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Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol

Protocol for designing and preparing gallium particles using cell membrane encapsulation for applications in melanoma cryoablation therapy

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SUMMARY

Liquid metals are increasingly applied in drug delivery, in vivo imaging, and biosensing. Herein, we describe a surface modification strategy, where cell membrane is introduced to encapsulate gallium (Ga) particles. We detail preparation steps of Ga microparticles by sonication, followed by Ga microparticles coating with purified tumor cell membranes and morphological assessment using TEM and cryo-TEM. We further describe cell uptake and establishment of tumor-bearing mouse models and steps to assess in vitro cytotoxicity and in vivo anti-tumor cryotherapy.

For complete details on the use and execution of this protocol, please refer to Wang et al. (2022).

BEFORE YOU BEGIN

As a kind of multifunctional biomedical materials, gallium-based liquid metals (LMs) have been developed in biomedical fields such as drug delivery, in vivo imaging and biosensing (Lu et al., 2015; Sun et al., 2020a, 2020b, 2021; Xie et al., 2021; Yan et al., 2018). However, the lack of LMs target for tumor (Wang et al., 2022) makes it difficult to retain at the tumor region, which seriously hinders the therapeutic efficiency.

Here we develop a protocol for the fabrication of LM Ga particles (GaPs). In this protocol, the surface modification of GaPs is administrated through introducing tumor cell membranes to encapsulate GaPs. With the assistance of cryo-electron microscopy, morphological variation of the membrane encapsulated Ga particles (Ga/MPs) is observed from spherical shape to spiky shape under freezing condition, which can puncture endosomal membrane to achieve highly efficient endosomal escape in in vivo cancer treatment. Therefore, excellent Ga/MPs target for tumor is obtained by introducing cell membrane to modify particles. The uptake capacity of antitumor drug paclitaxel-loaded Ga/MPs (Ga/M/PPs) to tumor cells is 2.25-fold to normal cells, and accordingly Ga/M/PPs exhibit enhanced tumor killing ability. It is evident that the employment of tumor cell membrane to the three Ga particles (GaPs, Ga/MPs, Ga/M/PPs) can enhance their target ability. Our protocol sheds light on a creative strategy based on LM particles to achieve high efficiency clinical tumor therapy.
Preparation one

© Timing: 1 week

Resuscitate and culture C8161 human melanoma cells, wild-type B16-F10 mouse melanoma cells and human normal epidermal cells (HaCaT).

1. Resuscitate the cell lines:
   a. Persevere Human melanoma cells (C8161), mouse melanoma cells (B16-F10), and human normal epidermal cells (HaCaT) in tubes in liquid nitrogen.
      i. To resuscitate these cells, take out the cryopreservation tubes from liquid nitrogen.
      ii. Thaw in a water bath at 37°C for 5 min.
   b. Transfer the thawed cell suspensions to 15 mL centrifuge tubes.
      i. Centrifuge cell suspensions for 5 min at 300 g (high-speed centrifuge, H1850, Cence, China).
      ii. Subsequently, aspirate the supernatant by 1 mL pipette (P1000, Eppendorf, German) without disturbing the pellet.

2. Culture the cell lines:
   a. Prepare the complete cell culture medium with the composition of 89% v/v Dulbecco’s Modified Eagle Medium (DMEM), 10% v/v certified fetal bovine serum (Biological Industries), and 1% v/v penicillin-streptomycin solution (Gibco).
      i. Use DMEM/F12 (Gibco) in C8161 complete medium.
      ii. Use RPMI-1640 (MultiCell) in B16 complete medium.
      iii. Use DMEM/High Glucose (HyClone) in HaCaT complete medium.
   b. Resuspend C8161 cells in 10 mL of C8161 complete medium.
      i. Resuspend B16-F10 cells in 10 mL of B16 complete medium.
      ii. Resuspend HaCaT cells in 10 mL of HaCaT complete medium.
      iii. Transfer all the cells to T75 flasks (Corning) and culture them in 37°C incubator (Carbon dioxide incubator, Thermo Fisher Scientific, USA) with 5% CO₂.

Preparation two

© Timing: 2 weeks

Establish tumor-bearing mouse models.

3. Build C8161 tumor-bearing mouse models (Wang et al., 2022).
   a. Purchase 56 BALB/c nude mice (8-week-old, female, average weight = 20 g) and raise them.

   Note: Raising condition of the mice: a specific pathogen-free laboratory animal room with sterile chow at a constant temperature of 26°C–28°C and relative humidity of 40%–60%.

   b. Culture and passage C8161 cells.
      i. Culture C8161 cells at T75 flasks.
      ii. Withdraw the cell culture medium when the cells overgrow the bottom of T75 flasks.
      iii. Then, add 1 mL trypsin (0.25% Trypsin-EDTA (1x), Gibco) in each T75 flask for 2 min to digest adherent cells.
      iv. Afterwards, add 3 mL of the complete medium to neutralize trypsin in each T75 flask.
      v. Collect all the liquid in the flasks in centrifuge tubes (tube volume: 15 mL, Corning) and centrifuge them at 300 g for 3 min (37°C).
      vi. Remove the supernatant and transfer the lower cells to one 15 mL centrifuge tube.
vii. Add 6 mL 1× PBS solution (PBS pH 7.4 basic (1×), Gibco) in the tube to dilute cells solution for cell solution with concentration of $2 \times 10^7$ /mL.

c. Subcutaneously inject 100 μL of C8161 cell solution on the back of each mouse.

**Note:** The number of injected cells for each mouse is about $2 \times 10^6$.

d. Measure the tumor length and width by Vernier calipers once every 2 days.

**Note:** Tumor volume is calculated by the formula $V=ab^2/2$, here $a$ is the tumor length and $b$ is the tumor width.

e. Implement *in vivo* tumor therapy when the tumor volume reaches $\sim 400$ mm$^3$.

△ **CRITICAL:** While injecting C8161 cells on the back of mice, the experimental operators should take special precautions as to not hurt themselves with needles. Importantly, it should be ensured that the cell solution is injected under the skin rather than into the muscle tissue, which displays obvious swelling at injecting site after injection.

**Institutional permissions**

Animal experiments in the protocol have gotten the permission from the Institutional Animal Care and Use Committee (IACUC) of Tsinghua University, Beijing, China, under contract no. SYXK(Jing) 2014-0024.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Gallium (Ga)        | Aladdin #7440-55-3 |
| Dulbecco’s modified eagle medium (DMEM) | MultiCell #319-006-CL |
| Penicillin-streptomycin | Gibco #15140-122 |
| 0.25% Trypsin-EDTA (1×) | Gibco #15050-065 |
| Certified fetal bovine serum | Biological Industries #04-001-1ACS |
| 1× PBS solution (PBS pH 7.4 basic (1×)) | Gibco # C10010000BT |
| Carbon-coated copper grids | Beijing Zhongjingkeyi Technology #BZ11022a |
| Uranyl acetate | Beijing Zhongjingkeyi Technology #GZO2625 |
| Sucrose | Aladdin #S112226 |
| EDTA-Na$_2$ | Beijinglinge N/S |
| N-2-hydroxyethylpiperazine-N-ethane-sulphonicacid (HEPES) | Sigma-Aldrich #H3375 |
| Protease inhibitor cocktail | Sigma-Aldrich #P8849 |
| Paclitaxel | Solarbio #IP0020 |
| 5 µm polycarbonate membrane | Whatman #110613 |
| 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Ex/Em: 549/565 nm) | Beyotime #C1036 |
| 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR, Ex/Em: 750/780 nm) | Kaijishengwu #KGMP0026 |
| LysoTracker Deep Red | Life Technologies #L12492 |
| DAPI staining solution | Beyotime #C1006 |
| Chitosan oligosaccharide | Yuanyeshwengwu #S31060 |

**Critical commercial assays**

| Cell counting kit-8 (CCK-8) kit | Solarbio #CA1210 |
| BCA Protein Assay Kit | Solarbio #PC0020 |

(Continued on next page)
**Step 1: Purification of C8161 cell membrane**

**Timing:** 2–3 h

1. Preprocess C8161 cells.
   a. Take out C8161 cells (cultured in Step [preparation one]) which are in the logarithmic growth phase (about $5 \times 10^6$ cells/T75 flask) from 37°C incubator and put them into the biosafety cabinet (Airtech, China).
   b. Remove the cell culture medium in T75 flask, and add 10 mL 1× PBS solution to wash C8161 cells for 2–3 times.
   c. Add 2 mL trypsin and incubate the cells in a 37°C incubator for 2 min.
   d. Then, transfer the cell culture dish to the inverted microscope stage, observe the cell digestion under the microscope until the cells become round and detach in large quantities.
   e. Add 2 mL C8161 complete medium to immediately stop the digestion.
   f. Transfer the cell suspension from the culture dish to a centrifuge tube, and then centrifuge at 300 g for 3–5 min.
   g. After centrifugation, move the centrifuge tube to the biosafety cabinet, then open the lid and remove the supernatant to obtain C8161 cell pellet.

2. Prepare the purified C8161 cell membrane.
   a. Disperse the C8161 cells pellet (about $1 \times 10^7$ of cells) in 50 mL lysis buffer (composed of 0.25 M sucrose, 1 mM EDTA, 20 mM HEPES, and a cocktail of protease inhibitors).
   b. Sonicate the C8161-lysis buffer suspension in a Cell Disruption Apparatus (JY92-IIIN, Ningbo Scientz) for 30 cycles on ice (power: 200 W; 3 s ON, 7 s OFF; 30 cycles).
   c. Centrifuge the mixture at 10,000 g for 25 min (4°C) to keep the supernatant and remove the cell sediment.
   d. Transfer the supernatant into a 26.3 mL Beckman ultracentrifuge tube. And centrifuge at 150,000 g for 35 min (4°C) by an ultracentrifuge (Beckman) to get cell membrane pellet.
   e. Disperse C8161 cell membrane pellet in 1× PBS (1 mL per tube). And then transfer C8161 cell membrane into a new 1.5 mL EP tube (Corning).
   f. Detect the protein concentration of C8161 cell membrane by BCA Protein Assay Kit.
Step 2: Obtain gallium particles (GaPs), cell membrane encapsulated gallium particles (Ga/MPs), and antitumor drug paclitaxel-loaded Ga/MPs (Ga/M/PPs)

© Timing: 4–6 h

3. Prepare chitosan oligosaccharide solution (Table 1).
   a. Weigh 2 g chitosan oligosaccharide (COS) by an analytical balance with the precision of 0.0001 g (METTLER TOLEDO).
   b. Dissolve COS in double distilled H₂O (ddH₂O) to get COS solution (40 mg/mL COS).

4. Prepare GaPs. Troubleshooting section (problem 1).
   a. Melt about 4 mL liquid metal-Ga in a 65°C DHG desktop blast drying oven (Shanghai Yiheng).
   Note: It is a good idea to use glass beaker to melt Ga since the glass beaker can resist high temperatures and metal corrosion. When Ga is in liquid state, it is confirmed that the Ga has been completely melted.
   b. Preheat COS solution in the 65°C DHG desktop blast drying oven.
   Note: Herein, COS solution is preserved in 15 mL centrifuge tube (Corning).
   c. Add 4 g melted Ga metal into 10 mL preheated COS solution by 1 mL pipette (Eppendorf).
   d. Disperse Ga in COS solution at 25°C by ultrasonic cell disruptor (JY92-IIN, Ningbo Scientz) to obtain GaPs solution with a concentration of 0.4 g/mL.
   Note: The ultrasonic cell disruptor has a probe which is the key to fabricate GaPs. The parameters of sonication dispersion method are as follows: power: 200 W; 4 s ON, 4 s OFF, 150 cycles.
   e. Centrifuge the above mixture at 150,000 g for 35 min (4°C) and suspended by COS solution to collect GaPs solution.

5. Assemble Ga/MPs.
   a. Isolate C8161 cell membrane by hypotonic cracking method (with the cells from step 1).
   b. Mix 160 mg GaPs and 1.6 mg C8161 cell membrane with a pipette.
   c. Sonicate the above mixture in the ultrasonic bath (power: 400 W, frequency: 45 kHz) at 25°C for 2 min.
   d. Squeeze the mixture with a 1 mL syringe for 12 times through the 5 μm polycarbonate membrane Avanti mini extruder (Avanti Polar Lipids) to obtain the membrane-coated Ga particles (Ga/MPs).

6. Assemble Ga/M/PPs. Troubleshooting section (problem 2, problem 3).
   a. Mix 160 mg GaPs and 1.6 mg paclitaxel and 1.6 mg C8161 cell membrane with a pipette.
   b. Sonicate the above mixture in the ultrasonic bath (power: 400 W, frequency: 45 kHz) at 25°C for 2 min.
   c. Squeeze the mixture by a 1 mL syringe through the 5 μm polycarbonate membrane for 12 times to obtain paclitaxel-loaded membrane-coated Ga particles (Ga/M/PPs).

Table 1. Preparation of chitosan oligosaccharide solution

| Reagent                  | Final concentration | Amount |
|--------------------------|---------------------|--------|
| Chitosan oligosaccharide (COS) | 40 mg/mL            | 2 g    |
| Double distilled H₂O (ddH₂O)   | N/A                 | 50 mL  |
| Total                    | 40 mg/mL COS        | 50 mL  |

A stock solution with 50 mL should be stored for no longer than 30 days at 25°C.
CAUTION: When taking out the glass beaker from the water bath, please wear insulated gloves because the oven temperature oven reaches up to 65°C.

Step 3: Morphological comparison of GaPs and Ga/MPs by TEM and cryo-TEM

Timing: 2 days

The Ga particles are characterized by transmission electron microscope (TEM) at 25°C (Figure 1A) and cryo-TEM at −196°C (Figure 1B) to capture their morphological variation in a frozen condition.

7. Prepare the samples of GaPs and Ga/MPs for TEM observation.
   a. Prepare two new 300 mesh carbon-coated copper meshes.
   b. Drop 5 µL GaPs solution and 5 µL Ga/MPs solution prepared in step 2 in 1 mL COS solution separately onto copper meshes.
   c. Incubate the sample for 1 min and then suck out the excess sample solution with clean filter papers.
   d. Drop 5 µL ddH2O onto the copper meshes by a 10 µL pipette to wash the samples for 2 times to remove the COS on top of the copper meshes.
   e. Stain Ga/MPs by 2% v/v uranyl acetate for 2 min.
   f. Dry the copper meshes at 25°C for 2 min.
   g. Observe the samples by transmission electron microscopy (TEM, HT7700, Japan) with the voltage of 80.0 kV.

8. Prepare the samples of GaPs and Ga/MPs for cryo-TEM observation.
   a. Spread out 5 µL GaPs solution and 5 µL Ga/MPs solution separately onto copper meshes and incubate for 1 min.
   b. Remove the excess sample solution with clean filter paper.
   c. Plunge the copper meshes into liquid ethane for rapid freezing at about −183.3°C.

Note: Ethane is the gaseous state at 25°C. Before the sample is prepared, ethane gas is first blown into a metal cup cooled in liquid nitrogen. The ethane changes from gas state to liquid state in the cup.

d. Store the prepared samples in liquid nitrogen at about −196°C for cryo-TEM (FEI Tecnai F20 TEM, Netherlands) observation, the parameters of the cryo-TEM are as follows: voltage: 200 kV, magnification: 3k.
CAUTION: Cryogenic liquids such as liquid ethane (boiling point: −183.3 °C) and liquid nitrogen (boiling point: −196 °C) need to be handled carefully to avoid frostbite.

△ CRITICAL: Rapid freezing treatment of samples under liquid ethane can promote the amorphous state ice formation without ice crystal, which keeps the frozen sample particles stay in their natural morphology state without any destruction to sample structure and electron diffraction interference during measurement as well.

Step 4: Cell uptake

© Timing: 2 days

9. Digest C8161/B16 cells.
   a. Take out C8161/B16 cells (cultured in Step [preparation one]) which are in the logarithmic growth phase (about 5 × 10^6 cells/T75 flask) from 37°C incubator and put them into the biosafety cabinet.
   b. Remove the cell culture medium in T75 flask, and add 10 mL 1× PBS solution to wash C8161/B16 cells for 2–3 times.
   c. Add 2 mL trypsin, incubate in the incubator at 37°C for 2 min.
   d. Transfer the cell culture dish to the inverted microscope stage, and observe the digestion of the cells under a microscope until the cells become round and detach in large quantities.
   e. Add 2 mL cell complete medium to immediately stop the digestion.
   f. Transfer the cell suspension from the culture dish to a centrifuge tube, and then centrifuge at 300 g for 3 min.
   g. After centrifugation, move the centrifuge tube to the biosafety cabinet, then open the lid and remove the supernatant.

10. Co-culture Ga particles and C8161/B16 cells.
   a. Suspend C8161/B16 cells by 5 mL 1× PBS, and count them with a hemocytometer.
   b. Seed C8161/B16 cells into confocal 8-well dishes (1.0 × 10^4 cells/dish, Corning) with the corresponding DMEM complete medium.
   c. Culture C8161/B16 cells in the incubator at 37°C for 12 h.
   d. Label Ga/MPs, Ga/M/PPs with 100 μM 1,10-dioctadecyl-3,3,30,30-tetramethylindo-carbocyanine perchlorate (DiI) in 1× PBS at 25°C for 5 min. Ga/MPs solution: Dil= 10:1, v/v. Ga/M/PPs solution: Dil= 10:1, v/v.
   e. Transfer the mixture into a 0.5 mL concentration tube (50 kDa), and centrifuge at 3000 g for 5 min (20°C) to remove the free Dil and get Dil-Ga/MPs and Dil-Ga/M/PPs.
   f. Add GaPs, Dil-Ga/MPs and Dil-Ga/M/PPs into confocal 8-well dish to co-incubate with C8161/B16 cells at 37°C for 4 h to prepare the co-incubated C8161/B16 cells.

11. Observe the co-incubation results of cells by confocal microscope.
   a. Remove the cell culture medium in the confocal dish, and wash the co-incubated C8161/B16 cells with 200 μL 1× PBS for 2–3 times to remove free GaPs, Dil-Ga/MPs and Dil-Ga/M/PPs.
   b. Fix the co-incubated C8161/B16 cells with 200 μL of 4% v/v of paraformaldehyde (Beyotime) for 10 min.
   c. Stain the nucleus of the co-incubated C8161/B16 cells with 200 μL DAPI solution for 10 min.
   d. Rinse the cells with 200 μL 1× PBS for 2–3 times.
   e. Observe the cells using 40× objective by the confocal laser scanning microscope (ZEISS).

Step 5: Temperature-dependent deformation and endosomal escape

© Timing: 2 days

12. Treat the co-incubated cells at different temperature.
   a. Digest C8161 cells as per step 9 in step 4.
b. Suspend the cells by 5 mL 1× PBS, and count them with a hemocytometer.
c. Seed C8161 cells into confocal 8-well dishes (1.0×10^4 cells/dish) with DMEM complete medium.
d. Culture C8161 cells in the incubator at 37°C for 12 h.
e. Label Ga/MPs with 10 μM Dil in 1× PBS at 25°C for 5 min.
f. Transfer the mixture into a 0.5 mL concentration tube (50 kDa), and centrifuge at 3000 g for 5 min to remove the free Dil.
g. Add Dil-Ga/MPs into the confocal 8-well dish to co-incubate with C8161 cells at 37°C for 4 h.
h. Place C8161 cells (defined as control group) at 25°C for 5 min. And place C8161 cells (defined as cryo group) at −80°C for 5 min.
i. Incubate all the cells at 37°C for 10 min to warm.
j. Aspirate cell culture medium with Dil-Ga/MPs, and wash C8161 cells with 200 μL 1× PBS for 2–3 times.

13. Observe the endosomal escape results of the co-incubated cells.
   a. Stain endosomes with Lysotracker Deep Red in DMEM medium for 20–30 min.
   b. Rinse C8161 cells by 200 μL 1× PBS for 2–3 times.
   c. Observe the co-localization of Ga/MPs and endosomes with a 63× oil immersion objective by a confocal laser scanning microscope.

Step 6: In vitro cytotoxicity

© Timing: 3 days

14. Seed cells into 96-well plate and do the frozen treatment.
   a. Digest C8161/B16/HaCaT cells as per step 9 in step 4 respectively.
   b. Suspend C8161/B16/HaCaT cells by 5 mL 1× PBS, and count C8161/B16/HaCaT cells by a hemocytometer.
   c. Seed C8161/B16/HaCaT cells into 96-well plate (4.0×10^3 cells/well) with the corresponding DMEM complete medium.
   d. Culture C8161/B16/HaCaT cells in 37°C incubator for 12 h.
   e. Add 1× PBS, GaPs (400 ng/μL), Ga/MPs (400 ng/μL), Ga/M/PPs (400 ng/μL) into 96-well plate to co-incubate with C8161/B16/HaCaT cells at 37°C for 24 h.
   f. Place C8161/B16/HaCaT cells (defined as control group) at 25°C for 5 min. And place C8161/B16/HaCaT cells (defined as cryo group) at −80°C for 5 min.

   Note: Here, both control group and cryo groups contains 12 samples: PBS-C8161, PBS-B16, PBS-HaCaT, GaPs-C8161, GaPs-B16, GaPs-HaCaT, Ga/MPs-C8161, Ga/MPs-B16, Ga/MPs-HaCaT, Ga/M/PPs-C8161, Ga/M/PPs-B16, Ga/M/PPs-HaCaT.

g. Incubate all the cells at 37°C for 10 min to warm.

15. Measure and calculate cell viability.
   a. Remove cell culture medium, and wash them with 100 μL 1× PBS for 2–3 times to clean 1× PBS, GaPs, Ga/MPs or Ga/M/PPs in the culture medium.
   b. Add 10 μL CCK-8 (Cell Counting Kit-8, Dojindo) reagent into each well of 96-well plate.
   c. After 1 h incubation, the multifunctional enzyme marker (Thermo Scientific) is applied to the optical density (OD) with the excitation wavelength of 450 nm.

   Note: The cell viability is calculated by the formula (OD<sub>eg</sub>-OD<sub>b</sub>)/(OD<sub>cg</sub>-OD<sub>b</sub>), where OD<sub>eg</sub> means the OD of cells cultured by GaPs, Ga/MPs, Ga/M/PPs with and without freezing treatments, OD<sub>b</sub> means the OD of wells filled with only CCK-8 solution.
Step 7: *In vivo* antitumor cryotherapy

**Timing:** 11 weeks

C8161 tumor-bearing mice are applied for *in vivo* cryotherapy with three Ga particles (GaPs, Ga/MPs and Ga/M/PPs) injected directly into tumor sites to achieve high performance of tumor ablation under freezing treatment.

16. Inject the pre-prepared three Ga solutions (GaPs, Ga/MPs and Ga/M/PPs, obtained from step 2) separately into tumor site 24 h ahead of *in vivo* cryo-treatments.
   a. Prepare 200 μL 1× PBS solution, 200 μL GaPs solution, 200 μL Ga/MPs solution, and 200 μL Ga/M/PPs solution.

   **Note:** The concentrations of the three Ga solutions are all 0.4 g/mL. 1× PBS and the three Ga solutions are separately mixed by a pipette before injecting into mouse tumor sites.

   b. 56 tumor-bearing mice prepared in Step [preparation two] are randomly divided into 8 groups and each group have 7 mice.

   **Note:** Here, 1- PBS group means inject 100 μL 1× PBS per mouse into the tumor tissue site, 2- PBS+Cryo group means inject 100 μL 1× PBS per mouse into the tumor tissue site with tumor cryoablation, 3-GaPs group means inject 100 μL GaPs solution per mouse into the tumor tissue site, 4-GaPs+Cryo group means inject 100 μL GaPs solution per mouse into the tumor tissue site with tumor cryoablation, 5-Ga/MPs group means inject 100 μL Ga/MPs solution per mouse into the tumor tissue site, 6-Ga/MPs+Cryo group means inject 100 μL Ga/MPs solution per mouse into the tumor tissue site with tumor cryoablation, 7-Ga/M/PPs group means inject 100 μL Ga/M/PPs solution per mouse into the tumor tissue site, 8-Ga/M/PPs+Cryo group means inject 100 μL Ga/M/PPs solution per mouse into the tumor tissue site with tumor cryoablation.

17. *In-vivo* cryo-treatments with cryoablation system. **Troubleshooting** section (problem 4, problem 5).
   a. Intraperitoneally inject 2.5% v/v Avertin solution for mice with dosage of 100 μL/10 g.
   b. Directly insert cryoprobe of HYG-II cryoablation system into tumor tissue (Figure 2).
   c. Treat tumor-bearing mice for 4 min for each time and twice totally with the interval of 7 days.
   d. Monitor body temperature of mice by infrared camera and thermocouple during treatment process.

Figure 2. The schematic of cryoablation treatment for tumor-bearing mouse

In order to perform cryoablation, cryoprobe is inserted into tumor site and cryosurgery system controls the ablation temperature by alternating cold medium liquid nitrogen and heat medium ethanol delivery for cooling and heating.
e. Measure body weight of mice.
f. Measure tumor length and width of mice by the Vernier caliper, and accordingly to calculate the tumor volumes.
g. Observe tumor growth by in vivo small animal optical imaging device (IVIS Spectrum, PerkinElmer, America) with Em: 540 nm, Ex: 500 nm, exposure time: 2 s.

**CRITICAL:** In the animal experiment, the Ga based drug dosage is 10 mg per mouse (500 mg/kg). Many studies have reported the safe dosage of metal Ga in living bodies, and 700 mg/kg has been identified as its maximum tolerated dose (MTD). (Yan et al., 2018). In this experiment, the Ga dosage in mice have met the requirement of MTD.

**EXPECTED OUTCOMES**

This proposal presents a specific cryo-facilitated Ga particles fabrication and the deformational behavior at low temperature. The obtained Ga particles can destroy the endosomal membrane through physical mechanical deformation (from spherical shape to spiky shape) of the particles. With cryo-stimulation, endosomal escape and drug release are achieved so as to enhance the efficacy of antitumor cryotherapy. Firstly, the membrane of C8161 tumor cells are collected and applied to encapsulate GaPs to fabricate Ga/MPs. The prepared Ga/MPs display a morphological transformation from sphere structure to cactus-like structure in cryogenic environment under Cryo-TEM observation (Figure 1). Furthermore, in order to efficiently kill cancer cells, a physical-mechanical procedure is used to create cryo-triggered Ga/MPs transformers that disrupt endosome membranes, thereby achieving effective endosomal escape. Under the in vivo antitumor treatments setting (Figure 2), Ga/MPs and Ga/M/PPs groups have exhibited good tumor growth inhibition (Figure 3). Besides, after the prepared solution is injected into the tumor site, both Ga/MPs and Ga/M/PPs groups demonstrate clear tumor images in vivo compared with the results of two control groups.
(PBS and GaPs) under X-ray (Figure 4). In Figure 4, GaPs are injected into the tumor site 24 h prior to X-ray imaging. GaPs cannot stay in the tumor for a long time due to the lack of targeting ability to tumor cells. Therefore, there is no particle contrast in GaPs group, which is similar to PBS group. Besides, when GaPs solution is injected into the hepatic portal vein to infuse the GaPs contrast agent perfusion of blood vessels into liver (Figure 5), high-resolution bio-imaging of vascular distribution can be achieved. In Figure 5, GaPs are injected into the hepatic portal vein to implement the GaPs contrast agent perfusion of blood vessels into the liver only 1 h prior to X-ray imaging. Thus, GaPs are still in the hepatic portal vein and accordingly blood vessels can be visualized perfectly.

QUANTIFICATION AND STATISTICAL ANALYSIS
To detect the in vivo antitumor effect of Ga-based particles, a software program, such as Living Image 4, is used to extract the fluorescence signals from C8161-bearing mice.

The X-ray images are captured by small animal micro-CT apparatus (Quantum GXmicro-CT, PerkinElmer, United States) with 90-kV voltages to scan the anesthetized animals. And the obtained images data could be processed by software such as 3D slicer.

LIMITATIONS
One potential disadvantage of Ga-based particles is that the batch-to-batch variability will affect experimental results. It is recommended to test Ga-based particles between new batches by measuring the particle size, charge, and deformability under low temperature stimulation of Ga-based particles from old and new batches.

In this protocol, we use a human-derived melanoma cell line to model melanoma. However, we have not been able to successfully establish a patient-derived xenografts model. Therefore, the methods described in this protocol may not be applicable to each melanoma subtype.

TROUBLESHOOTING
Problem 1
Easy solidification of liquid metal-Ga at room temperature (20°C–25°C).

The melting point of liquid metal-Ga is 29.76°C, making it easy to solidify at room temperature.
Potential solution
About 4 mL of liquid metal-Ga is incubated in a 65°C DHG benchtop blast drying oven (Shanghai Yiheng) to melt. Glass beakers are used to melt Ga since glass beakers are resistant to high temperatures and metal corrosion. When Ga become liquid, it is confirmed that Ga have completely melted.

Problem 2
Poor solubility of Ga-based particles (Wang et al., 2018).

Due to their poor solubility, Ga-based particles are prone to deposit to the bottom of the tube.

Potential solution
The strategy of encapsulating Ga-based particles with C8161 cell membrane can increase the solubility of Ga particles. Moreover, Ga particles are mixed by pipette before each administration.

Problem 3
Lack of tumor targeting of Ga-based particles.

Due to the lack of tumor targeting, Ga-based particles are difficult to retain at tumor site, which seriously reduce the efficiency of tumor treatments.

Potential solution
The strategy of encapsulating Ga-based particles by C8161 cell membrane can increase the tumor targeting of Ga particles.

Problem 4
Limited penetration capability of Ga-based particles into tumor cells.

The size of Ga-based particles is micro-sized level, and consequently the penetration capability is suboptimal.

Potential solution
Encapsulate Ga-based particles with C8161 cell membrane for specific targetability to tumor cells.

Intratumoral injection of Ga-based particles can ensure to penetrate deeper into the tumor.

Problem 5
Unsatisfactory cryo-treatment effect.
The cryoprobe tends to have difficulty in penetrating the skin outside the tumor site and inserting deeply into the tumor tissue, resulting in uneven cooling, poor cryotherapy effect, and easy frostbite to the skin.

**Potential solution**
First, in order to administer cryotherapy, a small incision is made on the skin above the tumor using scissors. A sharp cryoprobe is then inserted into the tumor tissue for treatment.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jing Liu (jlubme@mail.tsinghua.edu.cn).

**Materials availability**
This study did not generate new unique materials.

**Data and code availability**
The data presented in this work are available from the lead contact upon reasonable request.

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**AUTHOR CONTRIBUTIONS**
Conceptualization, J.L.; investigation, X.W. and J.C.; writing, X.W. and J.C.; language editing, J.Z.; supervision, J.C., X. L., and J.L.

**DECLARATION OF INTERESTS**
The authors declare no competing interests.

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