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Research paper

Nanostructured porous silicon microparticles enable sustained peptide (Melanotan II) delivery

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ABSTRACT

Peptide molecules can improve the treatment of a number of pathological conditions, but due to their physicochemical properties, their delivery is very challenging. The study aim was to determine whether nanostructured porous silicon could sustain the release and prolong the duration of action of a model peptide Melanotan II (MTII). Thermally hydrocarbonized nanoporous silicon (THCPSi) microparticles (38–53 μm) were loaded with MTII. The pore diameter, volume, specific surface area and loading degree of the microparticles were analyzed, and the peptide release was evaluated *in vitro*. The effects of MTII on heart rate and water consumption were investigated *in vivo* after subcutaneous administration of the MTII loaded microparticles. A peptide loading degree of 15% w/w was obtained. *In vitro* studies (PBS, pH 7.4, 37 °C) indicated sustained release of MTII from the THCPSi microparticles. *In vivo*, MTII loaded THCPSi induced an increase in the heart rate 2 h later than MTII solution, and the effect lasted 1 h longer. In addition, MTII loaded THCPSi changed the water consumption after 150 min, when the immediate effect of MTII solution was already diminished. The present study demonstrates that MTII loading into nanosized PSi pore structure enables sustained delivery of an active peptide.

1. Introduction

Peptides are promising but challenging molecules for drug delivery; many of their properties, such as poor oral bioavailability and restricted administration routes, limit their use. Due to their large molecular size, chemical characteristics and sensitivity to breakdown in the gastrointestinal tract, they are typically administered parenterally either in solutions or in particulate drug delivery systems. Furthermore, many of the new promising drug candidates purported to be beneficial in several pathological conditions, have short biological half-lives and are rapidly eliminated from the blood circulation [1].

Nanotechnology in medical and pharmaceutical sciences, including particulate drug carrier systems, has developed tremendously during the past decades. Particulate drug delivery systems are of interest, because they could e.g. increase bioavailability by protecting the drug molecule from degradation or by increasing the solubility, decrease side-effects by controlling the drug release or target the drug to specific tissue or cells [2–4]. Furthermore, particulate drug delivery systems can be adjusted to be suitable for all the delivery routes, including parenteral and enteral, systemic and local administration. In addition to be used for traditional drug molecules, they are offering a way to overcome the problems related to peptide administration [5]. In the case of therapeutical, short acting peptide molecules, one of the formulation development aims is to prolong the presence of the active molecule in blood circulation, leading to lower administration frequency and better patient compliance.

Porous materials have been used over a decade in tissue engineering and drug delivery. Recently, inorganic porous silicon (PSi), with a nanoporous structure, has been investigated as a novel material for several biomedical applications, including drug delivery since it has several advantages compared with the traditional drug delivery materials [6–9]. One of the drawbacks related to traditional polymeric particulate formulations is their limited

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capability to carry sufficiently high peptide loads [10–12]. In contrast, the extensive adsorbing surface area of PSi has the potential to carry and deliver large amounts of drug molecules within a relatively small carrier material mass (Fig. 1) [3,8]. In addition, pore size and surface chemistry can be adjusted to be suitable for the properties of the used drug molecule and furthermore modified to affect drug release appropriately; hence, the pore size, the particle surface and the loading level have been shown to affect the drug release [3,6,13–15]. Compared with the traditional particulate systems, the drug loading onto PSi is a simple and gentle procedure. Typically, drug is entrapped in drug carriers using methods with several different stages before reaching the final formulation, which might cause degradation or loss of bioactivity of the drug during the preparation [16–19]. On the contrary, onto PSi the drug can be loaded at room temperature in any solution into which the used drug compound is soluble, and the loading can be performed even at reduced temperature. These are important factors when loading sensitive drug molecules, such as peptides.

Melanocortin system mediates many of the physiological functions of melanocortins, such as cardiovascular effects and regulation of the energy and liquid homeostasis, and the system has been investigated in order to find new therapies for several pathological conditions, e.g. obesity, erectile dysfunction and inflammation [20–22]. Melanotan II (MTII) is a potent, unselective peptide agonist for melanocortin receptors and the pharmacological actions of MTII include inhibition of drinking and elevated heart rate [23,24].

The aim of the present study was to investigate *in vitro* and *in vivo* whether thermally hydrocarbonized (THCPSi) microparticles could serve as a sustained release system for peptides. THCPSi microparticles (size fraction 38–53 μm) were loaded with a synthetic model peptide, Melanotan II (MTII). The pore diameter, volume, specific surface area and loading degree of the THCPSi microparticles were analyzed, and the release of the MTII was examined *in vitro*. Furthermore, the duration of the action of MTII delivered via THCPSi microparticles on heart rate and water consumption was investigated and compared with corresponding MTII solution in rats and mice, respectively.

2. Materials and methods

2.1. Reagents

MTII (Ac-Nle-*cyclo* [Asp-His-D-Phe-Arg-Trp-Lys]-NH2, mw. 1024.2) was purchased from Peptides International Inc. (Kentucky, USA). The silicon wafers were bought from Cemat Silicon S.A. (Warsaw, Poland). Ethanol (99.5%) was purchased from Altia (Helsinki, Finland). Hydrofluoric acid (HF) (37–39%) was supplied by Merck KGaA (Darmstadt, Germany). Methanol for peptide loading was obtained from Merck KGaA (Darmstadt, Germany) and for *in vitro* experiments from JT Baker (Deventer, The Netherlands). The nitrogen (99.999%) and the acetylene (99.6%) gases were purchased from AGA (Espoo, Finland). Carboxymethylcellulose sodium (CMC) was bought from Sigma–Aldrich Chemie (Germany).

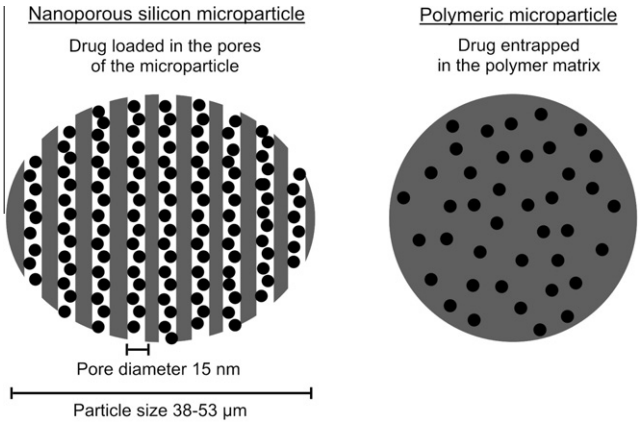

Fig. 1. Cross section of nanoporous silicon and polymer drug carriers. The scheme is not in scale.

HPLC reagents were acetonitrile (HPLC grade, JT Baker, Deventer, The Netherlands), trifluoroacetic acid (Sigma–Aldrich, St. Louis, MO, USA) and triethylamine (Fluka/Sigma–Aldrich, Steinheim, Germany). In the *in vitro* release experiments, 0.15 M phosphate-buffered saline was used as a buffer (PBS, pH 7.4, $\mu = 0.167$) containing 8.0 g sodium chloride (JT Baker Deventer, The Netherlands), 0.2 g potassium chloride (Merck KGaA, Darmstadt, Germany), 1.4 g disodium hydrogen phosphate (Merck KGaA, Darmstadt, Germany) and 0.2 g potassium dihydrogen phosphate (Merck KGaA, Darmstadt, Germany) in 1000 ml of deionized water. In addition, bovine serum albumin (BSA, Sigma–Aldrich, St. Louis, MO, USA) (0.1% w/v) was dissolved to the PBS in order to prevent adsorption of the model peptide onto the laboratory materials during the *in vitro* experiments.

2.2. Preparation of THCPSi microparticles

The preparation of free standing PSi films was made as described previously [6]. The free standing PSi films were ball milled and dry sieved to a 38–53 μm size fraction. After the dry sieving, the particles were washed on a 38 μm sieve with ethanol in order to remove any remaining small particles. Dry and wet sievings were performed in order to limit the lower and upper size of the particles between 38 and 53 μm. The microparticles were treated with a 1:1 HF:EtOH solution, to replace the oxidized surface formed during the milling with a hydrogen termination, and dried in 65 °C for 1 h. Thermal hydrocarbonization of the PSi microparticles was performed under 1:1 N_2:acetylene flow at 500 °C for 15 min as described earlier [25], in order to produce a more stable hydrocarbon surface. The pore volume, average pore diameter and specific surface area of the THCPSi microparticles were calculated from desorption branch of the nitrogen sorption measurements (Tristar 3000, Micromeritics) according to BJH-theory [26]. These values showed average pore diameter of 14.6 nm, specific surface area of 430 m^2/g and pore volume of 1.19 cm^3/g.

Two batches of THCPSi microparticles were loaded separately. The first batch was used in water consumption measurements in mice, and the second batch in rat heart rate and *in vitro* release experiments. MTII was dissolved in methanol, and the mesoporous THCPSi microparticles were immersed in the peptide solution (37 mg/ml) for 1.5 h at room temperature. The loading solution was treated with ultrasound three times during the loading to ensure homogeneity. The particles were filtered from the solution and dried for 3 h at room temperature additional 30 min in vacuum. The peptide loading degree was determined by thermogravimetric (TG) analysis (20 °C/min, 25–800 °C N_2 gas purge 200 ml/min, TGA 7, PerkinElmer) as described earlier [27]. Peptide loading degrees of the two batches were 14.8 ± 0.3% and 15.1 ± 1.4% (w/w) as measured by TG. In addition to the TG analysis, the loading degree of the second batch was analyzed with HPLC to be 15.3 ± 2.0% according to methanol extraction. The MTII particles were washed five times with 1 ml methanol, and the concentration of MTII in methanol extract was analyzed by high performance liquid chromatography (HPLC) as described in Section 2.4.

2.3. In vitro release

MTII loaded microparticles (2 mg THCPSi, containing 302 μg MTII) were placed in microcentrifuge tubes and suspended in

1.5 ml of pH 7.4 0.15 M PBS buffer containing 0.1% w/v BSA. The microcentrifuge tubes were placed in a water bath shaker with orbital shaking at a frequency of 120 strokes/min at +37 °C (Grant OLS200, Cambridge, UK). At pre-determined time intervals, the tubes were centrifuged for 2 min (13,000 rpm, 17,000g, Heraues Biofuge Fresco, Osterode, Germany), and supernatants were collected for the HPLC analysis of the MTII concentration. The microparticles were re-suspended in fresh pH 7.4 PBS buffer (+37 °C, 0.1% w/v BSA) after the supernatant collection, in order to maintain sink conditions throughout the experiment.

The Power Law with burst release ($M_t/M_\infty = at^n + b$, Eq. (1)) was applied in order to determine the release kinetics of the peptide, where M_t is amount of released drug in time t, M_∞ is initial drug amount and n is the diffusional exponent. The diffusional exponent (n) of 0.5 indicates a diffusional square-root-of-time release, a slope between 0.5 and 1.0 indicates an anomalous transport and a slope of 1 indicates zero-order release kinetics [28].

2.4. High performance liquid chromatography analysis of MTII

MTII was analyzed with a Gilson High Performance Liquid Chromatograph. The system consisted of an UV detector (UV/VIS-151), pump (321), autoinjector (234), interface (506C) and integrator (Unipoint 3.0). The mobile phase was a mixture of acetonitrile (36% v/v), water (64% v/v), trifluoroacetic acid (0.1% v/v) and triethylamine (0.15% v/v). The pre-column was a reverse-phase Pelliguard® LC-18 column (20 × 4.6 mm id, particle size 40 μm, Supelco, Bellefonte, PA, USA), and the analytical column was a reverse-phase Supelcosil® C-18 column (150 × 4.6 mm id, particle size 5 μm, Supelco, Bellefonte, PA, USA). Samples were diluted appropriately with the mobile solutions before their injection into the HPLC system. The injection volume was 100 μl, flow rate 1 ml/min and MTII was detected at 220 nm. The concentrations of MTII were determined by measuring peak areas, which were compared to a linear calibration curve prepared using known standard MTII concentrations (0.2–10 μg/ml).

2.5. Animals

Male Wistar rats and Balb/c × DBA2 hybrid male mice were purchased from National Laboratory Animal Center (Kuopio, Finland) at the age of 7–8 weeks. They were housed individually in a regulated environment; temperature 22 ± 1 °C, relative air humidity 55 ± 15% and 12/12 h light/dark cycle with lights on at 7 am. Commercial pellets (Lactamin R36, Sweden) and tap water were available *ad libitum*, unless otherwise indicated. The Institutional Animal Care and Use Committee of the Provincial Government approved the animal experiments. Experimental procedures were conducted in accordance with the guidelines set by the European Community Council Directives 86/609/EEC.

2.6. Telemetric monitoring of the heart rate in rats

Telemetry transmitters for monitoring the heart rate were implanted as described earlier [29]. Before the experiments, the rats were familiarized to the treatment procedure, in order to avoid stress induced blood pressure increase. All the injections were given subcutaneously in a volume of 700 μl. The dose of MTII was 3 mg/kg, and the administered THCPSi microparticle mass was ~8.1 mg/rat. To facilitate the injection procedure, the vehicle viscosity (0.9% NaCl) was increased by addition of 5 mg/ml carboxymethyl cellulose sodium (CMC).

Rats were divided into four different treatment groups: (1) vehicle (5 mg/ml CMC in 0.9% NaCl) (n = 6), (2) unloaded THCPSi microparticles suspended in the vehicle (THCPSi) (n = 6), (3) MTII loaded THCPSi microparticles suspended in the vehicle (THCPSi + MTII) (n = 6) and (4) MTII dissolved in the vehicle (MTII) (n = 6). Telemetry receivers (model RPC-1) were placed under individual cages for data acquisition with Aquisition A.R.T. software (DSI, MN, USA). Heart rate was measured each minute, starting 7 h before the injections to obtain a baseline and continued for 24 h after the treatment.

2.7. Monitoring of the water consumption in mice

Before the measurements, mice were acclimatized to conditions similar to the experiment for seven days and fasted overnight (16 h). Water was freely available. The mice were divided into four different treatment groups: (1) vehicle (5 mg/ml CMC in 0.9% NaCl) (n = 5), (2) unloaded THCPSi microparticles suspended in the vehicle (THCPSi) (n = 6), (3) MTII loaded THCPSi microparticles suspended in the vehicle (THCPSi + MTII) (n = 6) and (4) MTII dissolved in the vehicle (MTII) (n = 6). Injections were given subcutaneously in a volume of 300 μl. The dose of MTII was 3.7 mg/kg, and THCPSi mass ~0.58 mg/mouse. Water consumption was studied as a function of time for the next 24 h using the LabMaster®-system (TSE Systems, Germany).

2.8. Statistical analysis

In vivo data were evaluated with two-way Anova for repeated measurements followed by Bonferroni post-test (GraphPadPrism 4.03 for Windows, Graph-Pad Software Inc., San Diego, CA and SPSS 4.0 for Windows). The level of significance was set at $p < 0.05$.

3. Results

3.1. In vitro peptide release

The MTII release from THCPSi microparticles (loading degree 15.1% w/w) clearly demonstrate the sustained release, with a moderate burst release during the first 30 min in pH 7.4 PBS buffer containing 0.1% w/v BSA (Fig. 2). MTII release from the THCPSi microparticles followed closely square-root-of-time kinetics (n = 0.517 ± 0.001, mean ± SEM, n = 4) within 8 days, and the release rate constant was 0.196%/h ± 0.006 mean/SEM.

3.2. In vivo effects of MTII

3.2.1. Heart rate changes in rats

The rats received one of the following treatments; MTII loaded particles (THCPSi + MTII), unloaded particles (THCPSi), vehicle or

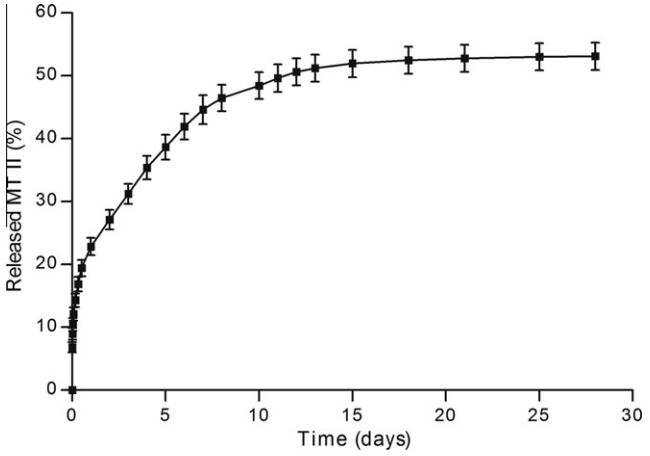

Fig. 2. Melanotan II *in vitro* release from THCPSi microparticles (38–53 μm, loading degree 15.1% w/w) in PBS, pH 7.4 (37 °C).

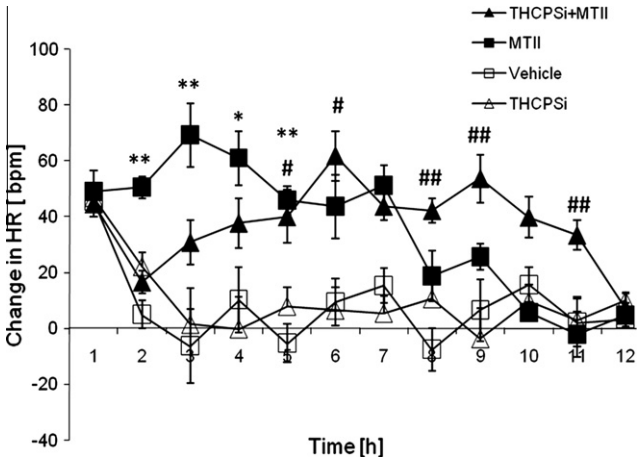

Fig. 3. The change in the heart rate is delayed after THCPSi + MTII treatment when compared with MTII treatment in rats (MTII dose 3 mg/kg, s.c.). Treatments: Vehicle = 5 mg/ml CMC in 0.9% NaCl, THCPSi = unloaded THCPSi microparticles suspended in the vehicle, THCPSi + MTII = MTII loaded THCPSi microparticles suspended in the vehicle; MTII = MTII dissolved in the vehicle. Data are presented as mean ± SEM, ($n = 6$), $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ THCPSi + MTII vs. vehicle; $^{*}p < 0.05$, $^{**}p < 0.01$ MTII vs. vehicle.

MTII solution (more detailed description of the treatments in the materials and methods section). Fig. 3 shows the effects of the different treatments on heart rate in rats as a function of time. The heart rate baseline before the treatments was 350.9 ± 13.4 beats per minute (bpm), which is in agreement with earlier studies [29,30]. Subcutaneous injections of peptide-free vehicle evoked a transient increase in the heart rate (45.6 ± 6.2 bpm), which normalized to the baseline 1 h after the injections. This temporary effect, due to the treatment protocol, is in accordance with the results from other groups [31]. A similar reaction was observed after injection of unloaded microparticles in the vehicle (THCPSi). MTII solution treatment caused an immediate and significant increase in the heart rate, and the effect of MTII lasted for 7 h after the injections. On the contrary, microparticles loaded with MTII did not evoke an immediate increase in the heart rate, but changed the curve profile. Instead of the immediate effect, the THCPSi + MTII treatment showed a delayed and sustained increasing effect on the heart rate, starting at 2 h after the treatments. After THCPSi + MTII injections, the heart rate increased until 6 h after the treatments and then decreased gradually, reaching a similar baseline of the heart rate as the other groups at 12 h after the injections. The maximal increases of the heart rate were observed at 3 h and 6 h after MTII and THCPSi + MTII injections, respectively. In addition, the heart rate stayed elevated 1 h longer period after THCPSi + MTII treatment when compared with the MTII injections. Therefore, the present results show that the THCPSi + MTII treatment caused a delayed and prolonged increase to the heart rate, which indicates sustained MTII release from the microparticles.

3.2.2. Water consumption changes in mice

The mice were treated with similar formulations to rats, and their water consumption was measured for 24 h. When compared with the vehicle, MTII and THCPSi + MTII tended to inhibit water consumption but did not reach statistical significance. However, a delayed and prolonged effect was achieved only with the THCPSi + MTII treatment, indicating sustained MTII release from the microparticles (Fig. 4A). In the first 50 min after the treatments, the water intake was similar in all the groups. MTII treatment started to inhibit water intake after 50 min, and the effect lasted until 160 min after the injection, as was expected (Fig. 4A). At a time of 160 min from the injections, the effect of the MTII

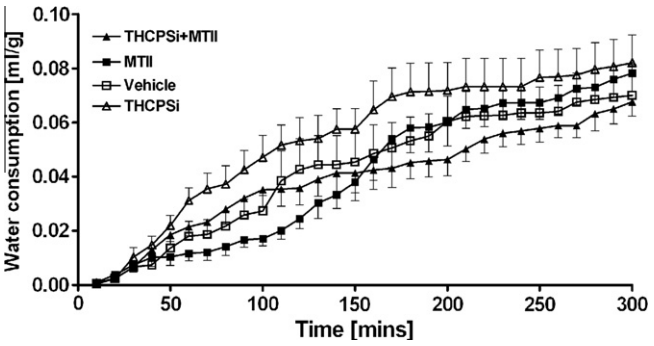

Fig. 4A. Cumulative water intake in mice. The inhibition in water consumption tends to be delayed and prolonged in THCPSi + MTII group when compared with MTII treatment. (MTII dose 3.7 mg/kg, s.c.) Treatments: Vehicle = 5 mg/ml CMC in 0.9% NaCl, THCPSi = unloaded THCPSi microparticles suspended in the vehicle, THCPSi + MTII = MTII loaded THCPSi microparticles suspended in the vehicle; MTII = MTII dissolved in the vehicle. Data are presented as mean ± SEM, $n = 6$, except vehicle $n = 5$.

treatment was already terminated and the water intake of the group increased rapidly, achieving the level of the control groups. On the contrary, at 210 min after the injections, the total water consumption was lower in THCPSi + MTII group when compared with the others (Fig. 4B). The THCPSi + MTII treated group did not substantially increase its water intake until 300 min when consumption reached that of vehicle and THCPSi, i.e. the control groups (Fig. 4A). The cross point, of the effects MTII and the THCPSi + MTII treatments, was at 150–160 min after the injections. The MTII treated mice compensated for their water consumption after the inhibitory effect, but in the THCPSi + MTII treated group, this compensatory phenomenon was lacking. Between 150 and 210 min, the water consumption of the THCPSi + MTII group was only 5% of that of the MTII treated group, evidence of a powerful effect of THCPSi + MTII treatment.

4. Discussion

In the present study, two independent *in vivo* measurement systems were used to monitor the effects of THCPSi + MTII formulation. The obtained *in vivo* results are evidence of sustained release of an active model peptide from the THCPSi microparticles, which is indicated by delayed and prolonged effects of MTII when

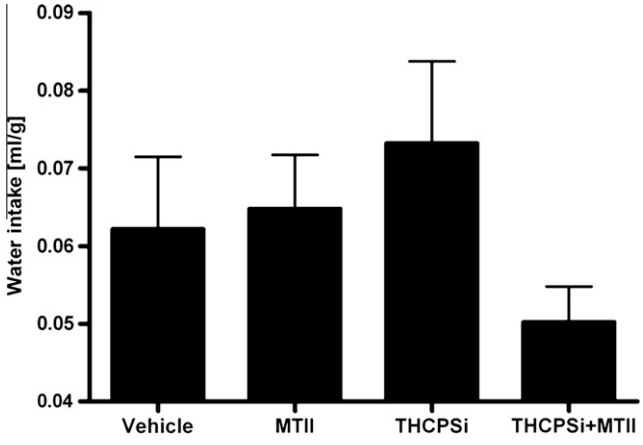

Fig. 4B. Total water intake at time 210 min after treatments in mice. The effect of THCPSi + MTII is present, while that of MTII has declined. Treatments: Vehicle = 5 mg/ml CMC in 0.9% NaCl, THCPSi = unloaded THCPSi microparticles suspended in the vehicle, THCPSi + MTII = MTII loaded THCPSi microparticles suspended in the vehicle; MTII = MTII dissolved in the vehicle. Data are presented as mean ± SEM, $n = 6$, except vehicle $n = 5$.

delivered in the nanostructured PSi. In rats, the increase in the heart rate started and achieved the maximum later and lasted longer after THCPSi + MTII treatment when compared with the MTII treatment (Fig. 3). In addition, THCPSi + MTII treatment decreased the water consumption in a sustained manner in mice (Figs. 4A and 4B). The sustained *in vivo* release of MTII from the THCPSi microparticles is also supported by the *in vitro* data of MTII release showing peptide release over several days in PBS buffer (Fig. 2).

Based on the changes in the measured *in vivo* parameters, the present results indicate that the MTII release was faster *in vivo* than *in vitro*. The changes in the heart rate and water consumption could be observed until 11 and 5 h in rats and mice, respectively, after the THCPSi + MTII treatments, when the effects of MTII solution were not anymore detectable. After 12 h, only 20% of the loaded MTII was released *in vitro* (Fig. 2) but the *in vivo* effects were already finished (Figs. 3, 4A and 4B). However, 20% release of the loaded MTII *in vivo*, from the administered THCPSi + MTII treatment during 12 h, would not have been sufficient to produce the observed responses. In an earlier publication [32], we have studied *in vivo* peptide (ghrelin antagonist) delivery via PSi but as far as we are aware, there are no other studies reporting *in vivo* peptide delivery using PSi as a sustained release system.

Silicon is a compound, which is a part of our every day diet, and PSi is considered to be safe material for drug delivery. PSi is biodegradable, and it degrades into orthosilicic acid, and the degradation rate is dependent on the porosity and the surface treatment [8,33]. It has been shown that PSi nanoparticles dissolve in hours *in vitro* and are totally cleared from the body in 4 weeks [9]. However, all the particle surface modifications might cause different reaction in the physiological system, but currently there is no evidence of any major toxic or immunogenic effects, encouraging further investigation of PSi for drug delivery [7,32,34].

Peptides are very promising molecules for the treatment of many severe illnesses, but unfortunately they typically cannot be compressed to tablets and administered per orally with a good bioavailability. Therefore, the micrometer drug carriers might offer a solution to the obstacles of peptide administration. In the present study, a high peptide loading degree was obtained (15% w/w). Nanoporous structure and large surface area of PSi has a great capability to adsorb and deliver high amounts of the peptide molecules, which has been a problem with the traditional polymer materials (Fig. 1).

In conclusion, the present *in vitro* experiment demonstrated sustained MTII release from the nanoporous THCPSi microparticles (38–53 μm). In agreement, *in vivo* experiments revealed delayed and prolonged effects of MTII, i.e. prolonged increase in the heart rate and decrease in the water consumption in rats and in mice, respectively, when administered via THCPSi microparticles. Therefore, these results clearly indicate that THCPSi microparticles have a capability to sustain the release of active peptides and thus to prolong their pharmacological effects *in vivo*. In addition, controllable porosity, tunable surface characteristics and favorable loading procedure for peptides enable the delivery of broad range of bioactive compounds and encourage further investigations PSi as a novel sustained nanostructured peptide carrier system.

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