Pharm Res (2013) 30:721–734
DOI 10.1007/s11095-012-0913-3

RESEARCH PAPER

Efficacy of Mucoadhesive Hydrogel Microparticles of Whey Protein and Alginate for Oral Insulin Delivery

Emmanuelle Déat-Lainé • Valérie Hoffart • Ghislain Garrait • Jean-François Jarrige • Jean-Michel Cardot • Muriel Subirade • Eric Beyssac

Received: 6 March 2012 / Accepted: 12 October 2012 / Published online: 24 October 2012

ABSTRACT

Purpose To evaluate the efficacy of mucoadhesive insulin-loaded whey protein (WP) /alginate (ALG) microparticles (MP) for oral insulin administration.

Methods Insulin-loaded microparticles (ins-MP) made of whey protein and alginate were prepared by a cold gelation technique and an adsorption method, without adjunction of organic solvent in order to develop a biocompatible vehicle for oral administration of insulin. *In vitro* characterization, evaluations of ins-MP in excised intestinal tissues and hypoglycaemic effects after intestinal administration in healthy rats were performed

Results The release properties and swelling behaviors, investigated in different pH buffers, demonstrated a release based on diffusion mechanism following matrix swelling. Mucoadhesion studies in rabbits and insulin transport experiments with excised intestinal rat tissues revealed that encapsulation in microparticles with mucoadhesive properties promotes insulin absorption across duodenal membranes and bioactivity protection. *In vivo* experiments reinforced the interest of encapsulation in whey protein/alginate combination. Confocal microscopic observations associated with blood glucose levels bring to light duodenal absorption of insulin biologically active following *in vivo* administration.

Conclusions Insulin-loaded WP/ALG MP with high quantities of drug entrapped, *in vitro* matrix swelling and protective effect as well as excellent mucohadesive properties was developped. Improvement of intestinal delivery of insulin and increased in bioavailability were recorded.

E. Déat-Lainé • V. Hoffart • G. Garrait • J.-F. Jarrige • J.-M. Cardot • E. Beyssac (✉)
Univ Clermont 1, UFR Pharmacie, ERT 18
Laboratoire de Biopharmacie
Clermont-Ferrand 63001, France
e-mail: eric.beyssac@u-clermont1.fr

M. Subirade
Institute of Nutraceuticals & Functional Foods (INAF),
Milk and Dairy Products (STELA Center),
Université Laval, Pavillon Paul Comtois
Sainte-Foy, Québec, Canada G1K 7P4

KEY WORDS alginate · insulin · microspheres · oral administration · whey protein

ABBREVIATIONS

ALG	alginate
EE	encapsulation efficiency
ins-MP	insulin-loaded microparticles
MP	microparticle
WP	whey protein

INTRODUCTION

Oral delivery is the most convenient and desired way for drug delivery, especially within the framework of chronical disease. Several research groups have been focusing on the development of delivery systems for peptide and protein oral administration (1), such therapeutics, i.e. insulin, being strongly limited by a rapid gastrointestinal enzymatic degradation and by low absorption rate. A number of different techniques, such as enteric coatings, microsphere encapsulations, use of permeation enhancers and protease inhibitors have been explored for protection and transport of insulin up to intestine (2,3). Some promising results were obtained showing clearly that oral administration of insulin, mimicking the physiological fate of insulin, is a real benefit for diabetic patients. However, most production techniques, on one hand, involve the use of organic solvents, heat or vigorous agitation potentially harmful to the structure and consequently to the biological activity of proteins, and on the other hand, lead to some problems of cytotoxicity (4).

These limitations could be effectively overcome by insulin encapsulation within hydrogel microparticles (MP) made of natural and mucoadhesive polymers. Biodegradable and biocompatible mucoadhesive polymers were reported promising to develop safe oral controlled delivery systems

with applications in pharmaceutics (5). Among natural polymers, one emerging polymer consists of whey proteins (WP), well-known in food industry because of their high nutritional value and their ability to form foam, emulsion and gel (6). Particularly, WP form a cold-induced gel, after a pre-heating step which ensures WP denaturation and polymerization of WP chains, by divalent cation addition (7). Several studies have explored and established WP potentialities as material to encapsulate compounds such as sensitive bioactive compounds (8–10) and living microorganisms (11,12) with protection in digestive tract.

Our laboratory works on the development of innovative drug delivery systems using WP combined with sodium alginate (ALG) as vehicles for active compounds in the human digestive tract. This association of polymers is interesting because both polymers exhibited antagonist mechanistic behavior at acidic pH: ALG matrix shrinkage counteracted swelling of WP. Moreover, previous studies showed the protection effect of this type of encapsulation toward enzymatic degradation (9) and the mucoadhesive properties of ALG (13) as well as WP (14). These properties could be effective to improve the oral bioavailability of insulin.

This study focused on the preparation of WP/ALG MP for delivery of insulin in order to maintain biological activity. MP were characterized in terms of size, morphology, encapsulation efficiency, swelling and *in vitro* release behavior. Then, evaluations of insulin-loaded MP (ins-MP) in excised intestinal tissues were performed in terms of mucoadhesivity, transport and bioactivity. Finally, the hypoglycaemic effects of ins-MP were examined after intestinal administration in healthy rats.

MATERIALS AND METHODS

Chemicals

Whey protein isolate (Alacen® 845), purified by ion exchange and ultrafiltration, was provided by NZMP (Wellington, New Zealand). Its protein content was 93% (dry matter basis) as determined by the Kjeldahl method (Nx6.38). Sodium alginate (Manucol® DH, >99% dry matter basis) was obtained from ISP (Wayne, New Jersey, USA). Acetonitrile, methanol, calcium chloride dihydrate ($CaCl_2$), sodium hydroxide (NaOH), hydrochloric acid (HCl), sulfate sodium anhydre (Na_2SO_4), phosphoric acid extra pure (H_3PO_4), trypsin from bovine pancreas, trifluoroacetic acid (TFA) and a human insulin ELISA kit were purchased from Fisher Scientific (Illkirch, France). Insulin solution (Umuline® rapide 100 UI/mL) was provided by Lilly France SAS. The Glucose Oxydase Kit® was purchased from R-Biopharm (Darmstadt, Germany). Ins–FITC labeled from bovine pancreas and α-chymotrypsin from bovine pancreas were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France) and Hoechst from Invitrogen (France).

Preparation of Microparticles

Preparation and Characterization of Polymeric Solutions

WP solutions (11.0% *w/w*) were prepared as follows: WP isolate powder was re-hydrated in de-ionized water by gentle magnetic stirring for 1 h at room temperature and then allowed to rest for 2 h to ensure complete hydration of proteins. WP solutions were adjusted at pH 7.0 with NaOH, heated and maintained at 80°C for 40 min to denature proteins completely. The solutions were cooled overnight at room temperature.

ALG solutions (3.0% *w/w*) were prepared in de-ionized water and stirred at room temperature.

Zeta potential measurements of WP, ALG and insulin solutions were performed, after dilution in de-ionized water by laser doppler electrophoresis using a Zetasizer nanoseries Nano ZS (Malvern Instruments Ltd., UK). Data analysis of Zeta potential is presented as mean ± standard deviation of three samples (with ten runs for each sample).

Unloaded Microparticle Preparation

Unloaded WP MP (11% *w/w*), ALG MP (3,0% *w/w*) or WP/ALG MP were obtained by an extrusion/cold gelation method using a simple extrusion device (syringe and 23G needle) according to a method previously described (9). In order to determine later the influence of WP/ALG ratio on insulin loading, polymer solutions with ratio of 60/40 and 80/20 WP/ALG (*w/w*) were prepared. These polymeric solutions were extruded and drops were collected into a 0.1 M $CaCl_2$ gelling bath. After filtration, unloaded MP were frozen at −80°C. Freeze-drying was performed for 48 h in a standard freeze-dryer as followed: the unloaded MP were placed in the pressure chamber (pressure less than 3 Pa), maintained at −34°C during 1 h and progressively heated to 10°C during 24 h. Then, during the past 24 h, temperature increased and was maintained at 25°C.

Insulin Loading

Insulin was loaded into MP by a diffusion loading method (15) to produce insulin loaded MP (ins-MP). Lyophilized

unloaded MP were hydrated in insulin solution (100 IU/mL, 12.8 mL/g of MP, pH 7.0) for 5 min at room temperature. Drug loading was determined by reverse-phase RP-HPLC MERCK-HITACHI, as follow: ins-MP (80 mg) were degraded into 50 mL of pH 6.8 USP buffer under vigorous magnetic stirring during 3 h. After centrifugation (5000 rpm, 5 min), 20 µL of the supernatant were injected via an autosampler (AS-2000 A) connected to an intelligent pump (L-62000 A), a diode array detector (L-45000) and an interface (D-6000). The LiChrospher RP-18 column (5 µm, 4×250 mm, Merck, Darmstadt, Germany) was equilibrated at 40°C, with column thermostat (L-5025), at a flow rate of 0.8 mL/min with 72% eluent A (56.8 g Na_2SO_4, 2 L ultra-pure water and pH adjusted at 2.3 with H_3PO_4) and 28% eluent B (acetonitrile). Absorbance of the eluent was recorded at 214 nm. The column was calibrated with insulin solutions of known concentrations. LOD and LOQ were determined respectively to be 0.05 and 0.1 µg/mL. The retention time of insulin was 5.81 min. All experiments were performed in triplicate.

Microparticle Coating

Ins-MP were further coated with ALG-insulin solution (1.5% ALG, insulin 25 UI/g) by immersion. After 5 min magnetic stirring, coated ins-MP were transferred into 0.1 M $CaCl_2$ solution for 5 min before microscopic observations.

Characterization of Microparticles

Microparticle Size and Morphology

All microparticle size and morphology were studied using an optical microscope (Nikon SMZ1000) and a digital camera (Olympus optical). Diameters of 90 MP (dry diameter, n=3) were measured by image analysis (Image Pro Plus).

Encapsulation Efficiency

Encapsulation efficiency (EE) was defined as total entrapped insulin (drug loading measured by RP-HPLC) to total volume of buffer solution absorbed (determined by swelling experiment). The volume of the solution absorbed was determined by swelling behavior as follow: accurately weighted lyophilized unloaded MP [mass (t_0), approx. 130 mg, n=3] were either hydrated into insulin solution (ins-MP) or into pH 7.0 USP buffer. These MP were withdrawn after 5 min, accurately weighed [wet mass (t)], measured as described above [wet diameter (t) of 30 MP] and dried to constant mass at 60°C [dry mass (t)]. The matrix weight loss (%), water uptake (%) and diameter changes (%) were calculated as follows (n=6):

$$\textit{Matrix weight loss}\,(\%) = \frac{mass(t_0) - dry\,mass(t)}{mass(t_0)} \times 100 \qquad (1)$$

$$\textit{Water uptake}\,(\%) = \frac{[wet\,mass(t) - dry\,mass(t)] - [wet\,mass(t) - mass(t_0)]}{wet\,mass(t) - mass(t_0)} \times 100 \qquad (2)$$

$$\textit{Diameter change}\,(\%) = \frac{wet\,diameter(t) - wet\,diameter(t_0)}{wet\,diameter(t_0)} \times 100 \qquad (3)$$

In Vitro Dissolution Studies

Insulin releases from ins-MP (approx. 390 mg), uncoated and coated, were investigated in 50 mL USP buffer pH 1.2, pH 4.5 and pH 6.8 at 37°C in Schott Duran® flask under stirring (50 rpm). Samples (1 mL), withdrawn at predetermined time intervals, were centrifuged (5000 rpm, 5 min) and insulin quantities were measured using the RP-HPLC method described above. After 6 h testing, undegraded ins-MP were collected by filtration and degraded in pH 6.8 to estimate the drug 100% value. All experiments were performed in triplicate.

Evaluations of swelling behaviors (%) during dissolution were determined under the same conditions at predetermined time intervals, as describe above (with mass (t_0), mass of ins-MP before dissolution studies).

Release Kinetic Model

Insulin releases were fitted to equation proposed by Harland *et al.* (16) allowing modelization of the release mechanisms.

$$\frac{Mt}{M\infty} = A\sqrt{t} + Bt \qquad (4)$$

In the above equations, Mt/M∞ is the fraction of drug released at time t, A and B are diffusion and erosion terms. When A>B, the diffusion factor prevails in the release system, while when A<B, erosion predominates. If A=B, the release mechanism includes both diffusion and erosion equally.

Insulin Degradation Studies

To evaluate the protective properties of the WP/ALG matrix towards enzymatic degradation of insulin, the ins-MP (50 MP) were dispersed in USP pH 7.4 buffer (20 mL) containing trypsin (10 IU/mL) and chymotrypsin (10 IU/mL). Samples were incubated at 37°C and at predetermined time points, an aliquot (1 mL) was withdrawn and the enzymatic reaction was stopped by the addition of 0.1% TFA in water. The insulin concentration in the buffer (insulin released) was estimated by RP-HPLC. Then, ins-MP were transferred and degraded in fresh buffer pH 7.4 to extract insulin. The drug content (insulin still entrapped) was estimated by RP-HPLC. The biological activity of these samples was also evaluated by the enzyme-linked immunosorbent assay (ELISA) technique as per the standard protocol. Results were obtained by reading the optical density at 450 nm using a microplate reader (Finstruments Microplate Reader). In parallel, a control system was maintained by placing a similar concentration of native insulin in enzymatic buffer. A similar set was maintained without enzymes for comparison and evaluation of percentage of insulin remaining undegraded. All experiments were performed in triplicate.

Evaluation of Ins-MP in Excised Intestinal Tissues

Animal protocols were approved by the Committee for Ethical Issues, CEMEA Auvergne (Clermont-Ferrand, France). Adult male Wistar rats and male Albino rabbits (Elevage Dépré, St. Doulchard, France), weighing 300 ± 20 g and 3.0 ± 0.2 kg respectively, were housed in a temperature-controlled room (22 ± 3°C) and maintained on a 12 h light/12 h dark cycle (lights on from 8:00 a.m. to 8:00 p.m.). Animals had free access to a commercial pelleted standard and tap water. After fasting for 12–16 h, rats were anesthetized using Imalgene 1000® and Valium 10® (150 µL and 50 µL/100 g of rat respectively) and locally with 1% Xylocaine®. Rabbits were anesthetized using Imalgene 1000® (0.30 mL/kg), Rompun® (0.25 mL/kg), Valium 10® (0.10 mL/kg) and urethane 50% (2 mL/kg).

Mucoadhesive Study on Duodenal Rabbit

Rabbits were divided into 4 groups of 3 animals. Mucoadhesion studies were done on rabbit duodenum mucosa as described by Rao and Buri (17) with different particles: glass beads (1000–1200 µm diameters), unloaded ALG or WP or WP/ALG (80/20 ratio) MP. Glass beads were considered adequate as a control for a surface area comparable to that of the tested polymeric MP. Briefly, intestinal tissue from duodenum (10 cm length) was taken, flushed with normal saline (0.9% NaCl) to remove free mucus on mucosal side and cut open longitudinally. The tissue was mounted on a plastic support at an angle of 45° and washed with normal saline. Twenty particles were spread in mucosal side and kept 5 min for particle-mucosal interactions. The tissue was then washed with pH 6.8 USP buffer for 30 min (flow rate 24 mL/min). The number of particles still on the duodenal tissue after 5, 10 or 30 min washing time was counted. Mucoadhesive capacity was calculated as the percentage of particles retained by the mucosal tissue at the end of the process. Experiment was performed in triplicate.

Insulin Transport Experiments with Excised Intestinal Rat Tissues

Rats were divided into 3 groups of 5 animals. The duodenum of each rat (segment of 10 cm between pylorus and ligament of Treitz) was quickly removed and serosal side was rinsed with 10 mL of normal saline maintained at 37°C. One end of the organ segment was ligated. Samples, ins-MP (corresponding to 8 IU of insulin) or free insulin solution (8 IU, 1 mL, control experiment) or unloaded MP (blank experiment), were introduced into the duodenal sac. Then the second end of the duodenum was ligated. Duodenal sacs were immediately transferred into a tissue chamber containing 15 mL of warmed (37°C), oxygenated (95% O_2/5% CO_2) Krebs Henseleit modified buffer (composition in mmol/L: 118.1 NaCl, 4.7 KCl, 2.2 $CaCl_2.2H_2O$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, and 11.1 glucose, pH 7.5). At predetermined times, 0.5 mL samples were collected from the serosal side, and replaced by fresh medium maintained at 37°C. Sampling was performed until 180 min incubation period (maximum viability for rat material (18)). Samples were analyzed by the RP-HPLC method as described above. At the end of the incubation period (180 min), duodenal segments were longitudinally open in order to collect the mucosal content. Collected ins-MP were quickly rinsed with normal saline and further degraded in 10 mL pH 6.8 USP buffer at 37°C under vigorous stirring during 3 h. After centrifugation, insulin extracted from ins-MP (corresponding to insulin unreleased at 180 min) were measured using the RP-HPLC method.

The apparent permeability coefficient (P_{app}) was calculated as follows:

$$P_{app} = \left(\frac{dQ}{dt}\right) \times \left(\frac{1}{AC_0}\right) \quad (5)$$

with dQ/dt, the flux of insulin from the mucosal to the serosal side of the mucosa; C_0, the initial concentration of insulin in the lumen compartment and A, the membrane

area (A=length × diameter). The absorption enhancement ratio was calculated as follows:

$$R = \frac{P_{app}(sample)}{P_{app}(control)} \quad (6)$$

Insulin Bioactivity Protection by Microparticles in Rat

Rats were divided into 2 groups (30 or 180 min incubation time) of 10 animals. The protection of insulin by ins-MP towards enzymatic degradation in the rat duodenal sacs (n=5) was evaluated. The bioactivity was assessed by subcutaneous administration to anesthetized rats (n=5). Again, ins-MP (8 IU) were introduced into duodenal sacs (as described above). For the first group, after 30 min, ins-MP were collected into the duodenal sacs (mucosal side) and degraded in 5 mL pH 6.8 USP buffer. The solution was filtered and 2 mL (corresponding to 0.40±0.02 IU/mL, determined by RP-HPLC) were subcutaneously injected to anesthetized and catheterized rats. For the second group, after 180 min incubation, the activity of insulin released from ins-MP and transported throughout the duodenal wall into the serosal side was estimated. The solution from the serosal side was concentrated by freeze-drying, reconstituted in normal saline and 2 mL (corresponding to 0.55±0.05 IU/mL, determined by RP-HPLC) were subcutaneously injected to anesthetized and catheterized rats. For catheterization, rats were anesthetized and a longitudinal incision from the base of the neck to the thorax was made. The sternohyoid muscle was dissociated and then carotid and pneumogastric nerve were separated. A cranial ligature was tied to stop blood flow. Then, a clamp was placed caudally to allow the introduction of catheter (25G needle) into artery. After the clamp was removed, the catheter was tied with a cranial ligature top hold it in place. One hundred microliters of heparinized saline was slowly injected through the catheter. At predetermined times blood samples (0.3 mL) were withdrawn and collected into heparinized tubes. Insulin activities were evaluated using a Glucose Oxydase Kit®. The initial blood glucose levels were regarded as 100% and all other levels were referred to them. Biological activity of samples (insulin solution or plasma) were also assessed using ELISA technique.

In Vivo Evaluation of ins-MP

Pharmacological Availability

Rats were divided into 4 groups of 6 animals. Due to the large size of ins-MP, direct oral administration was not performed. Instead, the efficacy of ins-MP was assessed by a duodenal administration method, which has previously been used for determining absorption efficiency in animals (19). Briefly, rat intestine was exposed through a midline abdominal incision (1.5 cm). A small longitudinal incision (0.5 cm) was made about 2 cm after the stomach. Four sets of experiments were performed: insertion through the opening into the lumen what made with i) Ins-MP (corresponding to 70 IU/kg body weight), ii) unloaded MP iii) free insulin solution (70 IU/kg body weight). The incisions were then sealed with suture. Reference test was performed by subcutaneously injection, after surgery, of free insulin solution to the remaining group (6 IU/kg body weight).

Blood samples were collected from the jugular vein, into heparinized tubes, before the experiments and at predetermined times. According to "guidelines for collection of bloods from experimental animals", for multiple blood draws a maximum of 1% of the animal's body weight can be removed, i.e. 3 mL for a 300 g rat. Based on these recommendations, the duration of the experiments was limited to 2 h and samples were done as follow: 0.6 mL (time 0,30,30 120 min) for insulin evaluation by a Glucose Oxydase Kit® and by ELISA and 0.3 mL (time 15,90) for only Glucose Oxydase Kit® determination. Effect of surgery on glucose level (group iv - receiving normal saline) was subtracted to all the experiments. The initial blood glucose levels were regarded as 100% and all other levels were referred to them.

The extent of hypoglycaemic effect was calculated as the area under the blood glucose level-time curve ($[AUC]_{S.C.}$, $[AUC]_{free\ ins}$, $[AUC]_{ins\text{-}MP}$) for 0–120 min. The relative pharmacological availability (PA% of ins-MP was calculated as follow:

$$PA\% = \frac{AUC_{SC}}{AUC_{Test}} * \frac{Dose_{SC}}{Dose_{Test}} * 100$$

Insulinemia at different times (0, 30, 60 and 120 min) was assessed using ELISA technique. For these time points, a supplementary sampling of 0.3 mL blood was done.

The relative bioavailability (BA% of ins-MP) was calculated from the area under the plasma level-time curve ($[AUC]_{S.C.}$, $[AUC]_{free\ ins}$, $[AUC]_{ins\text{-}MP}$) from 0–120 min.:

$$BA\% = \frac{AUC_{Test}}{AUC_{SC}} * \frac{Dose_{SC}}{Dose_{Test}} * 100$$

Microscopic Observation of Insulin in Rat Duodenum

Rats were divided into 3 groups (2 animals per group). Insulin-FITC was loaded into unloaded MP by the diffusion loading method as described above. Ins-FITC-MP were then introduced in duodenum of rat as described in the *in vivo* evaluation. Duodenal tissue not exposed to fluorescent-labeled MP (unloaded MP) was used as control to exclude the possibility of autofluorescence associated with tissues.

Negative control experiments were performed by the insertion of free insulin-FITC solution. After a 60 min incubation period, a segment of 2 cm from the experimented duodenum was quickly ligated at both end and then removed. Snap frozen duodenum in isopentan were embedded into optimal cutting temperature medium (OCT) and stored at −80°C. Seven micrometers of frozen duodenum were cut in a cryostat, cuts were performed in triplicate. Then Duodenum mucosa were fixed in 1% PFA for 20 min, washed in phosphate buffer saline (PBS) and nucleus were stained with Hoechst stain. Slides were mounted using Mountex-mounting medium (CellPath, Newtown Powys, UK). Tissues were visualized using a confocal microscope Zeiss LSM 510 Meta (Carl Zeiss, Inc).

Statistical Analysis

All experiments, except microscopic observation, were performed at least in triplicate. Values are given as mean ± standard deviation and statistical comparisons were made using analysis of variance (ANOVA). The level of significance used was $p<0.05$ (very significant at $p<0.01$).

RESULTS AND DISCUSSION

The formulation concept of insulin-loaded MP, prepared by ionic crosslinks of WP and ALG with Ca^{2+} ions, was develop in order to increase the oral bioavailability of insulin, by providing a protective environment for the drug and by improving intestinal absorption thanks to mucoadhesive polymer properties.

Preparation of Microparticles and Evaluation of Insulin Loading

Unloaded WP/ALG MP were obtained by a standardized and reproducible extrusion/gelation technique and subsequent freeze-drying. Insulin was loaded during a hydration step by absorption/adsorption. Freeze-drying and hydration preserved the spherical shape and the individually state of MP (Fig. 1). Ins-MP mean diameters were about 1450–1500 µm (Table I).

During the hydration step in pH 7.0, diameter of MP increased slightly (approx. 12%) with an increased in water uptake (approx. 43%), indicating that the morphological changes were the result of water flux (Table I). After hydration in pH 7.0, the weight loss was negligible (<4%), indicating absence of MP degradation. During hydration step in insulin solution (insulin loading), ins-MP diameter increased less than with pH 7.0 but water uptake was more pronounced for the 80/20 WP/ALG ratio (53.3%). The difference in swelling behavior between ratio 60/40 and 80/20 highlighted interactions between insulin and the polymeric matrix with probably an insulin repulsion effect stronger with ALG than WP. Indeed, insulin solution is negatively charged (Zeta potential: -38.6±3.0 mV). The surface properties of proteins, and especially the surface electrical properties, have an important influence on the ionic interactions with other biomacromolecules (20), insulin solution being less repulse by WP (−35.6± 2.8 mV) than by ALG (−96.1±7.5 mV). Consequently, the resulting insulin loading of 80/20 ins-MP was higher and about 870 IU/g of polymers (approx. more 10%, compared to 60/40, Table II). EE was linked to the swelling of the MP during hydration in insulin solution: higher swelling of ins-MP leading to higher EE (98.7% with 80/20 ratio).

Coating was further performed with an ALG-insulin solution onto ins-MP (Table II). Coating thickness, evaluated by comparison of coated to uncoated ins-MP diameters was therefore estimated by calculation at 37 and 39 µm for 60/40 and 80/20 ratio respectively. During the coating step, the drug diffusion was insignificant as indicated by comparison of EE.

Swelling Studies During *In Vitro* Dissolution and Insulin Release

Ins-MP swelling (Fig. 2) and insulin releases (Fig. 3) were evaluated, from ins-MP produced with 60/40 and 80/20 WP/ALG ratio, by using classical *in vitro* dissolution

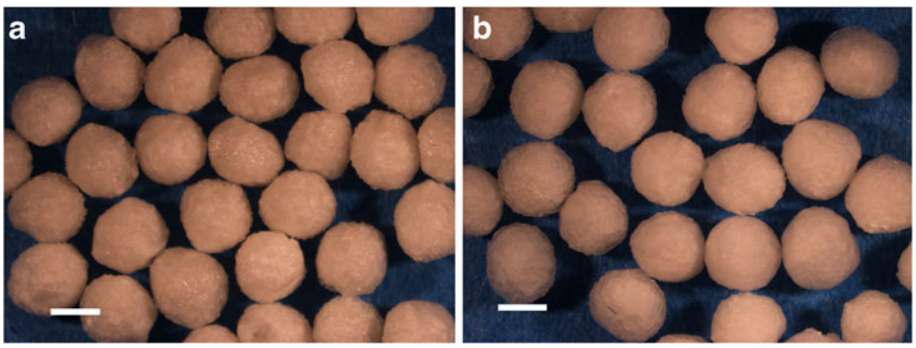

Fig. 1 Photographs of composite MP (80/20 WP/ALG) (**a**) unloaded (after freeze-drying) and (**b**) insulin-loaded (after hydration in insulin solution) taken using an optical microscope (scale bar represents 1 mm).

Table I Evaluation of Size and Swelling Behavior of Lyophilized MP (Unloaded) Prepared at Different WP/ALG Ratio (60/40 and 80/20) After 5 Min of Hydration in pH 7.0 Buffer or in Insulin Solution

Hydration step	MP Size (μm) before hydration	Swelling behavior (%)			MP Size (μm) after hydration
		Water uptake	Diameter change	Matrix weight loss	
In pH 7.0 buffer					
60/40 WP/ALG	1389 ± 114	42.8 ± 0.3	11.7 ± 2.6	3.6 ± 0.6	1551 ± 105
80/20 WP/ALG	1336 ± 111	44.7 ± 1.3	12.0 ± 2.0	3.3 ± 0.5	1496 ± 107
In Insulin solution					
60/40 WP/ALG	1389 ± 114	39.1 ± 2.6	7.8 ± 1.0	3.2 ± 0.4	1497 ± 112
80/20 WP/ALG	1336 ± 111	53.3 ± 2.0	9.8 ± 1.7	3.6 ± 0.6	1467 ± 101

Results are presented as mean ± standard deviation (n=90 for MP size and diameter change, n=6 for water uptake and matrix weight loss on samples of 130 mg)

method, in gastric (pH 1.2) and intestinal (pH 4.5, 6.8 and pH 7.4) pH. After 2 h in pH 1.2 buffer, ins-MP were resistant and still intact without any visible degradation (weight loss < 2%). Ins-MP swelled only slightly even if the swelling was more consequent with 80/20 ratio (water uptake 18.6% *vs.* 11.9% for 60/40 ratio). This behavior at pH 1.2 was already previously described for unloaded WP/ALG MP (21). At pH 1.2, carboxyl groups of ALG were protonized and hence the electrostatic repulsions among these groups lessened, favoring matrix shrinkage (22), while no matrix weight loss was detected.

In contrast, WP chains (isoelectric point of 5.2) were positively charged, resulting in repulsive forces with cation binding polymer chains (Ca^{2+}) (23) and subsequent higher matrix relaxation with higher WP content. In fact, insulin release profiles were not immediate release shape but were characterized by a higher release in 15 min for the 80/20 than for the 60/40 ratio, with 40% of the drug released. This released was followed by a gradual release during 6 h. Insulin release was clearly dependant on diffusion mechanism for the two ratio as demonstrated by the Harland equation (A>B, correlation coefficient r of approximately 0.987) and not by erosion. Nevertheless, the release step in acidic pH could be later easily overcome by using a classical gastroresistant capsule filled with ins-MP.

Table II Influence of Coating on the Encapsulation Efficiency (EE), the Insulin Loading and the Mean Size of Ins-MP Prepared with WP/ALG Ratio 60/40 and 80/20

WP/ALG ratio	EE (%)	Insulin loading (IU/g of polymers)	Mean size (μm)
60/40			
Uncoated	85.3 ± 3.7	819 ± 36	1497 ± 112
ALG-insulin coated	84.3 ± 4.1	729 ± 40	1571 ± 102
80/20			
Uncoated	98.7 ± 0.8	867 ± 7	1467 ± 101
ALG-insulin coated	97.2 ± 2.7	769 ± 21	1544 ± 98

Results are presented as mean ± standard deviation (n=3 for EE and insulin loading, n=90 for MP size)

In pH 4.5, water uptake, as well as swelling and degradation, were more limited with 60/40 ratio (<25% in 30 min) whereas ins-MP stabilities were still ensured (weight loss < 25% in all cases). Nevertheless, complete insulin releases were observed over 2 h. Again, insulin releases from ins-MP were clearly dependant on diffusion mechanism (A>B, correlation coefficient r comprised between 0.946 and 0.965) with released following matrix swelling.

Increasing pH to 6.8, increased water uptake, swelling and ins-MP degradation (but in lower proportion with the 80/20 ratio) and consequently increased drug release emphases by polymer degradation. In fact, at intestinal pH, ALG degraded rapidly due to exchange of Ca^{2+} ions binding to ALG carboxyl groups and phosphate ions of the medium (24). In comparison, as described in literature, WP appeared more stable, since no changes in water uptake and diameters were observed with WP MP within 1 h (23). It was plausible that above the isoelectric pH of WP (5.2), interactions between denatured protein chains and Ca^{2+} ions are reinforced by the negative charge of ionized carboxyl groups. These ionic interactions, combined with hydrophobic and electrostatic interactions pre-existing in the WP polymer network, could create a more stable structure. Despite the opposite behavior of WP and ALG matrices at intestinal pH, association of the two polymers in the same matrix still led to rapid degradation with fast increases in water uptake and diameter, probably due to the high sensitivity of ALG to intestinal pH (22). However, with insulin, the fast release observed *in vitro* was attributed not only to the swelling of ins-MP (matrix relaxation favoring diffusion, A>B, correlation coefficient r comprised between 0.965 and 0.983) and

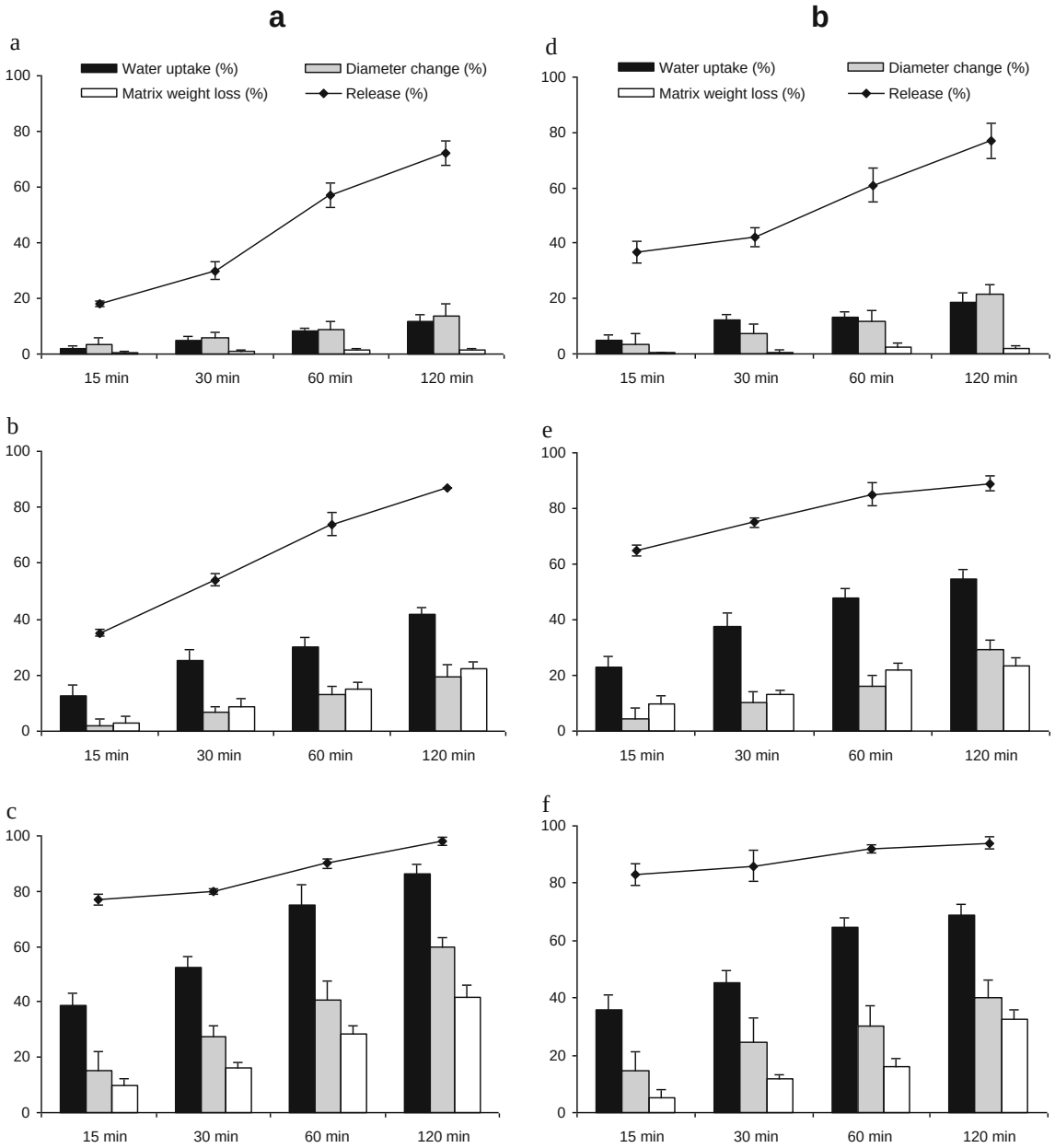

Fig. 2 Evaluation of the swelling behavior of ins-MP, prepared at different WP/ALG ratios (A: 60/40 and B: 80/20) during dissolution at pH 1.2 (**a**, **d**), pH 4.5 (**b**, **e**) and pH 6.8 (**c**, **f**) in comparison with *in vitro* insulin release in the same buffers. The results are presented as mean ± standard deviation (n=90 for diameter change, n=6 for water uptake and matrix weight loss on samples of 130 mg of MP, n=3 for drug release).

polymer degradation but also to insulin-matrix interactions. The observed burst release may be related to electrostatic repulsions between polymers and insulin. Insulin has an isoelectric point of 5.4, close to the one of WP (pI=5.2) and has the same charge as WP and ALG in function of pH. Repulsive interactions could occur between the different components leading to the relaxation of the matrix (24). Nevertheless, the swelling of our formulation observed, *in vitro* at pH 4.5 and pH 6.8, could potentially enhance insulin absorption through mechanical pressure as described by Dorkoosh *et al.* (25).

In order to improve further MP performances, the ins-MP were coated using ALG containing insulin. No differences in releases were observed in comparison to uncoated MP: the insulin release was fast and complete at pH 1.2 and pH 6.8 (Fig. 3). Coating was not effective on the contrary to a previous study performed with theophylline encapsulated into coated WP/ALG MP (9). Insulin created probably a steric hindrance in the MP and in the coating resulting in an unstable coating.

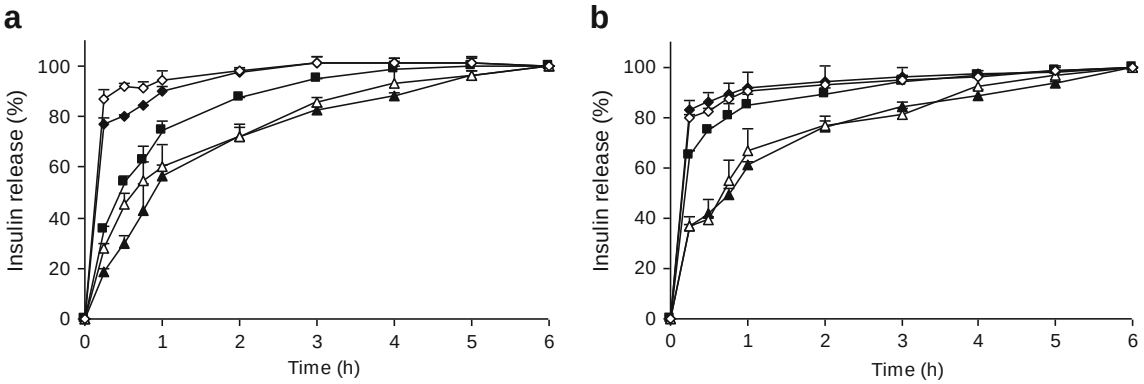

Fig. 3 *In vitro* release profiles of insulin from uncoated (black) and coated (white) ins-MP, composed of (**a**) 60/40 and (**b**) 80/20 WP/ALG, at 37°C. Experiments were performed in USP buffer at pH 1.2 (*black up-pointing triangle*), pH 4.5 (*black square*) or pH 6.8 (*black diamond*).Experiments with coated ins-MP were not performed at pH 4.5. The results are presented in % of insulin release, as mean ± standard deviation (n=3).

Given the results of EE (98.7%) and ins-MP being more stable with higher WP content, uncoated 80/20 MP were selected for further characterizations.

Determination of Protection Towards *In Vitro* Enzymatic Degradation

Insulin protection against enzymatic activities was investigated *in vitro* during 120 min. In spite of a rapid drug release at intestinal pH (Fig. 3), insulin was protected towards enzymatic degradation by ins-MP encapsulation (84% of insulin remaining intact *vs* 24% for free insulin solution) (Fig. 4). The benefice of encapsulation into WP/ALG MP was still significant at 60 min. Moreover, ELISA assays lead to prove that insulin unreleased at 60 min exhibited still a biological activity.

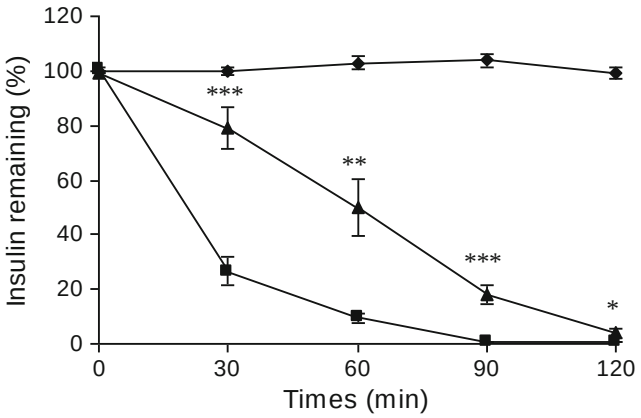

Fig. 4 Degradation studies in the presence and absence of polymeric MP. The results are presented in % of insulin remaining, as mean ± standard deviation (n=3). The experiments were performed in pH 7.4 buffer containing trypsin (10 IU/mL) and chymotrypsin (10 IU/mL) at 37°C. *Black diamond*, native insulin (untreated); *black square*, native insulin treated with enzymes; *black up-pointing triangle*, ins-MP treated with enzymes. Insulin remaining from ins-MP statistically different from native insulin: *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Mucoadhesion Studies

Mucoadhesive properties can potentially promote insulin absorption, even with a fast release on the preferential absorption site, upon condition that insulin is effectively protected from enzymatic degradation. For testing MP bioadhesive properties, Rao and Buri's method widely accepted was performed (17). The percentage of MP adhering to the intestine was considered as primary index of bioadhesive property (Fig. 5).

Glass beads used as control had no bioadhesive property. In contrast, MP made of ALG, WP and WP/ALG adhered to intestinal mucosa with more than 90% of the applied particles remaining stocked to the rabbit intestinal tissue after 30 min washing. WP based MP exhibited same mucoadhesivity than the already known ALG. Several theories described in the literature might explain the mechanism of mucoadhesion between adhesive materials and mucin. They included the theory of electrostatic adsorption (van der Waals, hydrogen

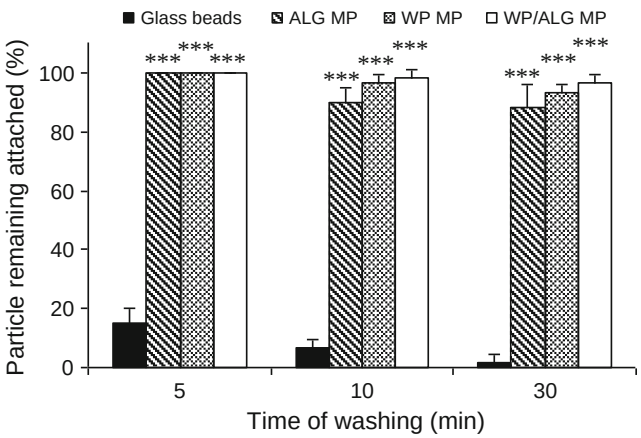

Fig. 5 Percentage of glass beads, pure ALG, pure WP and pure WP/ALG MP remaining attached on rabbit duodenum mucosa after washing with phosphate buffered saline for 30 min at an angle of 45°. The results are presented as mean ± standard deviation (n=3 per group). Statistically different from glass beads: ***$p<0.001$.

bonds), wetting, diffusion and fracture theories (26–28). ALG being anionic polymers, are good mucoadhesive agents, furthermore, polyanion polymers were more effective as bioadhesives than polycation polymers or nonionic polymers (29). WP polymers are also anionic polymers in intestinal pH, with interesting bioadhesive properties which could be able to improve drug intestinal absorption by enhancing the residence time of MP at the absorption sites (30).

Evaluation of Insulin Transport Across Duodenal Membranes

Insulin absorption by rat duodenal sac was investigated ex vivo during 180 min (Fig. 6).

After incubation with unloaded MP, no insulin was detected on the serosal or mucosal side. Following to incubation with free insulin solution, insulin was detected on the serosal side of duodenum, showing the capacity of insulin to be absorbed. Insulin absorption from solution was linear corresponding to a passive diffusion. However, this absorption was low (4.77% corresponding to approx 0.38 IU in 180 min). With ins-MP, larger amounts of insulin were detected in serosal content already in 30 min (7%, corresponding to approx 0.6 IU, significantly different from insulin administered in solution ($p<0.001$)). After 30 min, insulin amount increased linearly over time without reaching a steady state at 180 min (14% absorbed, about 1.12 IU). Nevertheless at 180 min, the entire drug was released from ins-MP (no insulin detected after ins-MP degradation). Insulin absorption from ins-MP was not linear corresponding certainly partially to a passive transport and partially to paracellular and transcellular transports (31).

The apparent permeability coefficient was increased to about 8.10^{-6} cm.s^{-1} with an absorption enhancement ratio of 2.9 showing that encapsulation into WP/ALG MP promotes insulin intestinal absorption (Table III). The absorption enhancement ratio is comparable to the one obtained in the literature with thiolated hydrogel MP for example (32).

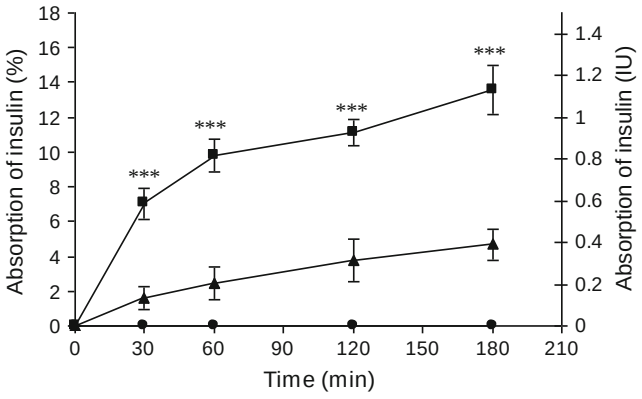

Fig. 6 Evaluation of insulin absorption into a tissue chamber from rat duodenal sac after introduction of insulin solution (*black up-pointing triangle*), ins-MP (*black square*) and unloaded MP (*black circle*) ($n=5$) during 180 min. Statistically different from insulin solution: ***$p<0.001$.

Determination of Bioactivity Protection by Microparticles

Ins-MP were introduced into rat duodenal sacs. In order to determine the protection provided by MP against ex vivo enzymatic degradation, both the amount of insulin (determined by RP-HPLC, Table IV) and the biological activity of insulin (determined by ELISA and after subcutaneous administration to rats, Fig. 7) were both estimated. These determinations were performed on the fraction of insulin released from ins-MP and transported into duodenal serosal side as well as on the fraction of insulin remaining encapsulated.

As observed during *in vitro* dissolution, about 24.7% of encapsulated insulin was unreleased in duodenal sacs and still encapsulated in 30 min (2.0 IU). This fraction of insulin was protected from duodenal enzymatic degradation as shown by i) identical retention time of insulin still encapsulated, of insulin extracted from ins-MP and of the insulin standard solution, no degradation products were observed (data not shown), ii) a biological activity of this insulin after subcutaneous injection (decrease of blood glucose level) (Fig. 7). These results suggested the maintenance of insulin stability and were confirmed by ELISA. The immunoreactivity values of insulin were comparable with those obtained from HPLC analysis and proved the protected effect of encapsulation (Table V).

The fraction of insulin absorbed by the rat duodenum in 180 min (1.1 IU) presents also chromatograms without degradation products and exhibited a biological activity i) after subcutaneous injection (limited by the presence of glucose inside Krebs Henseleit modified buffer used for experiments) (Fig. 7) and ii) after ELISA assay. These results reinforced the bioactivity protection from proteolytic enzymes by ins-MP not only during encapsulation process but although during intestinal residence and absorption.

The effective mucoadhesiveness of polymers seemed efficient and thus enhanced the insulin absorption by increase of the residence time and by close contact of the insulin at

Table III Apparent Permeability Coefficient (P_{app}) and Absorption Enhancement Ratio (R) for Insulin Across Excised Duodenum of Rats

Sample	P_{app} ($\times 10^{-6}$ cm.s^{-1})	*R*
Control (insulin solution)	2.7 ± 0.5	
Ins-MP (WP/ALG 80/20)	7.9 ± 0.8[a]	2.9

Results are presented as mean ± standard deviation ($n=5$). The control was the insulin solution introduced in intestine

[a] Statistically significant difference from control, $p<0.001$

Table IV Percentage and Amount of Insulin Released, Absorbed or Unreleased from Ins-MP After 30 Min or 180 Min Incubation Time in Excised Duodenum of Rat ($n=5$), Determination by RP-HPLC

	Ins-MP	Incubation time	Insulin unreleased	Insulin released[a]	Insulin absorbed
Insulin amount	8 IU	30 min	2.0 ± 0.1 IU[b]	6.0 ± 0.1 IU	0.6 ± 0.1 IU
	100%		24.7 ± 1.0%	75.2 ± 1.0%	7. 0 ± 0.3%
	8 IU	180 min	0.0 IU	6.9 ± 0.1 IU	1.1 ± 0.1 IU[b]
	100%		0%	86.5 ± 1.4%	13.5 ± 1.4%

[a] Obtained by calculation

[b] Values confirmed by ELISA

Table V Change in Plasma Insulin Level *Versus* Time Following Chirurgical Opening and: A) Subcutaneous Injection of Insulin (Ins-SC, 6 IU/kg), B) Intraduodenal Administration of Free Insulin Solution (Free-Ins, 70 IU/kg) and C) Intraduodenal Administration of Insulin-Loaded MP (Ins-MP, 70 IU/kg) to Healthy Male Wistar Rats

	ELISA Insulin (mIU/mL)		
Time (min)	Ins-SC	Free-ins	Ins-MP
0	–	–	–
30	86 ± 3	9 ± 3	35 ± 3
60	85 ± 4	16 ± 2	82 ± 3
120	40 ± 3	33 ± 2	104 ± 2

the absorption site. These results highlighted the interest of such formulation: the combination of WP and ALG procured insulin protection and promoted ex vivo local absorption.

In Vivo Evaluation ins-MP

The effectiveness of the oral ins-MP prepared with WP and ALG was also evaluated by the measurement of plasma glucose reduction in healthy rats and by ELISA assay (Fig. 8). Indeed, glycemia corresponding to the presence of insulin at the site of action can be considered as the accurate marker of insulin bioavailability whereas insulin blood concentration would be a surrogate marker of pharmacodynamic activity.

The efficacy of ins-MP was assessed by a duodenal administration method in rats, method previously used for determining absorption efficiency in animals (19). Insulin solution and unloaded MP, administered intraduodenally as well as the subcutaneously administered insulin solution after surgery served as controls. Figure 8 showed response curves obtained by plotting the percent blood glucose reduction from initial glucose levels *versus* time. The mean blood glucose baseline (initial glucose level) value was taken as the 100% level and all other blood glucose level/time data were calculated as a percentage of the baseline.

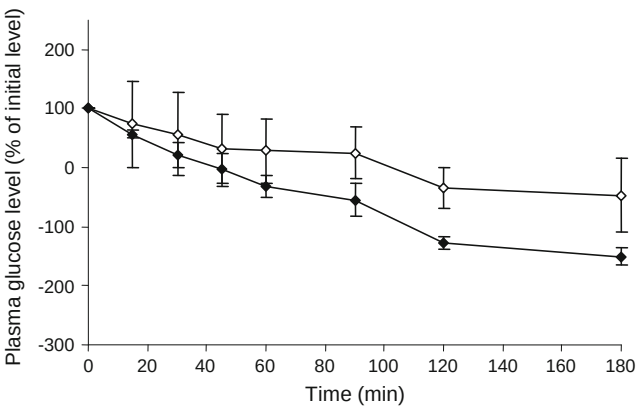

Fig. 7 Change in plasma glucose level *versus* time profiles following subcutaneous injection, in healthy male Wistar rats, of (*black diamond*) insulin extracted from ins-MP after 30 min in duodenal sacs or (*white diamond*) insulin collected in the serosal side 180 min after introduction of ins-MP in duodenal sacs. The injected dose of insulin was 3 IU/kg or 4 IU/kg respectively. The results are presented as mean ± standard deviation ($n=5$ per group).

The blood glucose levels after surgery (evaluation of surgery effect by intraduodenal administration of unloaded MP) were higher than the initials levels: this hyperglycemic effect, commonly described in the literature, was due to the stress associated with the surgery (33). The decrease in blood glucose associated with the subcutaneous insulin injection showed the hypoglycaemic effect of insulin: Concomitant subcutaneous administration of insulin counteracted the surgery hyperglycemic effect and normalized glycemia values . ELISA assay confirmed that the decreased in blood glucose level was due to human insulin (no cross reaction with rat insulin). The experiment was performed in animals on 120 min for ethical reasons (guidelines for collection of bloods from experimental animals). This time of experiment does not allow the curves to reach 100% glucose after 2 h especially in the case of uncoated MP (effect of surgery).Intestinal administered free insulin solution at a dose of 70 IU/kg body weight resulted in a slight fall in the blood glucose level within 2 h of administration; this weak hypoglycaemic effect was observed by other authors and confirmed the well-known inefficiency of insulin administered orally in solution (15,34). Indeed, the greatest barriers to the oral delivery of proteins lie in the gastro-intestinal tract. Dietary proteins do not normally cross the intestinal epithelium intact, but must first be broken down to their constituent free amino acids which are then absorbed. Of course, this route of protein absorption destroys physiological activity of the protein and explains why typical oral bioavailabilities of proteins are usually less than 1–2% (35). Bioavailability is defined in term of rate and extent and the best parameter to estimate the extent of absorption is the AUC or a fraction of AUC in our case. The insulin absorption was estimated of approximately 3,7% by glycemia measurement (PA) and 2,4% by ELISA assay (BA) in comparison to subcutaneous administration. As expected based on *in vitro* results, the ins-MP (70 IU/kg body weight) prepared with mucoadhesive WP and ALG polymers

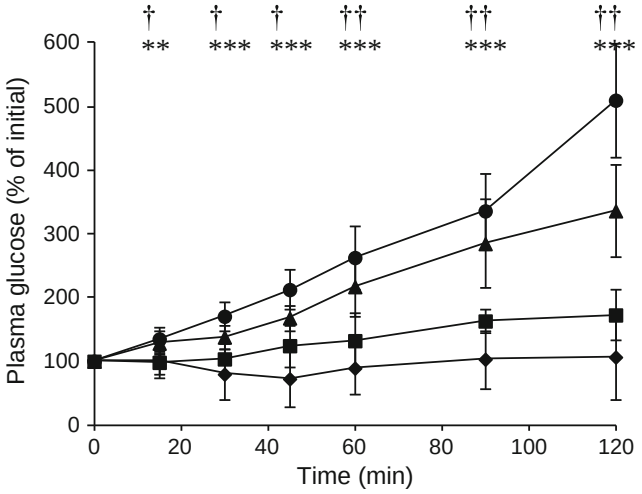

Fig. 8 Change in plasma glucose level *versus* time profiles following surgery and s.c. injection of insulin solution (6 IU/kg) (*black diamond*), intraduodenal administration of insulin solution (70 IU/kg) (*black up-pointing triangle*) intraduodenal administration of ins-MP (70 IU/kg) (*black square*) or intraduodenal administration of unloaded MP (*black circle*) in healthy male Wistar rats. Insulin s.c. injection and administration of insulin solution in duodenum were used as controls. The results are presented as mean ± standard deviation ($n=6$ per group). Ins-MP statistically different from unloaded MP: $^{**}p<0.01$; $^{***}p<0.001$ and from duodenal administration of insulin solution: $\dagger p<0.05$; $\dagger\dagger p<0.01$.

produced blood glucose reduction significantly higher, at each time point studied, from intestinal administration of free insulin solution (from $p<0.05$ to $p<0.001$). After 120 min, no significant difference in glycemia values was observed after subcutaneous administration of free insulin solution (6 IU/kg) and after intraduodenal administration of ins-MP (70 IU/kg). The $[AUC]_{ins\text{-}MP}$ and $[AUC]_{S.C.}$ from 0–120 min were 16113±2785 and11236±4738, respectively, indicating a relative pharmacological availability (PA) of 6,4%.

The oral bioavailability of ins-MP was estimated by ELISA assay. The $[AUC]_{ins\text{-}MP}$ and $[AUC]_{S.C.}$ from 0–120 min were 7845±53 mUI*h/mL and 7605±95 mUI*h/mL, respectively, indicating a relative bioavailability (BA) of 8,8%.

Confocal Microscopic Observations of the Duodenum Tissues After *In Vivo* Administration

In order to visualize insulin absorption, we followed the insulin uptake by confocale observations of section of the intestinal mucosa 60 min after administration of FITC-ins-MP; free FITC-insulin solution and unloaded MP were administrated as controls.

Confocal microscopic observations of the duodenal tissues demonstrated again the capability of ins-MP to promote insulin intestinal absorption. The nuclei were stained using Hoechst stain and appeared blue, while the green fluorescence corresponds to the free insulin-FITC. The substrates treated with unloaded MP or free insulin-FITC solution (Fig. 9a and b) were characterized by no fluorescence while the tissue treated with FITC-ins-MP showed green spots into the epithelial mucosa close to the nuclei (Fig. 9c). FITC-insulin was therefore protected from intestinal enzyme degradation. MP protection towards enzymatic degradation combined with mucoadhesive properties of MP can contribute to protect the encapsulated material and to allow an absorption by prolonging residence time and increasing concentration gradient of FITC-insulin near the intestinal cells which increased its absorption. In this way, release of insulin biologically active directly into the mucus layer can lead to two advantages. On one hand, in the mucus, the access to the drug of luminally secreted enzymes such as trypsin, chymotrypsin, carboxypeptidase, and elastase could be partly inhibited (36). Moreover, the enzymes, bound to the membrane of mucosal cells of the intestinal epithelium such as a large variety of exopeptidases, revealed significantly weaker enzymatic activity towards insulin than luminal enzymes. This effect was schown by Lueβen *et al.* (36): insulin was relatively stable against degradation by brush border membrane vesicles, which was due to its conformational structure where the terminal ends of the A- and B chains could be sterically protected from exopeptidase degradation. On the other hand, insulin transport after release from mucoadhesive polymers is dominantly via paracellular diffusion (37), which

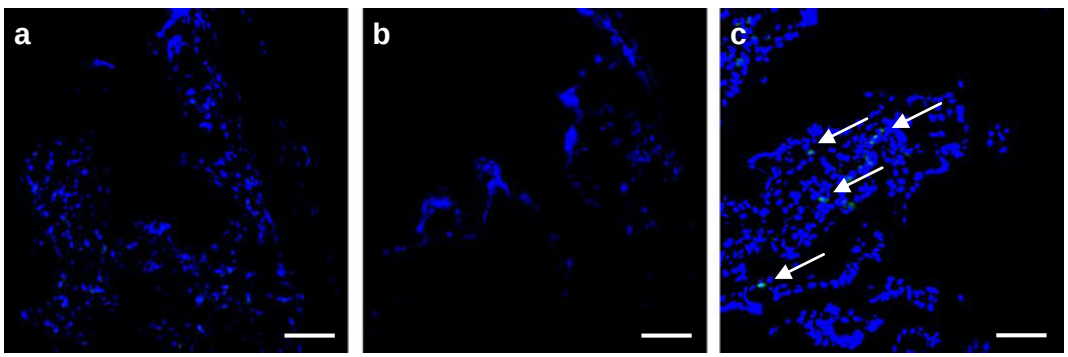

Fig. 9 Confocale microscopic observations of the duodenum tissues treated during 60 min with (**a**) unloaded MP, (**b**) insulin-FITC solution or (**c**) insulin-FITC-loaded MP in duodenum of healthy male Wistar rats. Administration of unloaded MP or insulin-FITC solution in duodenum, were used as controls. (scale bar represents 50 μm). Arrows referred to insulin-FITC.

was not related to cytosolic degradation. A high concentration gradient of insulin towards the absorption membrane represents the driving force of the passive paracellular uptake (38), thus favoring insulin transport. Moreover, other mechanisms increasing insulin absorption can be involved. Some mucoadhesive polymers exhibited (i) capacities of enzymatic inhibition, based on their Ca^{2+} binding abilities which could effectively protect the encapsulated material from the enzymes in the intestine and (ii) capacities of permeation enhancement as a result of opening of the tight junction (39).

CONCLUSIONS

This work has resulted in the development of insulin-loaded WP/ALG MP characterized by high EE. Despite fast release in intestinal buffers, *in vitro* experiments demonstrated useful properties for these ins-MP featuring matrix swelling and protection against enzymatic activities even if, ins-MP will have to be include inside classical gastroresistant capsule for *in vivo* delivery. Our ins-MP exhibited excellent mucoadhesive properties on rabbit's duodenum. Significant amounts of insulin biologically active were transported across duodenal membranes ex vivo as well as *in vivo*: the protective effects and the mucoadhesive properties of WP and ALG improved intestinal delivery of insulin. Confocal microscopic observations confirmed that WP/ALG MP were potentially useful as oral delivery vehicles for bioactive such as insulin.

REFERENCES

1. Tan ML, Choong PFM, Dass CR. Recent developments in liposomes, microparticles and nanoparticles for protein and peptide drug delivery. Peptides. 2010;31(1):184–93.
2. Wong TW. Design of oral insulin delivery systems. J Drug Targeting. 2010;18(2):79–92.
3. Lyer H, Khedkar A, Verma M. Oral insulin – a review of current status. Diabetes Obes Metab. 2010;12(3):179–85.
4. Khafagy ES, Morishita M, Onuki Y, Takayama K. Current challenges in non-invasive insulin delivery systems: a comparative review. Adv Drug Deliv Rev. 2007;59(15):1521–46.
5. Malafaya PB, Silva GA, Reis RL. Natural–origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications. Adv Drug Deliv Rev. 2007;59(4–5):207–33.
6. De Wit JN. Nutritional and functional characteristics of whey proteins in food products. J Dairy Sci. 1998;81(3):597–608.
7. Hongsprabhas P, Barbut S. Ca^{2+} -Induced cold gelation of whey protein isolate: effect of two-stage gelation. Food Res Int. 1998;30(7):523–7.
8. Beaulieu L, Savoie L, Paquin P, Subirade M. Elaboration and characterization of whey protein beads by an emulsification/cold gelation process: application for the protection of retinol. Biomacromolecules. 2002;3(2):239–48.
9. Hebrard G, Hoffart V, Cardot JM, Subirade M, Alric M, Beyssac E. Investigation of coated whey protein/alginate beads as sustained release dosage form in simulated gastrointestinal environment. Drug Dev Ind Pharm. 2009;35(9):1103–12.
10. Chen L, Subirade M. Alginate-whey protein granular microspheres as oral delivery vehicles for bioactive compounds. Biomaterials. 2006;27(26):4646–54.
11. Guerin D, Vuillemard JC, Subirade M. Protection of bifidobacteria encapsulated in polysaccharide–protein gel beads against gastric juice and bile. J Food Prot. 2003;66(11):2076–84.
12. Hebrard G, Blanquet S, Beyssac E, Remondetto G, Subirade M, Alric M. Use of whey protein beads as a new carrier system for recombinant yeasts in human digestive tract. J Biotechnol. 2006;127(1):151–60.
13. George M, Abraham TE. Polyionic hydrocolloids for the intestinal delivery of protein drugs: alginate and chitosan, a review. J Control Release. 2006;114(1):1–14.
14. Beyssac E, Touaref F, Meyer M, Jacob L, Sandouk P, Aiache JM. Bioavailability of morphine after administration of a new bioadhesive buccal tablet. Biopharm Drug Dispos. 1998;19(6):401–5.
15. Builders PF, Kunle OO, Okpaku LC, Builders MI, Attama AA, Adikwu MU. Preparation and evaluation of mucinated sodium alginate microparticles for oral delivery of insulin. Eur J Pharm Biopharm. 2008;70(3):777–83.
16. Harland RS, Gazzaniga A, Sangalli ME, Colombo P, Peppas NA. Drug /polymer matrix swelling and dissolution. Pharm Res. 1998;5(8):488–94.
17. Rao Ranga KV, Buri BA. A novel in situ method to test polymers and coated microparticles for bioadhesion. Int J Pharm. 1989;52(3):265–70.
18. Garrait G, Jarrige JF, Blanquet S, Beyssac E, Cardot JM, Alric M. Gastrointestinal absorption and urinary excretion of transcinnamic and p-coumaric acids in rats. J Agric Food Chem. 2006;54(8):2944–50.
19. Whitehead K, Shen Z, Mitragotri S. Oral delivery of macromolecules using intestinal patches: applications for insulin delivery. J Control Release. 2004;98(1):37–45.
20. Zhao Y, Li F, Carvajal MT, Harris MT. Interactions between bovine serum albumin and alginate: an evaluation of alginate as protein carrier. J Colloid Interface Sci. 2009;332(2):345–53.
21. Hébrard G, Hoffard V, Beyssac E, Cardot JM, Alric M, Subirade M. Coated whey protein/alginate microparticles as oral controlled delivery systems for probiotic yeast. J Microencapsul. 2010;24(4):292–302.
22. Pasparakis G, Bouropoulos N. Swelling studies and in vitro release of verapamil from calcium alginate and calcium alginate-chitosan beads. Int J Pharm. 2006;323(1–2):34–42.
23. Remondetto GE, Beyssac E, Subirade M. Iron availability from whey protein hydrogels: an in vitro study. J Agric Food Chem. 2004;52(26):8137–43.
24. Ouwerx C, Veilings N, Mestdagh MM, Axelos MAV. Physico-chemical properties and rheology of alginate beads formed with various divalent cations. Polym Gel Netw. 1998;6(5):393–408.
25. Dorkoosh FA, Borchard G, Rafiee-Tehrani M, Verhoef JC, Junginger HE. Evaluation of superporous hydrogel (SPH) and SPH composites in porcine intestine ex vivo: assessment of drug transport, morphology effect, and mechanical fixation to intestinal wall. Eur J Pharm Biopharm. 2002;53(2):161–6.
26. Mortazavi SA, Smart JD. An investigation of some factors influencing the in vitro assessment of mucoadhesion. Int J Pharm. 1995;116(28):223–30.
27. Burjak M, Bogataj M, Velnar M, Grabnar I, Mrhar A. The study of drug release from microspheres adhered on pig vesical mucosa. Int J Pharm. 2001;224(1–2):123–30.

28. Dobrozsi DJ, Smith RL, Sakr AA. Comparative mucoretention of sucralfate suspensions in an everted rat esophagus model. Int J Pharm. 1999;189(1):81–9.
29. George M, Abraham TE. Polyionic hydrocolloids for the intestinal delivery of protein drugs: alginate and chitosan. J Control Release. 2006;114(1):1–14.
30. Dodou D, Breedveld P, Wieringa PA. Mucoadhesives in the gastrointestinal tract: revisiting the literature for novel applications. Eur J Pharm Biopharm. 2005;60(1):1–6.
31. Kavimandan NJ, Peppas NA. Confocal microscopic analysis of transport mechanisms of insulin across the cell monolayer. Int J Pharm. 2008;354:143–8.
32. Sajeesh S, Vauthier C, Gueutin C, Ponchel G, Sharma CP. Thiol functionalized polymethacrylic acid-based hydrogel microparticles for oral insulin delivery. Acta Biomater. 2010;6 (8):3072–80.
33. Cilek A, Celebi N, Tirnaksiz F, Tay A. A lecithin-based microemulsion of Rh insulin with aprotinin for oral administration: investigation of hypoglycaemic effects in non diabetic and STZ-induced diabetic rats. Int J Pharm. 2005;298(1):176–85.
34. Kim BY, Jeong JH, Park K, Kim JD. Bioadhesive interaction and hypoglycaemic effect of insulin-loaded lectin-microparticles conjugates in oral insulin delivery system. J Control Release. 2005;102 (3):525–38.
35. Pauletti G, Gangwar S, Knipp GT, Nerurkar MM, Okuma FW, Tamura K, *et al.* Structural requirements for intestinal absorption of peptide drugs. J Control Release. 1996;41(1):3–17.
36. Lueβen HL, Bohner V, Perard D, Langguth P, Verhoef JC, de Boer AG, *et al.* Mucoadhesive polymers in peroral peptide drug delivery. V. Effect of poly (acrylates) on the enzymatic degradation of peptide drugs by intestinal brush border membrane vesicles. Int J Pharm. 1996;141(1–2):39–52.
37. Peppas NA. Devices based on intelligent biopolymers for oral protein delivery. Int J Pharm. 2004;277(1–2):11–7.
38. Schnurch AB, Kast CE. Chemically modified chitosans as enzyme inhibitors. Adv Drug Deliv Rev. 2001;52(2):127–37.
39. Yin L, Ding JY, Fei L, He M, Cui F, Tang C, *et al.* Beneficial properties for insulin absorption using superporous hydrogel containing interpenetrating polymer network as oral delivery vehicles. Int J Pharm Biopharm. 2008;350:220–9.