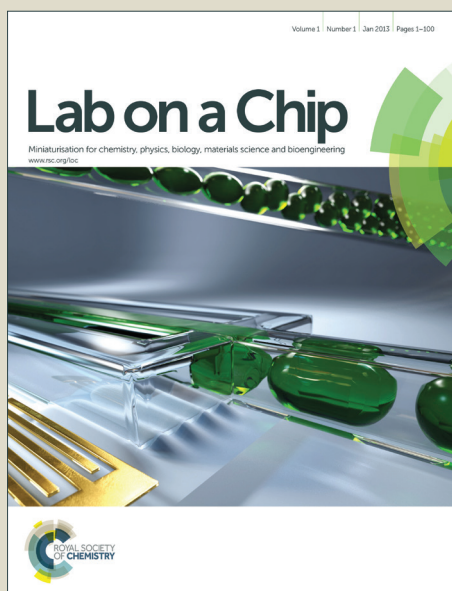


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COMMUNICATION

Microfluidic Assembly of Multistage Porous Silicon/Lipid Vesicles for Controlled Drug Release

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A reliable microfluidic platform for the generation of stable and monodisperse multistage drug delivery systems is reported. A glass-capillary flow-focusing droplet generation device was used to encapsulate thermally hydrocarbonized porous silicon (PSi) microparticles into the aqueous cores of double emulsion drops, yielding the formation of a multistage PSi/lipid vesicle. This composite system enables a large loading capacity for hydrophobic drugs.

Microfluidic technologies are becoming widely used methods for many applications ranging from material science¹⁻³ to biology.⁴ Particularly relevant for drug delivery applications is the controlled production of capsules⁵⁻⁷ or vesicles^{8,9} using microfluidic methods; these not only provide a higher encapsulation efficiency¹⁰⁻¹² than conventional production techniques, but also endow the delivery vehicles with extremely uniform sizes³ and very controlled chemical compositions. Widely used templates for capsules or vesicles are water-in-oil-in-water (W/O/W) double emulsion drops: their aqueous cores provide an ideal environment for the dissolution of hydrophilic drugs, whereas their oil shells can be loaded with hydrophobic molecules.¹³ After careful removal of the oil from the emulsion shells, double emulsion drops turn into capsules or vesicles, whose membranes protect the encapsulated material from the external environment.¹⁴

Due to their high biocompatibility, lipid vesicles are long-lasting recognized as promising drug delivery vehicles.¹⁵ If prepared using microfluidic technologies,¹⁶ they can efficiently encapsulate large amounts of drugs within their aqueous cores, particularly hydrophilic drugs.¹⁷ Unfortunately, due to the ultrathin nature of the lipid bilayer, the loading capacity of lipid vesicles for hydrophobic drugs is rather limited.¹⁴ Porous silicon (PSi) particles, which are also highly biocompatible delivery vehicles and widely studied for biomedical applications,¹⁸⁻²⁵ can overcome the limited hydrophobic loading capacity of the lipid vesicles. With typical pore sizes ranging from 2 to 50 nm, drug molecules confined within the mesopores maintain an amorphous conformation, avoiding extensive crystallization of the drugs, thereby leading to an increase in the drug

dissolution rates.^{18, 26, 21, 27, 28} However, PSi microparticles typically show a much faster release of their cargo,^{29, 30} which may cause a premature degradation of drugs in the body and a decrease in their therapeutic effect. The development of a new delivery platform that preserves the high biocompatibility of the lipid vesicles and PSi microparticles, provides a high loading capacity for both hydrophilic and hydrophobic drugs, and allows for a sustained release of cargo, is therefore highly desired.^{1, 31-35, 3}

Herein, we propose the combination of lipid vesicles with PSi microparticles as a multistage drug delivery system. Using a glass-capillary flow-focusing droplet generation device,^{10, 36} thermally hydrocarbonized PSi microparticles (THCPSiMPs)^{19, 30, 23, 37} were encapsulated within the aqueous cores of water-in-oil-in-water (W/O/W) double emulsion drops with ultrathin shells. Dewetting of the oil from the shell of the double emulsions induced the formation of a lipid bilayer.¹⁰ The presence of THCPSiMPs within the aqueous cores of these lipid vesicles not only provided the vesicles with higher loading capacity for hydrophobic drugs, as a result of the high porosity of the PSi microparticles, but also their synergistic effect endowed the vehicle of a sustained drug delivery capacity.

To fabricate lipid-stabilized double emulsion drops with ultrathin shells and simultaneously encapsulate THCPSiMPs within their cores, we used a glass-capillary microfluidic device that consisted of two tapered cylindrical capillaries of outer diameter 1 mm, carefully sanded to a tip diameter of 80 and 120 μm for injection and collection, respectively, inserted into the opposite ends of a square capillary of outer diameter 1 mm, as illustrated schematically in Figure 1a. An additional capillary inserted into the injection capillary allowed both, the inner water and middle oil phases, to co-flow within the injection capillary. The injection capillary was hydrophobic; this favored the contact of the middle oil phase with its wall. Large plug-like water drops containing the THCPSiMPs were thus formed within the injection capillary and broken-up at the tip into double emulsion drops stabilized by the thin layer of oil that was wetting the capillary wall (Figure 1a). The collection capillary

was hydrophilic; this prevented wetting of the oil shell of the emulsion on the wall of the collection capillary.

As inner water phase we used an aqueous suspension of THCPsiMPs with typical sizes of the order of 15 μm . To facilitate the dispersion of the THCPsiMPs, they were first wetted with ethanol and later suspended into an aqueous solution that contained 8 wt.% polyethylene glycol (PEG, 6 kDa) and 2 wt.% polyvinyl alcohol (PVA, 13–23 kDa) at a final concentration of 3 mg/mL. The suspension was sonicated and poured into a syringe together with a magnetic stirrer allowing for vigorous shaking of the suspension, thereby avoiding microparticle aggregation and sedimentation during the injection of the suspension within the microfluidic chip. Importantly, our microfluidic approach provides a hundred percent encapsulation efficiency as all the particles in the inner water phase ended-up in the cores of the double emulsions as shown in Figure 1b. The middle oil phase contained 4.6 mg/mL of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, Avanti) dissolved in a mixture of chloroform and hexane at a volume ratio of 1:1.8, eventually containing 0.25 mole-% of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (DHPE-Rh, Molecular Probes) to fluorescently label the lipid bilayer. The outer aqueous phase consisted of a 10 wt.% PVA (13–23 kDa) aqueous solution.

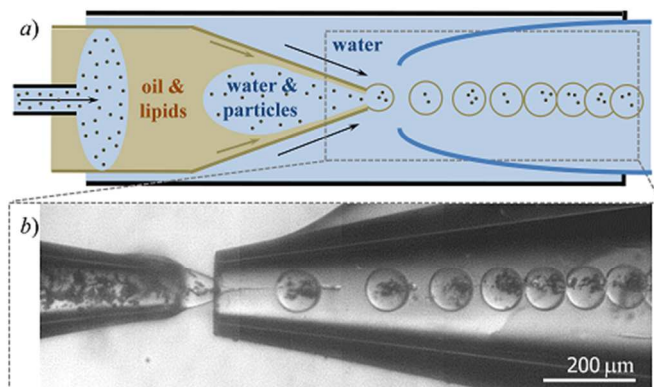


Figure 1. (a) Schematic illustration of the microfluidic device used to fabricate water-in-oil-in-water (W/O/W) double emulsion drops with ultrathin shells and P*Si* microparticles within their cores. (b) Optical microscope image of a typical production at flow rates of the inner, middle and outer phases of 700, 700, and 5000 $\mu\text{L}/\text{h}$, respectively.

The double emulsion droplets were collected in an aqueous solution of sucrose with the same osmolarity as the inner water phase (100 mOsm/L); this avoided any osmotic stress that may induce destabilization of the double emulsion droplets. Under these conditions, the chloroform in the middle oil phase easily evaporated from the shell of the double emulsions thereby increasing the ratio of hexane, which is a poor solvent for the lipids, within the oil shell of the double emulsion. This reduction in solvent quality induces an attraction between the two monolayers of lipids at the oil/water interfaces of the double emulsions, which leads to the dewetting of the hexane from the inner cores of the double emulsion droplets; this ultimately results in the formation of the lipid bilayer. The dewetting process occurs in about a couple of minutes.¹⁰ The small amount of

residual solvent present in the vesicle after dewetting remains concentrated in a very small region of the bilayer, allowing the bilayer to behave similar to vesicles produced by conventional approaches.¹⁰ Moreover, it evaporates further in the next days.

The resultant THCPsiMPs-lipid vesicle system had a mean diameter of $114 \pm 8.4 \mu\text{m}$ as shown in the optical microscope image in Figure 2a. Each delivery vehicle consisted of a lipid bilayer, which appears fluorescently labeled in the microscope image shown in Figure 2b, encapsulating the THCPsiMPs within its aqueous core. A detailed scanning electron microscope (SEM) image of a THCPsiMP such as those within the vesicles is shown in Figure 2c. The residual oil after the dewetting process is also visible by optical microscopy as pointed by the white region in Figure 2b.

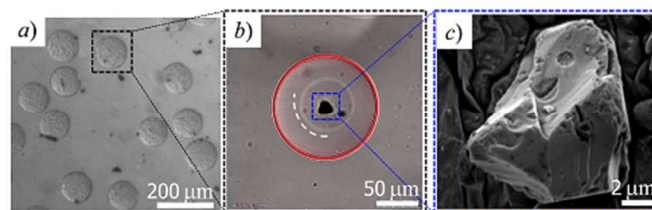


Figure 2. (a) Optical microscope image showing a suspension of THCPsiMPs-lipid vesicles in an aqueous solution of sucrose. Note that since the lipid vesicles are in aqueous suspension there is more than one focal level, and thus, only few droplets are focused at a time in the picture. (b) Detailed optical microscope image of one of the vesicles. A confocal fluorescence microscope image is overlaid to highlight the lipid bilayer (in red). An oil pocket (white region) is still visible in the micrograph. The vesicle stably encapsulates the THCPsiMPs as pointed by the blue region. (c) Detailed SEM of a typical THCPsiMP such as those encapsulated within the lipid vesicles.

To test the potential of THCPsiMPs-lipid vesicles for drug delivery applications, the THCPsiMPs were loaded with a model drug, piroxicam. This drug belongs to the Biopharmaceutical Classification System class II, i.e. it is highly hydrophobic, poorly water-soluble and highly permeable across biological membranes. To load piroxicam, the THCPsiMPs were stirred in an acetone solution of piroxicam at 15 mg/mL for 2 h. Then, the suspension was centrifuged at $11,300 \times g$ for 4 min and the pellet was washed 3 times with 0.5 mL of MilliQ-water; water allows to remove the excess of piroxicam from the surface of the particles, while avoiding a premature release of the drugs from the pores. Finally, the loaded microparticles were suspended into the inner water phase and the production proceeded as described previously.

The drug release tests were performed by introducing the resultant THCPsiMPs-lipid vesicles in a dialysis bag, immersed into a buffer solution. We estimate that the number of THCPsiMPs-Lipid vesicles loaded in each dialysis bag is approximately 90,000, and each droplet containing an average mass of THCPsiMPs of 0.0023 μg . The solution was placed in an orbital incubator shaker at 37°C and 45 rpm. Ten milliliters of two saline phosphate buffer solutions with different pH-values of 7.4 and 6, were used as release medium. Samples aliquots of 200 μL were taken at different time points, and

the same volume of pre-warmed fresh release medium was immediately replaced to keep constant the dissolution volume. The concentration of piroxicam in the samples was analyzed by spectrophotometry at a maximum absorbance wavelength of 333 nm (NanoDrop, Thermo Scientific) and the results are shown in Figure 3. The drug (piroxicam) loading degree in the system was 19%. THCPsiMPs-lipid vesicles showed sustained release compared to the bare THCPsiMPs. Moreover, while at pH 6 the whole payload was released after 3 h, at pH 7.4 the drug release was completed only after 6 h. These differences could be due to the heterogeneous stability behavior of the lipid bilayer towards different pH values. Although no morphology changes were observed on the vesicles over time (ESI 1.†), breakage of vesicles was significant after approximately 4 h; this explains the faster release observed after 200 min in Figure 3.

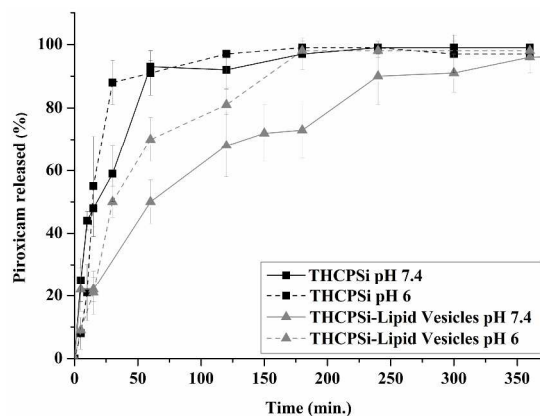


Figure 3. Piroxicam release profile from THCPsiMPs, in black, and from THCPsiMPs-lipid vesicles, in grey, in saline phosphate buffer at pH-values of 7.4 and 6 37°C. Each experiment was performed in triplicate and the vertical lines represent the standard deviation of each value ($n = 3$).

In order to evaluate the cell viability of the particles, mucus-secreting intestinal HT-29 (5.0×10^5 cells/well)^{29,30} cells were selected and the cells cultured as described in ESI 2.†. Briefly, a suspension of cells in Dulbecco's modified Eagle medium was seeded into 96-well plates (100 μ L/well, PerkinElmer Inc.) and allowed to attach overnight. The THCPsiMPs and THCPsiMPs-lipid vesicles suspensions at concentrations of 0.05–0.5 mg/mL were added into the wells (100 μ L/well). After 3 h incubation, the viability was assayed with CellTiter-Glo (Promega Corporation) using a Varioskan Flash fluorometer (Thermo Fisher Scientific).^{18–20, 24–26, 28–30}

As a result of the micrometric size of these microvehicles, they are envisaged to be applied for oral drug delivery, and thus, we evaluated their cytocompatibility with gastrointestinal tract-related HT-29 cancer cell lines. In general, and as expected,^{29,30} we observed a decrease in the cell viability as the concentration of the particles increased. There were no statistically significant differences ($p < 0.05$) on cell viability between the bare THCPsiMPs and THCPsiMPs-lipid vesicles, which

demonstrates the cytocompatibility of both systems in mucus-secreting intestinal HT-29 cells.

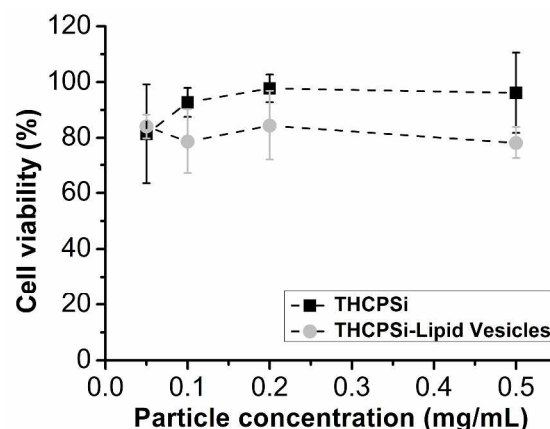


Figure 4. Cell viability of intestinal HT-29 cancer cells after 3 h incubation with different concentrations (mg/mL) of the bare THCPsi and THCPsi-Lipid vesicle microparticles assessed by a luminescence-based assay. All experiments were conducted at 37 °C. Errors bars represent the mean \pm SD ($n = 4$). After removing the medium and washing the wells twice with fresh $1 \times$ Hanks Balanced Salt Solution pH 7.4 (HBSS), the samples were added to the wells and incubated for 3 h. For positive and negative controls, HBSS (pH 7.4; 100% cell viability) and triton X-100 (<1% cell viability) were used. Statistical analysis was made by an one-way analysis of variance (ANOVA), followed by a Dunnett's multiple comparison test was used to analyze the data using GraphPad Prism v. 5.01 (GraphPad Software). The level of significance was set at probability of $*p < 0.05$.

Conclusions

In conclusion, we have successfully produced a multistage drug delivery system comprised by THCPsiMPs, with high drug loading capacity, embedded within the aqueous core of lipid vesicles. The microfluidic technology made possible the production of THCPsiMPs-lipid vesicles in a highly reliable, reproducible, and efficient manner. The delivery system was characterized by optical microscopy and the drug release profiles were obtained at different pH-values. The results showed that the encapsulation of the THCPsiMPs within lipid vesicles with a mean diameter of 114 μ m is highly efficient and stable. The drug loading degree of the system was 19%, and the encapsulated drug showed a sustained release from the THCPsiMPs-lipid vesicles compared to the bare THCPsiMPs, particularly at pH 7.4, where the whole payload was released only after 6 h. Due to its potential biocompatibility, production reliability, large loading capacity for hydrophobic drugs, and sustained drug delivery capacity, we propose the THCPsiMPs-lipid vesicle composite as a promising drug delivery system for multiple biomedical applications.

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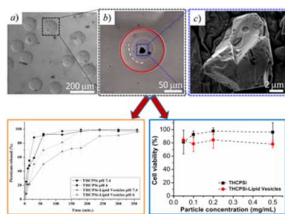
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†Electronic supplementary information (ESI) available: ESI 1: morphological changes over time of the THCPsiMPs-Lipid vesicles during release experiments. ESI 2: cell viability experiments. See DOI

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Thermally hydrocarbonized porous silicon microparticles are encapsulated into the aqueous cores of double emulsion lipid vesicle drops to form advanced multistage drug delivery systems.