SUPPORTING INFORMATION

Spheroid-based skin-on-a-chip platform for the evaluation of the toxicity of small molecules and nanoparticles

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1. Size distribution of multilayer skin spheroids (MSSs)

To validate the ability to generate multilayer skin spheroids (MSSs) with uniform dimensions, we performed MSS analysis of their brightfield microscopy images. As shown in **Fig. S1A** and the corresponding histogram in **Fig. S1B**, the MSSs had high size uniformity across the quadruplet. The average diameter of the MSSs was 182 ± 7 μm.

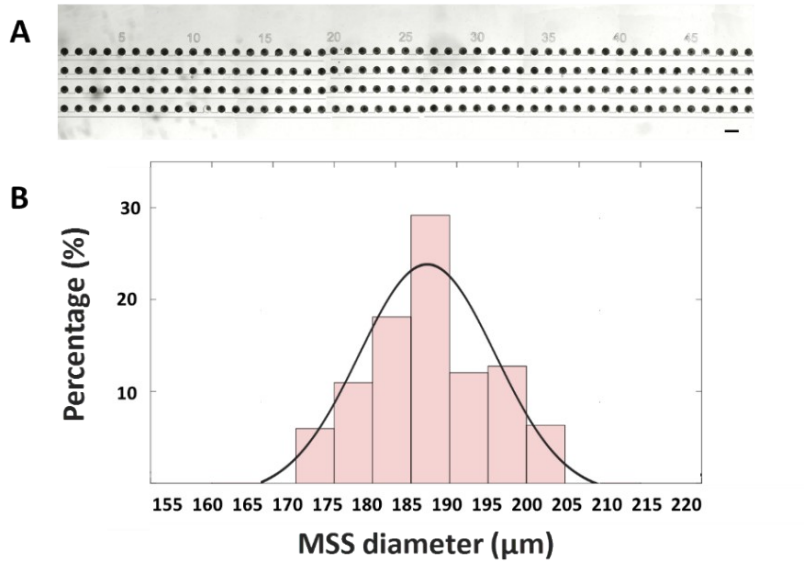

Fig. S1 Size distribution of MSSs. (A) Brightfield image of 200 MSSs in a quadruplet, formed on day 2 in the MF device. **(B)** Distribution of MSS diameters. Measurements were performed for n = 280 MSSs obtained from three independently prepared arrays, with a minimum of 80 MSSs analyzed per array. The scale bar is 500 µm.

2. Barrier function of MSSs

To assess the barrier function of the MSSs, we examined the penetration of a fluorescent dye, 5 µM CellTracker Red CMTPX (molecular weight 687 Da), into spheroids with and without an epidermal shell. As shown in **Fig. S2**, fibroblast-only spheroids were fully stained after approximately 40 min. In contrast, the penetration of the dye into the MSSs with epidermal shells was significantly slower, and the spheroids remained incompletely stained even after 60 min perfusion of the dye solution. The delayed dye penetration suggested the formation of a functional barrier in MSSs with an epidermal shell.

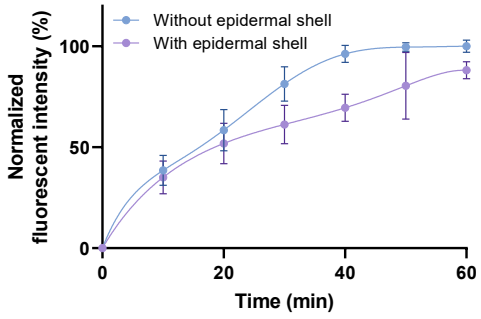

Fig. S2 Characterization of skin-like barrier properties of skin spheroids. Dye penetration into skin spheroids with or without an epidermal shell of keratinocytes. The data are shown as means ± SD for n = 10 spheroids. Scale bars, 200 μm.

3. Synthesis of carbon dots

To synthesize blue-emissive carbon dots (C-dots), citric acid (3 mmol) and urea (10 mmol) were dissolved in 10 mL of *N,N*-dimethylformamide (DMF) under vigorous stirring for 20–30 min[1,2]. The reaction mixture was transferred to a polytetrafluoroethylene (PTFE)-lined stainless-steel autoclave and heated at 140 °C for 4 h in a furnace. After cooling for 1 h under r.t., the reaction mixture was centrifuged at 8,000 *g* for 10 min in a 50 mL tube. The supernatant was collected and purified using a 1:1 mixture of petroleum ether (20 mL) and ethyl acetate (20 mL). The purified C-dots were separated using a phase separation funnel and dried under vacuum overnight at r.t.. The C-dots were synthesized with a yield of 68 % (0.8 g) and stored in a sealed vial at -19 °C prior to experiments conducted in the microfluidic (MF) device[1,2].

4. Uptake and retention of nanoparticles in MSSs

After the formation of MSSs on day 2, the dispersion of C-dots or fluorescent liposomes was perfused through the MF device at an average flow rate of 0.06 mL h^{-1}. Fluorescence microscopy images of the MSSs in the microwells were taken 2, 15, 30, 45, and 60 min after perfusing the dispersion of C-dots or fluorescent liposomes. The images were taken using Nikon Ti Eclipse fluorescent microscope (Nikon Instruments Inc., U.S.A.) using the excitation wavelengths of 570 nm and 488 nm, respectively. In a separate experiment, the fluorescence images of the microwells were taken after the dispersion of C-dots or fluorescent liposomes was perfused through the MF device not compartmentalizing the MSSs. The intensity of photoluminescence, I_{PL}, of the nanoparticles in the MSSs was measured by analyzing fluorescence microscopy images of the MSSs in the microwells and normalizing it by the $I_{PL,60}$ of the nanoparticle dispersion in the MSS-free microwells of the MF device. The ratio I_{PL}-$I_{PL,0}$/$I_{PL,\ 60,\ microwell}$ was used to characterize the uptake of the nanoparticles by the MSSs, where I_{PL} is intensity of photoluminescence of the MSSs exposed to the nanoparticle dispersion at a particular time, $I_{PL,0}$ is the photoluminescence intensity

of the MSSs before perfusing nanoparticle dispersion, and $I_{PL,\ 60,\ microwell}$ is the photoluminescence intensity of the dispersion of nanoparticles perfused in the spheroid-free microwells after 60 min. Nanoparticle retention in the MSSs was determined following the permeation of the MSSs by the dispersion of C-dots or fluorescent liposomes. Cell culture medium was perfused through the MF device at an average flow rate of 0.06 mL h^{-1}, and fluorescence images of the MSSs were taken at 2, 15, 30, 45, and 60 min after the beginning of perfusion of cell culture medium. Image analysis and normalization of the photoluminescence intensity were similar to those used for nanoparticle uptake characterization, that is, the ratio I_{PL}-$I_{PL,0}$/$I_{PL,\ 60,\ microwell}$ was utilized to determine nanoparticle retention in the MSSs.

Exemplary images of the MSSs permeated with 2 wt.% dispersion of C-dots after 2- and 60-min perfusion of C-dots, followed by 60-min perfusion of the cell culture medium are illustrated in **Fig. S3**.

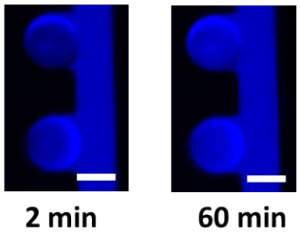

Fig. S3 Uptake of C-dots by MSSs. Fluorescence microscopy images of the MSSs after 2- and 60-min exposure to 2 wt.% C-dot dispersion. Scale bars are 200 μm.

5. Liposome preparation

A solution containing cholesterol (40 mM) and phosphatidylcholine (400 mM) in absolute ethanol, with a 1:10 mass ratio of cholesterol to phosphatidylcholine, was supplied into the central channel of the MF device (**Fig. S4**) at a flow rate of 0.181 mL min^{-1} (1:5 flow rate ratio of lipid solution to buffer)[3]. This solution was hydrodynamically focused by two side streams of Dulbecco's Phosphate-Buffered Saline (DPBS) solution, which were introduced into the side microchannels at a flow rate of 0.905 mL min^{-1}. Subsequently, the dispersion of liposomes was filtered using Amicon® ultra centrifugal filters with a 30 kDa MWCO (Millipore Sigma, product number: 803024). The dispersion was stored at 4 °C. For fluorescent liposomes, 10 mol. % of the cholesterol in the lipid solution was substituted with cholesterol labeled with the boron

dipyrromethene (TopFluor®). To encapsulate metaflumizone (BASF, product number: 7969-231) in the liposomes, metaflumizone at mass ratio of 1:20 to the lipids was dissolved in absolute ethanol, which was followed by the liposome self-assembly procedure.

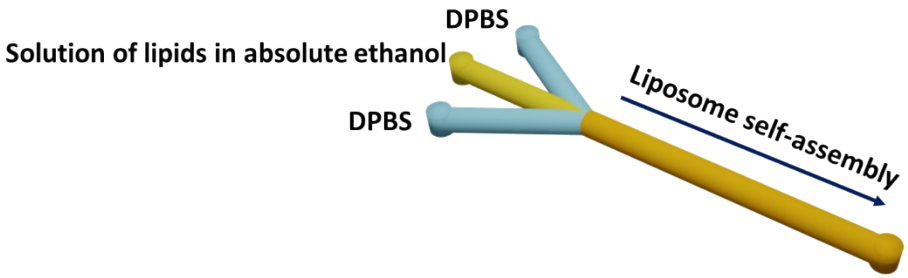

Fig. S4 Schematic of the generation of liposomes in the MF device.

6. Determination of encapsulation efficiency of metaflumizone

Metaflumizone solutions with different concentrations were prepared in absolute ethanol. The dependence of extinction intensity on the metaflumizone concentration was plotted as a calibration graph (**Fig. S5**).

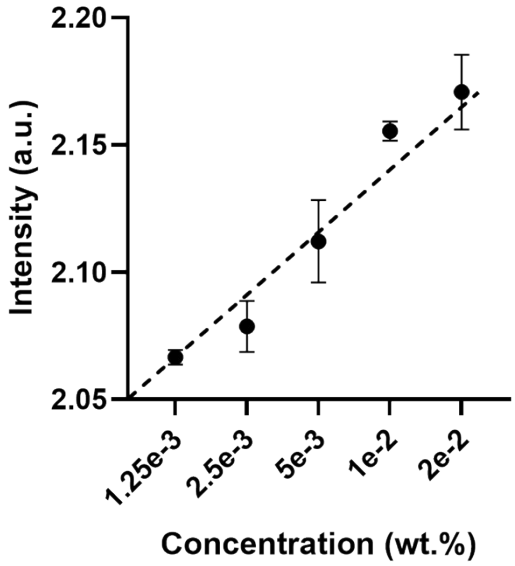

Fig. S5 Variation in extinction intensity of metaflumizone solution in absolute ethanol, plotted as a function of metaflumizone concentration. The data are shown as mean ± SD from n = 3 independently prepared solutions of metaflumizone in absolute ethanol.

In a separate experiment, metaflumizone was encapsulated in the liposomes using a MF procedure illustrated in **Fig. S4.** The lipids and metaflumizone were co-dissolved in ethanol. The concentrations of cholesterol and phosphatidylcholine were 40 and 400 mM, respectively, with the total mass of the lipids of 0.64 g. The concentration of the metaflumizone, c_{sol}, was 1.25×10^{-3}, 2.5×10^{-3}, 5×10^{-3}, 1×10^{-2}, or 2×10^{-2} wt.%. Following the encapsulation, the supernatant was separated by filtering the liposome dispersion using Amicon® ultra centrifugal filters (MWCO: 30 kDa). The extinction spectra were acquired to determine the concentration of metaflumizone, c_s, in the supernatant using a calibration graph. The encapsulation efficiency of metaflumizone in the lipids was determined as

$$\text{Encapsulation efficiency} = \frac{C_{sol} - C_s}{C_{sol}} \times 100\%$$

The average encapsulation efficiency was 70 ± 2%, corresponding to the mass ratio, R, of the encapsulated metaflumizone/lipid of 0.035, calculated by dividing the mass of encapsulated metaflumizone by the total mass of lipids in the dispersion.

7. Screening of MSSs for 60-min perfusion of metaflumizone-loaded liposomes

Following a 60-min exposure of the MSSs to dispersions with different concentrations of metaflumizone-loaded liposomes and a 60-min perfusion of the cell culture medium for the MSS recovery, live/dead staining was performed for the cells in the MSSs. **Fig. S6** shows the concentration-response curve of the MSSs after a 60-min perfusion of different concentrations of metaflumizone-loaded liposomes (0.08, 0.8, 4, and 8 wt.%). Corresponding cell viability was 101 ± 1, 99 ± 2, 98 ± 3, and 97 ± 2%, respectively. Given that cell viability exceeded 50% for all examined concentrations of liposome-encapsulated metaflumizone, determining the IC50 value was not applicable. We attribute high cell viability to the limited release of metaflumizone from the liposomes and uptake by the cells in MSSs within 60 min.

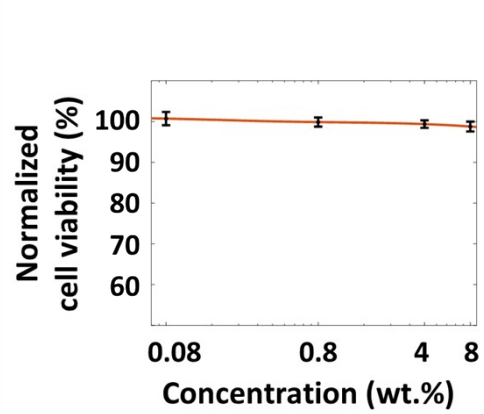

Fig. S6 Concentration-response curve of MSSs exposed to the liposome dispersion carrying metaflumizone at varying concentrations after 60-min perfusion and 60-min of recovery. The data points are shown as means ± SD. Each data point represents measurements from a total of at least 470 MSSs, collected from three independently prepared arrays.

8. Uptake of liposomes by MSSs following extended perfusion of the liposome dispersion

To examine liposome uptake by the MSSs, perfusion of 8 wt.% dispersion of metaflumizone-free fluorescent liposomes was extended from 60 min to 24 h. **Fig. S7A** shows representative fluorescence images of the MSSs after 2-min, 60-min, and 24-h perfusion of the liposome dispersion. After 24-h perfusion, the MSSs were fully permeated by the fluorescent liposomes. The normalized I_{pl} of the MSSs noticeably increased, compared to that after 2- and 60-min perfusion.

To further validate the liposome uptake by the MSSs, confocal microscopy experiments were performed using Leica SP8 STELLARIS confocal microscope for the MSSs subjected to 60-min or 24-h perfusion of 8 wt.% dispersion of metaflumizone-free fluorescent liposomes, followed by the recovery step. Images were taken 48 μm above the middle plane of the MSSs. As shown in **Fig. S7B**, **left**, after 60-min perfusion, the liposomes permeated only a fraction of the MSS that was close to their periphery, however, after 24-h perfusion, the distribution of the liposomes within the MSS was significantly more uniform (**Fig. S7B, right**).

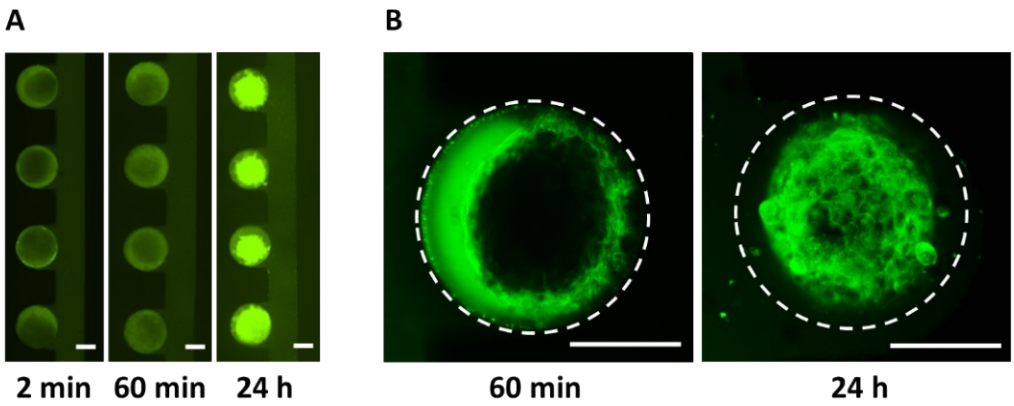

Fig. S7 Uptake of liposomes by the MSSs. (A) Widefield fluorescence images of MSSs after 2-min, 60-min, and 24-h perfusion of 8 wt.% metaflumizone-free fluorescent liposome dispersion. **(B)** Confocal fluorescence images of MSS after 60-min and 24-h perfusion of 8 wt.% metaflumizone-free fluorescent liposome dispersion. The white dashed line shows the contour of the microwells. Scale bars in A and B are 100 μm.

9. Calculation of metaflumizone concentration in the liposome dispersion

In the toxicity tests, a stock dispersion of metaflumizone-loaded liposomes at the concentration of 8.0 wt.% was used. The dispersions with concentrations of metaflumizone-loaded liposomes of 4.0, 0.8, and 0.08 wt.% were prepared by diluting the 8 wt.% dispersion with DPBS. The concentrations of metaflumizone in 8.0, 4.0, 0.8, and 0.08 wt.% of metaflumizone-loaded liposomes for the mass ratio, R, were 2.8×10^{-1}, 1.4×10^{-1}, 2.8×10^{-2}, and 2.8×10^{-3} wt.%, respectively.

10. Cell viability of MSSs after perfusion of Dulbecco's phosphate-buffered saline and dispersion of metaflumizone-free liposomes

To normalize cell viability after 24-h perfusion of metaflumizone-free and metaflumizone-loaded liposomes, Dulbecco's phosphate-buffered saline (DPBS) was perfused through the channels of the MF device for 1 or 24 h, followed by the perfusion of cell culture medium for recovery. The results of live/dead staining showed that after 1 and 24 h of DPBS perfusion, cell viability in the MSSs was 87 ± 3 and 79 ± 3%, respectively (**Fig. S8A**). The reduction in cell viability in the MSSs

after 24-h perfusion, compared to 1-h perfusion of DPBS, presumably occurred due to insufficient supply of nutrients by the buffer.

To examine the effect of the liposomes on cell viability, an 8 wt.% dispersion of metaflumizone-free liposomes was perfused through the MF device for 1 or 24 h, followed by perfusion of the media for 1 h. Cell viability in MSSs was assessed by live/dead staining. The results were normalized by the cell viability after 24 h-perfusion of DPBS and 1-h perfusion of Trition-X 100.

Fig. S8B shows that the MSSs maintained normalized cell viability of 96 ± 4 and 85 ± 5% after 1- and 24-h perfusion of metaflumizone-free liposomes, respectively. Comparison of these results with the IC50 curve for 24-h perfusion of metaflumizone-loaded liposomes indicates that the liposome carriers only weakly reduced the viability of cells in the MSSs. Thus, the reduction of cell viability was dominated by the release of metaflumizone from the liposomes when the MSSs were subjected to the dispersion of metaflumizone-loaded liposomes.

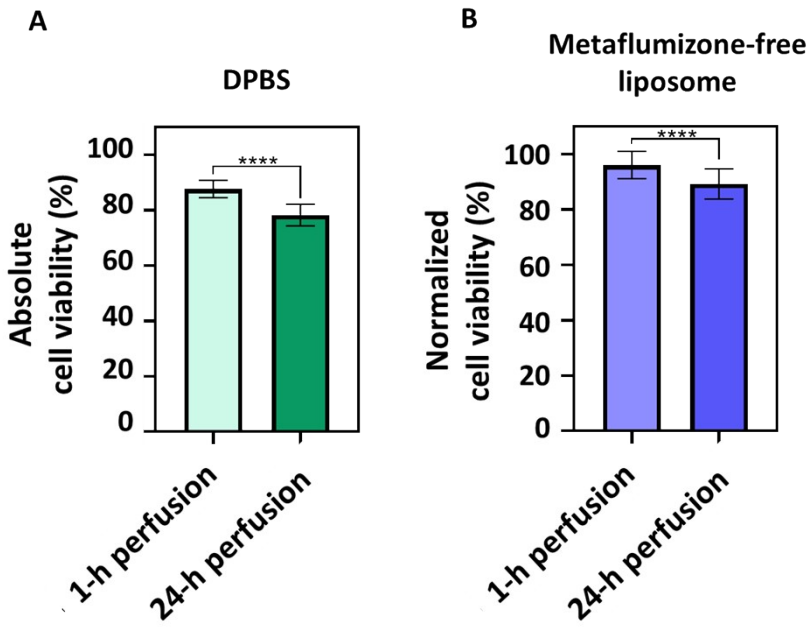

Fig. S8 Cell viability in MSSs after exposure to DPBS and the dispersion of metaflumizone-free liposomes. (A) Viability of cells in MSSs after 1- and 24-h perfusion of DPBS**. (B)** Cell viability in MSSs after exposure to the dispersion of metaflumizone-free liposomes for 1 h and 24 h**.** The data points are shown as means ± SD. Each data point represents measurements from a total

of at least 560 MSSs, collected from three independently prepared arrays; ****: $p < 0.0001$, using paired t-test.

References

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