



Cyclodextrin complexed insulin encapsulated hydrogel microparticles: An oral delivery system for insulin

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ABSTRACT

An oral insulin delivery system based on methyl- β -cyclodextrin (MCD) complexed insulin encapsulated polymethacrylic acid (PMAA) hydrogel microparticles was evaluated in this investigation. Poly(methacrylic acid)-chitosan-polyethylene glycol (PCP) microparticles were prepared by ionic gelation method. The insulin–MCD (IC) complex prepared was characterized by fluorescence spectroscopic and isothermal titration micro-calorimetric (ITC) methods. MCD complexed insulin was encapsulated onto PCP microparticles by diffusion filling method. Loading and release properties of the complexed insulin from microparticles were evaluated under *in vitro* conditions. The effect of MCD complexation on the permeability of insulin was studied using Caco 2 cell monolayers and excised intestinal tissue with an Ussing chamber set-up. *In vivo* experiments were carried on streptozotocin induced diabetic rats to evaluate the efficacy of MCD complexed insulin encapsulated PCP microparticles to deliver insulin by the oral route.

IC complex formation was established by fluorescence and ITC investigations. Insulin loading and release properties from the hydrogel matrix was rather unaffected by the MCD complexation. However MCD complexation was effective in enhancing insulin transport across Caco 2 cell monolayers, when applied in combination with the PMAA hydrogel system. Both insulin and MCD complexed insulin encapsulated PCP microparticles were effective in reducing blood glucose level in diabetic animal models. Cyclodextrin complexed insulin encapsulated hydrogel microparticles appear to be an interesting candidate for oral delivery of insulin.

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1. Introduction

β Cyclodextrins (CD) are cyclic oligosaccharides capable of forming non-covalent inclusion complexes with a variety of drugs, including proteins [1]. CD complex formation often improves the physicochemical and biological properties of guest molecules [2,3]. CD complexes with protein drugs such as insulin, might drastically reduce their state of aggregation in solution [4]. The hydrophobic domains in the protein molecule can penetrate into the non-polar CD cavity, leading to the formation of non-covalent inclusion complexes [4]. Apart from the complexation aspects, hydrophobic derivatives of CD, such as MCD are also known to enhance the absorption of hydrophilic molecules across biological barriers [5,6].

Combining CD complexed systems with polymeric carriers seems to be an interesting strategy in improving oral drug delivery [7,8]. CD complexes may help in enhancing drug stability/absorption, while the particulate delivery system may serve as a platform for the encapsulation of the complexed drugs [9,10]. In this view, it can be assumed that the combination of hydrogel carriers with CD complexes

might enhance the efficacy of orally administered insulin [11,12]. Polymethacrylic acid (PMAA) based hydrogel particles were identified as a potential candidate for oral insulin delivery, as they can enhance the paracellular permeability of hydrophilic compounds and can also inhibit the protease enzymes [13].

The present study aims to investigate the efficacy of MCD complexed insulin entrapped in PCP microparticles. At first, the formation of IC complex was characterized using fluorescence spectroscopy and ITC method. Secondly, the MCD complexed insulin was encapsulated into PCP hydrogel particles and loading/releasing properties were evaluated *in vitro*. Permeability of insulin was studied across a monolayer of Caco 2 cells and through excised intestinal tissue with an Ussing chamber set-up. Finally, the performance of the system to deliver active insulin *in vivo* by the oral route was evaluated on model diabetic rats induced by streptozotocin (STZ).

2. Materials and methods

2.1. Materials

Methacrylic acid (MAA), ethylene glycol dimethacrylate (EDMA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

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(MTT), polyethylene glycol (20 kDa), potassium persulfate, streptozotocin (STZ) were purchased from Sigma–Aldrich. Chitosan (CS) with approximate molecular weight of 270 kDa and 85% deacetylation was obtained from India Sea Foods (India). Methyl- β -Cyclodextrin (MS 1.8, MW 1313) was from Cyclolab Ltd. (Hungary), and Human insulin Incelligent™ AF was obtained from Millipore (Kankakee, IL). In vitro release experiments were conducted in enzyme free simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.4), prepared according to USP standards. All animal experiments were conducted with the approval of Institute Ethics Committee. The agreements for animal experiments were A92-019-01 (Université Paris Sud-11) and B 1342009 V (SCTIMST).

2.1.1. Analytical methods

Insulin concentration was analyzed by high performance liquid chromatography (HPLC) using Waters 2690 separations module, with Waters 2487 dual absorbance detector (Waters, Guyancourt, France) and a C18 column (YP5C18-25QS Interchim, 250 × 4.6 mm, 5 μ m). The mobile phase consisted of two solutions; solution A was water with 0.1% (v/v) trifluoroacetic acid (TFA) and solution B was acetonitrile/water (50:50) with 0.1% (v/v) TFA and gradient elution was performed with a flow rate of 1 ml/min over 0–30 min (linear gradient from 50% A/50% B to 100% B in 15 min and 50% A/50% B in 20–30 min.) [13]. UV detection was performed at a wavelength of 220 nm and 50 μ l of sample was injected for each analysis. For determining the linearity of the method, different concentrations of insulin in the range from 0.1 mg/ml to 0.005 mg/ml was prepared and analyzed. The limit for the correlation coefficient was set to ≥ 0.999 .

2.2. Synthesis of PCP hydrogel microparticles

PCP microparticles were prepared by a modified ionic gelation method as previously described [14,15]. In a typical experiment, MAA (3 g) was polymerized with 0.2 g EDMA in the presence of 0.01 g CS and 0.1 g polyethylene glycol, by using potassium persulfate as the initiator. Reaction was carried out for 6 h at 60–70 °C and particle suspension so obtained was centrifuged at 10,000 rpm (6708 g) for 10 min; particles isolated were dried under vacuum. The hydrodynamic mean diameter of the particles was determined by dynamic light scattering using Zetasizer (Nano-ZS, Malvern, UK) at a wavelength of 633 nm at 25 °C with an angle detection of 90°. The particles were dispersed in phosphate buffer (pH 7.0) prior to this analysis.

2.3. Preparation and characterization of insulin–MCD (IC) complex

2.3.1. Preparation of the IC complex

Insulin solution (10 ml) in phosphate buffer pH 7.0 (1 mg/ml) was mixed with MCD at room temperature and the complex was stirred for 30 min under magnetic stirring. Complex was kept for another hour at room temperature. In this investigation insulin and MCD were mixed in three different weight ratios, 1:2.5, 1:5, 1:10 insulin to MCD (w/w) and were designated as IC I, IC II and IC III respectively.

2.3.2. Fluorescence spectroscopic investigation

Fluorescence emission spectrum of insulin solution (0.5 mg/ml) was recorded by the excitation of the sample at 280 nm and emission was recorded from 290–350 nm. Increasing concentration of solid MCD (1, 2.5, 5, 10 mg) was added to the same insulin solution in a step-wise manner and fluorescence emission intensity was measured with Varian Cary Eclipse fluorescence spectrophotometer.

2.3.3. Isothermal titration micro-calorimetric (ITC) studies

2.3.3.1. Preparation of solutions for ITC experiments. MCD (50 mM) solutions were prepared by dissolving the corresponding weight of the MCD powder in phosphate buffer (pH 7.0). Insulin solution was

prepared by dissolving insulin powder in 200 μ l of 1 M HCl and the pH of the solution was adjusted to 7.0 with the addition of 1 M NaOH solution. Thereafter, insulin solution was diluted with phosphate buffer to obtain a concentration of 1 mg/ml (0.1768 mM). For the preparation of insulin–MCD solution, neutral insulin solution was diluted with phosphate buffer containing 50 mM MCD to obtain a final insulin concentration of 1 mg/ml.

2.3.3.2. ITC experiments. An isothermal calorimeter instrument (MicroCal Inc., USA) was periodically calibrated either electrically using an internal electric heater, or chemically by measuring the dilution enthalpy of methanol in water. To investigate the interaction between insulin and MCD, 10 μ l of insulin solution (1 mg/ml) contained in the stirring syringe (283 μ l), was titrated with an aqueous solution of MCD contained in the calorimetric cell. Intervals between the injections were 600 s and agitation speed was 220 rpm [16,17]. Temperature of the experiment was fixed to 25 °C. Data consisted of a series of heat flows as a function of time. They were collected automatically and analyzed by the Windows-based Origin 7 software package supplied by MicroCal.

ITC was further used to study the energy of dissociation of insulin (0.1768 mM) in the presence of MCD (50 mM). In a typical dilution experiment, 10 μ l of insulin dissolved in buffer or buffer/MCD mix, were injected into the calorimeter reaction cell (1.43 ml) containing the identical buffer or buffer/MCD mixture. Integrated heat data, after correction for control (consisting on the dilution of insulin solution into phosphate buffer) were analyzed by non-linear regression in terms of a monomer–dimer equilibrium model leading to the determination of the apparent equilibrium constant (K_{diss}) and the enthalpy of dissociation (ΔH_{diss}^0 per mole of dimer). Other thermodynamic parameters (ΔG_{diss}^0 and ΔS_{diss}^0) were calculated as follows:

$$\Delta G_{\text{diss}}^0 = -RT \ln K_{\text{diss}} = \Delta H_{\text{diss}}^0 - T\Delta S_{\text{diss}}^0$$

2.4. Insulin loading and release experiments

2.4.1. Insulin encapsulation efficiency

A known amount of dried PCP particles (100 mg) was kept in either insulin or IC II (1:5 weight ratio) complex solution for remote loading. After specified time interval (8 h), particles were taken out and insulin loaded particles were dried under vacuum. PCP particles containing IC complex in a weight ratio of 1:5 was denoted as PCP-IC II, while PCP with unmodified insulin was referred as PCP-ins. Insulin content inside these microparticles was determined by suspending 50 mg in 20 ml SIF (pH 7.4). After 24 h, an aliquot (200 μ l) was withdrawn and insulin content was analyzed by HPLC [13].

Encapsulation efficiency (EE) was calculated as follows

$$EE = \frac{(\text{Total amount of insulin used} - \text{Amount of unloaded insulin})}{\text{Total amount of insulin used}} \times 100$$

2.4.2. In vitro release studies

For the insulin release experiments, 100 mg of insulin loaded particles (PCP-ins and PCP-ICII) were suspended separately in 20 ml of SGF and SIF (pH 1.2 and 7.4 respectively). At regular intervals of time, an aliquot of sample (200 μ l) was withdrawn from this release medium and insulin content was estimated by HPLC method. The release medium was replaced with fresh buffer to maintain total volume after each withdrawal.

2.5. Cytotoxicity studies

The colonic adenocarcinoma cell line, Caco 2 was obtained from the American Type Culture Collection (Rockville, MD) and was grown

as described elsewhere [18]. Caco 2 cells used for MCD cytotoxicity assay were seeded on 96 well culture plates at seeding density of 2×10^4 cells/well and grown for 5–6 days. Once a monolayer was obtained, culture medium was replaced with MCD (100 μ l) test solution in PBS and cells were incubated at 37 °C for 4 h. Test medium and sodium dodecylsulfate (SDS) (2% w/v) were used as negative (non-cytotoxic) and positive (cytotoxic) controls, respectively. Thereafter, the medium were removed and 100 μ l of MTT solution (0.5 mg/ml) was added to each well and the cells were incubated for another 3 h at 37 °C. The reaction products were then solubilized in 200 μ l of DMSO, before quantifying the color of reaction product using a microplate reader (Multiscan MS) at 570 nm. Data were expressed as the percentage of viable control cells calculated from the absorbance at 570 nm by comparing the test with the control systems.

2.6. Insulin transport experiments

2.6.1. Transport experiment with Caco 2 cell monolayers

Caco 2 cell monolayer for the transport studies, were grown for 21 days on a porous polycarbonate filter membranes with a pore size of 0.4 μ m and 13 mm diameter (Costar Transwell®). On the day of the transport experiments, the culture medium was replaced with an equal volume of Hank's balanced salt solution containing calcium and magnesium chloride (HBSS, Gibco) and cells were incubated with the transport medium for an hour prior to the experiment. Permeation experiments were carried out in two different series. In the first series, 0.5 ml IC complex were applied to the Caco 2 cell monolayers at three different compositions (IC I, IC II, IC III). Insulin concentration at the donor compartment was maintained at 1 mg/ml. In the second series of experiments, 2.5 mg PCP microparticles were applied to the monolayers and HBSS containing either insulin or IC II complex at a concentration of 1 mg/ml was introduced to the donor compartment. In both types of experiments, an aliquot of sample (50 μ l) was withdrawn from the receptor compartment at time periods of 0, 30, 60, 90 and 120 min and concentration of insulin was assessed by HPLC technique.

2.6.2. Transport experiments with excised intestinal tissue

Small intestinal tissue from male Wistar rats (250–300 g) (Charles River, Paris) was excised and mounted on an Ussing chamber experimental set-up [19]. Thereafter, 5 mg PCP microparticles was applied to the mucosal surface and Krebs–Ringer buffer (5 ml) containing either insulin or IC II complex at a concentration of 0.5 mg/ml was introduced to the donor compartment. At pre-set time intervals, an aliquot of 200 μ l was recovered from the acceptor chamber and replaced with the same volume of fresh medium. Assays were carried out for 2 h and four tissue portions were used to evaluate each formulation.

2.6.3. Analysis of permeation experiments

The apparent permeability coefficient (P_{app}) for Caco 2 cells and Ussing chamber experiments was calculated using the following equation

$$P_{app} = (dQ/dt) \times (1/A \cdot C_0)$$

Where dQ/dt is the flux of insulin from the mucosal to the serosal side of the Caco 2 cell monolayer or of the intestinal mucosa, C_0 is the initial concentration of insulin in the donor compartment and A is the area of the membrane (1 cm² for Ussing chamber and 1.12 cm² for Caco 2 cell monolayers).

Absorption enhancement ratio (R) was calculated as follows

$$R = P_{app}(\text{sample}) / P_{app}(\text{control}).$$

2.7. In vivo evaluation on diabetic animals

Male Wistar rats (200–250 g average body weight) were obtained from Mahaveera Enterprises (Hyderabad, India). STZ was dissolved in citrate buffer (pH 4.5) and diabetes was induced in rats by injecting STZ (35 mg/kg) intraperitoneally [20]. After 3 days of injections, diabetic values were checked and animals having diabetic level above 250 mg/dl were selected for these experiments. Animals were kept for 16 h fasting, prior to the start of experiments. Rats were divided into five groups each containing minimum of 5 rats. First two groups received either PCP-ins or PCP-IC II formulations at a dose of 50 IU/kg animal body weight. The next two groups were kept as diabetic control and placebo respectively. Control experiments were performed without the administration of any polymer particles, while placebo experiments were performed with the administration of PCP particles containing MCD (without insulin). A fifth group received a subcutaneous injection of insulin solution at a dose of 1 IU/kg. Particles were fed to the rats using an oral gavage tube and blood glucose level was determined using glucose oxidase reagent. Percentage reduction of blood glucose level (BGL) was calculated by comparing the initial glucose value and glucose level at a given point of time.

The extent of glycemia reduction response was calculated using the trapezoidal method [21] to determine the area above the curve (AAC). The relative pharmacological availability [PA] of the orally administered insulin was calculated as follows

$$PA(\%) = [AAC_{\text{oral}} / \text{dose}_{\text{oral}}] \times 100 / [AAC_{\text{s.c.}} / \text{dose}_{\text{s.c.}}]$$

where $[AAC_{\text{oral}}]$ was the area determined after oral administration of insulin loaded particles and $[AAC_{\text{s.c.}}]$ was the area determined after subcutaneous injection of 1.0 IU/kg insulin solution.

2.7.1. Statistical analysis

Statistical analysis was performed using Student's t-test and differences were judged to be significant at $p < 0.05$.

3. Results and discussion

3.1. Preparation of hydrogel carriers

In this study, hydrogel microparticles based on PCP was used for the oral delivery of insulin. PCP particles were prepared by template assisted polymerization of methacrylic acid in the presence of polyethylene glycol and CS. Particles were obtained spontaneously during the polymerization process without the addition of any stabilizers. The hydrodynamic diameter of PCP particles dispersed in phosphate buffer pH 7.0 was 1030 nm with a polydispersity index of 0.346.

3.2. Characterization of insulin–MCD complex

IC complex was characterized by fluorescence spectroscopic studies. Fluorescence spectroscopy of proteins is a sensitive and reliable technique in highlighting their conformational variations, following an interaction with a second component. The technique can be useful to investigate the formation of non-covalent complex between insulin and the MCD. The fluorescence of insulin molecule arises mainly due to the presence of tyrosine residues. The fluorescence emission intensity of insulin with and without MCD is given in Fig. 1. The formation of a host–guest complex is usually accompanied by an increase in the fluorescence emission intensity [22]. The internal cavity of CD provides a less polar environment than water, an increase in fluorescence intensity may result upon complex formation [23]. From the study, it was observed that addition of MCD to insulin solution enhanced the fluorescence emission intensity. Importantly, as the concentration of MCD in the insulin solution was

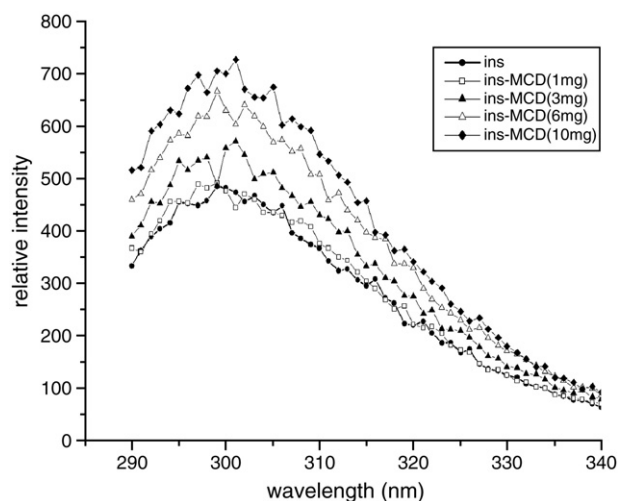


Fig. 1. Fluorescence emission spectra of insulin with and without MCD (λ_{Ex} 280 nm, λ_{Em} 290–350 nm). \circ — insulin solution (0.5 mg/ml), \square — insulin solution (0.5 mg/ml) with 1 mg MCD, \triangle — insulin solution (0.5 mg/ml) with 3 mg MCD, \diamond — insulin solution (0.5 mg/ml) with 6 mg MCD, \bullet — insulin solution (0.5 mg/ml) with 10 mg MCD.

increased, the fluorescence intensity was further enhanced. This phenomenon is in favor of the occurrence of an interaction between insulin and MCD.

The interaction between insulin and MCD was further confirmed by isothermal titration microcalorimetry experiments (ITC). ITC is becoming the leading method of choice for characterizing intermolecular interactions and recognizing reactions with exquisite sensitivity, as low and high affinity interactions can be quickly and accurately characterized using ITC [24]. A typical ITC titration curve corresponding to the binding interaction of insulin and MCD is presented on Fig. 2A. During insulin addition into MCD solution, heat release was observed indicating an interaction between these two compounds. The exothermic heat was directly proportional to the amount of added insulin and as the MCD in the cell became saturated with insulin, the heat signal diminished progressively (Fig. 2A). Heat flow which was released after the successive injections of 10 μl aliquots of insulin into the solution of MCD were integrated and expressed as a function of the molar ratio between the two reactants (Fig. 2B).

The probable mode of binding mechanism is expected to involve the interaction of a less polar region of the guest molecule, i.e. insulin, with the cyclodextrin cavity, while the more polar- and often charged-groups of the guest molecule are attracted towards the bulk solvent, outside the wider opening of the cavity. It was previously demonstrated that interaction of cyclodextrin with insulin occurs on specific amino acids, including tyrosine residues [25]. The results observed using ITC and fluorescence spectroscopy were both supporting the occurrence of an interaction between MCD and insulin. In solution, insulin is known to occur in a variety of oligomer states depending on the concentration, pH, temperature, divalent ion concentration and other ionic conditions [26]. The interaction of CDs with insulin through the hydrophobic amino acids is expected to suppress the aggregation and to improve protection against degradation by proteases, if sites sensitive to protease attack are sterically masked by CD.

Dilution of a series of small aliquots of insulin into phosphate buffer contained in the measurement cell of the ITC apparatus gave a sequence of endothermic heat flows which could be attributed to the molecular dissociation of insulin oligomers (Fig. 2C). As observed in Fig. 2C, dilution of insulin into a mixture of phosphate buffer and MCD at high concentration (50 mM) induced more pronounced endothermic peaks. Data can be analyzed by non-linear regression in terms of a monomer–dimer equilibrium model leading to the determination of the apparent equilibrium constant $K_{\text{diss}} = 16 \mu\text{M}$ and thermodynamic

parameters of $\Delta H_{\text{diss}}^0 = 55 \text{ kJ mol}^{-1}$, $\Delta G_{\text{diss}}^0 = 27.36 \text{ kJ mol}^{-1}$ and $T\Delta S_{\text{diss}}^0 = 27.64 \text{ kJ mol}^{-1}$. It is noteworthy that the calorimetric events were too weak in the absence of MCD to allow accurate determination of the thermodynamic parameters for the dissociation of eventual oligomers of insulin. In our work conditions (pH 7.4), the results obtained from the ITC measurements agreed well with those of Lovatt et al. [27] supporting the hypothesis that insulin oligomers were dissociated in the presence of MCD. (27).

3.3. Insulin loading and release experiments

Encapsulation efficiency (EE) of complexed and uncomplexed insulin encapsulated onto PCP microparticles was evaluated (Table 1). No significant difference was observed with these two different systems. With 0.5 and 1 mg/ml insulin concentration, the EE was 87% and 82% respectively with PCP-IC II formulation. On the other hand, 90% and 85% efficiency was obtained with the PCP-ins system.

Cyclodextrins and their derivatives are known to enhance the loading efficiency and alter the release profile of drugs from polymeric nano/microparticles [28,29]. Formation of non-covalent inclusion complexes often improves the solubility of poorly soluble drugs and this in turn improves their loading efficiency. A similar effect was not obvious in this study, possibly due to the solubility of insulin in water and also due to high protein incorporation efficacy of swelling controlled hydrogel matrices.

In vitro release profile of insulin from complexed and non-complexed insulin encapsulated PCP particles is given in Fig. 3. Similar to the encapsulation studies, significant variation in the release properties of insulin was not observed between PCP-ins and PCP-IC II systems. Release of insulin for 3 h was observed at pH 7.4 and around 10% of total insulin loaded was released at pH 1.2 within 2 h of the study. The release profile was quite similar with one that obtained with PCP-ins formulation.

3.3.1. Cytotoxicity studies

The ability of MCD to extract lipid components from cell membranes often results in cellular damage [30]. Thus, the cytotoxicity of MCD was evaluated on Caco 2 cell monolayers (Fig. 4). MCD concentration of 25 mM induced 100% of cell death. In contrast, the concentration in MCD lower than 10 mM were non-cytotoxic on Caco 2 cells. The results obtained were in good agreement with the literature [31]. PCP hydrogel particles used in the study were non-cytotoxic on Caco 2 cells in a range of concentrations from 0.25 to 2 mg/ml in agreement with previous work [18].

3.3.2. Permeation experiments

The ability of MCD to enhance insulin transport across Caco 2 cells was studied by preparing insulin–MCD in three different weight ratios, P_{app} and R values were calculated in each case (Table 2).

Although the obtained P_{app} and R values were low, the values corresponding to IC II complex were higher than one obtained with IC I composition. On the other hand P_{app} and R values obtained with IC III were quite similar than those obtained with IC II complex. This indicates that the addition of more MCD did not improve further the permeability of insulin through the Caco 2 cell monolayer. Thus the complex IC II appeared to give the best compromise keeping the amount of MCD rather low, but giving the maximum benefit in terms of permeability improvement across the cellular barrier. Based on these observations, the complex IC II formed with a weight ratio 1:5 between insulin and MCD was selected for the rest of the study.

In the next step, insulin transport properties of PCP-ins and PCP-IC II systems were compared on Caco 2 cell monolayers (Table 2). This study was performed to investigate the synergic effect of polymeric particles and MCD complexation, on the transport properties of insulin across the Caco 2 monolayers. The use of hydrogel particulate formulations significantly enhanced the P_{app} value of insulin across

the Caco 2 cell monolayers. A higher P_{app} value was obtained with the PCP-IC II in comparison with the PCP-ins formulation in this study. Indeed, 3 and 4.4 fold increase in insulin permeation was observed with PCP-ins and PCP-IC II formulations respectively (Table 2). The amount of insulin transported through the cell monolayers was also

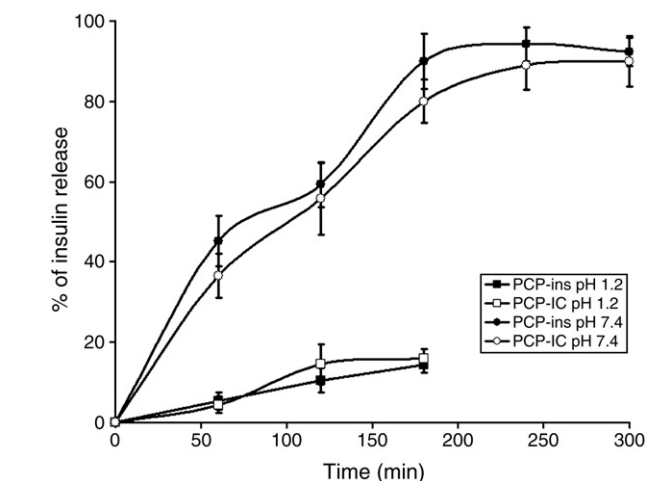
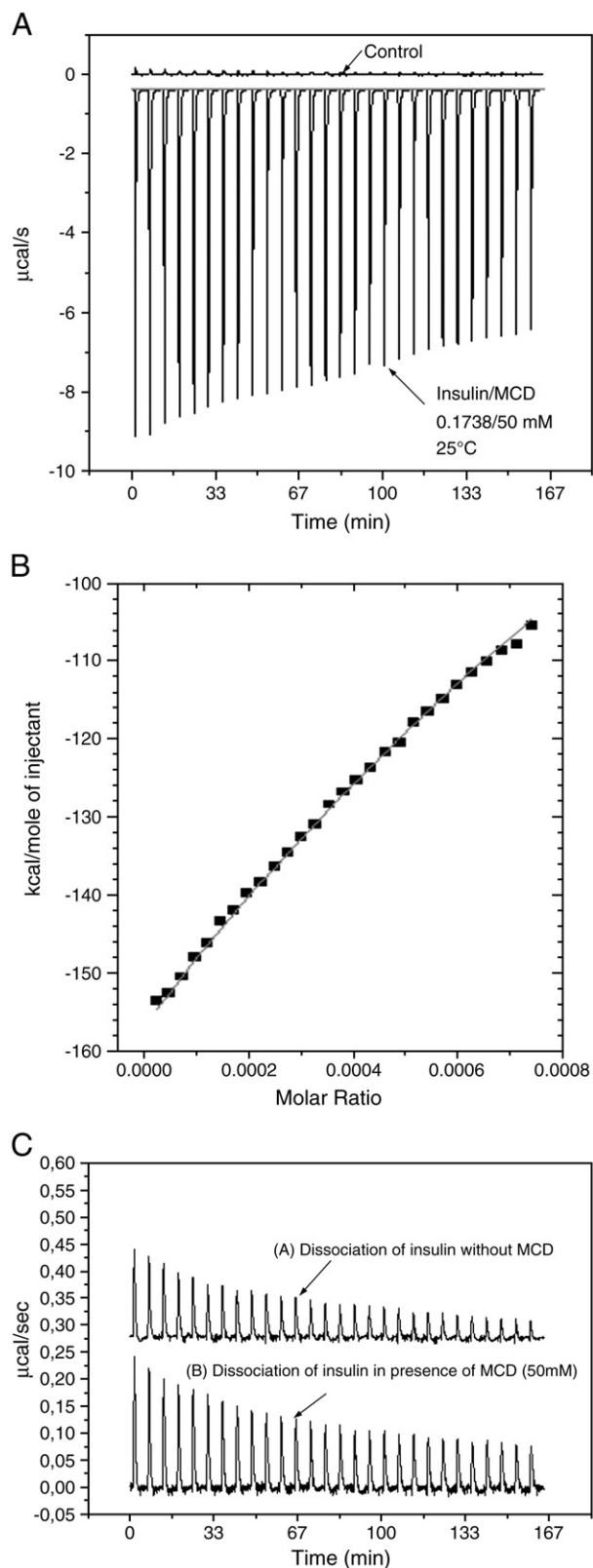


Fig. 3. In vitro release profile of insulin from PCP hydrogel microparticles at gastric and intestinal pH ($n = 3$). -○- PCP-ins at pH 7.4, -●- PCP-IC II at pH 7.4, -■- PCP-ins at pH 1.2, -□- PCP-IC II at pH 1.2.

increased when complexed insulin was used along with the hydrogel system (Fig. 5A).

Further permeation experiments were performed by placing freshly excised rat intestinal tissue between the apical and basolateral halves of the Ussing chamber. In these experiments, PCP-IC II was evaluated in comparison with a formulation of PCP-ins (Table 3). No significant improvement in the P_{app} value was observed in case of PCP-IC II in comparison with PCP-ins in this set of study. R values of 2.5 and 2.8 were obtained with PCP-ins and PCP-IC II respectively. The amount of insulin which permeated through the intestinal membrane (Fig. 5B) was rather unaffected by the formulation of insulin as a complex with the MCD.

3.4. In vivo studies

Fig. 6 shows the variation of glycemia in diabetic rats expressed as a percentage of the animal's initial glycemia after oral administration of either an insulin-containing formulation or placebo. No reduction in glycemia was observed with the groups of control and placebo, indicating the stable diabetic level in the STZ induced experimental animals. A single dose of insulin (50 IU/kg) was given to each animal by oral gavage and the pharmacological response (reduction in the glycemia) was monitored with time. After 2 h, the diabetic rats which received the polymeric dosage form containing insulin (PCP-ins, PCP-IC II) showed a marked reduction in glycemia. PCP-ins formulation induced a reduction of 15% in the initial value of the glycemia. The effect was observed after 2 h and was even more pronounced after 6 h of the experiment. Then the glycemia of the rats increased again to reach the initial level after 10 h of the study. A marked reduction in glycemia was also observed with the PCP microparticles loaded with IC II. After 2 h, the formulation was able to

Fig. 2. Typical ITC data corresponding to the binding interaction of insulin (0.1738 mM, 1.441 ml cell volume) with MCD (50 mM). Temperature of the experiment was fixed to 25 °C. (2A) Figure shows exothermic heat flows which are released upon successive injection of 10 µl aliquots of insulin into MCD solution. (2B) Figure shows integrated heat data. Control consisted in successive injections of the insulin solution in solely phosphate buffer solution. Heat flows accounting for dilution effects were further subtracted from each experimental heat flows. (2C) Dissociation of insulin in the absence and presence of MCD. Injection of insulin dissolved in a mixture of buffer/MCD. Final concentration of insulin was 1 mg/ml (0.1738 mM) and the concentration of MCD was 50 mM. The fit of the differential binding curve to a simple monomer-dimer equilibrium model led to the determination of the following parameters. $K_{diss} = 16 \mu\text{M}$, $\Delta H_{diss}^0 = 55 \text{ kJ mol}^{-1}$, $\Delta G_{diss}^0 = 27.36 \text{ kJ mol}^{-1}$ and $T\Delta S_{diss}^0 = 27.64 \text{ kJ mol}^{-1}$.

Table 1
Insulin encapsulation efficiency of PCP particles with insulin and complexed insulin (n = 3).

Formulation	Encapsulation efficiency	
	With 0.5 mg/ml insulin	With 1 mg/ml insulin
PCP-ins	90 ± 1.5	85 ± 1.5
PCP-IC II	87 ± 2	82 ± 1.5

reduce the initial blood glucose level by 30% and the blood glucose level was maintained at this level up to 6 h. Although the glycemia of the rats increased again, it remained 10% below that of the control group even after 10 h. Comparing the two microparticulate formulations, both were able to reduce the glycemia of diabetic rats to a maximal reduction of 30%, but the PCP-IC II microparticles showed a better profile with a faster reduction and a longer period of stable glycemia at a low level. After administration of the insulin solution subcutaneously at a dose of 1 IU/kg, the blood glucose level in diabetic animal decreased significantly within 30 min. The maximum decrease in blood glucose level (70% of initial glucose level) was reached within 2 h after administration; subsequently, the blood glucose level increased with time. The relative pharmacological bioavailability which can be calculated from these experiments was 1.8 and 1.95 respectively for PCP-ins and PCP-IC II formulations.

The increase of the bioavailability of insulin by oral administration with the microparticulate formulations developed in this work can be explained by a combination of favorable effects. Insulin administered via oral route under the formulation of microparticles was delivered in the small intestinal region, thanks to the pH sensitive release mechanism of PMAA based hydrogel systems [32]. At the intestinal pH, these particles swell, and allow insulin to be rapidly released in the duodenum and jejunum portion of the small intestine (pH 6.0–7.4). Thus, it can be suggested that the rapid dispersion of the polymeric hydrogel particles in the basic environment of the intestine promotes the release of the encapsulated insulin. Secondly, PMAA based particles have good mucoadhesion capability on the mucus layer of the gastro-intestinal tract (GIT). This effect may help the particles to adhere on the mucosal surfaces for a longer duration [33]. The adhesion of the particles on the mucus layers may also protect the encapsulated insulin from proteolysis degradation, as insulin will be released closer to the surface of the intestinal epithelial cells, without exposing them directly to the GIT fluids.

Thirdly, the protection of insulin against proteolysis can be further reinforced by the proteolytic inhibitory activity of PMAA on protease enzymes [34]. Indeed, PMAA based hydrogels can bind Ca^{+2} ions, hence reduces the local concentration of calcium available in the GIT fluids. The activity of the calcium dependant proteases are reduced by this effect [34]. As calcium ions are also crucial to maintain the integrity of the epithelial tight junctions, local reduction in calcium ion concentration can induce the opening of the tight junctions, by a

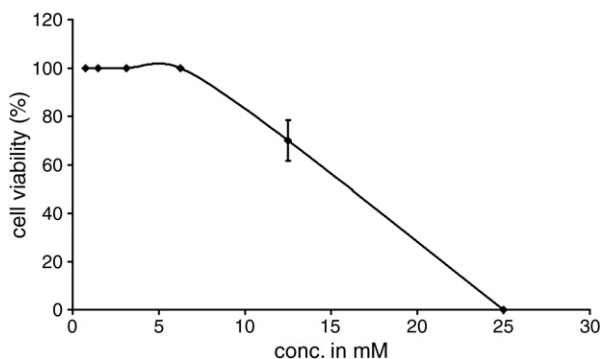


Fig. 4. Survival curve of the Caco 2 cell monolayer treated with different concentrations of MCD. Percentage of viable cells was quantified by MTT assay (n = 3).

Table 2
Apparent permeability coefficient and absorption enhancement ratio for insulin across Caco 2 cell monolayers (n = 3).

Formulation	$P_{app} \times 10^{-6}$ cm/s	R
Control	0.5 ± 0.3	–
IC I	0.6 ± 0.2	1.2
IC II	0.74 ± 0.15	1.48
IC III	0.73 ± 0.2	1.48
PCP-ins	1.5 ± 0.2	3
PCP-IC II	2.2 ± 0.3**	4.4

** Statistically different from corresponding PCP-ins group.

rather complex mechanism involving the activation of the protein tyrosine kinases and the phosphorylation of tight junction protein–occludin [35]. This phenomenon can improve the paracellular transport of hydrophilic macromolecules, like insulin.

As a subsequent effect, MCD complexation is known to enhance absorption of the drug across epithelial barriers [36]. Results from permeation experiments performed on the Caco 2 cell monolayers highlighted that the complexation of insulin with MCD enhanced insulin transport across the model epithelium. Hydrophobic derivatives of CDs are known to enhance the permeation of hydrophilic molecules across biological membranes. The mechanism behind this effect can be explained by the fact that MCD extracts lipids components (such as cholesterol) from cell membranes and increases

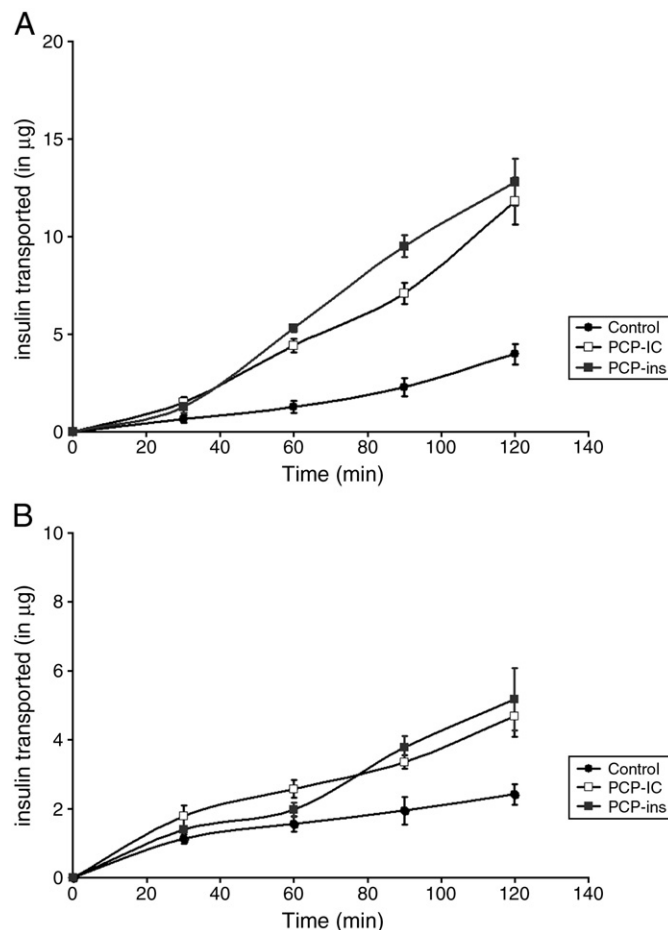


Fig. 5. A. Amount of insulin transported across Caco 2 cell monolayer during the permeation experiments (n = 3). -●- control, -■- PCP (2.5 mg) with insulin, -□- PCP (2.5 mg) with IC II complex. B. Amount of insulin transported across rat intestinal tissue during the permeation experiments (n = 3). -●- control, -■- PCP (5 mg) with insulin, -□- PCP (5 mg) with IC II complex.

Table 3

Apparent permeability coefficient and absorption enhancement ratio for insulin/complexed insulin in combination with PCP particles on rat small intestinal tissue (n = 3).

Formulation	$P_{app} \times 10^{-7}$ cm/s	R
Control	1 ± 0.1	–
PCP-ins	2.5 ± 0.2	2.5
PCP-IC II	2.8 ± 0.15	2.8

the cell membrane fluidity and permeability. Depletion of cholesterol in Caco 2 cell membrane due to its extraction by MCD causes redistribution of tight junction proteins claudins 3 and 4 and occludin, resulting in the loss of junctional integrity [37]. Due to the strong affinity of MCD for lipid components, the above mentioned phenomenon can also lead to the displacement of relatively hydrophilic guest molecule (i.e. insulin) complexed in the CD cavity, resulting in its detachment from the CD complex [38]. Additionally, cyclodextrins can stabilize insulin in its non-aggregated form by reducing its self-aggregation tendency, which in turn is favorable to promote the insulin absorption.

From our results, it can be suggested that the PCP hydrogel microparticles may serve as a platform for the CD complexed insulin, when used in combination. With its pH sensitivity, PMAA based particles can trigger the release of MCD complexed insulin in a control manner at the close proximity of the intestinal epithelium surface, allowing the complexed insulin to interact directly with the epithelial surfaces. The combination of favorable effects brought with the new formulation could improve the bioavailability of the insulin after oral administration to rats.

4. Conclusion

An oral insulin delivery system consisting of MCD complexation strategy with mucoadhesive delivery system was evaluated in the study. The formation of a complex between MCD and insulin could be established based on fluorescence and ITC investigations. MCD was effective in enhancing insulin transport across Caco 2 cell monolayers, and a synergic effect was observed when used in combination with hydrogel particles composed of PMAA. Finally, MCD complexed insulin encapsulated in the PCP hydrogel microparticles induced a better biological response in diabetic animals as compared to the microparticles containing unmodified insulin. The cyclodextrin

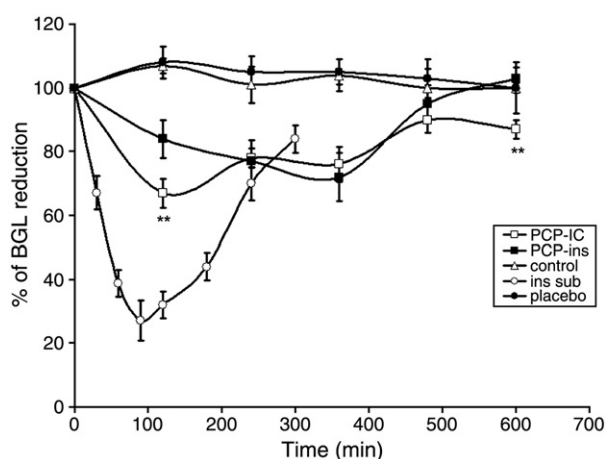


Fig. 6. Hypoglycemic effect following oral administration PCP particles containing either insulin or MCD complexed insulin and subcutaneous injection of insulin solution (1 IU/kg) on diabetic rats (n = 5). -Δ- control diabetic group, -●- placebo (PCP-MCD without insulin), -■- PCP-ins, -□- PCP-IC II group, -○- subcutaneous dose of 1 IU/kg. **Statistically different from corresponding PCP-ins group.

complexed insulin encapsulated in PMAA hydrogel microparticles is worth considering as a suitable system of oral insulin delivery.

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