Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Evaluation of aconitine cardiotoxicity with a heart-on-a-particle prepared by a microfluidic device

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Experimental procedures

Materials

An SU-82050 negative photoresist and developer were purchased from MicroChem Corporation (Newton, Massachusetts, USA). PDMS prepolymers and initiators were brought from Dow Corning (Midland, Michigan, USA). 1H,1H,2H,2H-Perfluorooctyl trichlorosilane, sodium alginate and doxorubicin were purchased from Sigma-Aldrich (Missouri, USA). Fluorescently labeled polystyrene nanoparticles (B200, G200, and R200) were purchased from Thermo Fisher Scientific (Waltham, MA). The magnetic nanoparticle was purchased from Macklin (Shanghai, China). HL-1 cell lines were purchased from Saibaikang Biotechnology Co., Ltd. (Shanghai, China). HUVEC cell lines were purchased from the National Infrastructure of Cell Line Resource (Beijing, China). Dulbecco's modified Eagle medium (DMEM), 0.25% trypsin with ethylene diamine tetraacetic acid, phosphate buffer saline, fetal bovine serum, dimethyl sulfoxide (DMSO), and lactic acid (HPLC≥98%, SL8750), were purchased from Beijing Solarbio Science &Technology Co., Ltd. (Beijing, China). Succinic acid (purity>98%, RFS-H05911804026), malic acid (purity>98%, RFS-P01302010028), fumaric acid (purity>98%, RFS-F01611804026), and citric acid (purity>98%, RFS-N01211812016) were purchased from Chengdu Herb Purify Co., Ltd. (Chengdu, China). Anti-cardiac troponin (cTnT), CD31monoclonal antibody, and anti-mouse IgG H&L (Alexa Fluor 555) were purchased from Thermo Fisher Scientific (Waltham, MA). Tirton-X and antibody diluent was bought from Beyotime Co., Ltd. (Beijing, China). A lactate dehydrogenase (LDH) assay kit, was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Reactive oxygen species (ROS) assay kit and Fluo-4, AM ester were purchased from UElandy Biotechnology Co., Ltd (Suzhou, China). Live/Dead assay kit and Hoechst 33342 were obtained from Gibco Corporation (New York, USA). All reagents were of analytical reagent grade and used without further purification.

A microplate reader (Varioskan Flash, Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for absorbance measurement. An inverted fluorescence microscope (Leica DMi8, Wetzlar, German) and confocal microscope (Carl Zeiss LSM 980, Carl Zeiss, German) were used for cell imaging. A liquid chromatography-mass spectrometer (LC-MS 8050, Shimadzu, Kyoto, Japan) was applied to analyze chemical components with a C_{18} column (CAPCELL PEK C_{18}; Japan).

Fabrication of a Microfluidic Device.

The microfluidic device consists of the upstream microfluidic chip and the downstream gas-liquid needle (Figure S1). The soft lithography technology was used to fabricate microfluidic chips. The SU-8 2050 negative photoresist was coated on the cleaned silicon wafer, which was spun at 500 rpm for 20 s and 1000 rpm for 1 min. Then, the silicon wafer was heated at 5 °C for 3 minutes and baked at 95 °C for 10 minutes. After using a transparent photomask to apply UV exposure to patterned silicon wafers, continue to heat the same method for 13 minutes and develop with an SU-8 developer. Next, it was silanized overnight to obtain a mold. Later, the degassed 10:1 mixture of PDMS and initiators was poured onto the mold and cured at 75 °C for 3 h. Once the curing device was prepared, the PDMS stamp was peeled off from the mold pattern, and the channel inlet and outlet were manufactured using precision stamping machines. Finally, the obtained PDMS chip was sealed by oxygen plasma and bonded with a glass sheet to obtain the microfluidic chip. The gas-liquid needle is a commercial product composed of a set of axially arranged steel needles, divided into inner needles (inner diameter: 220 μm, outer diameter: 400 μm) and outer needles (inner diameter: 1050

μm, outer diameter: 1500 μm). After connecting the upstream microfluidic chip outlet with the downstream inner needle inlet, fix the entire device with a hot melt adhesive.

Cell culture and treatment

HL-1 cells and HUVEC cells grew in DMEM with 10% FBS added, under culture conditions of 5% CO_2 and 37℃, and the medium was changed every 2 days. Aconitine was dissolved in DMSO to create a stock solution with a concentration of 50 mmol/L. This stock solution was used to prepare a range of concentrations, which were then mixed with the culture medium for subsequent experiments.

Generation of six-compartmental particles

Six-compartmentalized particles were prepared accordingly using a six-channel integrated microfluidic device. Each individual dispersed phase was prepared by mixing a 2% alginate solution with different fluorescently labeled polystyrene nanoparticles (B200, G200, or R200) in a 9:1 volume ratio. The volume flow rate of the six dispersed phases was 3500 μL/h, while the volume flow rate of the continuous phase was 1.2 L/min (Airflow can be adjusted with a gas flow meter).

We prepared the heart-on-a-particle models using the same method as above, with six dispersions: two HL-1 cell suspensions, one HUVEC cell suspension, one magnetic nanoparticle, and two softened waters, all mixed with sodium alginate solution in a 9:1 volume ratio. Among them, magnetic nanoparticles were used for localization, with HL-1 cells on both sides and HUVEC cells on the opposite side. The prepared particles were removed and washed twice with DMEM medium, after which the particles were incubated in Petri dishes containing 5 mM $CaCl_2$ in DMEM medium at 37 °C with 5% CO_2. Afterward, the same number of particles are removed and administered in groups for 24h, and the assay is performed by adding the appropriate reagents. See Fig. S9 for detailed steps.

Cell survival rate and immunofluorescence analysis in the particles

On the first day, third day, and fifth day after the generation of the heart-on-a-particle, cell apoptosis was detected using the calcein-AM/propidium iodide (PI) double staining kit. For immunofluorescence staining, 500 μL of the culture medium containing heart-on-a-particle that had been cocultured for 3 days was collected. The supernatant was discarded, and the precipitate was washed three times with normal saline containing 0.1 M calcium chloride. Subsequently, it was fixed in 500 μL of 4% polymer formaldehyde for 15 minutes. After washing three times to remove the supernatant, 200 μL of 0.5% Triton-X was added to permeabilize for 10 minutes. The supernatant was then removed after another three washes. Next, 100 μL of blocking solution was added and incubated for 1 hour. cTnT and CD31 were used as primary antibodies. They were diluted with the primary antibody diluent (1:1000), and 500 μL of this dilution was added to the sample, which was kept at 25 °C overnight. After the incubation, the supernatant was removed, and the sample was washed three times. Afterwards, 500 μL of goat anti-mouse IgG H&L (Alexa Fluor 555) diluted with the secondary antibody diluent (1:500) was applied as the secondary antibody. It was incubated at 25 °C for 2 hours. The supernatant was removed after three additional washes. Then, the sample was added with DAIP, incubated for 10 minutes, and observed using confocal microscopy.

Cytotoxicity assay

HL-1 cells (1×10^5 cells) in logarithmic growth phase were inoculated in 96-well plates for 24 h and incubated with different concentrations of aconitine (0, 0.05, 0.1, 0.2, 0.5, 0.8, 1.2, 1.5, 2.0, and 2.5 mmol/L) for 24 h. Then, at 37 °C, 10 μL of CCK-8 assay was added to each well, which was contained in 100 μL of culture medium for 2 hours. Subsequently, the optical density (OD) of each well was measured using an enzyme-linked immunosorbent assay at 450 nm.

LDH release assay

The prepared heart-on-a-particle was cultured for 24h. Subsequently, stimulated with different concentrations of aconitine for 24h, the LDH release in the particles was detected according to the manufacturer's protocol, and the OD level was measured at 450nm with a microplate reader.

Measurement of ROS and Ca^{2+} level

After stimulating the heart particles with different concentrations of aconitine for 24 hours, they were incubated with DCFH-DA diluted 1000-fold at 37 °C for 20 minutes to determine the presence of ROS. The Ca^{2+}-specific fluorescent probe Fluo-4 AM diluted to 4 μmol/L was loaded for 40 min at 37 °C in the dark. Finally, both were analyzed for fluorescence by applying the inverted fluorescence microscope after staining the nucleus with Hoechst 33342.

Detection of cell apoptosis

After aconitine treatment for 24 h, apoptosis was detected with the calcein-AM/PI double staining kit. The fluorescent probe incubated the cells in darkness at 37 °C for 20 minutes, and then the fluorescence intensity was observed under a Leica fluorescence microscope. The image was analyzed using NIH ImageJ software.

LC-MS/MS

The supernatant of the culture medium collected 24 hours after the stimulation of the heart particles with varying concentrations of aconitine, was extracted using acetonitrile. The test substance was obtained afterwards by means of centrifugation. The column temperature remained at 40 °C. In the analysis of cell metabolites, the mobile phase consisted of 0.2% (*V/V*) aqueous formic acid (solvent A) and 0.2% (*V/V*) formic acid in acetonitrile (solvent B) flowing at a rate of 0.3 mL/min. The gradient elution proceeded as follows: 5%B (0-2.49 min), 5%-50% B (2.49-2.75 min), 50% B (2.75-3.5 min), 50%-5% B (3.5-4.0 min). The sample injection volume was 1 μL. Mass spectrum parameters are as follows: spray voltage: 3.00 kV, interface temperature: 300 °C, nebulizer gas (N_2) flow rate: 3 L/min, and heating gas (N_2) and drying gas (N_2) flow rate: 10 L/min. Each component was analyzed using MRM. The negative ion mode of the electrospray ionization source was used. The MS/MS transitions of each compound analyzed are detailed in Table S1.

To create the standard curve, the standard substance stock solution of cell metabolites (10 mmol/L) was diluted to different concentrations. A range of concentrations were produced: from 50 to 5000 μmol/L for lactic acid, from 0.1 to 10 μmol/L for citric acid, from 0.5 to 50 μmol/L for succinic acid, from 0.5 to 50 μmol/L for fumaric acid, and from 0.1 to 10 μmol/L for malic acid.

Data and statistical analysis

The quantitative data were replicated at least three times and expressed as the mean ± standard deviation. The statistical analysis performed in GraphPad Prism 9.5.0 included a one-way ANOVA test and Tukey's multiple comparison test. For all statistical analyses, the significance level was defined as $P < 0.05$.

Supplementary Figures

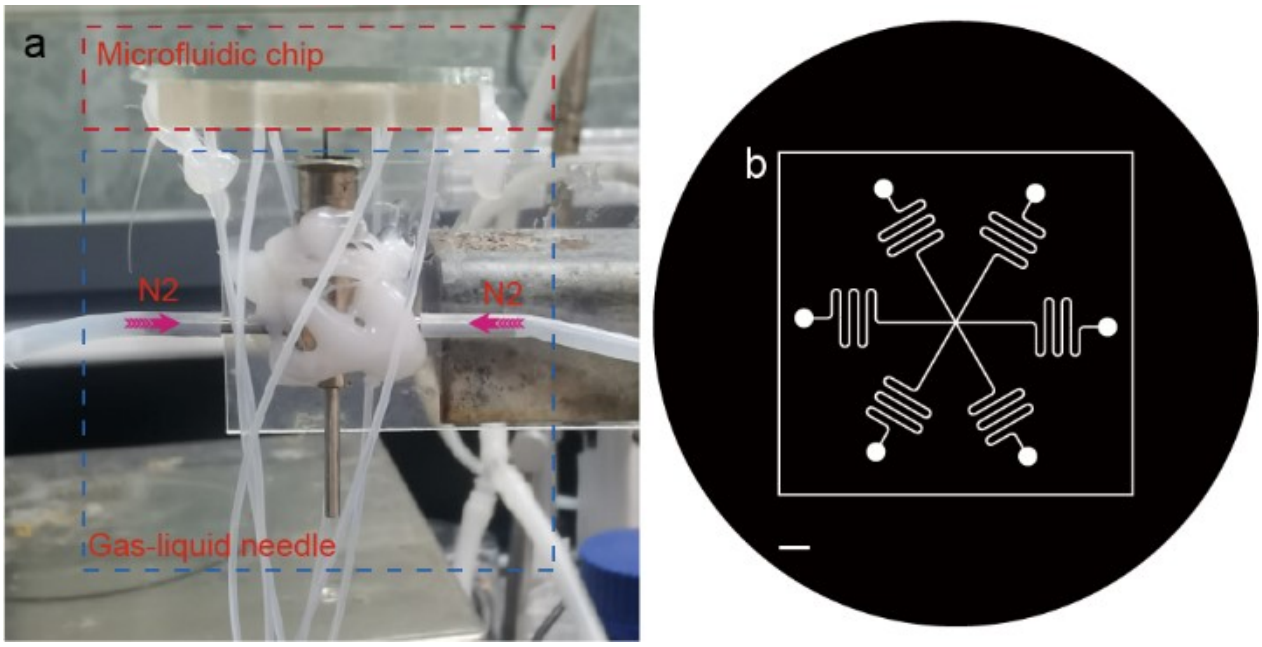

Fig. S1. Schematic illustrations of the microfluidic device. (a) Image of the microfluidic assembly system for the generation of six-compartmental microspheres. The device includes the upstream microfluidic chip component and the downstream gas-liquid needle. (b) microfluidic chip. Scale bars, 2 mm.

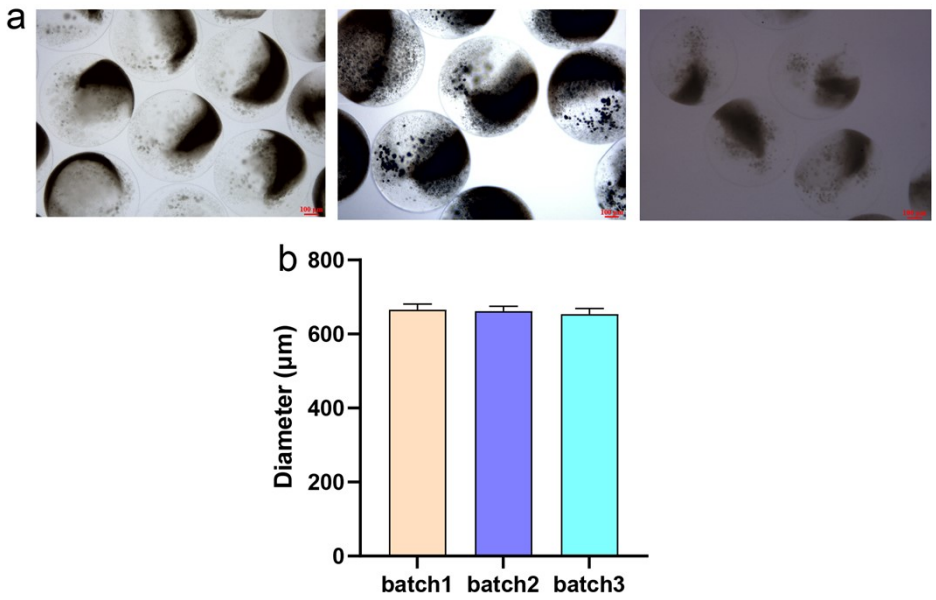

Fig. S2. Reproducibility of particles. (a) Bright field images of 3 batches of different particles; (b) Comparison of the

outer diameter size of the particles in the 3 batches showed no significant difference, n=10.

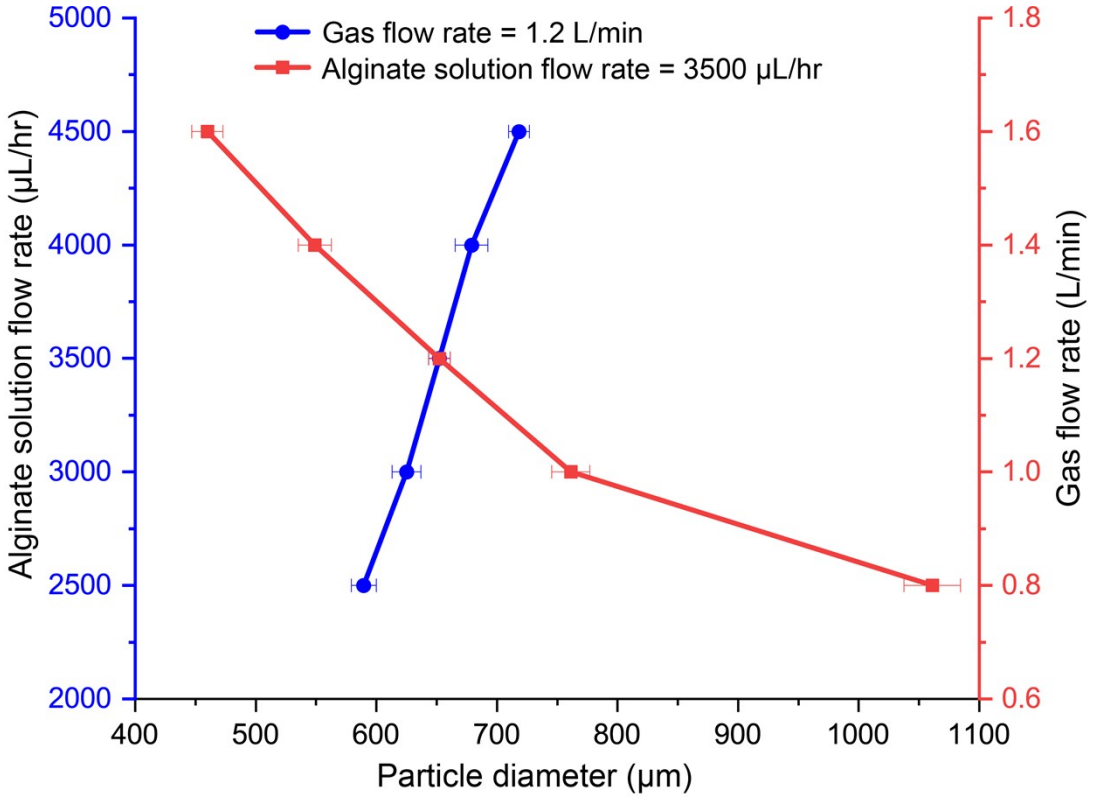

Fig. S3. Relationship between the diameter of the particles and the flow rate of alginate solution (blue color, with the gas flow rate of 1.2 L/hr) and gas (red color, with the alginate flow rate of 3500 µL/hr).

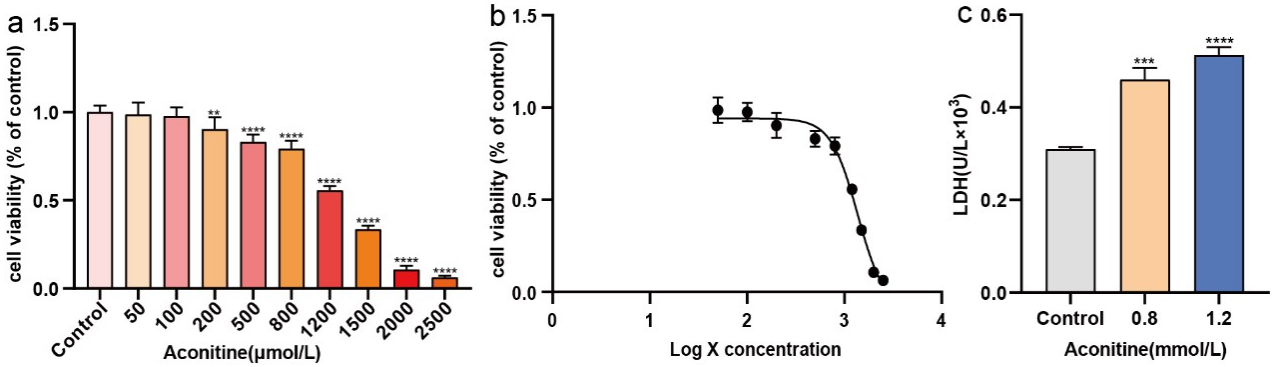

Fig. S4. Viability of HL-1 cells and LDH levels in the heart-on-a-particle stimulated with different concentrations of aconitine. Data are shown as mean ± SD, n=5 for each protocol of the experiment. (a) The mortality of HL-1 cells presented a significant difference with the control group when the concentration of aconitine was over 200 µmol/L; (b) Aconitine of 1307 µmol/L was the 50% inhibitory concentration (IC_{50}). (c) LDH levels of different concentrations of aconitine stimulated in the heart-on-a-particle. **: P<0.01, ***: P<0.005, and ****: P<0.001, comparison between groups of different aconitine concentrations and control group.

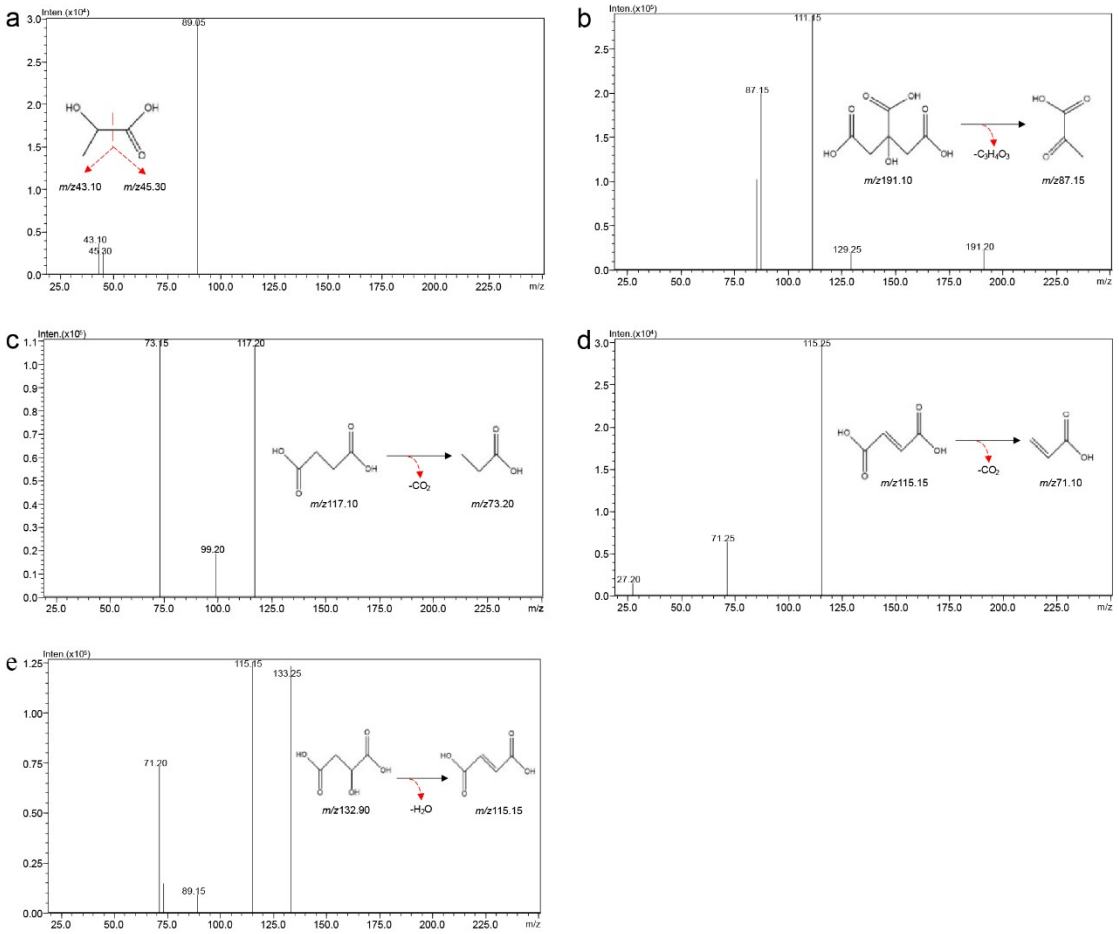

Fig. S5. The Characteristic fragment structure of five metabolites. (a) lactic acid, *m/z* 89.20/ 43.10/45.30; (b) citric acid, *m/z* 191.10/ 87.15; (c) succinic acid, *m/z* 117.10/ 73.20; (d) fumaric acid, *m/z* 115.15/ 71.10; and (e) malic acid, *m/z* 132.90/ 115.15.

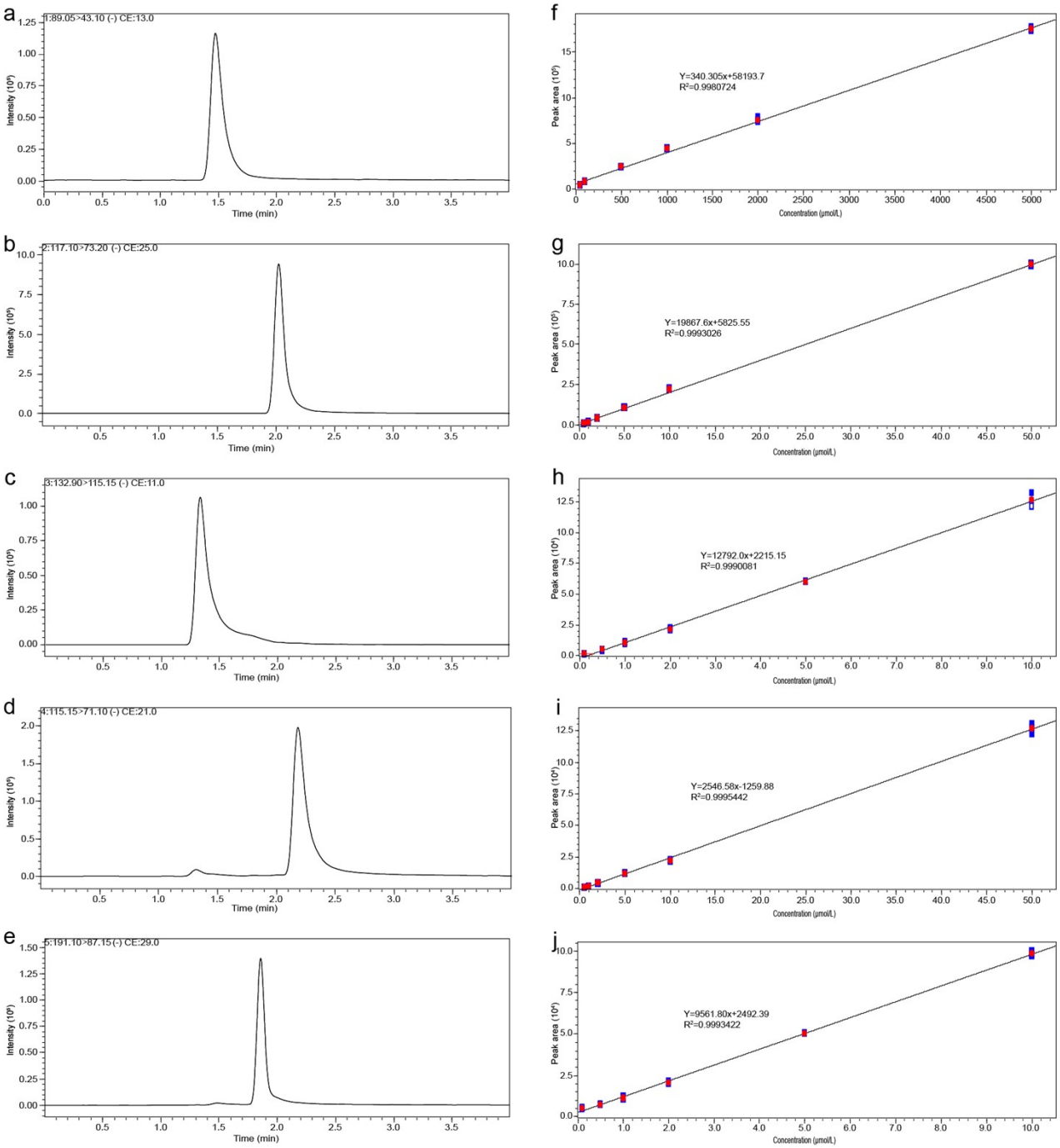

Fig. S6. The multi-reaction monitoring (MRM) mode and the standard curves of five target metabolites in standard substances. Five target metabolites screened including (a) lactic acid, (b) succinic acid, (c) malic acid, (d) fumaric acid, and (e) citric acid detected in the standard substances through MS/MS spectra under the MRM mode respectively. Each standard curve was constructed by the mean value of series concentrations, with each concentration detected 3 times. The peak area of metabolites changed linearly with the concentration increasing steadily, and the fitting formula is written as follows: (f) lactic acid, Y = 340.305 x+58193.7 (R^2=0.9981); (g) succinic acid, Y = 19867.6 x+5825.55 (R^2=0.9993); (h) malic acid, Y = 12792.0 x-2215.15 (R^2=0.9990); (i) fumaric acid, Y = 2546.58 x-1259.88 (R^2=0.9995); (j) citric acid, Y = 9561.80 x+2492.39 (R^2=0.9993).

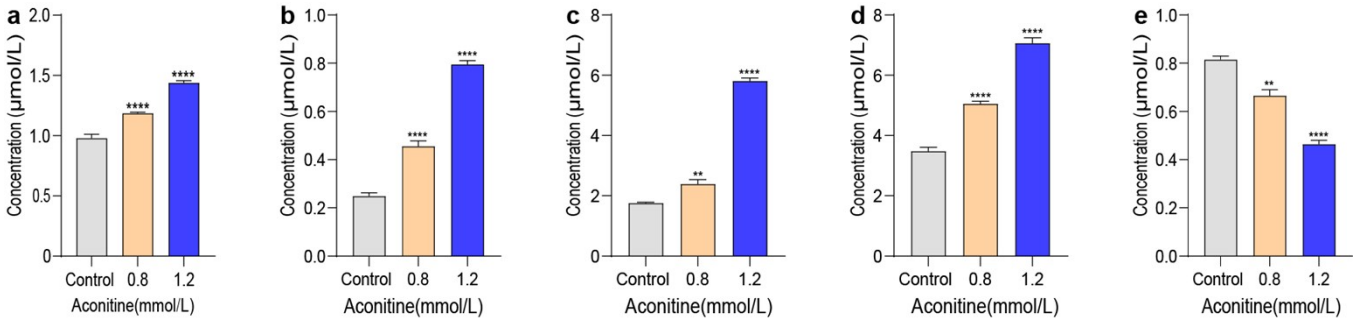

Fig. S7. Determination of five metabolites in the heart particle. Data are shown as mean ± standard deviation (n = 3) for each protocol of the experiment. The difference analysis of peak area of (a) lactic acid, (b) citric acid, (c) succinic acid, (d) fumaric acid, and (e) malic acid under stimulation of aconitine at different concentrations. **: *P*<0.01 and ****: *P*<0.0001, comparison between groups of different aconitine concentrations and control group.

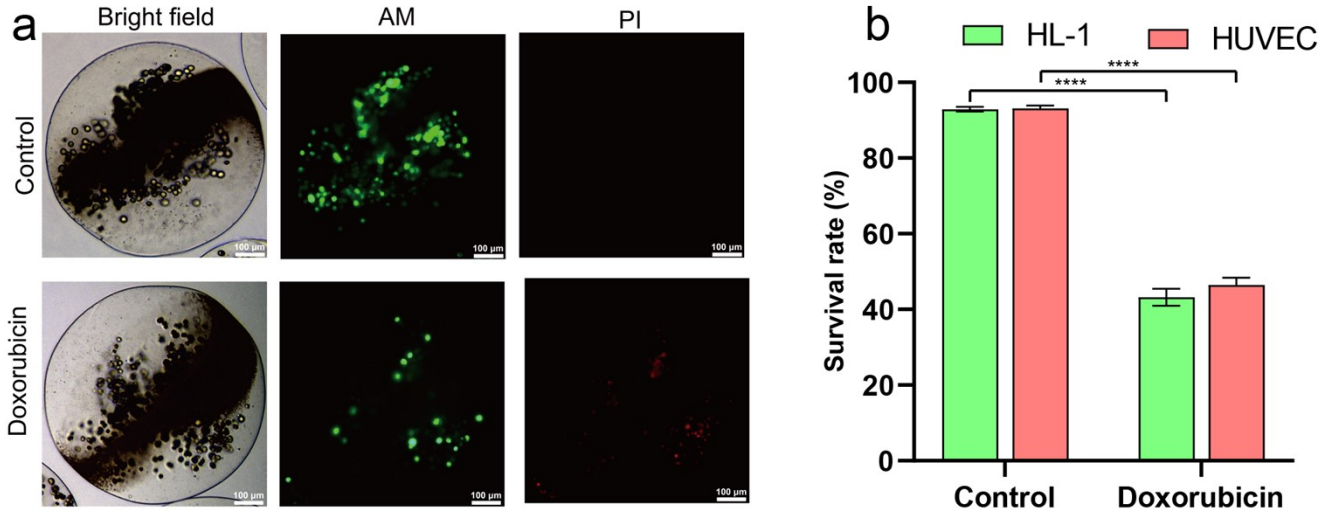

Fig. S8. Effects of doxorubicin on apoptosis of the heart-on-a-particle. Data are shown as mean ± standard deviation (n = 3) for each protocol of the experiment. Fluorescence imaging of (a) live/dead levels after being stimulated by 10 μM doxorubicin. (b) Cell survival rate stimulated by doxorubicin. ****: *P*<0.0001.

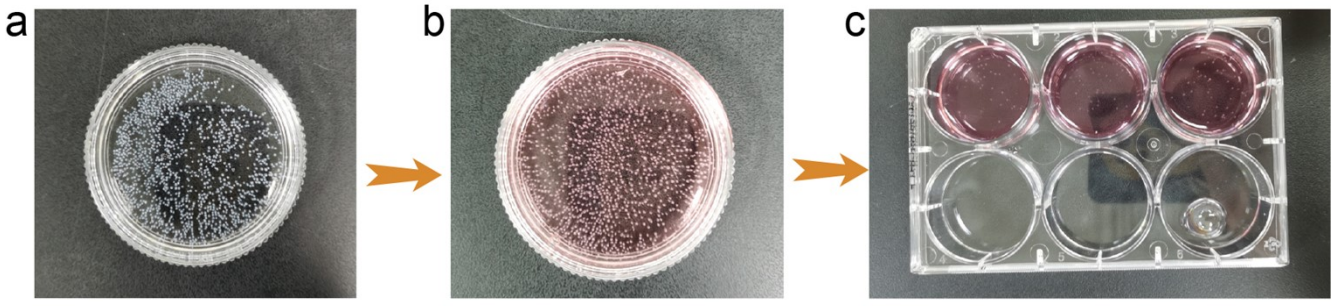

Fig. S9. Steps for working with particles. (a) heart-on-a-particle in 100 Mm $Cacl_2$ solution prepared by microfluidic devices. (b) The $Cacl_2$ solution was aspirated and the particles were washed twice with DMEM medium, then added to DMEM medium supplemented with 5 mM $Cacl_2$ and incubated for 24 hours. (c) The particles were administered and tested in groups in six-well plates

Table S1. The MRM mode parameters of target metabolites.

Compounds (positive/negative mode)	tR	MRM(m/z)	CE (eV)
Lactic acid (-)	1.553	89.05>43.10	13.0
Succinic acid (-)	2.012	117.10>73.20	25.0
Malic acid (-)	1.315	132.90>115.15	11.0
Fumaric acid (-)	2.173	115.15>71.10	21.0
Citric acid (-)	1.855	191.10>87.15	29.0