Supplementary Information

Mitochondria Targeting IR780-based NanoGUMBOS for Enhanced Selective Toxicity Towards Cancer Cells

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Experimental Methods

Materials. IR780 iodide, trifluoromethanesulfonate (OTf) sodium salt (98%), sodium L-ascorbate (98%) (Asc), dichloromethane (DCM), chloroform, methanol, and anhydrous acetonitrile were purchased from Sigma Aldrich and used as received. Lithium bis(perfluoroethylsulfonyl) imide (BETI) was obtained from Ionic Liquids Technologies (Tuscaloosa, AL). Cell viability MTT (3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) and Mitochondrial ToxGloTM Assay kits were purchased from Promega Corporation (Madison, WI). Triply deionized water (18.2 MΩ•cm) from an Elga model PURELAB ultra water filtration system (Lowell, MA) was used for ion exchange reaction and nanoGUMBOS preparations. A model 08849-00 cleaner (Cole-Parmer Instrument Company) was used for preparation of nanoGUMBOS.

Synthesis of IR780-based GUMBOS and characterization. The IR780-based GUMBOS were synthesized by an anion exchange method. First, 1 to 1.2 molar ratio of [IR780][I] and the counter-ion salts were dissolved in a mixture of dichloromethane (DCM) and deionized water (2:1, v/v) and allowed to stir for 48 h at room temperature (Figure 1). The organic phase was washed with fresh water several times to remove any byproducts. The organic layer was removed in vacuo, followed by freeze-drying to remove traces of water. The obtained GUMBOS were confirmed by use of ESI-MS (Table S1 and Figure S2). Hydrophobicity of each GUMBOS was determined by use of octanol-water partition. A standard calibration curve of peak absorbance (y=790 nm) versus concentration of GUMBOS in 1-octanol was constructed by using
a UV/VIS spectrometer (PerkinElmer, Lambda 750). A known concentration of IR780-based GUMBOS in 1-octanol was then mixed with equal volume of water and shaken for 24 h. The upper 1-octanol layer was analyzed by use of UV/Vis near-IR spectrophotometer and quantified using the standard calibration curve. The equation, \( K_{(o/w)} = \frac{[\text{GUMBOS}] \text{in octanol}}{[\text{GUMBOS}] \text{in water}} \), was used to calculate the partition coefficient. It is worth noting that the 1-octanol used in the partition experiments was pre-saturated with water overnight before use to correct for mutual solubility of the two solvents.

**Synthesis of IR780-based nanoGUMBOS and characterization.** NanoGUMBOS were prepared by use of a simple and additive-free reprecipitation method. Typically, 20 µL of various concentration of GUMBOS dissolved in DMSO was rapidly injected into 980 µL of cell media (DMEM containing 10% fetal bovine serum), followed by sonication for 5 min in an ultrasonic bath (55 kHz). These nanoGUMBOS were aged in the dark for 30 mins to complete growth. Formation of nanoGUMBOS can be easily controlled by concentration of GUMBOS in DMSO, volume ratio of solvent (DMSO) and non-solvent (cell media), as well as sonication time. For nanoGUMBOS characterization, the average particle size and morphologies of prepared nanoGUMBOS were determined by use of transmission electron microscopy (TEM). These TEM micrographs were recorded using an LVEM 5 transmission electron microscope (Delong America, Montreal, Canada). A few microliters of nanoGUMBOS suspension were drop-casted on a carbon-coated copper grid and allowed to air-dry at room temperature. Upon drying, the grids were then washed with fresh water several times to remove cell media and left to dry before TEM imaging. Dynamic size of nanoGUMBOS at physiological pH 7.4 were also measured via Dynamic light scattering (DLS) by a Zetasizer Nano ZS (Malvern Instruments, U.K.).
NanoGUMBOS in cell media were centrifuged down at 35K rpm, using a Beckman ultra-centrifuge, to obtain nanoparticle pellets, which were then re-suspended in 0.01M phosphate buffered saline (PBS) for measurement. Zeta potential measurements in 0.01M PBS with ionic strength 0.15M at various pH (pH=7.4 and pH=6.5) were also performed by use of Zetasizer Nano ZS.

**Spectroscopic studies of GUMBOS and nanoGUMBOS.** All absorbance measurements were conducted using a UV/VIS spectrometer (PerkinElmer, Lambda 750). All fluorescence emission spectra were obtained using a Spex Fluorolog-3 spectrofluorimeter (model FL3-22TAU3); Jobin Yvon, Edison, NJ). A 1 cm quartz cuvette (Starna Cells) was used to collect the absorbance and fluorescence relative to an identical cell filled with relevant solvent as the blank. Each GUMBOS was dissolved in acetonitrile to make 2 µM solutions that were used for characterization of their spectral behavior including absorption and fluorescence. Nanoparticles formed in serum-DMEM were diluted to 20 µM for measurements. Serum-DMEM solution without nanoparticles was also measured as a control. Colloidal stability of nanoparticles in serum-DMEM was monitored over 48 h by recording their absorption spectrum at different time intervals.

**Cell culture.** Hormone-independent human breast adenocarcinoma (MDA-MB-231, ATCC no. HTB-26), hormone-dependent human breast adenocarcinoma (MCF7, ATCC no. HTB-22), normal human breast fibroblast (Hs578Bst, ATCC no. HTB-125), normal human breast epithelial (HMEC, ATCC no. PSC-600-010) and hormone-dependent pancreatic adenocarcinoma (MIA PaCa-2, ATCC no. CRL-1420) cell lines were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA). All cell lines were cultured in a humidified incubator at 37 °C...
with 5% CO\textsubscript{2} and grown to 90% confluence as per ATCC’s specifications before use in further experiments.

**Cell viability assay.** Cytotoxicity of synthesized nanoparticles was assessed on different cell lines by use of MTT assay (Promega Corporation, Madison, WI, USA). Briefly, 100 µL MDA-MB-23, MCF-7, Mia Paca-2 and Hs578Bst were seeded in 96-well plates (~5000 cells/well) and allowed to grow 24 h for cell adhesion at 37 °C and 5% CO\textsubscript{2} in a humidified incubator. Old culture medium was removed, and 0.1 mL of new culture medium containing various concentrations of nanoGUMBOS (0-12.5 µM) was introduced to the cells. After incubation with nanoGUMBOS for 48 h, cells were washed twice with fresh cell media and 18 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added. After an additional 2 h of incubation, 100 µL of stop buffer solution (SDS/HCl) was added to each well and incubated overnight. Cell viability was determined by measuring the absorption at 570 nm using a microplate reader (PlateReader AF2200, Eppendorf). Cell viability was then calculated as the percentage of absorbance from treated cells subtracted from the background absorbance, and absorbance of untreated cells (control) subtracted from the background absorbance such that the control was 100% cell viability. IC\textsubscript{50} value was calculated by use of non-linear regression with least square fit in the GraphPad Prim 7 software.

**Fluorescence microscopy.** Fluorescence images of live cells were recorded on a fluorescence microscope (Leica, TCS SP5, Mannheim, Germany) equipped with a 40× water-dipping objective. Briefly, 200K cells in 2 ml of cell media were seeded on a 35 mm glass cover slip-bottomed petri dish (10 mm micro cell; Ashaland, MA, USA) and allowed to grow for 24 h for cell adhesion at 37 °C and 5% CO\textsubscript{2} in a humidified incubator. To study co-localization of nanoGUMBOS with
mitochondria, 10 nM of the MitoTracker Green (Beyotime Biotechnology Co., Ltd.) was used to stain mitochondria for 30 min. Cells were then washed several times with fresh media, followed by introduction of 1 µM nanoGUMBOS. After incubation for 30 min, cells were washed several times with PBS and studied by fluorescence microscopy. A GFP filter tube (excitation: 450 nm to 490 nm; emission: 500 nm to 550 nm) was used for MitoTracker Green, and a CY7 filter tube (excitation: 672 nm to 748 nm; emission of 765 nm to 855 nm) was employed for IR780-based nanoGUMBOS. Multicolor images were captured by high-speed frame sequential imaging. Merged fluorescence images of MitoTracker Green dye with nanoGUMBOS were used for the determination of sub-cellular localization of nanoGUMBOS. The same protocol has been used for determination of cellular uptake of nanoparticles into cancer and normal cells.

**Quantitative cellular uptake measurement.** Cellular uptake of different nanoGUMBOS into MDA-MB-231 cancer cells were quantified by use of UV/VIS spectrometer (PerkinElmer, Lambda 750) using similar protocol reported in literature. In this case, 12.5 µM of nanoGUMBOS in 2 mL cell media were added to a petri-dish with ~200,000 cells. After incubation for 4 h, free particles in cell media were removed and cells were washed thoroughly at least three times with PBS. The cells were digested by use of 3.5 mL DMSO, leading to the exposure and dissolution of internalized nanoGUMBOS in this DMSO solution. The resulting solution was analyzed by measuring absorbance from the GUMBOS against a DMSO-digested untreated cell reference and quantitated using a standard calibration curve.

**Determination of mitochondrial responses.** A Mitochondrial ToGloTM Assay kit, developed by Promega Corporation (Madison, WI), was used to determine whether the drug is mitochondrial toxin. This assay was performed in accordance with the manufacturer’s protocol. The
experiment consists of two parts: 1) membrane integrity, and 2) ATP level after introduction of the test compound. Cell membrane integrity (MI) was measured using a fluorogenic peptide substrate (bis-AAD-R110). This substrate cannot across the membrane of live cells, and therefore, produces fluorescence that is typically proportional to dead cells (cytotoxicity). Cellular ATP levels was measured by use of the ATP detection reagent, which leads to viable cell lysis and generate a luminescent signal proportional to the present ATP amount. Briefly, 10,000 cells/well of MDA-MB-231 were plated using standard media in a white clear 96-well plate (Falcon®) and allowed to grow for 24 h for cell adhesion. To restrict ATP production to oxidative phosphorylation, cells were washed in serum-free, glucose-free, and galactose supplemented DMEM medium. All test compounds were prepared in this medium. After washing, cells were incubated with test compounds containing various concentrations (0-100 µM) for 2 h. Subsequently, 20 µL of a 5x diluted fluorogenic peptide substrate (bis-AAF-R110) was added to each well and mixed by orbital shaking for 1 min at 600 rpm to ensure reagent/sample homogeneity. After 30 min of incubation at 37 °C, fluorescence was measured at 530 nm using excitation at 485 nm by use of Wallac 1420 Victor2 microplate reader (Perkin Elmer) to assess membrane integrity (cytotoxicity). To determine the amount of ATP, the sample plate was equilibrated to room temperature for 5-10 min. Then, 100 µL of ATP detection reagent were introduced to each well and mixed on an orbital shaker at 600 rpm for 5 min. Luminescence was measured for determination of ATP level. Data was expressed as percentage of vehicle control containing no test compound.

**Bio-TEM preparation.** To study the intracellular stability of nanoparticles, bio-TEM was employed. In brief, 1 µM of nanoparticles were incubated in 200,000 cells/dish for 30 min. The
cells were washed with PBS, scrapped from the culture plates, and collected into microcentrifuge tubes. After that, fixative including 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH=7.4) were added into each tube and wait for 10 min. The cell suspension was then centrifuged, followed by removal of the supernatant. Same fresh fixative was added into the tube on shaker for another 2 h and centrifuged again. The obtained cell pellets were mixed with equal amount of 3% agarose, then transferred to a LM slide and was cut to cubes after it solidified. The cell cubes were washed with phosphate buffer containing 0.08M glycine several times. Following that, cells were fixed by 2% osmium tetroxide in phosphate buffer in dark for 2 h and rinsed with deionized water. Then, cells were dehydrated through a graded ethanol series for 20 min each, and infiltrated with 1:1 EtOH and LR white and 100% LR White for 2 h, respectively. Cell samples were then embedded in LR white and polymerized in an oven at 65 °C for 24 h. Ultra-thin sections (90nm) for TEM were cut on a Leica EM UC7 Microtome, and then transferred to a copper grid for Bio-TEM observation.
Figure S1. Chemical structures of anions used: (a) ascorbate (Asc), (b) trifluoromethansulfonate (OTf), (c) bis(perfluoroethylsulfonyl) (BETI)
Figure S2. ESI-mass spectra of all GUMBOS at positive mode and negative mode.

Figure S3. Z-averaged diameter and PDI for IR780-based nanoparticles through DLS at 25 °C.
Figure S4. Absorbance spectra corresponding to IR780-based nanoparticles in serum-DMEM as a function of time showing transition from J-aggregation ($\lambda = 820$ nm) to randomly oriented aggregation ($\lambda = 790$ nm) at the beginning of the experiment.

Figure S5. Characterization of particle sizes and morphology for IR780-based nanoparticles after 48 h storage in cell culture medium using TEM, all scale bars represent 500 nm.
Figure S6. Cell viability of HMEC and MCF-7 cell lines upon treatment after being incubated with IR780-based nanoparticles for 48 h. For some points, the error bars are shorter than the symbol, and thus are not visible in the graph. Statistical significance analysis was assessed by SPSS via one-way ANOVA test; (**P ≤ 0.05, ***P ≤ 0.001, ****P ≤0.0001)
Figure S7. Cellular uptake of IR780-based nanoparticles in MDA-MB-231 cancer cells and Hs578Bst normal cells. Fluorescence microscope was used to detect the accumulation of nanoparticles.

| GUMBOS  | Theoretical mass (positive mode) | Experimental mass (positive mode) | Theoretical mass (negative mode) | Experimental mass (negative mode) |
|---------|----------------------------------|-----------------------------------|----------------------------------|----------------------------------|
| [IR780][Asc] | 540.2 | 539.3 | 175.2 | 175.0 |
| [IR780][OTf] | 540.2 | 539.2 | 149.1 | 149.0 |
| [IR780][BETI] | 540.2 | 539.3 | 378.1 | 379.9 |

Table S1: ESI-mass spectrometry analysis of all GUMBOS
### Table S2: Yields, melting points, logarithm of 1-octanol/water partition coefficients of IR780 and its GUMBOS.

|                | [IR780][I] | [IR780][Asc] | [IR780][OTf] | [IR780][BETI] |
|----------------|------------|--------------|--------------|---------------|
| Yields (%)     | N/A        | 90           | 95           | 97            |
| Melting point (°C) | 232.5     | 246.3        | 212.6        | 150.8         |
| Log(P)         | 0.93 ± 0.07| 0.34 ± 0.09  | 0.74 ± 0.03  | 1.3 ± 0.1     |

### Table S3: Zeta potential of IR780-based nanoparticles in PBS buffer with ionic strength I=0.15M

|                | [IR780][I] | [IR780][Asc] | [IR780][OTf] | [IR780][BETI] |
|----------------|------------|--------------|--------------|---------------|
| pH = 7.4       | -26 ± 2 mV | -25 ± 3 mV   | -22 ± 5 mV   | -24 ± 4 mV    |
| pH = 6.5       | -15 ± 5 mV | -26 ± 2 mV   | -16 ± 4 mV   | -21 ± 5 mV    |

### Table S4: Z-averaged diameter for IR780-based nanoGUMBOS through DLS at 37 °C

|                | [IR780][I] | [IR780][Asc] | [IR780][OTf] | [IR780][BETI] |
|----------------|------------|--------------|--------------|---------------|
| pH = 7.4       | 151 ± 10 nm| 85 ± 10 nm   | 176 ± 14 nm  | 167 ± 8 nm    |
| pH = 4         | 221 ± 4 nm | 215 ± 8 nm   | 245 ± 7 nm   | 276 ± 11 nm   |

### Table S5: Pearson’s coefficients for co-localization of IR780-based nanoGUMBOS and MitoTracker green calculated using Fiji Coloc2

|                | [IR780][I] | [IR780][OTf] | [IR780][Asc] | [IR780][BETI] |
|----------------|------------|--------------|--------------|---------------|
| Pearson’s coefficient | 0.91       | 0.88         | 0.77         | 0.92          |
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