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Abstract

Novel treatment methods for obesity are urgently needed due to the increasing global severity of the problem. Gastrointestinal hormones, such as GLP-1 and PYY, are secreted by the enteroendocrine cells, playing a critical role in regulating food intake. Digested nutrients trigger the secretion of these hormones, which have a very short half-life. α -Linolenic acid (α LA) has been shown to stimulate GLP-1 secretion, however, chemical instability and fast uptake in the small intestine hinder its use in body weight management. We developed a novel delivery system based on inorganic mesoporous particles for α LA to increase secretion of gastrointestinal peptides. α LA was loaded to thermally hydrocarbonized porous silicon particles (THCPSi). $47.9 \pm 3.84\%$ and $30.7 \pm 2.86\%$ of α LA was released during 6 h from 3.0% and 9.2% loading degree (w/w) samples *in vitro*, respectively. Native α LA (50 μ M) significantly increased GLP-1 secretion from enteroendocrine STC-1 and GLUTag cell lines. α LA loaded THCPSi significantly and dose dependently stimulated GLP-1 secretion from STC-1 cells, whereas empty particles did not. We demonstrated *in vitro* that THCPSi particles have the potential to be used as a controlled delivery system for nutrients such as α LA, increasing GLP-1 secretion. Our results justify further *in vivo* investigations.

Keywords: controlled delivery, α -linolenic acid, nutrients, porous silicon, GLP-1, enteroendocrine cells, food intake

Introduction

The increase of overweight and obesity has been a serious problem for our societies for last three decades worldwide. According to the World Health Organization (WHO), obesity has nearly tripled since 1975, and 39 % of the over 18 years old were overweight, and 13 % were obese in 2016 (1). Increase of food intake and decrease of physical activity are considered to be the causing factors to this pandemic, which affects the health of the individuals and the economic burden of the societies.

Nutrients induce secretion of several gastrointestinal hormones, such as Glucagon-like peptide-1 (GLP-1), peptide YY (PYY), and cholecystokinin (CCK), which play an important role in regulation of appetite, blood glucose level, and insulin secretion (2, 3). Although gastrointestinal hormones work as efficient appetite regulators, they have very short half-lives: GLP-1 and CCK; 1 to 2 minutes, and gastric inhibitory peptide (GIP) and PYY; 8 to 10 minutes (4, 5, 6). Currently, the pharmaceutical industry is focusing on developing different agonists of these peptides to increase the stability and prolong their duration of action (7). Another approach is to endogenously stimulate the enteroendocrine system by utilizing a delivery system which carry nutritional compounds specifically to the receptors of enteroendocrine cells (e.g. L-cells) without a significant addition of the caloric load. This might be an alternative and a more feasible approach to release the peptides endogenously in a sustained manner. These cells, secreting GLP-1 and PYY, are mainly located in the distal part of the gastrointestinal tract, while most nutrients are absorbed in proximal intestine before reaching the required site of action. Therefore, we aimed in this study to load nutritional compounds into a carrier system to stimulate enteroendocrine signals in sustained manner. Porous silicon (PSi) was selected as carrier material for the nutrient due to its ability to carry high payloads and protect sensitive compounds from degradation (8). Previously, the potential of PSi particles has been studied for different biomedical applications, tissue engineering and as well as for controlled drug delivery (9, 10, 11). The pore size and surface chemistry can be modified to be suitable for the payload molecules, and PSi has been widely tested for its safety *in vitro* and *in vivo* (12, 13), thus it is considered as a safe material. In fact, silicon is an important micronutrient, playing a role in bone and skin well-

being (14). PSi particles are degraded into silicic acid in aqueous conditions and the biodegradation rate is strongly affected by the surface modification (15) and pH of the environment.

The unmodified PSi surface is chemically unstable and requires stabilization. PSi particles with different surface modifications, such as thermally oxidized PSi (TOPSi), thermally hydrocarbonized PSi (THCPSi), and undercyclic acid treated thermally hydrocarbonized PSi (UnTHCPSi) have different interactions with the payload based on their surface charge and hydrophilicity (15, 16, 17). These properties affect adsorption as well as desorption of the cargo compounds (18). THCPSi particles have a hydrophobic surface due to the hydrocarbon covered surface, and the wettability of these particles are low (19). This makes the particles suitable for loading and delaying the release of hydrophobic molecules, such as fatty acids. Therefore, this PSi type was selected to be investigated with the selected model nutrient.

α -Linolenic acid (α LA), an omega-3 Long Chain Fatty Acid (LCFA), was selected as a model nutrient. It is an essential fatty acid and widely present in vegetable oils like canola, soy, and flaxseed oils. Importantly, LCFAs, including α LA, stimulate secretion of several gut hormones, such as GLP-1 and PYY (20, 21). Furthermore, it has been shown that GPR120 receptors, expressed in endocrine L-cells, are specific for LCFAs stimulating GLP-1 release (20, 22). Hence, we aim to load α LA into PSi particles and evaluate the effects of α LA loaded PSi particles on GLP-1 secretion *in vitro*, using two enteroendocrine cell lines: STC-1 cell line, which was originally established from murine enteroendocrine tumor cells, and GLUTag cell line, which was derived from colonic tumors of transgenic mouse expressing large T antigen under the control of the proglucagon promoter (23, 24). Both cell lines are commonly used for studying the effects of compounds on secretion of gastrointestinal hormones (20, 25, 26, 27).

Materials and Methods

Materials

Silicon wafers (p+ type, 0.01-0.02 Ωcm) were donated by Okmetic Ltd. (Vantaa, Finland). Ethanol (99.5 %) used in the etching solution was purchased from Altia Oyj (Rajamäki, Finland). The acetylene (99.6 %) and nitrogen (99.999 %) gases for surface modification of porous silicon were obtained from AGA (Espoo, Finland). α -Linolenic Acid (Cat. #: L2376), Dulbecco's phosphate buffered saline (Cat. #: D1408), Hydrofluoric acid (HF) (38-40 %) and Dulbecco's modified Eagle medium (Cat. #: F0435) were acquired from Merck (Darmstadt, Germany). Fetal Bovine Serum (Cat. #: 10270106), penicillin-streptomycin (Cat. #: 15140-122), glutamine (Cat. #: 25030-024), horse serum (Cat. #: 16050-122), cell culture inserts (Cat. #: 140656), and Pierce LDH Cytotoxicity Assay kit were obtained from Thermo Fisher Scientific Inc. (Roskilde, Denmark). GLP-1 (Active) ELISA kit (Cat. #: EGLP-35K) was bought from Millipore Co. (MA, USA).

Fabrication of PSi particles

Porous silicon was made by electrochemically etching a silicon wafer in 1:1 EtOH: HF mixture with current density of 40 mA/cm² for 2400s. High current pulses were applied at the end of the etching to remove the porous film from the wafer. The films were dried for 2 h at 65 °C. The films were milled with a planetary ball mill and sieved into 10 – 25 μm size fraction.

Before the stabilization of the surface, the microparticles were immersed in the 1:1 EtOH: HF mixture for one minute, filtered out from the solution and dried for 2 h at 65°C. Thermally hydrocarbonized porous silicon particles were prepared by first placing the HF-treated particles into a quartz tube under nitrogen flow (1 l/min) for 30 min. Then, acetylene gas flown (1 l/min) into the tube first at room temperature for 15 min and then at 500 °C for 15 min. The particles were cooled down to room temperature under the nitrogen flow (28). Two similar batches of THCPSi particles were prepared for the experiments.

Loading α LA to THCPSi particles

α -Linolenic acid (α LA) was loaded in the THCPSi particles with the impregnation method (29). Briefly, α LA was dissolved in methanol with the concentration of 12 mg/ml. PSi particles were weighted into a glass tube. α LA/methanol solution was added to the tube and incubated for 1 h under mild stirring condition. The volume of α LA/methanol solution used was calculated based on targeted loading degrees of 5% and 10% (w/w), assuming all α LA would be adsorbed onto PSi surface. After incubation, remaining methanol solution was evaporated completely by nitrogen flow for 1 h. The loading degree was analyzed with thermogravimetry (TG).

Thermogravimetric analysis

Thermogravimetric measurements were carried out with a TA Q50 instrument under nitrogen environment with flow rate of 200 ml/min. The samples were placed on a platinum pan and the temperature was raised to 40 °C. The sample was kept 30 min at this temperature in order to remove adsorbed moisture. Subsequently the measurement was carried out up to 700 °C with a heating ramp of 20 °C/min.

Nitrogen sorption analysis

Prior the nitrogen sorption measurements, the samples were degassed for 2 h at 65 °C. The measurements were performed using a Micromeritics Tristar 3020 II instrument at the liquid nitrogen temperature. The surface area was calculated using the Brunauer Emmett Teller theory, pore size distribution (from desorption branch) using the Barrett Joyner Halenda theory and pore volume using a single point in the isotherm at relative pressure of 0.95.

Fourier transform infrared spectroscopy analysis (FTIR)

Fourier transform infrared spectroscopy measurements were performed with a Thermo Scientific Nicolet iS50 spectrometer using the Smart Performer ATR stage. The measurements were performed at spectral range of 500-5000 cm^{-1} with resolution of 4 cm^{-1} .

Scanning electron microscopy analysis

Scanning electron microscopy imaging was performed with Zeiss Sigma HD VP instrument using acceleration voltage of 3.72 kV. Secondary electron detector was used for imaging overall morphology of the particles with lower magnification and InLens detector for imaging porous structure of the surface of the particles with higher magnification.

In vitro release of α LA from PSi particles

1 mg of THCPSi $\pm$ α LA particles were weighted into a tube and dispersed in 1.0 ml PBS buffer (pH of 1.2, 4.5, 6.8 or 7.4). The buffer pH was selected to mimic the conditions in the gastrointestinal tracts of human and mouse (30, 31). α LA (250 $\mu\text{g}/\text{ml}$) and PBS buffer were used as control. The tubes were placed in a water bath with orbital shaking at frequency of 120 strokes/min at 37 $^{\circ}\text{C}$. At predetermined time points (0.5, 1, 2, 3, 6, 9 and 12h), the tubes were centrifuged, and supernatants were collected for analysis of α LA concentration by High Performance Liquid Chromatography (HPLC). After collecting the supernatants at the final time point, the particles were washed with 250 μl hexane for 4 times to extract the unreleased α LA from the particles. Hexane solution was collected into a 1.5 ml tube and evaporated in under vacuum conditions overnight. The samples were analyzed by HPLC (Beckman System Gold, Programmable Solvent Module 126, Programmable Detector Module 166, Beckman Coulter, CA, USA) with C18 column (Vydac 218PT, 5 μm , 300 $\text{\AA}$, 4.6 $\times$ 250 mm,

Alltech Associates Inc., Illinois, USA) with acetonitrile (ACN) and water in the ratio 87: 13 at a flow rate of 1 mL/min and α LA was detected at $\lambda=208$ nm. The samples were diluted with 87% ACN, and α LA concentration was calculated according to the standard curve, which was made by measuring α LA with known concentration at each measurement. In order to evaluate the release kinetics of α LA from the particles, the diffusion equations which is known as Korsmeyer-Peppas equation ($M_t/M_\infty=kt^n+b$, Eq. (2)) was applied, where M_t/M_∞ is the fraction of drug released in time t , k is the kinetic constant, and n is release exponent (32). The n value represents the release mechanism from polymeric films, that $n = 0.5$ means a diffusional square root of time release, $0.5 < n < 1$ indicates the non-Fickian transport, $n = 1.0$ shows zero order release.

GLP-1 secretion from STC-1 and GLUTag cell line

GLUTag cells were a gift from Dr. D. J. Drucker, University of Toronto, Canada. Before evaluating the effects of THCPsi $\pm$ α LA on GLP-1 secretion, 0 to 50 μ M α LA were tested on the STC-1 (25) and GLUTag cell lines for its ability to induce GLP-1 secretion. α LA was dissolved in ethanol and 0 to 50 μ M α LA solutions were prepared with Krebs-Ringer Bicarbonate Buffer (KREBS; 118 mM NaCl, 4.7 mM KCl, 25 mM NaHCO₃, 1.25 mM CaCl₂, 1.2 mM MgSO₄, and 1.2 mM KH₂PO₄) at pH 7.4. The final concentration of ethanol was 0.1%. In addition, 0.25% DPP-4 inhibitor (Cat #. DPP4-010, Millipore Co., MA, USA) was added for prevent degradation of GLP-1. STC-1 and GLUTag cells were seeded into the 24-well plates and incubated for 72 hours. The cells were washed twice with KREBS buffer, and incubated 1 h with KREBS buffer for acclimatization. Next, the samples were added to the cells, and incubated with STC-1 and GLUTag cells for 1 and 2 h, respectively. The supernatants were collected, and their GLP-1 concentrations were measured by ELISA kit (Millipore Co., MA, USA) according the manufacturer's instructions. The intra-assay variation was 7.4%, and inter-assay variation was 8.0%.

GLP-1 secretion was studied with pure α LA and α LA loaded THCPsi particles. THCPsi $\pm$ α LA were weighted into the cell culture inserts (Cat. #: 140656, Thermo Fisher Scientific, Roskilde, Denmark). The inserts were

used for collecting sample solution without particles, to avoid physical contact of particles and cultured cells, which may cause cell detachment (33). The inserts were inserted to the host plate, 500 μ l of KREBS buffer was added to insert wells and 800 μ l to host plate wells. KREBS buffer and 50 μ M α LA were used as controls. All the samples contained 0.1% ethanol to aid dissolving α LA into the aqueous buffer. The plates were incubated for 3 h in a water bath with orbital shaking at frequency of 120 strokes/min at 37 °C. After 3h incubation, buffer from the host plate well were collected. Collected sample solution were tested with STC-1 cell lines for 1h, and GLP-1 level was measured.

Cytotoxicity test

Lactate dehydrogenase (LDH) release from incubation of STC-1 cells with loaded THCPsi particles. Shortly, 1×10^4 STC-1 cells/well were seeded into a 96-well plate and incubated for overnight. 10 μ l of sample solution from the loaded THCPsi particles from the GLP-1 secretion study and the appropriate controls (50 μ M α LA, Empty THCPsi, and buffer) were added to the cells and incubated for 1h. The spontaneous LDH release and maximum (after lysis buffer) LDH releases were studied with distilled water and 10X Lysis buffer respectively. After 1h incubation, 50 μ l of each sample was transferred to a 96-well plate, and measured LDH level according the manufacturer's instructions.

Statistical analysis

Data are represented as means $\pm$ standard errors of means (SEM). Multi group comparisons were conducted by one-way ANOVA followed by Tukey's multiple comparisons test and two-way ANOVA followed by Dunnett's multiple comparisons test (Graph-Pad Prism 7, Graph Pad Software Inc., CA). Values of $p < 0.05$ were considered statistically significant.

Results

Particle characterization

The surface chemistry of the THCPSi particles was characterized with FTIR, the spectra showed Si-C and C-H_x vibration bands. Batches were named THCPSi-1 and THCPSi-2 (**Supplementary Fig. 1**). Existence of C-H_x vibrations proved the successful thermal carbonization that makes the particles hydrophobic and suitable to α LA loading. The porous structure was characterized by nitrogen sorption. The average pore diameters of the batches were approximately 15 nm and the specific pore volumes between 1.2 and 1.4 cm³/g, which provides good capacity for cargo loading. The pore size distributions are shown in **Fig. 1** and the results of two batches are summarized in **Table 1**, demonstrating similar properties of the two THCPSi batches.

Morphology of fabricated PSi particles was characterized with scanning electron microscopy (**Fig. 2**), demonstrating the irregular shape of fabricated PSi particles. Openings of the parallel pores can be seen on the surface of the particles in higher magnification image (**Fig. 2B**).

Loading α LA in THCPSi particles

α LA was loaded in THCPSi particles by impregnation method with two different targeted loading degrees of 5% and 10%, which were calculated by assuming complete adsorption of the α LA to particles. The actual loading degrees were measured by TG analysis. α LA was successfully loaded into THCPSi particles with 3.0 and 10.2 % loading degrees (**Table 2**).

In vitro release of α LA from THCPSi particles

The released α LA (%) from PSi particles at pH 1.2, 4.5, 6.8 and 7.4 are presented in **Supplementary Fig. 2A** and **B**. The different buffer pH did not affect the release profile of α LA from carrier particles, but the detectable

amounts of α LA decreased depending on incubation time and buffer acidity. Therefore, the incubation was set for PSi + α LA in pH 7.4 PBS buffer up to 6 hours. PSi + α LA particles with two loading degrees (3.0% and 9.2%) were used for the experiments, and 250 μ g/ml α LA and PBS buffer were used as controls. The percentage of released α LA from PSi particles are shown in **Fig. 3A**. The loaded α LA was gradually released into the buffer throughout the incubation time from both samples. From 3.0% α LA loading degree particles, a total of $47.9 \pm 3.84\%$ of α LA was released from particles at 6 h, and release mechanism followed Fickian diffusion ($n=0.299 \pm 0.058$, $n=3$). Similarly, from 9.2% loading degree sample, $30.7 \pm 2.86\%$ of α LA was released into the buffer in 6 h, following Fickian diffusion ($n=0.377 \pm 0.053$, $n=3$). The amount of unreleased α LA was $49.9 \pm 6.29\%$ and $25.7 \pm 1.53\%$ ($n=3$) in THCPSi particles with loading degree 3.0% and 9.2%, respectively (**Fig. 3B**). The detected α LA amount from the positive control (250 μ g/ml α LA) is shown in **Fig. 3C**. After 6 h incubation, the detected α LA levels from positive controls were significantly decreased compared to their 1 h values.

Effect of α LA loaded THCPSi particles on GLP-1 secretion in vitro

Before evaluating the effect of α LA loaded THCPSi particles on GLP-1 secretion from enteroendocrine cell lines, 0-50 μ M of α LA was tested with STC-1 and GLUTag cell line, to confirm its effect on GLP-1 secretion and select the best cell line for further experiments. 25 μ M of α LA significantly increased GLP-1 secretion from both cell lines, however the secreted amount of GLP-1 from STC-1 cells was 4 times higher than that from GLUTag cell line (**Fig. 4A and 4B**). Since expression of LCFA receptors are crucial for the physiological response of these enteroendocrine cells, we investigated the expression level of GPR120 in both cell lines, and found that STC-1 cells expressed higher GPR120 protein levels than GLUTag cells (data not shown). Therefore, we selected STC-1 cell line for our further investigation on the effect of α LA loaded THCPSi particles. The results demonstrated that THCPSi + α LA increased GLP-1 secretion from STC-1 cell line significantly, approximately 1.5 times, when compared with the buffer (**Fig. 4C**). The empty THCPSi did not stimulate GLP-1 secretion, whereas 50 μ M α LA significantly increased GLP-1 secretion to similar level than THCPSi + α LA

compared to buffer group. In addition, the GLP-1 secretion levels were dose dependently increased by THCPsi + α LA amounts (**Fig. 4D**). There was not significant difference of in LDH release among buffer and particles loaded incubations from the enteroendocrine cells (**Supplementary Fig. 3**).

Discussion

In the present study we demonstrate the successful loading of α LA into THCPsi particles with good loading degrees (**Table 2**). The loaded α LA was gradually released from the carrier particles during the incubation time (**Fig. 3A**), protecting α LA from degradation. The release of α LA was controlled by diffusion, which is in line with our previous results (17). Interestingly, detectable amount of α LA by HPLC decreased along with increasing incubation time and acidity of the buffer (**Fig. 3C** and **Supplementary Fig. 2B**). The decreasing trend was most likely due to the oxidation of α LA that is accelerated at low pH (34). The time period that α LA stays in the stomach after ingestion depends on meal constancy, which in case of liquids would be app. 30 min. Within 2-4 hours the meal will reach the distal small bowel. The pH in the stomach varies from pH 1-2.5 in the fasted phase to pH 5 after a meal (30, 35). Therefore, the chosen pH and incubation times are well within the physiological range of the gastrointestinal tract (36, 37). Pure α LA significantly increased GLP-1 secretion in both cell lines, however, STC-1 cells were more responsive to α LA than GLUTag cells (**Fig. 4A** and **4B**). α LA has a higher affinity with GPR120 (19, 38), which were more expressed in the STC-1 than GLUTag cells (data not shown). Hence, we selected STC-1 cell lines for the further experiment. PSi $\pm$ α LA significantly increased GLP-1 secretion from STC-1 cells, whereas empty PSi particles did not increase GLP-1 secretion (**Fig. 4C**). The secreted amount of GLP-1 by pure α LA differs between **Fig. 4A** and **C**, because of the oxidation of α LA during incubation 3h-pre-incubation in **Fig. 3C**.

Several studies have been utilizing α LA for treatment of overweight or obesity (39). However poor stability, shown also in our results (**Fig. 3C**), bitter taste, uncomfortable smell and early reabsorption in the proximal gastrointestinal tract have prevented utilization of α LA in pharmaceutical formulation. The location of

enteroendocrine cells in the distal part of gastrointestinal tract has been an essential problem for targeting those by drugs or nutritional compounds. Gut targeted delivery systems have been focusing more on improving the colonic drug administration to treat colonic bowel disease or enhancing the absorption of certain drugs from the colon. Most studies have reported only the use of nutritional compounds without any specific formulation, despite existing technologies for colonic drug delivery (40, 41). Our inorganic particles could solve these practical problems as novel delivery system using α LA as a model compound. Our particles could travel through the gastrointestinal tract to colon, which is the optimal site for stimulating peptide release from the enteroendocrine cells in humans (13). L-cells are existing through the gastrointestinal tract from duodenum to colon, however, they are more localized in the distal colon in humans and mice, while in rats they are more highly distributed in the ileum (42, 43). The loading process of α LA to PSi particle is easy and fast, and does not require the use of high temperature which would affect the chemical structure or stability of α LA. Furthermore, the PSi particles chemical surface can be further modified to optimize the release and adhesion e.g. to the mucus layer (44).

In conclusion, α LA was successfully loaded for the first time into a mesoporous carrier, thermally hydrocarbonized porous silicon. Native α LA stimulated GLP-1 secretion from both STC-1 and GLUTag cells, with the STC-1 cells more sensitive to α LA than GLUTag cells. α LA was gradually released from THCPSi and significantly increased GLP-1 secretion from STC-1 cell lines. Thus, α LA loaded PSi particles have the potential to be used as controlled delivery system for decreasing food intake via increasing GLP-1 secretion by gradually releasing loaded nutrients. The present study is a proof of concept *in vitro* study, and our results justify further *in vivo* investigations.

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Conflict of interest

The authors declare no conflict of interest.

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Figure 1

Pore size distributions of the unloaded porous silicon particles.

Particles from different batches are named as THCPSi-1 and THCPSi-2.

Figure 2

Scanning electron microscopy images.

A THCPsi microparticles and **B** the porous surface of it after fabrication

Figure 3

***In vitro* release of α LA from THCPsi**

A *In vitro* release of α LA from THCPsi particles with loading degree 3.0% and 9.2%. The graph presents released α LA (%) from THCPsi particles in PBS buffer (pH 7.4) after 0.5, 1, 2, 3, and 6 hours incubation. Each value represents the mean $\pm$ SEM for n=3. **B** The unreleased α LA% from THCPsi particles after 6 hours incubation. Each value represents the mean $\pm$ SEM for n=3. **C** 250 μ g/ml α LA incubated in PBS (pH 7.4) up to 6 hours. Each value represents the mean $\pm$ SEM for n=3. Value with * is significantly different at $P < 0.01$ for compared to 1 h value by one-way ANOVA followed by Dunnett's multiple comparisons test.

Figure 4**Effect of α LA and α LA released from THCPsi particles on GLP-1 secretion**

Effect of α LA (0-50 μ M) on GLP-1 secretion from STC-1 cells (A), and GLUTag cells (B). Each value represents the mean $\pm$ SEM for n=6. C The effect of α LA released from THCPsi particles on GLP-1 secretion from STC-1 cells. Each value represents the mean $\pm$ SEM for n=5 (Buffer, α LA 50 μ M, and α LA_THCPsi) and n=4 (Empty_THCPsi). D The dose-response effect of THCPsi + α LA on GLP-1 secretion from STC-1 cells. Each value represents the mean $\pm$ SEM for n=5. Values not sharing a common letter are significantly different at $P < 0.05$ by Tukey-Kramer multiple comparisons test.

Table 1. Porous properties of the PSi particles

Sample	A [m ² /g] ^a	V [cm ³ /g] ^b	D [nm] ^c
THCPSi -1	334±4	1.39±0.01	15.9±0.1
THCPSi -2	337±1	1.19±0.01	14.7±0.1

^a BET surface area.^b Single point pore volume.^c Pore diameter based on the average of the pore size distribution calculated from the desorption isotherm using the BJH theory.**Table 2. Loading degree of α LA measured with TG**

Loading degree (measured with TG)	Target loading degree
3.0 %	5 %
10.2 %	10 %

α LA is released from THCPSi particles by diffusion