Supporting Information

All-in-one microfluidic assembly of insulin-loaded pH-responsive nano-in-microparticles for oral insulin delivery

Clarinda Costa[a,b,c], Zehua Liu[a], João P. Martins[a], Alexandra Correia[a], Patrícia Figueiredo[a], Antti Rahikkala[a], Wei Li[a], Jani Seitsonen[d], Janne Ruokolainen[d], Sami-Pekka Hirvonen[e], Ana Aguiar-Ricardo[b], M. Luísa Corvo[c,*], and Hélder A. Santos[a,f,*]

[a]Drug Research Program, Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki, FI-00014, Helsinki, Finland

[b]LAQV-REQUIMTE, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

[c]Instituto de Investigação do Medicamento (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, Avenida Professor Gama Pinto, 1649-003 Lisboa, Portugal

[d]Nanomicroscopy center, Aalto University, FI-00076, Aalto, Finland

[e]Department of Chemistry, Faculty of Science, University of Helsinki, FI-00014, Helsinki, Finland

[f]Helsinki Institute of Life Science (HiLIFE), University of Helsinki, FI-00014, Helsinki, Finland

E-mail: helder.santos@helsinki.fi; lcorvo@ff.ulisboa.pt

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Experimental Section:

Materials

Egg-phosphatidylcholine (E-PC) and distearoylphosphatidylethanolamine-poly(ethyleneglycol)$_{2000}$ (DSPE-PEG$_{2000}$) were obtained from Lipoid (Germany). Cholesterol (Chol), recombinant human insulin (rhIns), medium viscosity chitosan (*M*w= 190 000–310 000 Da, 75–85 % deacetylated), 2-(4-(2-hydroxyethyl) piperazin-1-yl) ethanesulfonic acid (HEPES), paraformaldehyde (PFA), citric acid and sodium chloride were purchased from Sigma-Aldrich (USA). Poloxomer 407 (Kolliphor® 407) was purchased from BASF (Germany). MF grade of HPMCAS was kindly provided from ShinEtsu (Japan). Hank's balanced salt solution (HBSS) and phosphate buffer saline (PBS) were purchased from Life Technologies (USA). Fasted state simulated intestinal fluid (FaSSIF) was purchased from Biorelevant.com Ltd (London, UK). Triton® X-100 was purchased from Merck Millipore (Germany). Dulbecco's Modified Eagle medium (DMEM), L-glutamine, non-essential amino acids, penicillin (100 IU mL^{-1}) and streptomycin (100 mg mL^{-1}), ethylenediamine tetraacetic acid (EDTA) and trypsin–EDTA were purchased from HyClone (USA). Human colon carcinoma (Caco-2) and the human colorectal adenocarcinoma modified with methotrexate (HT29-MTX) cells were purchased from ATCC (USA). 4',6-Diamidino-2-phenylindole (DAPI-405), 4-[4-(dihexadecylamino)- styryl]-N-methylpyridinium iodide (DiA) and Gibco™ Versene Solution were purchased from Thermo Scientific (USA). CellMask Red® was purchased from Invitrogen (USA). CellTiter-Glo® assay reagent was purchased from Promega Corporation (USA).

Preparation of insulin-loaded liposomes (InsLip)

The recombinant human insulin was encapsulated into the inner aqueous space of liposomes by a microfluidics glass-capillary method. Briefly, E-PC, DSPE-PEG$_{2000}$ and Chol (molar ratio 1.85:0.15:1, respectively) were dissolved in ethanol in a concentration of 43.2 µmol mL^{-1}. In parallel, rhIns was dissolved in saline citric acid solution (145 mM of NaCl, 10 mM of Citric Acid, pH 2.0) in a final concentration of 100 µg mL^{-1}. The insulin-loaded liposomes (InsLip) were achieved using a co-flow microfluidic device. Briefly, a borosilicate glass capillary with an inner diameter of 70 µm was inserted into a glass capillary with an inner diameter of 1000 µm and they were coaxially aligned. The lipidic solution was injected into the inner phase, while the insulin solution was injected into the outer phase, both with flow rates of 10 mL h^{-1} and a flow rate ratio (FRR) of 1. The non-encapsulated protein was separated from the liposomes by ultracentrifugation twice, after a dilution of 20 times (Optima L-80, XP Ultracentrifuge, Beckman Coulter, CA, USA), at 135 000*g* at 15 °C for 2 h. Then, the InsLip pellet was re-suspended in a saline citric acid solution at pH 2. The experiment was performed in triplicate.

Coating of InsLip by chitosan (InsLip-CHT): The InsLip was coated with chitosan by physical adsorption. Briefly, chitosan solution (10 mg mL^{-1}) was prepared dissolving chitosan powder in 1 % of acetic acid solution (v/v). The solution was kept under stirring overnight. Then, the pH was increased to pH 5.5 with 0.01 of NaOH and the final solution was centrifuged (Hettich EBA 21, Tuttlingen, Germany) at 4 020 *g* for 15 min [1] to remove the precipitated chitosan. Afterwards, the InsLip suspension was added dropwise to the chitosan solution at the same volume ratio and kept under stirring for 6 h. Then, the dispersion containing chitosan-coated liposomes (InsLip-CHT) was centrifuged (Optima MAX, Beckmann Coulter, USA) twice at 27 000 *g* for 4 min. The experiment was performed in triplicate.

Preparation of insulin-loaded microparticles

The InsLip-CHT nanoparticle encapsulation into MF was performed by water-in-oil-in-water (W/O/W) double emulsion, also using the microfluidic technique [2]. The nanoparticles were dispersed in a MF (10 mg mL^{-1} in ethyl acetate) in a volume ratio of 1:10 and sonicated (inner oil fluid), in order to obtain a homogeneous suspension. In parallel, the outer aqueous solution was prepared by dissolving Poloxamer 407 in Milli-Q water (10 mg mL^{-1}, pH 4.0). Afterwards, a flow focusing microfluidic chip, as previously described [3], was used. To achieve the double emulsion, the inner and outer flow rates of 0.5 mL h^{-1} and 3.0 mL h^{-1} were employed. The formed droplets were collected in 10 mg $mL^{-1}$of Poloxamer 407 aqueous solution, pH 4.0. Finally, the nano-in-microparticles (Ins@MPs) were collected and were washed for three times with Milli-Q water, using the centrifuge (Hettich EBA 21, Tuttlingen, Germany) at 3 000 rpm for 5 min. The experiment was performed in triplicate.

Characterization of liposomes and insulin-loaded nanoparticles

The mean size, determined as Z-average, polydispersity index (PDI) as a measure of the particle size distribution that can range from 0 (monodisperse) and 1.0 (polydisperse), and the surface charge (zeta-potential, ζ) of InsLip and InsLip-CHT NPs dispersions were determined by Zetasizer Nano ZS (Malvern Panalytical Ltd., Malvern, UK). The morphology of the liposomes was confirmed using cryo-transmission electron microscope (Cryo-TEM, JEOL JEM-3200FSC, JEOL, Tokyo, Japan). Briefly, prior to use, vitrified specimens were prepared using an automated FEI Vitrobot device, and Quantifoil 3.5/1 holey carbon copper grids with a hole size of 3.5 µm. Then, an aliquot of liposomal suspension was applied on the grid and it was blotted twice for 5 sec and then vitrified in a 1:1 mixture of liquid ethane and propane at −180 °C. The grids with the vitrified liposomes were kept in liquid nitrogen temperature and then cryo-transferred to the microscope.

Imaging was carried out using a field emission cryo-TEM (JEOL JEM-3200FSC), operating at 200 kV. Images were taken in the bright field mode and using zero loss energy filtering

(omega type) with a slit width of 20 eV. Micrographs were recorded using a Gatan Ultrascan 4000 CCD camera (Gatan Inc., Pleasanton, CA, USA). The specimen temperature was maintained at −187 °C during the imaging. The images were treated using Gatan Microscopy Suite Software (Gatan Inc).

The encapsulation efficiency (E.E.) was calculated based on the amount of insulin encapsulated into the liposomes, previously disrupted with 2 % (v/v) Triton® X-100, as follows (Eq. 1):

$$E.E.\ (\%) = \frac{Ins_{Liposomal}\ (\mu g)}{Ins_{Liposomal}\ (\mu g) + Ins_{free}\ (\mu g)} \times 100 \quad (1)$$

Where the $Ins_{Liposomal}$ is the insulin encapsulated into liposomes and Ins_{free} is the insulin present in the supernatant from the ultracentrifugation, described above. Both were determined using a high-performance liquid-chromatography (HPLC; Agilent 1260, Agilent Technologies, Santa Clara, CA, USA), using a C18 column, 15 cm × 4.6 mm, 5 µm (SUPELCO Discovery®, Sigma Aldrich, St. Louis, MO, USA), and the method as previously described [4].

Characterization of insulin-loaded microparticles

The dimensional analysis of the Ins@MPs were performed using microscopy. Briefly, an aliquot of Ins@MPs was dispersed on a 35 mm Petri-dish with a thin bottom and imaged using a microscope, with a 10× objective (Leica SP5 II HCS A, Leica, Wetzlar, Germany). The chemical modification upon encapsulation in MF was evaluated using attenuated total reflectance Fourier transformed infrared (ATR-FTIR) spectrometry. The spectra were recorded from 3 600 to 690 cm^{-1}, with a resolution of 2 cm^{-1}.

The amount of insulin loaded into the nano-in-microparticles was evaluated dissolving and disrupting them with a solution of PBS (pH 6.8) and 2% (v/v) Triton® X-100, in a ratio of 1:1 (v/v) and analyzed by HPLC, as described above. The loading degree (L.D.) was then calculated as follows (Eq. 2):

$$L.D.\ (\%) = \frac{Total\ mass\ of\ insulin\ loaded\ in\ microparticles\ (\mu g)}{Total\ mass\ of\ NPs\ (\mu g) + Total\ amount\ of\ polymer\ (\mu g)} \times 100 \quad (2)$$

In vitro drug release studies

The insulin release profile from Ins@MPs was evaluated by mimicking the gastrointestinal tract conditions at 37 °C under stirring. First, 2 mg of Ins@MPs were dispersed in 4 mL of simulated gastric fluid (SGF) at pH 1.2. SGF solution was prepared with 0.2 % (w/v) sodium chloride and 0.7 % (v/v) hydrochloric acid, without pepsin to avoid insulin degradation, during the release studies. After 2 h, the solution was centrifuged (EBA 21, Andreas Hettich GmBH & Co, Tuttlingen, Germany) at 6 000 *g* for 15 min, the pellet was resuspended and dispersed into 4 mL of FaSSIF (pH 6.8) for the following 24 h. The studies were conducted taking 100 µL at determined time-points, being the solution replaced at the same volume in order to keep the volume constant.

Then, the samples were centrifuged (Micro Centrifuge, Model 5415D, Eppendorf, Hamburg, Germany) at 16100g for 5 min. The insulin, released from the nanoparticles, was determined using the HPLC method, as described above. All the experiments were performed at least in triplicate.

Cell lines and cell culture conditions

HT29-MTX (passage #30) and Caco-2 (passages #35-40) were separately cultured in a 75 cm^2 culture flask in DMEM containing 10 % of fetal bovine serum, 1 % (v/v) of L-glutamine, penicillin, streptomycin and 1 % (v/v) of NEAA. For further cell growth, the conditions were maintained at 37 °C in 5 % of CO_2 and relative humidity of 95 %. The cell culture medium was changed every other day. Sub-culturing was performed using trypsin-PBS-EDTA when confluency reached 80 %.

In vitro cytotoxic studies

The cell viability studies were carried out using CellTiter-Glo® assay reagent diluted with HBSS−HEPES buffer (pH 7.4), in a ratio of 1:1 (v/v)). Briefly, 5×10^4 cells of Caco-2 and HT29-MTX cell lines were individually seeded in 96-well plates (Corning Inc., USA), and left to attach for 24 h. Afterwards, the medium was discarded, and the cells were washed with HBSS-HEPES buffer at pH 7.4. Then, 100 µL of microparticles with a concentration from 50 to 1000 µg mL^{-1} were added to the cells. The cells were incubated at 6 h or 24 h, under 37 °C. After the incubation time, cells were washed twice with fresh HBSS−HEPES (pH 7.4), and 100 µL of CellTiter-Glo® was added. The plates were lightly shaken for for 2 min. HBSS−HEPES and 1 % (v/v) Triton® X-100 solutions were used as positive and negative controls, respectively. The luminescence values were measured using a Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Waltham, MA, USA). All the experiments were performed at least in triplicate.

Drug permeability across Caco-2/HT29-MTX cell monolayer

The insulin permeability across the Caco-2/HT29-MTX cell monolayer was evaluated as previously described [4,5]. Briefly, Caco-2 and HT29-MTX cells were seeded on 12-Transwell™ filter membranes (3 µm pore-size; Corning Inc., USA) inserts in a ratio of 9:1 (v/v), respectively, at a seeding density of 5×10^4 cells per cm^2. For 21 days, the medium was replaced every other day until the complete monolayer formation. At the 21st day, the transepithelial electrical resistance (TEER) was measured using a Millicell® ERS-2 volt-ohm-meter with STX01 electrodes (Millipore, Burlington MA, USA).

The insulin permeability across the cell monolayer from the apical (donor) to basolateral (receiving) compartment was evaluated as follows. Briefly, 2.0 mg of Ins@MPs (equivalent to 15.6 µg mL^{-1} of insulin) and the equivalent concentration of free insulin (as control), were added on the apical part with FaSSIF (0.5 mL) at pH 6.8. HBSS-HEPES

(1.5 mL) at pH 7.4 was added to the basolateral compartment in order to simulate the physiological condition. The experiment was evaluated, in triplicate, at 37 °C and shaking at 100 rpm [6]. At different time-points, 200 µL of the basolateral compartment were withdrawn and replaced with the same volume of pre-warmed HBSS-HEPES (pH 7.4). The permeated insulin was then quantified by HPLC, as described above, and the apparent permeability P_{app} was calculated according to Eq. 3:

$$P_{app}\,(cm\,s^{-1}) = \frac{dQ}{dt)} \times \frac{1}{C_0 A} \quad (3)$$

Where $\frac{dQ}{dt}$ is the steady-state flux (µg s^{-1}), C_0 is the initial drug concentration on the apical compartment (µg mL^{-1}) and A is the surface area of the membrane (cm^2). The TEER values were measured in all time-points. All the experiments were performed at least in triplicate.

Cell–nanoparticle interaction studies

The interaction studies between cells and liposomes, as well as liposomes-coated chitosan were evaluated. Briefly, a hydrophobic dye, DiA, was entrapped by microfluidics into the liposomal bilayer (DiALip), in a mass ratio 100:1 (Lip:DiA), as described above. Then, the liposomes were coated by CHT (DiALip-CHT). The morphological properties of DiALip and DiALip-CHT were evaluated in order to keep the same properties that the InsLip and InsLip-CHT, respectively. Afterwards, the interactions between the Caco-2 and HT29-MTX cell lines and DiA-loaded NPs, were quantitatively and qualitatively evaluated by flow cytometry and confocal microscopy, respectively.

For evaluation by flow cytometry, 0.4 mL of the cells were seeded in 24-well plates at a density of 1 × 10^5 cells per well and left to attach overnight. Then, the cell culture medium was removed, and the cells were washed once with PBS buffer (pH 7.4). Afterwards, 0.3 mL of 500 µg mL^{-1} of DiALip and DiALip-CHT suspensions were incubated with the cells for 6 h at 37 °C. After removing the nanoparticles and washing the cells with PBS buffer in order to remove the non-adherent nanoparticles, the cells were detached with Gibco™ Versene Solution for 5 min (0.48 mM). The cells were then washed once with PBS buffer and suspended with PBS-EDTA (pH 7.4) for flow cytometer analysis. Then, the cells were incubated with trypan blue (0.005 %, v/v) during 4 min, washed twice with PBS-EDTA (pH 7.4), and suspended with PBS-EDTA. Flow cytometry was performed with the LSR II flow cytometer (BD Biosciences, San Jose, CA, USA), using a laser excitation wavelength of 488 nm and a FACS Diva software. After collecting 2 × 104 events, the data was analyzed using FlowJo VX software (Tree Star, Ashland, OR, USA). The association of nanoparticles was measured before cells incubation with trypan blue, whereas the uptake was measured after incubation.

Regarding the confocal microscopy, 200 µL of 5 × 10^4 cells per well were seeded in Lab-Tek 8-chamber slides (Thermo Fisher Scientific, USA) and left to attach overnight. After

removing the cell culture medium, 200 µL of 500 µg mL^{-1} of DiALip and DiALip-CHT suspensions in PBS buffer (pH 7.4) were added to the cells and incubated for 6 h at 37 °C. The cells were then washed twice with PBS buffer (pH 7.4). The plasma membrane was stained by adding 200 µL of CellMask Red (5 µg mL^{-1}) and incubated for 3 min at 37 °C. In order to remove the excess of staining solution, it was washed once with fresh PBS buffer. Afterwards, the cells were fixed using 4% paraformaldehyde (PFA), for 10 min at room temperature. Finally, the nuclei staining was done by adding 200 µL of DAPI-405 (2.8 µg mL^{-1}) and incubated for 5 min at 37 °C. The localization of nanoparticles was observed with a Leica SP5 inverted confocal microscope (Leica Microsystems, Germany), using a 63×/1.2-0.6 oil immersion objective. All the experiments were performed in triplicate.

Insulin structure stability

The structure stability of insulin, after permeability across the monolayer, was studied by J-815 (Jasco Co Ltd, Hachioji, Japan) far UV circular dichroism spectroscopy. For each experiment, quartz cuvettes with 10 mm were used. Each spectrum was recorded from 400 to 190 nm, with a data pitch of 1 nm and using a scanning speed of 100 nm min^{-1} at 20 °C. All the records were performed in triplicate.

Statistical analysis

All results are expressed as mean ± standard deviation (S.D.). To analyze the data, analysis of variance (ANOVA) followed by Bonferroni post test (GraphPadPrism, GraphPad software Inc., CA, USA) was used. The level of significance was set at the probabilities of *p< 0.05, **p< 0.01 and ***p< 0.001.

Supporting Figures

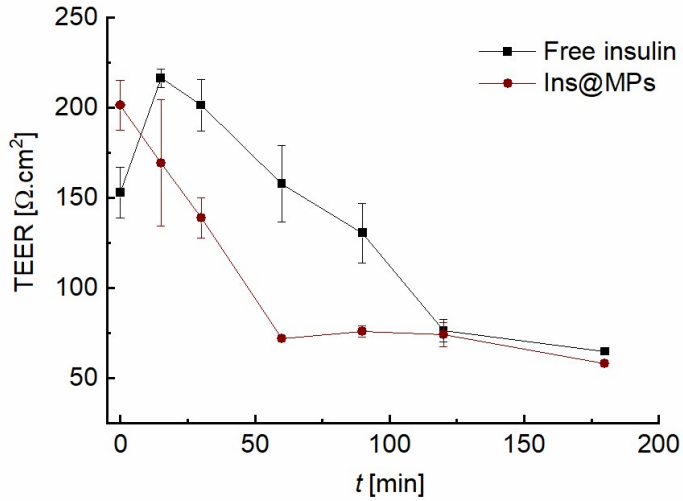

Figure S1: TEER values, at different time-points, for Caco-2/HT29-MTX monolayer after incubation, at 37°C and for 3 hours, with insulin-loaded nano-in-microparticles (Ins@MPs) and free insulin. The results are expressed as mean ± S.D. (n = 3).

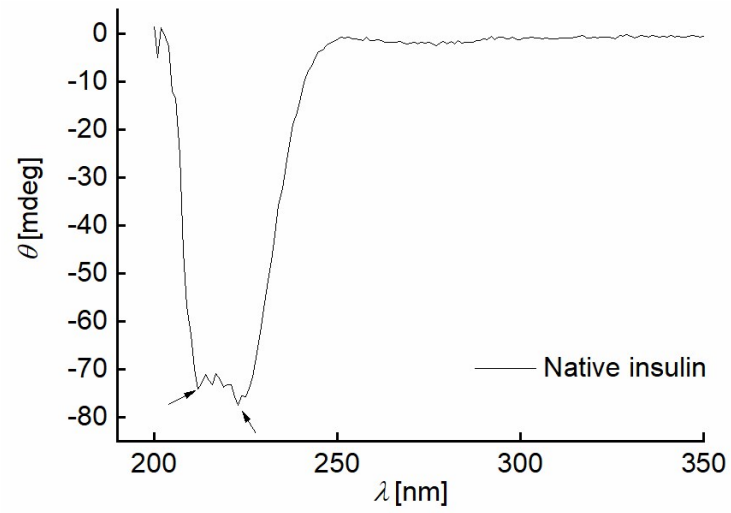

Figure S2: Circular Dichroism of insulin in its native form at 1.0 mg mL^{-1}, in HBSS-HEPES (pH 7.4). The results are expressed as mean ± S.D (n = 3).

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