Smart Protein-Based Formulation of Dendritic Mesoporous Silica Nanoparticles: Toward Oral Delivery of Insulin

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Experimental Procedures

Materials

Cetyltrimethylammonium chloride (CTAC, 25 wt% H₂O), Tetraethylorthosilicate (TEOS, 99%), Insulin (human recombinant, 5800 Da), (3-mercaptopropyl)triethoxysilane (MPTS, 95%), (3-aminopropyl)triethoxysilane (APTS), Fluorescein isothiocyanate (FITC, > 90%), Rhodamine isothiocyanate (Rho) were obtained from Sigma Aldrich (Austria). Triethanolamine (TEA, 98%) was purchased from Alfa Aesar (USA). 50 % succinylated β-lactoglobulin (BL) was provided by Aventus Innovations (Levis, QC, Canada). Materials for cell culture and cytotoxicity experiments were purchased from Gibco Invitrogen (Karlsruhe, Germany), Lonza Group Ltd (Basel, Switzerland), Sigma-Aldrich Chemie GmbH (Munich, Germany) and Sarstedt AG&Co (Nuembrecht, Germany).

Methods

Synthesis of Dendritic Mesoporous Silica Nanoparticles (DMSNs). DMSNs with various pore sizes were synthesized by adapting the procedure previously reported.[1] First, the aqueous solution was prepared: 8 ml of CTAC, 360 mg of TEA and 72 ml of H₂O were mixed together for 1 h at 60 °C (stirring plate Heidolph, stirring rate 150 rpm). Then, the organic solution was prepared: 32 ml of organic solvent (hexane, cyclohexane or toluene) and 8 ml TEOS were mixed and subsequently added to the first solution. The solution was kept at 60 °C under the same stirring rate overnight. Prior to the centrifugation at 10000 rpm for 20 min, the organic phase was removed. After drying at 100 °C overnight, the as-made nanoparticles were extracted for 2 h with 100 ml of EtOH and 1 drop of HCl (37%). After drying the extracted nanoparticles at 100 °C overnight, they were calcined under air at 550 °C for 5 h. The resulting calcined nanoparticles are named DMSN-Hex, DMSN-Cyclo or DMSN-Tol.

Functionalization of DMSN-Hex. DMSN-Hex were functionalized with MPTS. Typically, 500 mg of calcined DMSN-Hex were dispersed in 50 ml of anhydrous toluene under stirring and N₂ atmosphere at 115 °C for 2 h. Then, 0.5 ml of MPTS was added and the mixture was allowed to proceed overnight. The functionalized DMSN-Hex were recovered by centrifugation at 9000 rpm for 20 min, washed twice with toluene and once with ethanol and dried at 80 °C overnight. The resulting functionalized DMSN-Hex are named DMSN-SH.

Labeling of DMSNs. DMSN-Hex and DMSN-SH were labeled with Rhodamine isothiocyanate based on previously reported method.[2] For DMSN-Hex labeling, first Rho-APTS silane was prepared. Rhod was reacted with APTS: 10 mg of Rhod was dissolved in 0.5 ml of anhydrous DMSO and 0.1 ml of APTS was added at RT, under stirring and N₂ atmosphere. The reaction was left 24 h in the dark. Then, 100 mg of DMSN-Hex was dispersed in 10 ml anhydrous toluene for 2 h at 110 °C. A volume of 0.5 ml of Rho-APTS was added to the dispersion of DMSN-Hex and the grafting was further carried out overnight. For DMSN-SH labeling, 8.2 mg of Rhod was added to a suspension of 100 mg of DMSN-SH in EtOH (25 ml) and the reaction between thiol and isothiocyanate further proceeded in the dark under stirring for 24 h. DMSN-Hex was also labeled with FITC as follows: DMSN-Hex was first grafted with APTS and 50 mg of the resulting amino-modified DMSN-Hex was reacted with 17.6 mg of FITC in 20 ml EtOH in the dark for 24 h. The samples were recovered...
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by centrifugation at 9000 rpm for 20 min, washed several times with EtOH and dried at 80 °C overnight. The resulting labeled materials are named DMSN-Rhod, DMSN-SH-Rhod and DMSN-FITC.

Labeling of Insulin. Insulin was coupled with FITC according to standard protocol.[3] Briefly, 10 mg of insulin was first dissolved in 1 ml of Na₂CO₃ 0.1 M. In parallel, 5 mg of FITC was dissolved in 0.5 ml of DMSO. The FITC solution was added to insulin by increment of 50 μl and the mixture was stirred at RT for 1 h and protected from light exposure. After semi-preparative HPLC purification, the coupling was confirmed by mass spectrometry. The resulting labeled insulin is named Ins-FITC.

Loading of Insulin. Insulin, human recombinant, was introduced into the pores of DMSNs through an electrostatic attraction technique.

First, 40 mg of Insulin was dispersed in V = 20 ml of nanopure H₂O and the pH was brought to 3 with HCl 0.01 M until complete dissolution is observed. Then, the pH was increased to 4 using NaOH 0.2 M. In the meantime, a suspension of 100 mg of either DMSN-Hex, DMSN-Tol or DMSN-SH is prepared using V = 5 ml of a solution at pH 4. The solution of insulin was added to the different suspensions of DMSN-Hex, DMSN-Tol and DMSN-SH and the mixtures were placed in a mechanical rocker for 1.5 h. The resulting DMSNs loaded with insulin were recovered by centrifugation (9000 rpm, 10 min), frozen overnight and lyophilized for 2 days. The obtained DMSNs are named DMSN-Hex-Ins, DMSN-Tol-Ins and DMSN-SH-Ins.

Characterization of the materials. N₂ physisorption isotherms were measured at -196 °C (77 K) using an Autosorb-IQ sorption analyzer (Anton Paar, Boynton Beach, USA). Prior to the analysis, calcined DMSNs, functionalized DMSN-SH and insulin loaded DMSNs were outgassed 10 h at 150 °C, 12 h at 80 °C and 20 h at 35 °C, respectively. The specific surface area (Sₐ BET) was determined using the Brunauer-Emmett-Teller (BET) equation in the relative pressure range 0.05 - 0.2. The total pore volume was determined at P/P₀ = 0.95. The pore size distributions were calculated using the non-local density functional theory (NLDFT) method considering a model of silica with cylindrical pores. Transmission Electron Microscopy (TEM) images were recorded with a Philips CM200 microscope at an accelerating voltage of 200 kV using suspensions of DMSNs in EtOH (4 μl) deposited on a carbon-coated copper grid. Dynamic light scattering (DLS) analyses were performed on a Malvern DTS Nano Zetasizer 173 ° (equilibrium time set at 3 min, 3 measurements for each sample). The samples were dispersed in H₂O with a concentration of 0.7 mg ml⁻¹, shaken and sonicated prior to the analysis. The Zeta Potential measurements of the DMSNs and insulin over the pH range 2 – 6 were obtained using a titrator coupled to a Malvern DTS Nano Zetasizer. Thermogravimetric (TG) and Differential Scanning Calorimetry (DSC) analyses were performed on a Netzsch STA-449 F3 Jupiter instrument under airflow of 20 ml min⁻¹ with a heating rate of 10 °C min⁻¹, from 40 to 700 °C. The percentage of mass loss was assessed in the temperature range 120 – 700 °C. Wide angle powder X-ray diffraction (WXRD) measurements were performed using a Panalytical Empyrean diffractometer (Malvern Panalytical, UK) in reflection geometry (Bragg–Brentano HD) using Cu Kα₁,₂ radiation operated at a voltage of 45 kV, a tube current of 40 mA, with a fixed divergence slit of 0.05 mm. The measurements were performed in a continuous mode with a step size 2θ of 0.013° and a time per step of 200 s. The quantification of insulin was performed using a Vanquish ultra-high-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific, Bremen, Germany) coupled with a LTQ Orbitrap Velos mass spectrometer equipped with an electrospray ion source with the voltage 2.1 kV and the ion transfer capillary temperature was 300 °C. The retention of insulin was carried out on a C18 analytical column Acclaim 120™ (Thermo Scientific, 2.1 x 150 mm, 3 μm, 10 nm) at a flow rate of 0.4 ml min⁻¹ and a temperature of 40 °C. The injection volume was 5 μl. The mobile phases were: A: 100 % H₂O, 0.1 % FA and B: 100 % ACN, 0.1 % FA. A gradient method was applied as follow: 0.0 – 8.0 min 5 - 53 % B, 8.0 - 8.5 min 53 - 95 % B, 12.5 - 13.0 95 - 5 % B and re-equilibration was made until 17 min. The full MS scans were acquired in positive ion mode at 400 – 2000 m/z range at a resolution of 60000 (FWHM at 400 m/z).

Tablet formulations. Tablets of BL combined with either insulin (BL-Ins) or insulin loaded in the different DMSNs (BL-DMSN-Hex-Ins, BL-DMSN-Tol-Ins, BL-DMSN-SH-Ins) were prepared using a single punch press (PerkinElmer, UK). Prior to the compression, the powders were mixed together in a vial with a spatula, this refers to the mixed formulation. For comparison purposes, a layer-by-layer formulation (lbl) was also tested and in this case the tablet of BL-Ins (lbl) was prepared as follows: 100 mg of BL was first placed at the bottom of the compression tool followed by a layer of 30 mg of DMSN-Hex-Ins and finally the second layer of BL was placed at the top. Unless otherwise stated, all the tablets were prepared through the mixed formulation.

Insulin release experiments. The solution at pH 1.2 and 7.4 were prepared according to previously reported protocols.[4] For the solution at pH 1.2, 2 g of NaCl, 7 ml of HCl (37 %) and 1 l of nanopure H₂O were mixed. For the solution at pH 7.4, first 6.8 g of KH₂PO₄ was dissolved in 250 ml of nanopure H₂O and then combined to 190 ml of NaOH 0.2 M and 400 ml of nanopure H₂O. If needed, the pH was adjusted using NaOH 0.2 M and the volume was completed to 1 l with nanopure H₂O. For each dissolution test, 40 ml of the solutions at pH 1.2 or 7.4 were introduced into a 500 ml erlenmeyer, subsequently placed inside an incubator set at 37 °C and stirring at 150 rpm was allowed prior to the addition of the tablets. The tablets were first soaked 2 h at pH 1.2, were then taken out and directly placed in 5 ml of PBS for few minutes to prepare the solution at pH 7.4. Once transferred in pH 7.4, the test continued until 24 h. Aliquot of 1 ml were taken out at adequate period of time and replaced by 1 ml of the fresh solution to maintain the same volume. The concentration of insulin released in both solutions was quantified using UHPLC-MS system. Triplicates were only realized for BL-Ins, BL-DMSN-Hex-Ins and BL-DMSN-SH-Ins, experiments made for comparison purposes i.e., BL-Ins (lbl), BL-DMSN-Tol-Ins were performed once. Statistical analysis was performed with OriginPro 9.55 (OriginLab) applying one-way ANOVA with Fisher test for pairwise comparison (threshold value p < 0.05).
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**Cell culture.** Non-tumorigenic human colonic epithelial cells (HCEC) were cultivated in DMEM supplemented with 2% medium 199 (10X), 2% cosmic call serum, HEPES 20 mM, gentamycin (50 μg ml⁻¹), insulin-transferrin-selenium-G (10 μg ml⁻¹), recombinant human EGF (20 ng ml⁻¹), hydrocortisone (1 μg ml⁻¹). The cells were incubated in a humidified incubator with 5% CO₂ at 37 °C. The HCEC cells (HCEC-1CT) were kindly provided by Prof. Jerry W. Shay (UT Southwestern Medical Center, Dallas, TX, USA) and cultivated as previously described.[5,6]

**Fluorescent microscopy experiments.** For the live cell imaging experiment, a confocal LSM microscope Zeiss 710 was equipped with ELYRA PS.1 and a Water Plan Apochromat 63x/1.2 objective. HCEC were seeded in 96-well slides, 6000 cells/well and incubated at 37 °C for 48h before the live cell imaging experiments. Prior to their incubation, the DMSNs, i.e., DMSN-FITC, DMSN-SH-Rhod and DMSN-Rhod were dispersed in DMEM, vortexed for 15 sec and sonicated for 30 min, this procedure was repeated three times and a concentration of 200 μg ml⁻¹ was applied to the cells. Ins-FITC was dissolved in the cell culture media at a concentration of 10 μg ml⁻¹, equivalent concentration of insulin was used for DMSN-Ins-FITC. In the case of DMSN-Ins-FITC, the dispersion procedure was only applied once to avoid premature release of Ins-FITC. At the end of the treatments, the cells were washed three times with LCI to remove non-internalized DMSN-FITC, DMSN-SH-Rhod, DMSN-Rhod, Ins-FITC or DMSN-Ins-FITC. Then, the cells were stained with CellMask™ Deep Red Plasma Membrane Stain (1:1000 dilution).[7] Imaging was performed in Live Cell Imaging Solution (Molecular Probes, Life Technologies, Thermo Fisher Scientific, USA).

**Insulin starvation of HCEC.** HCEC are normally cultured with insulin in their media at the concentration of 10 μg ml⁻¹. Thus, the removal of insulin-transferrin-selenium-G from their culture media has permitted to test the metabolic activity of the cells in presence of the different insulin-containing DMSNs and compare with the unformulated insulin purchased. To achieve so, 8000 cells per well were seeded in a 96-well black side plate at 37 °C for 48h. Once confluency could be observed, the culture media was removed, replaced by an equivalent volume lacking insulin-transferrin-selenium-G and the cells were further incubated at 37 °C for 1h. Then, the cells were treated with insulin, i.e., the unformulated purchased one, at a concentration of 10 or 15 μg ml⁻¹ in DMEM or the insulin-containing DMSNs, i.e., DMSN-Hex-Ins or DMSN-SH-Ins at an equivalent concentration of insulin of 15 μg ml⁻¹ in DMEM. The samples were vortexed and sonicated to ensure the dissolution of unformulated insulin or the suspension of DMSN-Hex-Ins and DMSN-SH-Ins. A volume of 100 μl was added to the insulin starved cells; the wells were then completed with 100 μl of culture media lacking insulin. The incubation was continued for a time period of either 1 or 6h. For comparison purposes, the same experiment was conducted in normal culturing conditions, i.e., cells were not insulin-starved and they were incubated with a full media. At the end of the incubation, cells were washed twice with 100 μl of DPBS prior to the Alamar Blue assay.

**Alamar Blue (AB) assay.** AB assay was used following methods previously reported with slight modifications.[8] Briefly, a solution of 10% AB in DMEM (v/v) was prepared and 100 μl of this solution was added to each well and the reaction was allowed for 40 min in the incubator. The fluorescence was measured at 530/560 nm (excitation/emission) using a Cytoation 3 Imaging Multi Mode Reader (BioTek, Bad Friedrichshall, Germany). Cells incubated were compared to the respective control cells and the ratio treated over control was calculated in %.

Statistical analysis was performed with OriginPro 9.55 (OriginLab) applying one-way ANOVA with Fisher test for pairwise comparison (threshold value *p* < 0.05).
Results and Discussion

Characterization of the DMSNs

Table S1. Physicochemical parameters of the pure DMSNs, the functionalized DMSNs and the insulin-loaded DMSNs.

|                  | Size[^a] (m² g⁻¹) | Pore volume[^a] (cm³ g⁻¹) | Pore size[^a] (nm) | Particle size[^b] (nm) | Mass loss[^c] (%) |
|------------------|--------------------|---------------------------|-------------------|------------------------|-------------------|
| DMSN-Hex         | 823                | 1.5                       | 7.6               | 151                    | -                 |
| DMSN-Cyclo       | 868                | 1.60                      | 8.4               | 154                    | -                 |
| DMSN-Tol         | 949                | 2.70                      | 11.7              | 238                    | -                 |
| DMSN-SH          | 615                | 1.15                      | 7.0               | 159                    | 8.6               |
| DMSN-Hex-Ins     | 600                | 1.15                      | 7.0               |                        | 20.4              |
| DMSN-Tol-Ins     | 589                | 1.75                      | 11.7              |                        | 17.8              |
| DMSN-SH-Ins      | 633                | 1.15                      | 7.0               |                        | 20.2              |

[^a] The specific surface area S_{BET}, pore volume and pore size were obtained from the N₂-physisorption analysis. [^b] The particle size has been measured using DLS. [^c] Mass loss values were calculated in the temperature range of 120 - 700 °C from the thermogravimetry analysis.

Whilst the pore sizes of the DMSNs have been tailored upon changing the nature of the organic solvent, the particle sizes of the resulting nanoparticles are only slightly changed. As seen in Table S1, while the DMSNs made with hexane or cyclohexane have very similar particle sizes of approximately 150 nm and comparable porosity characteristics, i.e., pore sizes of 7.6 nm for DMSN-Cyclo and 8.4 nm for DMSN-Hex, the ones made with toluene have bigger particle diameter of almost 240 nm and much larger pores of almost 12 nm.
Figure S1. Physicochemical characterization of the thiol-functionalized DMSNs: (a) $N_2$ physisorption isotherm of DMSN-SH measured at $-196$ °C, (b) Pore size distribution of DMSN-SH calculated using NLDFT method considering the adsorption of $N_2$ in silica materials with cylindrical-like pores, (c) Hydrodynamic diameter of the particles in suspension in water measured by DLS; (d) TG-DSC analysis of DMSN-SH.

DMSN-Hex have been further functionalized with thiol groups using (3-mercapto)triethoxysilane (MPTS) which decreased the pore size from 7.6 to 6.9 nm, the surface area from 823 to 615 m$^2$ g$^{-1}$ and the pore volume from 1.50 to 1.15 cm$^3$ g$^{-1}$. However, the particle size did not change and stayed centred at 159 nm. As it can be seen from Figure S1d, the combustion of the thiol-silane occurs at approximately 275 °C and around 9 wt% has been anchored on the surface through post-synthesis grafting.
Insulin loading strategy

As it can be seen in Figure S2a, the size of insulin in an aqueous solution is around 6 nm, therefore, larger pore sizes of DMSNs were used for loading. Then, the effect of the charge of insulin and the DMSNs in solution on the loading efficiency was assessed. To begin with, insulin has a positive charge in the pH range 2 – 3, as determined by the results of the Zeta potential measurements depicted in Figure S2b. On the other hand, both the pure and the thiol-functionalized DMSNs have nearly a neutral charge at this pH. The loading of insulin at pH 3 was first attempted followed by an attempt at pH 4.

Figure S2. (a) Diameter of insulin in pH 7.4 measured by DLS; (b) Zeta potential of insulin, DMSN-Hex and DMSN-SH measured over the pH range 2-6.
Insulin loading results

Figure S3. (a) Mass loss and (b) DSC profiles of the different materials: insulin, insulin loaded at pH 4 in pure DMSN-Hex and DMSN-Tol or thiol-functionalized DMSN-Hex (DMSN-SH). For comparison purpose, insulin loaded at pH 3 in DMSN-Hex is shown.

A mass loss of around 10 wt% was calculated for the loading of insulin inside both DMSN-Hex and DMSN-Tol (data not shown for DMSN-Tol) at pH 3. Further optimization has led to the loading of insulin at pH 4 since, at this pH, insulin has a positive charge of around +10 mV and the pure or thiol-DMSN have a negative charge of around –20 mV. The important difference in the charges makes the electrostatic attraction between both parts stronger. Indeed, the mass loss increased to 20 wt% (Table S1) for DMSN-Hex-Ins and DMSN-Tol-Ins. Using the same amount of insulin and silica materials, lower loading was detected for the thiol-functionalized DMSNs, i.e., 12 wt% (Figure S3a and Table S1). In this case, the initial amount of insulin was increased to 60 mg while keeping an equal quantity of DMSN-SH. By doing so, the loading of insulin reached also around 20 wt% (data not shown). The structure of insulin was maintained during the loading procedure as confirmed by MS spectrometry. The effect of the confinement of insulin inside the mesopores of the different DMSNs can be appreciated from the Figure S3b. While the combustion of insulin confined in the mesopores occurs during one exothermic event at T = 340 °C, insulin itself is decomposed throughout several exothermic and endothermic processes.
Figure S4. (a) Isotherms and (b) pore size distributions obtained from N<sub>2</sub> physisorption analysis (-196 °C) of insulin loaded into DMSN-Hex, DMSN-Tol and DMSN-SH. Physicochemical values are reported in the Table S1.
The wide-angle powder X-ray diffractogram presented in Figure S5 shows that insulin itself can be considered as a semi-crystalline peptide with diffraction peaks in the region $5 < 2\theta < 15^\circ$. Differently, when incorporated inside the pores of either DMSN-Hex or the thiol-functionalized version, DMSN-SH, no diffraction peaks can be seen, therefore insulin is confined as an amorphous protein in the pores.

Figure S5. Wide-angle powder X-ray diffractograms of the starting DMSN-Hex and DMSN-SH, the insulin-loaded into DMSN-Hex and DMSN-SH and insulin alone.
As it can be observed, the release of insulin from the pure DMSNs does not seem to depend on the pore sizes as similar performances have been observed for both DMSN-Hex (pore size: 7.6 nm) and DMSN-Tol (pore size: 11.7 nm). However, changing the formulation of the BL tablet resulted in drastically decreasing the elution of insulin at pH 7.4. This might originate from the fact that, in the layer-by-layer formulation, the DMSN-Hex are located in the center of the tablet and therefore more time is needed to navigate from the center to the media. Nevertheless, this gives an interesting insight supporting the assumption that after 1h at pH 7.4, the DMSNs in the mixed formulation start to reach the media and would be ready to be transported through the intestinal barrier.
Cellular uptake experiments

**Figure S7.** Live cell fluorescence images (a) HEC control cells, (b) FITC control, (c) Ins-FITC incubated for 45 min; 3D stack after 3 h incubation of Ins-FITC (d) upper and (e) bottom view. The plasma membrane is represented in red, the nucleus in blue and Ins-FITC in green.

FITC was solubilized in DMSO and applied to the cells, the images were taken immediately after the incubation. Thus, it can be concluded that FITC alone goes quickly in the cells and reach the nucleus (Figure S7b). Insulin coupled with FITC does not seem to penetrate the cells and this up to 3h. From the upper view, Ins-FITC dispersed in the medium can be observed, however from the bottom view, no Ins-FITC was seen to approach the cells.
Figure S8. Live cell fluorescence images of the uptake of Rhod-labeled DMSNs after 20h of incubation of (a) DMSN-SH-Rhod and (b) DMSN-Rhod with HCEC. The plasma membrane is represented in white and the signal from Rhod in red.

It can be seen that the thiol function seemed to enable a more uniform distribution of the nanoparticles in the cells. Because of the presence of non-reacted amine groups at the particle surface, DMSN-Rhod expressed a positive charge (see Figure S9 for Zeta potential measurements) which led to agglomeration of these particles and decreased cellular uptake. With their negative charges, DMSN-FITC and DMSN-SH-Rhod were equally capable of entering the cells.
Figure S8. (a) Zeta potential measurements of the different labelled-DMSNs: DMSN-FITC, DMSN-Rhod, DMSN-SH-Rhod. The positive charge of DMSN-Rhod might originate from remaining non-coupled APTS. (b) MS spectrum of Ins-FITC after the synthesis (bottom) and confirmation of the structure of insulin in live cell imaging solution (LCI) added to the cells.

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Author Contributions

E.J. has performed the experimental work, treated the data and written the original draft. G.D.F. has contributed to the cell studies and confocal microscopy data acquisition and treatment. R.C. has contributed to the investigation and formal analysis of the pH-gating system based on beta-lactoglobulin. E.J., R.C. F.K. have worked together on the materials chemistry experiment design, analysis and interpretation. E.J., G.D.F., D.M., F.K. have worked together on the biological studies, acquisition and interpretation. D. M. has provided additional supporting supervision for the cell studies. F.K. is lead PI and project administrator, and main coordinator and supervisor of the work. All the authors have contributed to the revision of the original draft.