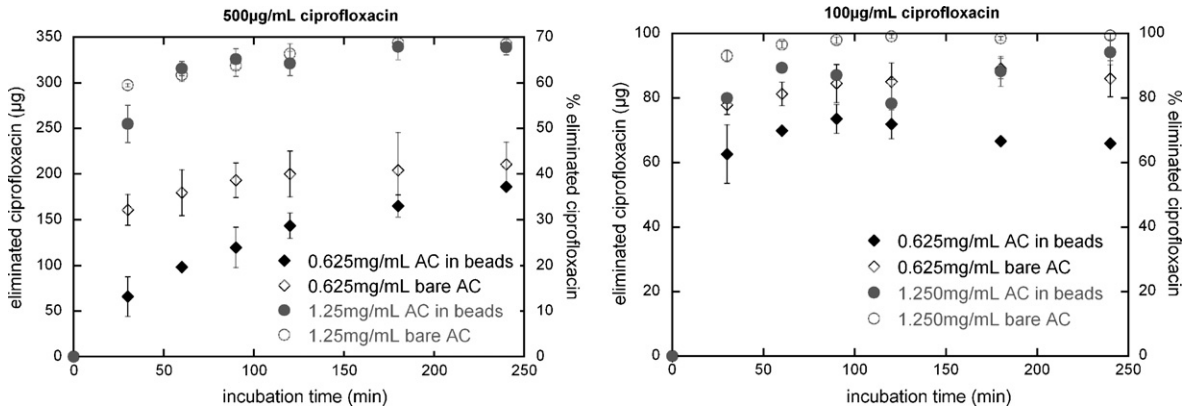

Fig. 7. Amount of ciprofloxacin adsorbed by (0.650 and 1.250) mg/mL of bare AC and AC loaded within pectin beads versus incubation time in SCM with enzymes. Initial concentration of ciprofloxacin is 500 μg/mL (left) and 100 μg/mL (right).

tions of ciprofloxacin. For an initial ciprofloxacin concentration of 100 μg/mL, the amounts of ciprofloxacin adsorbed by 0.625 and 1.25 mg AC are 70 and 95 μg respectively. For an initial ciprofloxacin concentration of 500 μg/mL, the amounts of ciprofloxacin adsorbed by 0.625 and 1.25 mg AC are 200 and 350 μg respectively. The adsorption profiles can be described into two regions. The first region corresponds to a fast adsorption rate followed by the second slow adsorption until equilibrium is achieved. One can notice that the equilibrium is achieved later when AC is encapsulated within pectin beads owing to the time required for beads disintegration. However, the adsorption capacity of AC is not modified by its encapsulation within pectin beads.

Adsorption kinetics studies were then carried out first in HEPES-SIM containing pancreatic enzymes and ciprofloxacin. After that the intestinal medium was replaced by the colonic one containing the same initial concentration of ciprofloxacin. Incubation time was 5 h in each medium. Fig. 8 presents a typical adsorption experiment where the cumulated amount of eliminated ciprofloxacin versus incubation time was plotted. One can observe that about 50 μg of ciprofloxacin was eliminated from the intestinal medium after only 2 h of incubation despite the fact that beads remained quite intact. After 5 h of incubation in SIM, a total of 75 μg of ciprofloxacin was adsorbed. When the intestinal medium was replaced by the colonic one, beads rapidly disintegrated releasing AC that adsorbed another 185 μg of ciprofloxacin, close to what was adsorbed in the experiment described above. Although the AC is not released in the intestinal medium due to the good bead stability, ciprofloxacin was adsorbed in this medium. Most probably, ciprofloxacin is interacting with AC present on bead surface or even encapsulated within beads via diffusion. Adsorption in the intestinal medium is not wanted since it may modify the pharmacokinetics of ciprofloxacin and as a result weakens treatment efficacy. Beads coating with low permeability to antibiotics and non-adsorbent layer was therefore considered to prevent the premature adsorption of ciprofloxacin in the intestinal medium.

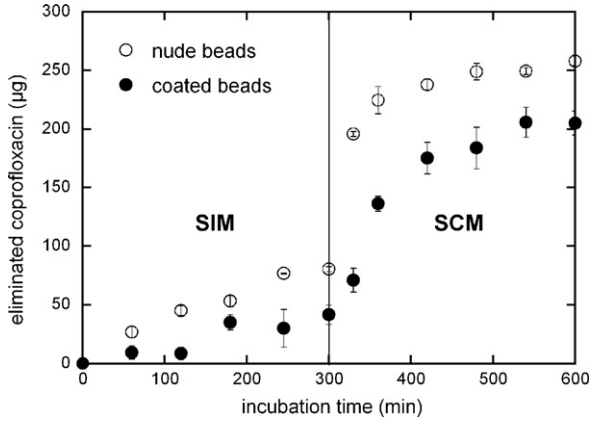

Fig. 8. Cumulated amount of ciprofloxacin eliminated by 0.650 mg/mL of AC loaded in nude or coated beads versus incubation time in SIM followed by SCM with enzymes. Initial concentration of ciprofloxacin is 500 μg/mL.

3.6. Ciprofloxacin adsorption kinetic by poylectrolyte-coated bead-entrapped active charcoal

Coating by the formation of polyelectrolyte complexes was obtained by the reaction between the positively charged DEAE dextran or positively charged poly-L-lysine and the negatively charged pectin. Beads coated by the polyelectrolyte complex were similar to the uncoated ones from the size and weight point of view. Bead stability in SCM containing pectinases was used as an indirect indicator of the formation of polyelectrolyte complexes.

On one hand, coated beads were in general more stable when incubated directly in SCM than uncoated ones. This could be due to the formation of the polyelectrolyte layer which would prevent or delay the entrance of the enzymes. On the other hand, the stability did not increased when beads were pre-incubated in SIM probably due to the dramatic ion-exchange that beads undergo in SIM. Two methods were used to prepare DEAE dextran coated beads but none of them changed beads properties. Adding the coating agent together with zinc ions could have provided a competition mechanism and could have modified beads properties. This was not the case since numerous carboxyl groups available on pectin, competition is unlikely to occur. In addition, coating with polyelectrolyte complexes did not prevent the adsorption of ciprofloxacin by beads in SIM. About 75 μg ciprofloxacin was adsorbed in SIM as for nude AC-loaded beads. Neither the increase of DEAE dextran concentration, nor the modification of the coating method of coating could prevent ciprofloxacin adsorption in SIM. The complex layer may be porous enough to allow ciprofloxacin entrance in the matrix and its later adsorption by AC.

3.7. Ciprofloxacin adsorption kinetic by Eudragit RS®-coated bead-entrapped active charcoal

It is reported that drug delivery systems prepared from Eudragit® RS, a water-insoluble polymer, show a pH-independent sustained drug release (Haznedar and Dortunc, 2004). Therefore coating with Eudragit® RS was proposed to delay the adsorption of ciprofloxacin by beads until its complete absorption in the small intestine. After oral dosing, ciprofloxacin is absorbed well

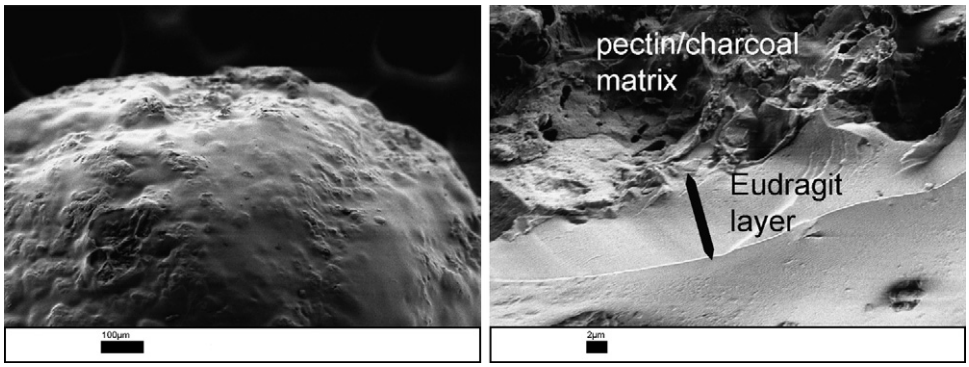

Fig. 9. SEM images of coated bead surface with 10% of weight increase. Scale bars represent 100 μm (left) and 2 μm (right).

and rapidly and exhibits excellent tissue penetration (Crump et al., 1983). Since peak plasma concentrations of ciprofloxacin occur at 47 ± 20 min (Lubasch et al., 2000), to ensure total efficacy of treatment, it is crucial to prevent ciprofloxacin adsorption in the small intestine for at least 2 h until complete absorption of the drug.

Two coating percentages were tested: 5 and 10% of the bead weight. Coated beads were shiny whereas uncoated ones were dull. SEM images of coated bead surface (10% coating) show a continuous layer of about 10 μm thick (Fig. 9). By contrast, for 5% coating was not sufficient to create a homogenous and continuous layer (not shown).

Fig. 8 shows that coating beads with Eudragit® RS (10%) prevents completely the adsorption of ciprofloxacin by beads during the first two hours of incubation in SIM. Similar results were obtained with other concentrations of ciprofloxacin. We estimate that this time is sufficient to allow complete absorption of ciprofloxacin in the small intestine. Interestingly even after bead disintegration in the SCM some intact pieces of the coating layer were observable in the medium.

Coated beads were first incubated for 5 h in HEPES-SIM containing ciprofloxacin followed by incubation in SCM containing ciprofloxacin at the same concentration. The cumulated amount of ciprofloxacin adsorbed after incubation is reported for three ciprofloxacin concentrations and two AC concentrations in Fig. 10. Results show that the higher the AC concentration, the more ciprofloxacin is adsorbed. For a fixed AC concentration, the higher the ciprofloxacin concentration, the more ciprofloxacin adsorbed by beads as adsorption in a cooperative process. A Student's *t*-test was performed to assess that results were significantly different one from another as the AC concentration was doubled ($p < 0.05$). Another Student's *t*-test was performed to assess that results were significantly different one from another as the initial ciprofloxacin concentration varied ($p < 0.05$). For 1.25 g AC/mL, beads may adsorb up to 450 μg ciprofloxacin. In the clinics, 500–750 mg of ciprofloxacin are given by oral route twice daily. Approximately 32.50% of the oral dose is recovered in the faeces, unmetabolized (Beermann et al., 1986). Assuming that the colon volume is about 600 mL, 30% of 750 mg therefore correspond to 375 μg/mL ciprofloxacin in the colon. This theoretical residual concentration is on the order of what is found experimentally after a 7 day regimen: 185–2200 μg/mL of faeces (Ramon et al., 1994). The good *in vitro* adsorption capacity of AC-loaded beads makes them ideal candidates for *in vivo* experiments to eliminate residual ciprofloxacin in the colon and prevent bacterial resistance. However, it should be noted that antibiotics could be reactivated by desorption once excreted in the environment. Therefore, by opposition with systems based on enzymatic inactivation, our adsorbent-based inactivation system would be mostly useful to prevent the selection of new resistance in the colonic microflora.

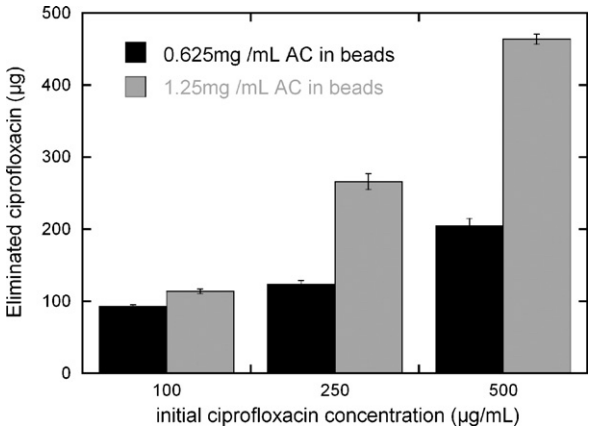

Fig. 10. Cumulated amount of ciprofloxacin eliminated after 5 h-pre-incubation in HEPES-SIM and 5 h-incubation in SCM, as a function of ciprofloxacin concentration and bead concentration, expressed as equivalent AC concentration for Eudragit® RS coated beads. Experiments were performed in triplicate.

4. Conclusion

Activated charcoal was efficiently encapsulated within zinc-pectinate beads. The optimal formulation corresponds to a 5/3 (w/w) AC/pectin ratio. Beads were stable after a 2-h incubation in simulated gastric medium and then disintegrated fast in simulated intestinal medium. Bead protection by gastro-resistant capsules was therefore considered. Beads were stable after a 5-h incubation in an HEPES-SIM and disintegrated fast once put in presence of SCM containing pectinolytic enzymes. Despite their stability, coating with Eudragit RS® was needed to prevent the early adsorption of antibiotics in the intestinal medium. Adsorption studies in the simulated colonic medium show that the adsorption capacity of AC is not modified after its encapsulation within pectin beads. Beads can eliminate up to 450 μg of ciprofloxacin making the elimination of ciprofloxacin reaching the colon clinically feasible.

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