

Supplementary Materials

*Research article*

**Transformation of Theranostic Alginate-based Microbubbles from Raspberry-like to Core-Shell-like Microbubbles and In Vitro Studies**

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## Experimental

**Materials:** ALG was obtained from Sigma-Aldrich (low viscosity, cat. no. A1112-100G, MO, USA). Anhydrous calcium chloride (99%, J. T. Baker, NJ, USA), ICG (0.25 g, lot-0000028885, Biosynth Carbosynth, UK), shikonin (SHK,  $\geq 98.0\%$ , 10 mg, PHL89791-10 mg, Sigma-Aldrich, MO, USA), dimethyl sulfoxide (DMSO,  $\geq 99.9\%$ , Sigma-Aldrich, MO, USA), phosphate buffered saline (PBS, 10X, ECHO Chemical, Taiwan), and agarose (Sigma-Aldrich, St Louis, USA) were purchased from the respective manufacturers and used without further purification.

**Preparation of the raspberry-like or core-shell-like SHK-ICG ALG MBs:** For the synthesis, 2.9 mg shikonin powder and 10 mg ICG fluorescent-dye powder were dissolved in 4.08 mL water–DMSO co-solvent (4 mL deionised water and 80  $\mu$ L DMSO). This mixture was kept in dark and frothed with a milk frother for 1–2 min. During the frothing process, 0.15 g of ALG was slowly added to the mixture over 2 min. After all components completely dissolved in the co-solvent, the resultant solution was withdrawn using a micro-syringe, which was equipped with an ES system, as described in our previous studies.<sup>11</sup> Firstly, the freshly prepared stock solution was supplied using a syringe-pump apparatus (NE-300 Just Infusion; New Era Pump Systems, Farmingdale, NY, USA). A 25-gauge flat-tipped needle was used as the spray needle in the ES procedure. A 15 kV positive voltage was applied to the spray needle using a direct current high-voltage-power supply (Bertan Model 205B-20R; Spellman High Voltage Electronics, Hauppauge, NY, USA) to establish an electrical field between the spray needle and the electrically grounded collection solution (10 mL of the 2 wt.%  $\text{CaCl}_2$  aqueous solution kept under 1000 rpm stirring). The spray needle was placed approximately 4 cm above the surface of the collection solution. The ES modes of the system were monitored by observing the shape of liquid meniscus at the outlet of the spray needle. Additionally, the meniscus was illuminated with light from an optical cable, and its droplet shape was recorded using a microscopic system comprising a microscopic lens (model: DFK22AUC03, The IMAGING SOURCE, Germany), digital camera (model STC-620PWT; Sentech, Carrollton, TX, USA), and high-resolution liquid-crystal-display panel. The duration of the ES procedure was 5 min for each run of the MB production. At the end, the obtained MBs floated on the top of the collection solution with a green-coloured appearance. For light-microscope characterisation, a 200  $\mu$ L micropipette was used to draw the top layer of the collection solution, and the sample was transferred onto a glass slide. The specimen was quickly visualised, and the shape of the produced MBs was determined and recorded. The internal composition of the produced MBs was filled with gas, thus the shape and internal structure of the MBs was easily differentiated under light illumination. The cumulative release of the total encapsulated SHK was determined after being triggered by ultrasound supply. The supernatant at each release stage was analysed via spectrophotometry using a SPECTROstar Nano instrument (BMG LABTECH, Ortenberg, Germany).

**Evaluation of the drug release profile:** The real-time drug release profile for the SHK/ICG ALG MBs was determined in a reciprocal shaking water-bath tank that was heated to 37 °C and at a speed of 100 rpm shaking. In the typical procedure, 10 mL of the 2 wt%  $\text{CaCl}_2$  aqueous solution with dispersed SHK/ICG ALG MBs was kept in a 20 mL glass-sample vial. At predetermined time intervals, 2 mL of the supernatant was withdrawn, and the dispersion was replenished with 2 mL of the fresh  $\text{CaCl}_2$  aqueous solution. The supernatants harvested at each release stage were analysed by spectrophotometry using a SPECTROstar Nano instrument (BMG LABTECH, Ortenberg, Germany).

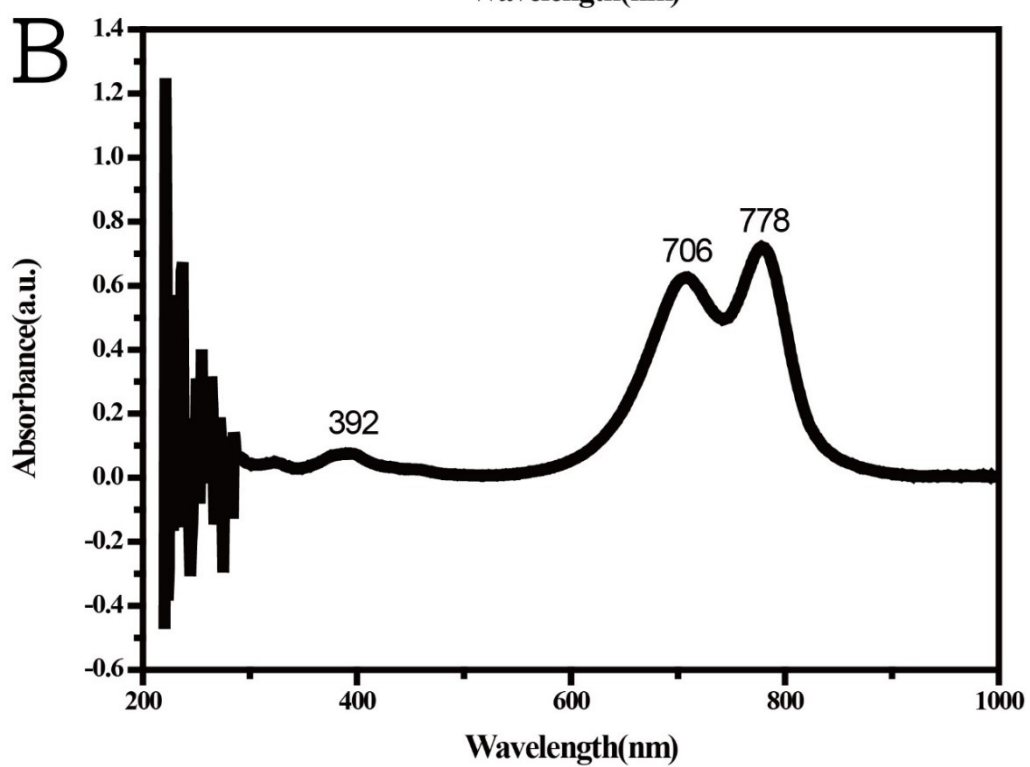
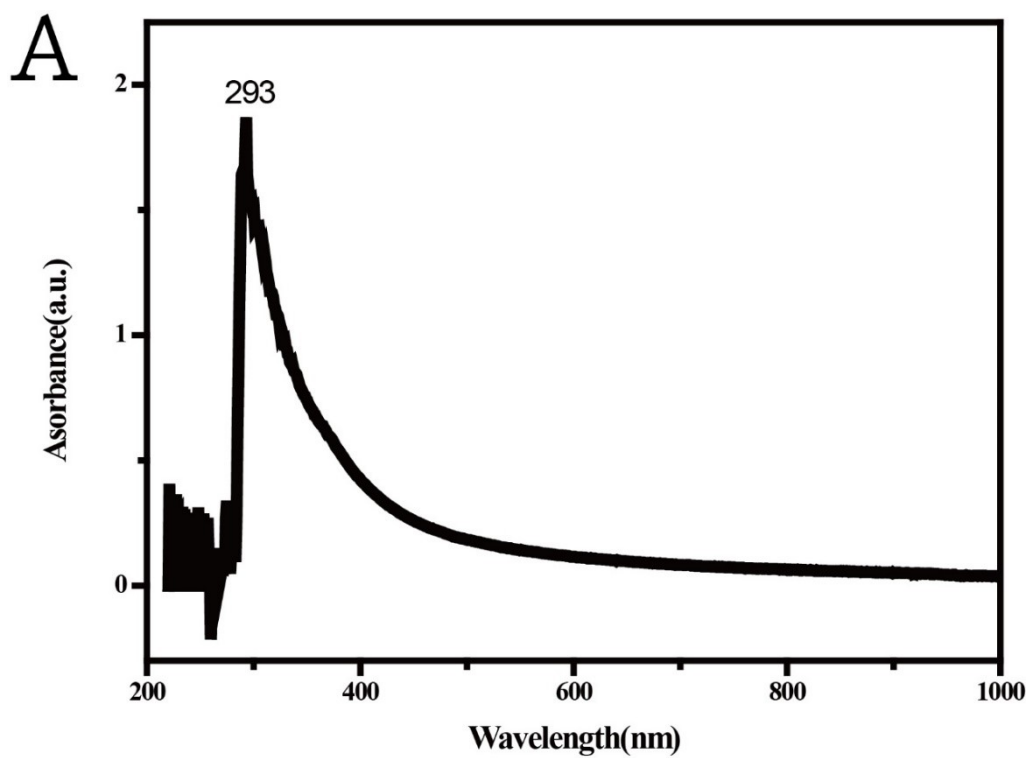
**Acquisition of Ultrasound Image:** A cylindrical-empty pit was moulded in 2% agarose. Generally, 2% agarose is considered a low-backscattering medium. It was used to hold the aqueous suspension of the MBs for acquiring the ultrasound images. Acoustic images were acquired using a high-frequency ultrasound-imaging system (GE®, Voluson E8) with a 9 L high-frequency 2D probe. The in situ ultrasound images were stored as digital files for visualising the differences and comparing them with the blank sample, i.e., the agarose mould. All images were mapped to the grayscale values using custom software plugins for ImageJ (W. H. Rasband, NIH, Bethesda, MD).

**Acquisition of Fluorescent Image:** A cylindrical-empty pit was moulded in 2% agarose. Generally, 2% agarose is considered a low-backscattering medium. It was used to hold the aqueous suspension of the MBs for acquiring the ultrasound images. Acoustic images were acquired using a high-frequency ultrasound-imaging system (GE®, Voluson E8) with a 9 L high-frequency 2D probe. The in situ ultrasound images were stored as digital files for visualising the differences and comparing them with the blank sample, i.e., the agarose mould. All images were mapped to the grayscale values using custom software plugins for ImageJ (W. H. Rasband, NIH, Bethesda, MD).

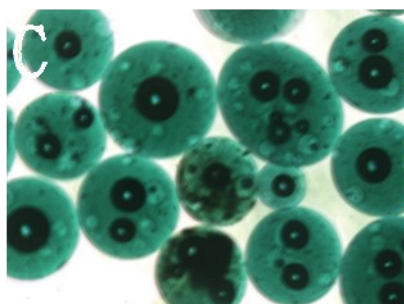
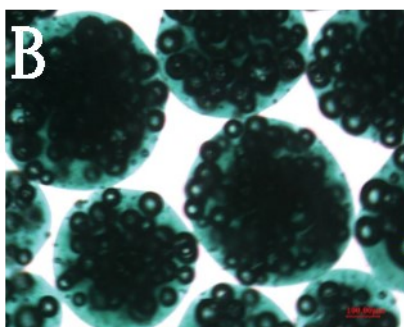
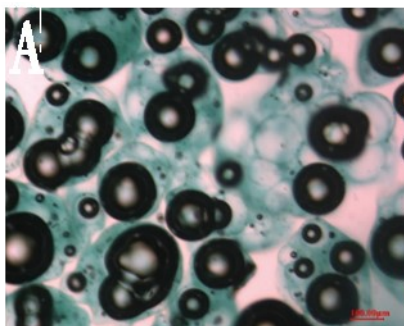
**Table S1** Atomic ratio percentage of SHK-ICG ALG MB measured from the energy dispersive spectrum shown in Figure 1.

Element	Wt%	Wt% Sigma	Atomic %
C	38.76	1.36	47.98
N	1.46	2.00	1.55
O	50.56	1.51	46.98
Na	0.11	0.15	0.07
S	0.37	0.12	0.17
Ca	8.74	0.46	3.24
Total:	100.00		100.00

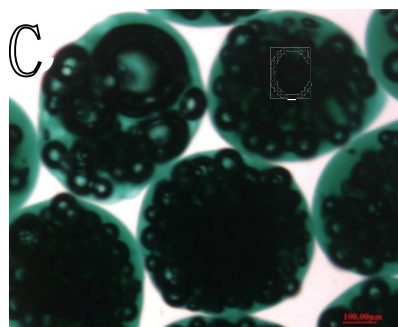
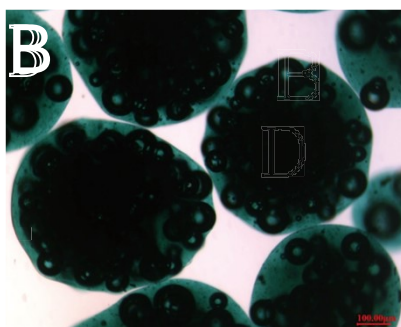
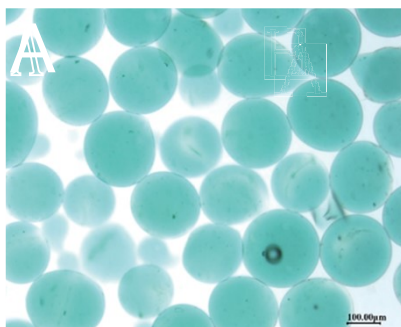
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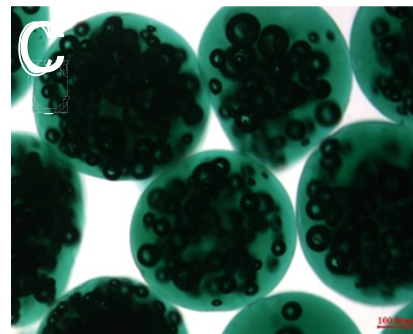
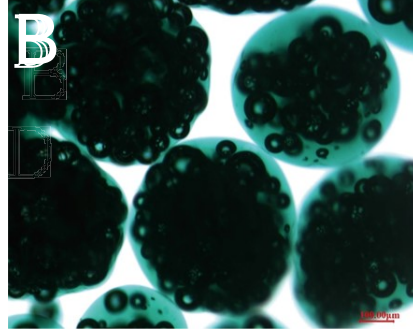
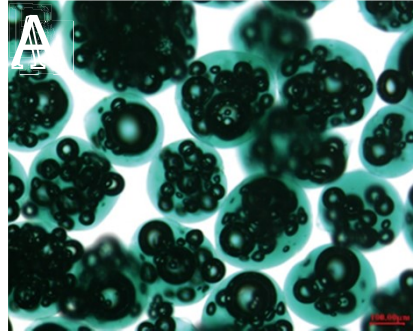
**Figure S1** UV-vis spectra of (A) pure ALG stock solution and (B) ALG stock solution mixed with ICG solution.



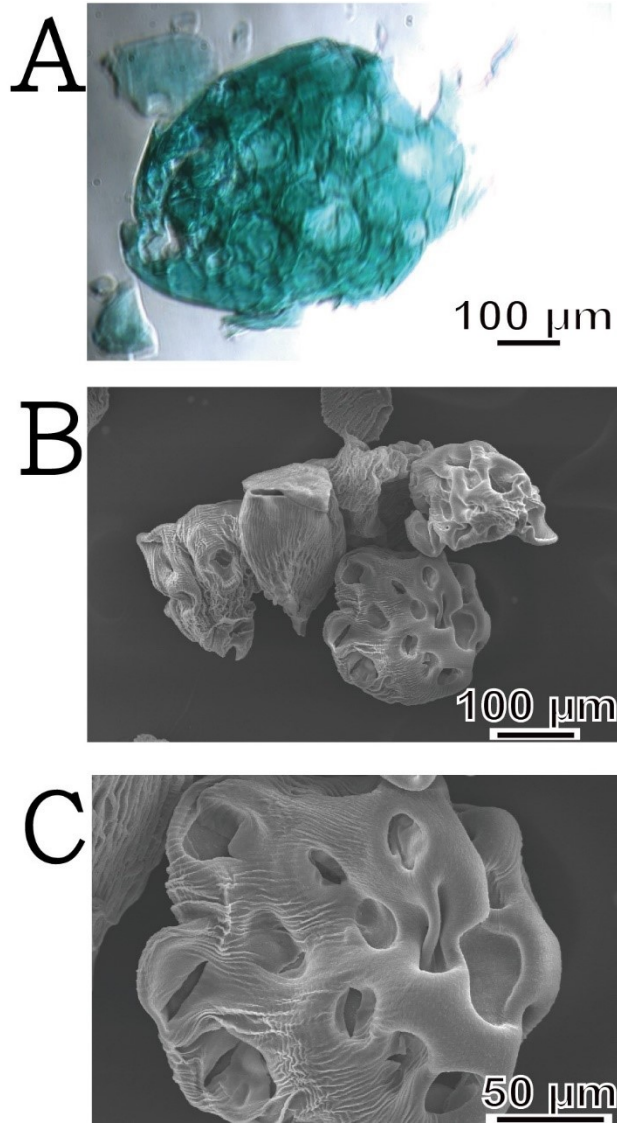
**Figure S2** Optical Images of SHK-ICG ALG MBs produced from stock solution of (A) 0.1 g ALG dissolved in 4.08 mL water-DMSO cosolvent, (B) 0.15 g ALG dissolved in 4.08 mL water-DMSO cosolvent and (C) 0.2 g ALG dissolved in 4.08 mL water-DMSO cosolvent.



**Figure S3** Optical Images of SHK-ICG ALG MBs produced from stock solution of (A) 5 mg ICG dissolved in 4.08 mL water-DMSO cosolvent, (B) 10 mg ICG dissolved in 4.08 mL water-DMSO cosolvent and (C) 20 mg ICG dissolved in 4.08 mL water-DMSO cosolvent.

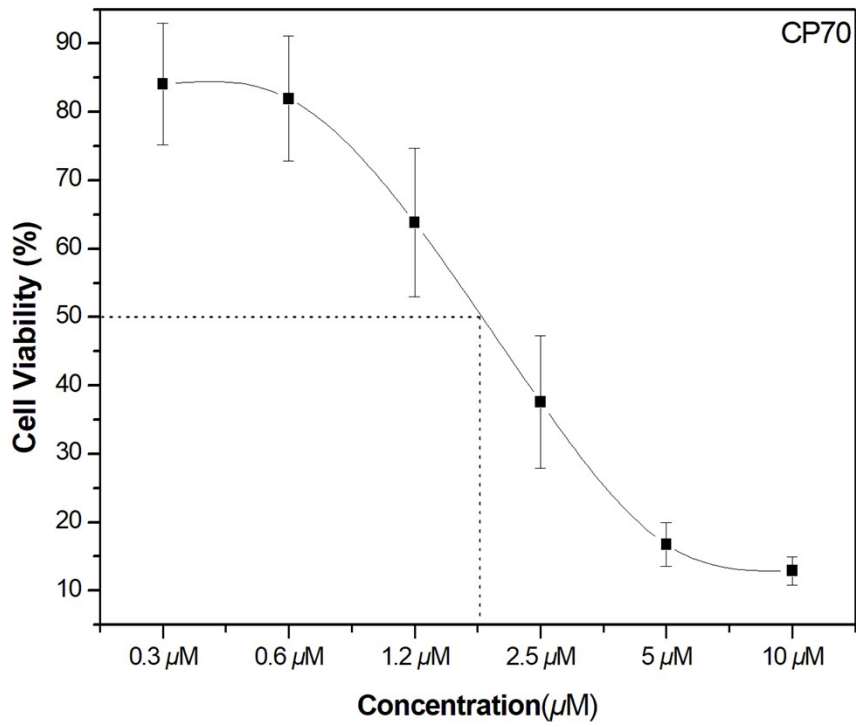


**Figure S4** Optical Images of SHK-ICG ALG MBs produced from electrospray system with (A) 2 cm working distance, (B) 4 cm working distance and (C) 6 cm working distance between spray needle and collection substrate.

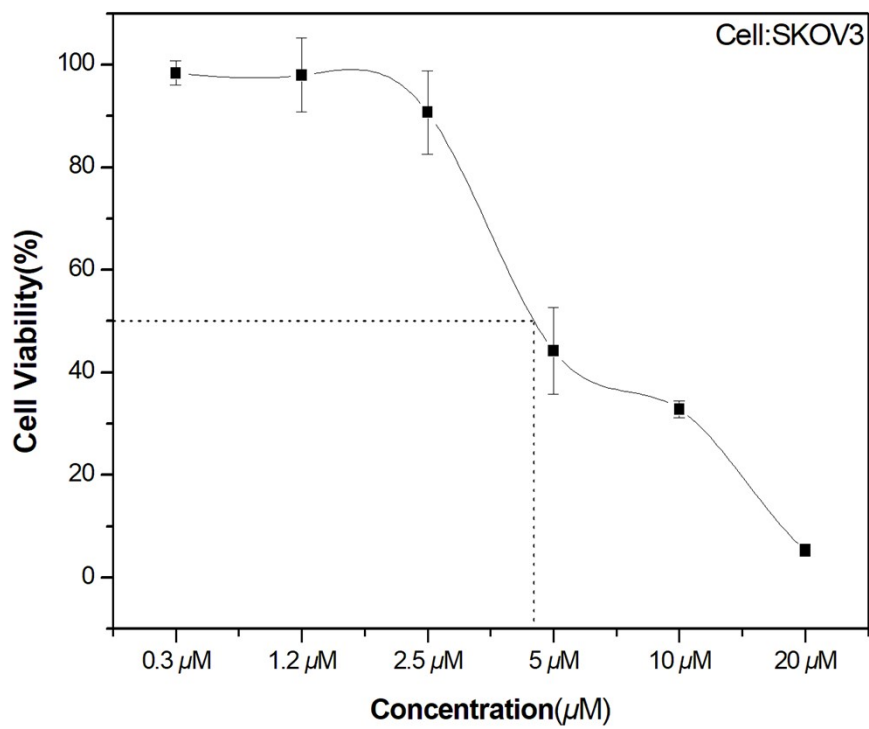


**Figure S5** (A) in-situ optical images of SHK/ICG ALG MBs after being subject to 40 kHz ultrasonication and (B-C) scanning electron microscope images of the same sample after being freeze-drying; (B) 200 X and (C)500 X.

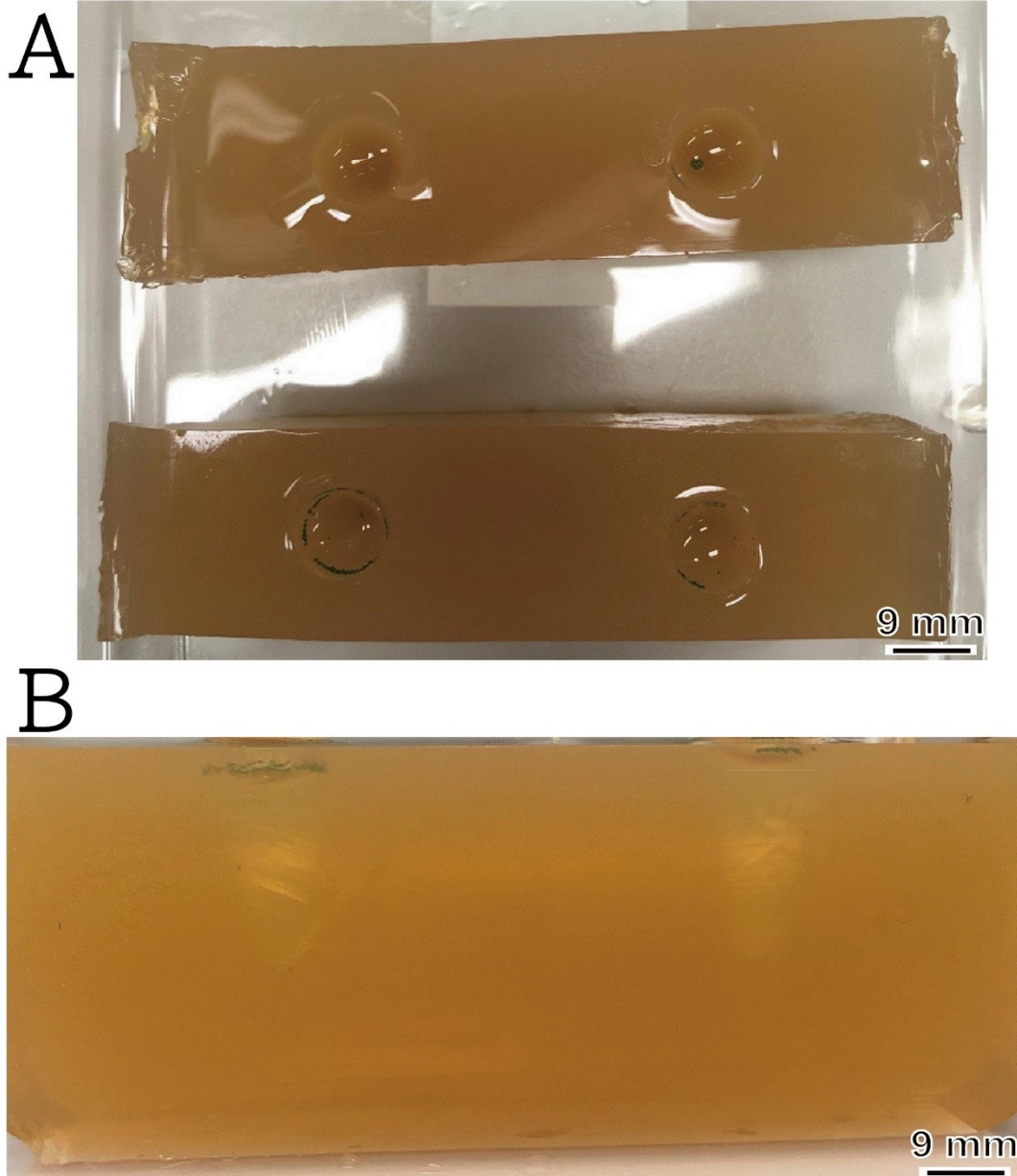




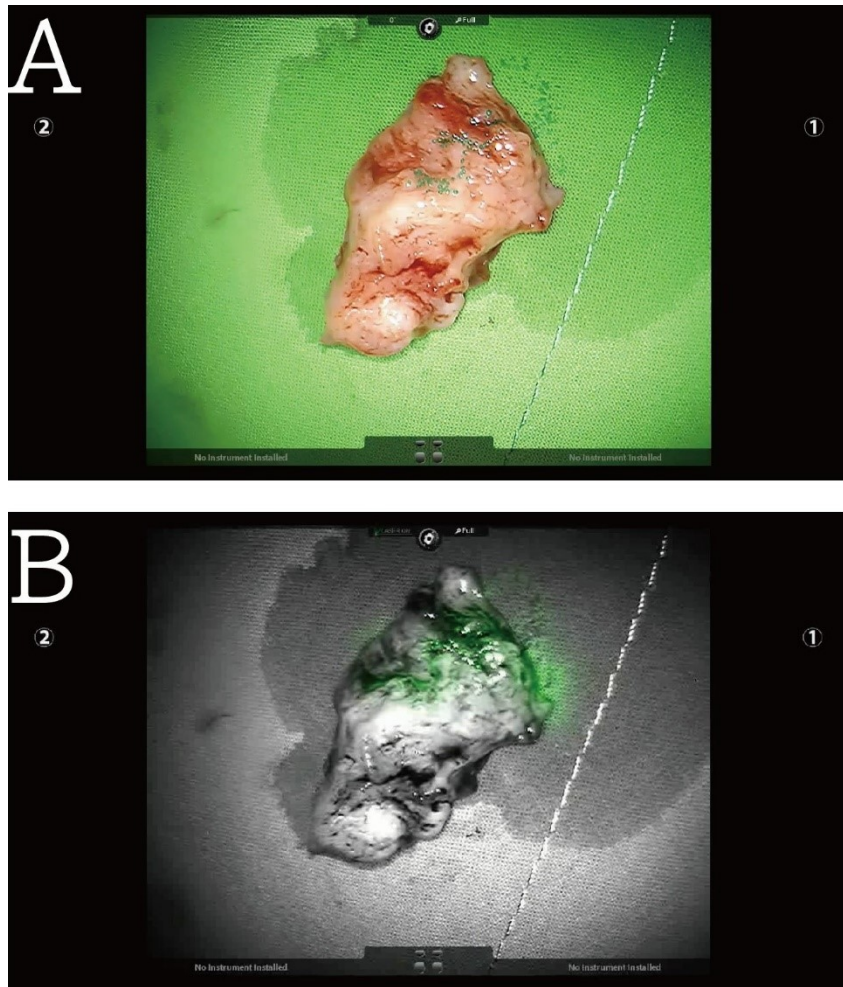
**Figure S6** Cell viability assay with spline fitting of SHK/ICG ALG MBs against CP70 cells. IC<sub>50</sub> is determined by the intersection point of two dash lines at 2.08  $\mu\text{m}$ .



**Figure S7** Cell viability assay with spline fitting of SHK/ICG ALG MBs against SKOV3 cells. IC<sub>50</sub> is determined by the intersection point of two dash lines at 4.43  $\mu\text{m}$ .



**Figure S8** A self-made agarose phantom model was constructed for ultrasound measurements in this study: (A) top view of the agarose phantom with SHK/ICG ALG MBs suspension filling in the pit. (B) cross-section view of the agarose phantom with SHK/ICG ALG MBs suspension filling in the pit.



**Figure S9** *In vitro* (A) bright field image and (B) fluorescence Imaging of a uterine myoma using near-infrared light system equipped on a Da Vinci surgery system.