In Vivo Toxicity Study of Engineered Lipid Microbubbles in Rodents

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Differential scanning calorimetry of the freeze-dried powder.

The lyophilized cake of lipids (see “Naked MBs” in Methods), described in the main text, was analyzed as a powder using a TA Q200 (Waters, Milan, Italy) differential scanning calorimeter (DSC). A known amount of the cake, typically 2 mg, was placed in an aluminum pan and sealed. The scans were performed from 10 to 100 °C at heating rate of 1.00 °C/min under a flux of 50 mL/min of dry nitrogen. A lipid phase melting peak is detected at 60 °C with an enthalpy of 167 J/g (see Figure S1), due to the PEG 4000 component, present as predominant compound in the cake.
Figure S1. Differential scanning thermogram of the cake of DSPC and DPPG-Na.

**Microbubbles reconstitution**

The injection of 5 mL of saline (NaCl 0.9%) through the septum followed by the hand agitation of the vial results in the formation of a milky suspension of SF$_6$ microbubbles (Figure S2).

![Figure S1. Differential scanning thermogram of the cake of DSPC and DPPG-Na.](image)

Figure S2. Milky suspension of SF$_6$ lipid microbubble in saline; part of the bubbles floated on the surface.

**Microbubble size distribution**

Size distribution of SF$_6$-encapsulated lipid microbubbles prepared as described in the main text have been characterized by bright field transmission microscopy of the focused equatorial plane of microbubbles using a Nikon Inverted Microscope Eclipse Ti-E, equipped with Spectra Physics Ar ion laser (488 nm) source. MBs were visualized with a Plan Apo 60× oil immersion objective (Nikon, Japan). The outer average diameter and standard deviation were calculated on a population of 200 microbubbles, using the Nikon software EZ-C1 (version 3.9). The average diameter is 2.8 µm with a standard deviation of ± 1 µm (see Figure S3)
**Figure S3.** Transmission micrograph on a dispersion of lipid MBs, left; size distribution of the sample of lipid MBs. Average diameter: 2.8±1 µm

**Table S1.** The toxicology study plan on 54 eight-week-old male Sprague-Dawley® rats weighing 300 mg±20%

| Group No.&Sex | Animal No. | Treatment       | Dose Level | Dose volume & Route | Sacrifice Time Post-Dosing |
|--------------|------------|-----------------|------------|---------------------|---------------------------|
|              | 1,2,3      | Saline (Control)| 0ml/kg     | 20ml/kg IV slow Injection | 10 min 7days               |
|              | 4,5,6      |                 |            |                     |                           |
| M2           | 7,8,9      | Naked-MB (Test Item I) | 2ml/kg25   | ml/kg2 IV Bolus Injection | 10 min 7days               |
|              | 10,11,12   |                 |            |                     |                           |
| M3           | 13,14,15   |                 | 20ml/kg25  | ml/kg20 IV slow Injection | 10 min 7days               |
|              | 16,17,18   |                 |            |                     |                           |
| M4  | 19,20,21 MBs-ICG (Test Item II) | 2ml/kg25 ml/kg2 IV Bolus Injection | 10 min | 7days |
|-----|---------------------------------|-----------------------------------|--------|-------|
| 22,23,24 |                                |                                   |        |       |
| M5  | 25,26,27                        | 20ml/kg25 ml/kg20 IV slow Injection | 10 min | 7days |
| 28,30,29 |                                |                                   |        |       |
| M6  | 31,32,33 MBs-RGD (Test Item III) | 2ml/kg25 ml/kg2 IV Bolus Injection | 10 min | 7days |
| 34,35,36 |                                |                                   |        |       |
| M7  | 37,38,39                        | 20ml/kg25 ml/kg20 IV slow Injection | 10 min | 7days |
| 40,41,42 |                                |                                   |        |       |
| M8  | 43,44,45 MBs-ICG-RGD (Test Item IV) | 2ml/kg25 ml/kg2 IV Bolus Injection | 10 min | 7days |
| 46,47,48 |                                |                                   |        |       |
| M9  | 49,50,51                        | 20ml/kg25 ml/kg20 IV slow Injection | 10 min | 7days |
| 52,53,54 |                                |                                   |        |       |
Table S2: Hematological changes in rats treated with naked MBs, MBs-ICG, MBs-RGD, MBs-ICG-RGD or saline

|                | WBC  | LY  | MO  | NE  | EO  | BA  | RBC | MCV | HCT | MCH | MCHC | HGB | RDW | FLT |
|----------------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|-----|-----|-----|
| Saline 20ml/kg | 10min| 12.8±0.4 | 85.2±2.3 | 2.3±0.7 | 9.6±2.4 | 2.3±0.2 | 0.3±0.3 | 7.4±0.5 | 44.7±1.5 | 31.4±1.4 | 1410±5 | 65±239 |      |
| 7 days         |      | 14.8±2.5 | 86.8±4.2 | 2.6±0.4 | 8.6±1.8 | 1.4±0.2 | 0.2±0.2 | 8.1±0.3 | 60.1±0.6 | 48.6±1.6 | 31.44±6.6 | 15.3±0.6 |      |
| Naked-MB 2ml/kg| 10min| 11.2±1.7 | 85.8±4.2 | 0.7±0.2 | 10.3±3.7 | 2.3±1.2 | 0.4±0.1 | 7.8±0.2 | 41.2±0.7 | 48.6±1.6 | 31.7±0.2 | 14.9±0.6 |      |
| 7 days         |      | 14.0±4.2 | 83.8±4.2 | 2.5±1 | 10.8±0.3 | 2.1±0.5 | 0.3±0.2 | 8.2±0.4 | 61.0±0.5 | 50.7±2.3 | 18.5±0.1 | 15.2±0.9 | 710±111 |
| Naked-MB 20ml/kg| 10min| 13.7±4.1 | 96.9±5.5 | 0.4±0.6 | 6.4±1.5 | 1.5±0.4 | 0.5±0.2 | 8.5±0.1 | 62.8±2.1 | 54.3±2.1 | 18.3±0.7 | 16.3±0.9 | 36±33  |
| 7 days         |      | 17.7±1.5 | 82.1±4.2 | 2.6±0.4 | 12.1±0.6 | 1.8±0.7 | 0.2±0.1 | 7.9±0.2 | 62.1±0.8 | 49.2±1.4 | 18.3±0.5 | 16.3±0.2 | 117±196 |
| MB-ICG 2ml/kg  | 10min| 15.7±6.2 | 81.9±3.0 | 2.4±0.5 | 14.9±2.7 | 0.6±0.7 | 0.3±0.1 | 7.5±0.3 | 7.4±0.2 | 62.9±0.6 | 47.5±1.9 | 19.4±0.6 | 30.7±0.5 | 14.5±0.3 |
| 7 days         |      | 36.1±6.4 | 87.4±3.7 | 1.9±1.6 | 9.4±1.0 | 0.8±0.9 | 0.2±0.3 | 6.7±0.2 | 7.4±0.6 | 65.4±1.0 | 45.2±1.0 | 18.3±0.5 | 28.9±0.6 | 12.7±0.6 |
| MB-ICG 20ml/kg | 10min| 13.5±7.2 | 90.1±4.0 | 0.6±0.2 | 7.3±2.7 | 1.4±0.1 | 0.2±0.1 | 6.7±0.3 | 6.8±1.0 | 62.0±1.3 | 46.4±1.7 | 19.9±0.6 | 31.4±1.1 | 16.4±0.2 |
| 7 days         |      | 17.8±4.3 | 79.2±6.0 | 3.4±0.5 | 14.2±0.0 | 2.4±0.5 | 0.2±0.1 | 6.7±0.7 | 61.2±0.5 | 52.8±1.3 | 18.9±0.4 | 18.6±0.4 | 30.2±0.6 |
| MB-RGD 2ml/kg  | 10min| 14.0±6.2 | 85.4±3.4 | 1.9±0.5 | 11.8±0.5 | 0.4±0.1 | 0.3±0.0 | 6.5±0.1 | 61.3±0.7 | 46.9±0.1 | 19.6±0.6 | 30.9±0.4 | 16.4±0.3 |
| 7 days         |      | 21.5±8.6 | 84.9±3.6 | 2.7±0.9 | 9.6±2.5 | 0.7±0.5 | 0.2±0.1 | 6.7±0.6 | 64.3±3.0 | 52.1±2.6 | 18.9±0.5 | 18.6±0.4 | 30.2±0.5 |
| MB-RGD 20ml/kg | 10min| 11.6±3.7 | 92.2±1.7 | 0.3±0.1 | 5.6±1.5 | 0.6±0.5 | 0.2±0.1 | 6.5±0.1 | 59.7±0.1 | 56.2±0.6 | 18.9±0.5 | 18.6±0.4 | 30.2±0.5 |
| 7 days         |      | 15.5±1.4 | 84.6±2.4 | 2.6±0.9 | 10.4±0.5 | 0.7±0.5 | 0.1±0.1 | 6.7±0.7 | 61.9±1.0 | 50.2±1.5 | 18.1±1.7 | 18.6±0.5 | 30.2±0.5 |
| MB-ICG-RGD 2ml/kg| 10min| 12.3±3.7 | 88.6±4.9 | 1.3±0.4 | 7.7±3.1 | 1.0±0.4 | 0.3±0.1 | 7.5±0.6 | 66.1±1.7 | 50.1±1.3 | 18.1±0.5 | 18.7±0.3 | 30.2±3.5 |
| 7 days         |      | 18.3±4.0 | 78.9±4.7 | 3.1±0.6 | 15.2±0.6 | 1.1±0.5 | 0.2±0.1 | 8.1±0.3 | 67.8±2.4 | 51.4±2.4 | 18.2±1.6 | 18.6±1.5 | 30.1±3.5 |
| MB-ICG-RGD 20ml/kg| 10min| 15.3±3.3 | 87.3±4.7 | 1.1±0.5 | 9.4±1.3 | 0.7±0.2 | 0.2±0.1 | 6.7±0.3 | 65.4±2.4 | 52.8±3.1 | 18.1±1.6 | 18.6±1.2 | 30.2±4.5 |
| 7 days         |      | 16.7±4.3 | 79.6±4.0 | 3.8±1.6 | 12.3±1.4 | 2.7±1.0 | 0.2±0.1 | 6.7±0.6 | 62.6±4.0 | 52.8±3.1 | 18.2±1.7 | 15.4±0.9 | 106±436 |

Table S3: Blood biochemistry values in rats treated with naked MBs, MBs-ICG, MBs-RGD, MBs-ICG-RGD or saline
| Name         | 20ml/kg  | 7 days | 30ml/kg  | 7 days | 50ml/kg  | 7 days | 75ml/kg  | 7 days | 100ml/kg | 7 days | 150ml/kg | 7 days | 200ml/kg | 7 days | 250ml/kg | 7 days | 300ml/kg | 7 days | 350ml/kg | 7 days | 400ml/kg | 7 days | 450ml/kg | 7 days | 500ml/kg | 7 days | 550ml/kg | 7 days | 600ml/kg | 7 days | 
|--------------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|
Experimental methodology for measuring the attenuation of bubbly suspensions.

Figure S4 shows a schematic overview of the experimental set-up for an ultrasound transmission experiment through bubbly media. In a 2×2×4 cm measuring cell containing the liquid suspension or the suspending medium (control) the liquid was continuously and gently stirred by a magnetic stirrer underneath the cell. The cell had openings at the two opposite sides, where the emitting and receiving transducers were placed in contact with the liquid. All measurements were carried out using flat 10 MHz central frequency unfocused transducers (Olympus, V311) for emission and reception, separated by a distance of 2 cm. To investigate the dispersion characteristics, ultrasonic signals were produced by means of a waveform generator as consecutive sinusoidal bursts in the frequency range from 0.5 to 20 MHz, with 250 kHz steps. At each frequency, the burst signal was driven by an input voltage of 6 Vpp, generating a pressure level below 62 kPa, as calibrated by means of a needle hydrophone (Precision Acoustics, Dorchester, UK) with sensitivity between 371 mV/kPa and 496 mV/kPa in the range of frequencies considered. The received signals were processed by comparison with the signals from a reference medium (saline solution). All steps are controlled by LabView (National Instruments).

The ultrasound attenuation spectrum of the reconstituted microbubbles displays a maximum at the resonance frequency. As rule of thumb, the resonance frequency, f_r, has an inverse proportionality with the size, independently from the model used to describe the dynamics of the oscillations of the microbubbles in a megasonic field. Therefore a collection of microbubbles with a broad size distribution will be characterized by a peak in the attenuation spectra covering a wide range of frequencies. In order to limit such broadening, 5 fractions of reconstituted microbubbles were collected from the starting suspension by dropping from the bottom of a glass syringe used as floatation column the MBs at different floating times, i.e. 0, 5, 13, 20 and 30 minutes. The longer floatation time corresponds to the smaller microbubbles. The attenuation spectra recorded are reported in Figure 4S.
Figure S5. Measured attenuation coefficients for the five SF$_6$ MB suspensions at different floating times; ○: 0 min; □: 5 min; ◇: 13 min; ×: 20 min; +: 30 min.