Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.
This journal is © The Royal Society of Chemistry 2023

Supporting information

For

Calixarene-based Cryoprotectants for Ice Recrystallization Inhibition and Cell Cryopreservation

Bing Hu[a], Juan-Juan Li[b], Yan-Bin Ren[a], Tian-Xing Zhang[c], Li-Bin Chen[a], Xiao-Liu Li[a], Dong-Sheng Guo[b,d]*, Ke-Rang Wang [a,]*

[a] College of Chemistry and Materials Science, Key Laboratory of Medicinal Chemistry and Molecular Diagnosis (Ministry of Education), State Key Laboratory of New Pharmaceutical Preparations and Excipients, Key Laboratory of Chemical Biology of Hebei Province, Hebei University, Baoding, 071002, China. E-mail: kerangwang@hbu.edu.cn.

[b] College of Chemistry, Key Laboratory of Functional Polymer Materials (Ministry of Education), State Key Laboratory of Elemento-Organic Chemistry, Collaborative Innovation Center of Chemical Science and Engineering, Nankai University, Tianjin 300071, China. E-mail: dshguo@nankai.edu.cn.

[c] College of Chemical Engineering, Inner Mongolia Engineering Research Center for CO_2 Capture and Utilization, Key Laboratory of CO_2 Resource Utilization at Universities of Inner Mongolia Autonomous Region, Inner Mongolia University of Technology, Hohhot 010051, China.

[d] Xinjiang Key Laboratory of Novel Functional Materials Chemistry, College of Chemistry and Environmental Sciences, Kashi University, Kashi 844000, China.

S1. Experimental section

1.1 Materials and measurements

IRI activity analysis was performed on a Nikon polarized optical microscope (LV100ND, Japan) equipped with a Linkman (LTS420) cooling stage.

1.2 Ice recrystallization inhibition (IRI) activity

IRI activity analysis was performed via a splat cooling method. The experimental apparatus used to investigate the IRI activity was equipped with a Nikon polarized optical microscope (LV100ND, Japan) and a Linkman (LTS420) cooling stage. In a typical IRI activity measurement, a 10 μL droplet of solution at room temperature (25°C) was dropped onto the surface of a silicon substrate precooled to -60.0°C with liquid nitrogen from a height of 1.5 m. The droplet quickly formed to a thin layer of ice. Then the temperature was increased to -6.0°C at a rate of 15°C min^{-1}, and the samples annealed at this temperature for 30 min. The ice wafer was imaged randomly with a digital camera (Nikon Y-TV55, Japan) to obtain the grain size of the ice crystals. Image J software was used to calculate the average number of ice crystals in the fields of four photos, and the mean grain size (MGS) values relative to that of the PBS solution were calculated.

1.3 Dynamic ice shaping (DIS) activity with an improved sucrose-assisted dynamic ice shaping experiment

An improved sucrose-assisted DIS experiment was performed using a Nikon polarized optical microscope (LV100ND, Japan), controlling the temperature with a Linkman LTS 420 cooling stage. Each compound was dissolved in 45.0 wt% sucrose solution with the final concentrations of 10 mg/mL, 20 mg/mL, and 40 mg/mL, respectively. 45.0 wt% Sucrose solution was as control group. One microlitre of 45 wt% sucrose solution was placed in the center of a circle on a microscope slide, then a cover slide was covered, and which was sealed with a flap sealant. The microscope slide was placed on the heating/cooling stage and cooled to -50°C at a rate of

25°C/min. The temperature was remained at -50°C until ice nucleation was formed, followed by heating up to -8°C at a rate of 5°C/min, and further warmed up at a rate of 0.5°C/min to melt most of the ice crystals. A single ice crystal was observed, then which was cooled at a rate of 0.1°C/min to a point at which the size of the ice crystals remains constant. After that, the single ice crystal was continued to cool at the same rate of 0.1°C/min. Photographs of ice crystal shaping was observed at 0.2°C intervals, and the corresponding growth rate (γ) was calculated.

1.4 Sucrose-sandwich IRI assay

Sucrose-sandwich IRI assay was performed using a Nikon polarized optical microscope (LV100ND, Japan), controlling the temperature with a Linkman LTS 420 cooling stage. Each compound was dissolved in 45.0 wt% sucrose solution with the final concentrations of 10 mg/mL, 20 mg/mL, and 40 mg/mL, respectively. 45.0 wt% Sucrose solution was as control group. Samples were dissolved in 45 wt% sucrose solution, which were sandwiched with two cover slides and sealed with a flap sealant. Samples were cooled to -50°C at a rate of 25°C/min on a heating/cooling stage, then maintained for 2 min. The temperature was increased to -8°C at a rate of 5°C/min and held for 60 min. The ice crystal shape was observed.

1.5 Cytotoxicity of macrocycle molecules in human cells

MCF-7, hucMSCs and A549 cells were seeded in a 96-well plate at a density of 7 × 10^3 cells per well (with 180 μL of DMEM). After 24 h of incubation, 20 μL of medium containing different concentrations of macrocycle molecules was added to the wells with cells. Then, all the cells were cultured for an additional 48 h. Cell

viability was assessed by the MTT method. First, cells were incubated with MTT (0.5 mg mL^{-1}) for 4 h at 37°C. During this incubation period, water-insoluble crystals formed, which were then dissolved by the addition of 100 μL of a hydrochloric acid-isopropanol mixed solution to each well. The optical density at 490 nm in each well was measured using an enzyme-linked immunosorbent assay plate reader. Wells containing culture medium and MTT but no cells acted as blanks. The percent of cell viability was calculated as follows: $(A_{compound} - A_{blank})/(A_{control} - A_{blank}) \times 100\%$.

1.6 Cryopreservation of human cells

MCF-7, hucMSCs and A549 cells were cultured under standard conditions (5% CO_2, 37°C). MCF-7, hucMSCs and A549 cells were grown in DMEM supplemented with 10% FBS. After 48 h of incubation under standard conditions (5% CO_2, 37°C), the cells were washed from the plate using trypsin and centrifuged for 3 min at 1200 rpm. For cryopreservation, the cells were diluted into the cryoprotectants (87.5% DMEM, 10% FBS and 2.5% DMSO) containing different concentrations of macrocycle molecules (0.25, 0.5 and 1.0 mg/mL) at a density of 5×10^5 cells·mL^{-1}. The mixtures were first cooled from room temperature to 4°C and equilibrated for 5 min. Then, they were further frozen in a -20°C freezer and equilibrated for 0.5 h. Finally, the mixtures were frozen in a -80°C freezer and equilibrated for 24 h to completely freeze the cells. To thaw the cells, the mixtures were placed in a water bath at 37°C for 2 min. After removing of the cryoprotectants, the cells were cultured in DMEM with 10% FBS under standard conditions. After 24 h of incubation, adherent cells (alive) were counted, and the proportion of surviving cells compared to

the total number of cells was calculated and compared with the group without compound. Six parallels in each group were repeated three times.

S2. Additional figures

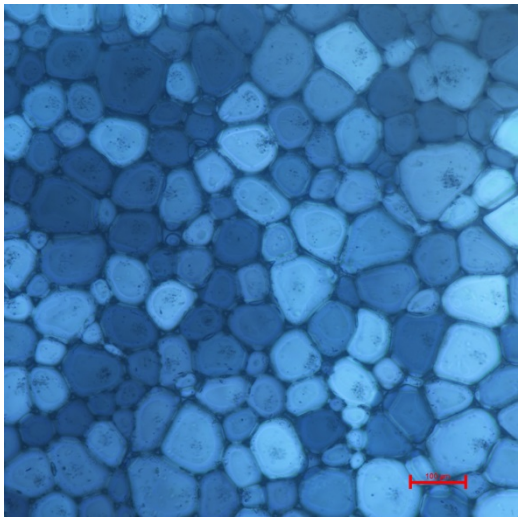

Fig. S1 Representative polarized light microscopy images of ice wafers for PBS buffer (pH = 7.3) after 30 min annealing at -6°C. The scale bar was 50 μm.

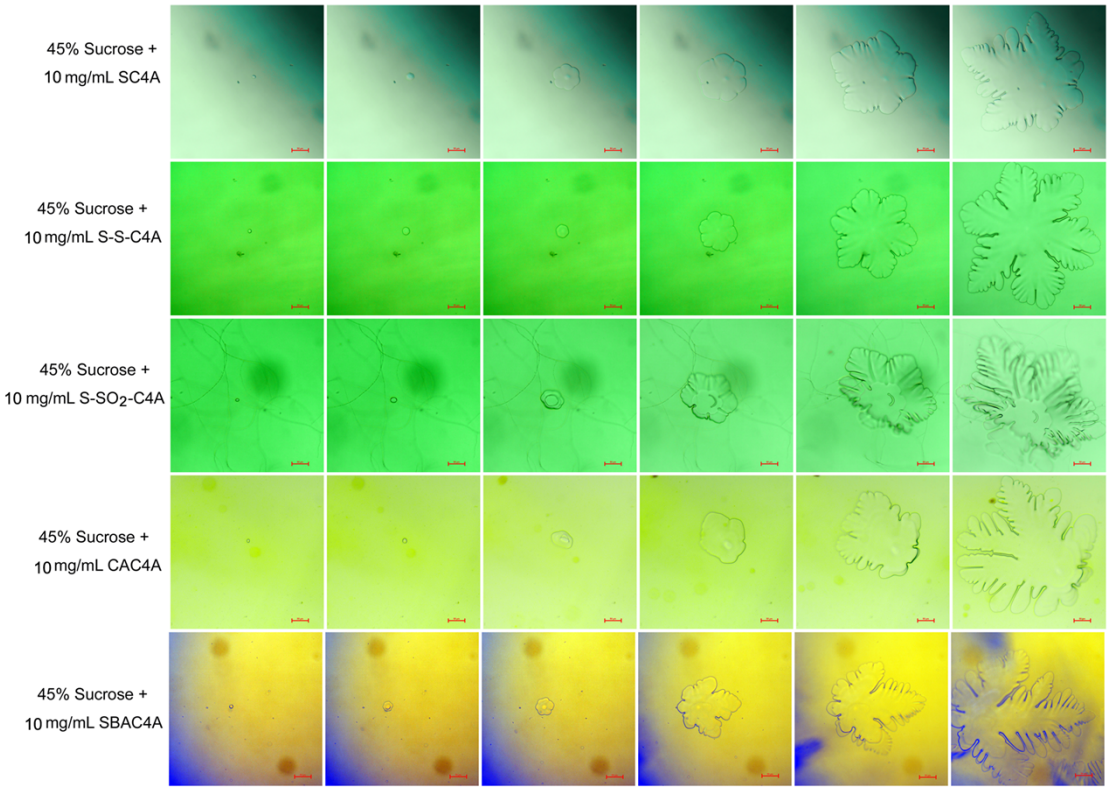

Fig. S2 Sucrose-assisted DIS experiment of macrocycle molecules (10 mg/mL) along

with 45 wt% sucrose. The scale bar was 50 μm.

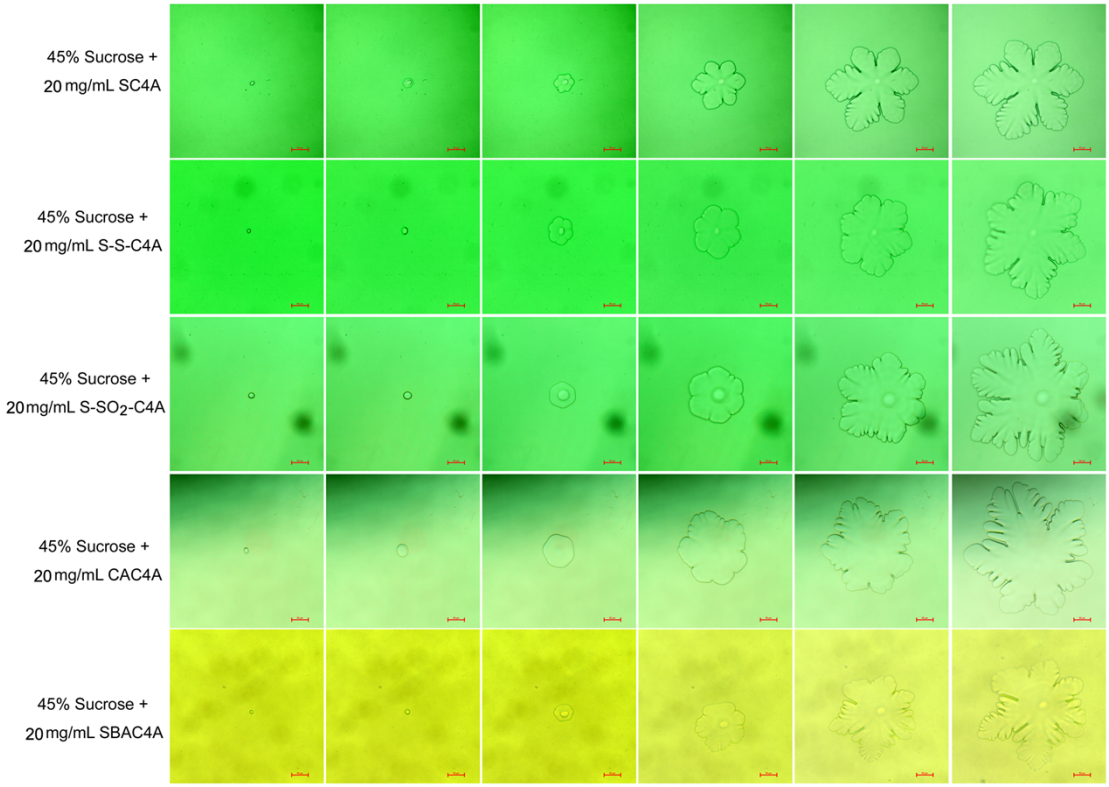

Fig. S3 Sucrose-assisted DIS experiment of macrocycle molecules (20 mg/mL) along with 45 wt% sucrose. The scale bar was 50 μm.

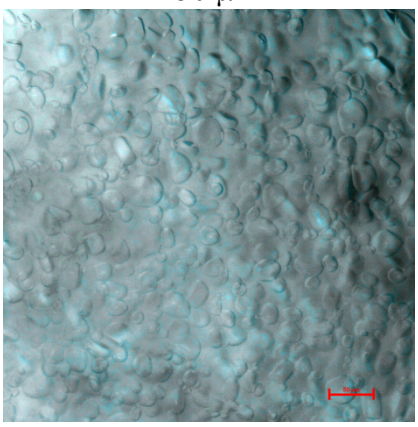

Fig. S4 Microphotograph of 45 wt% sucrose annealed at -8.0°C for 60 min. The scale bar was 50 μm.

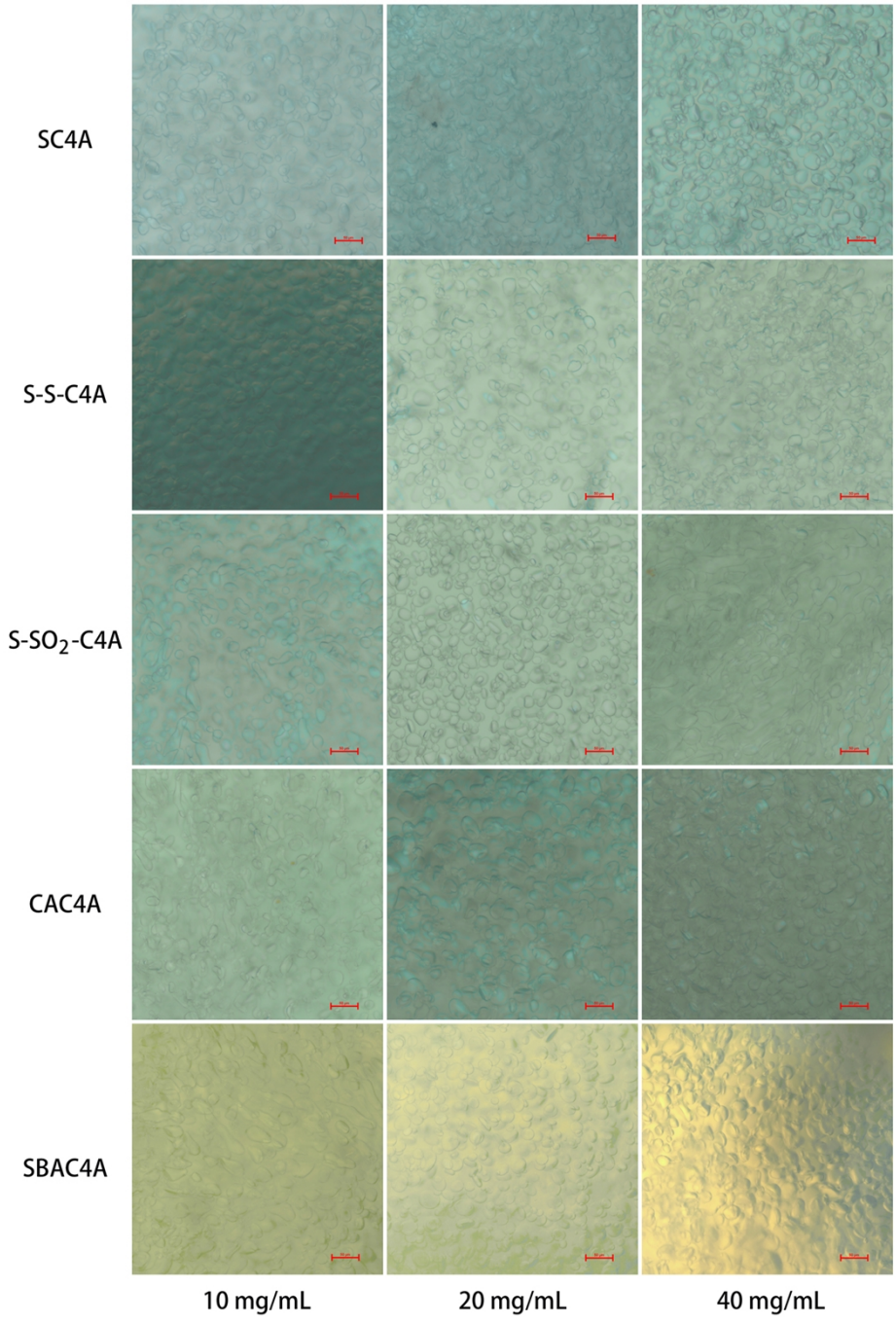

Fig. S5 Microphotographs of 45 wt% sucrose annealed at -8.0°C for 60 min with different concentrations (10, 20 and 40 mg/mL) of calixarene-based CPAs (**SC4A**, **S-S-C4A**, **S-SO_2-C4A**, **CAC4A** and **SBAC4A**). The scale bar was 50 μm.

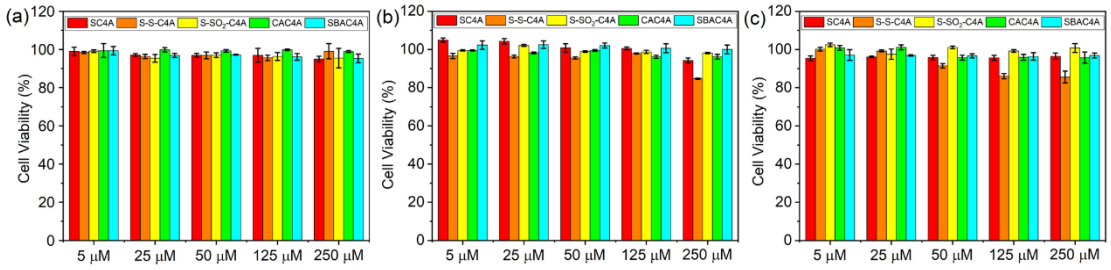

Fig. S6 Cell viability of calixarene-based CPAs (**SC4A**, **S-S-C4A**, **S-SO$_2$-C4A**, **SBAC4A**, and **CAC4A**) against MCF-7 (a), hucMSCs cells (b) and A549 (c) cells at different concentrations (5.0, 25.0, 50.0, 125.0 and 250.0 μM). Each value represents the mean ± SEM (n = 6).

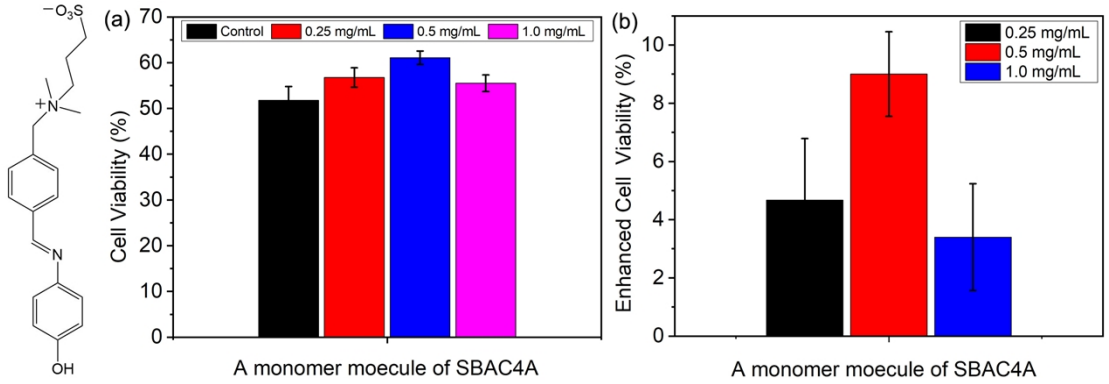

Fig. S7 The structure of a monomer molecule of **SBAC4A**, and its cell viability (a) and its enhanced cell viability (b) of hucMSCs cells: cryopreserved for 24 h in the solution of 2.5% DMSO, 87.5% DMEM and 10% FBS (control), and containing 0.25 mg/mL, 0.5 mg/mL and 1.0 mg/mL. Each value represents the mean ± SEM (n = 6).