Supplementary Information

Anti-apoptosis Effect of Amino Acid Modified Gadofullerene via Mitochondria Mediated Pathway

Tong Yu[1,2], Mingming Zhen[1,2,], Jie Li[1,2], Yue Zhou[1,2], Haijun Ma[1,2], Wang Jia[1,2], Chunru Wang[1,2*]*

[1]Beijing National Laboratory for Molecular Sciences, Key Laboratory of Molecular Nanostructure and Nanotechnology, CAS Research/Education Center for Excellence in Molecular Sciences,Institute of Chemistry,Chinese Academy of Sciences, Beijing 100190, China

[2]University of Chinese Academy of Sciences, Beijing 100049, China

E-mail: zhenmm@iccas.ac.cn, crwang@iccas.ac.cn

Contents

1. Experimental Section

1.1. Materials and Cell Culture. Solid Gd@C_{82} was purchased from Xiamen Funano New Material Technology Co., Ltd (Xiamen, China). β-Alanine and 2,2′-(1,8-Dihydroxy-3,6-disulfonaphthylene-2,7- bisazo) bisbenzenearsonic acid (Arsenazo Ⅲ), 5,5-dimethyl-1-pyrroline N-oxide (DMPO) and H_2O_2 were purchased from Sigma-Aldrich (Shanghai, China). Gadolinium standard solution was purchased from Guobiao (Beijing) Testing & Certification Co., Ltd (Beijing, China). Human liver cell line L02, obtained from National Infrastructure of Cell Line Resource (Beijing, China), was cultured in RPMI-1640 (Corning, USA) supplemented with 10% fetal bovine serum (FBS, Corning, USA) and 1% (v/v) penicillin-streptomycin (Corning, USA) in a humidified atmosphere with 5% CO_2 at 37℃. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo China Co., Ltd (Shanghai, China). 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) was purchased from Thermo Fisher Scientific (China) Co., Ltd (Shanghai, China). Annexin V-FITC/PI Apoptosis Detection Kit and Mitochondrial Membrane Potential Assay Kit with JC-1 were purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). The antibodies anti-Bcl-2, anti-Bax, anti-Bcl-xL and anti-caspase 3 were purchased from Abcam Trading (Shanghai) Company Ltd (Shanghai, China).

1.2. Preparation and Characterization of GF-Ala. The metallofullerene Gd@C_{82} was synthesized via a nucleophilic reaction of β-alanine and hydroxyl with Gd@C_{82}. Solid Gd@C_{82} were grinded for 10, 30, 60 and 90 minutes in advance and then observed by scanning electron microscopy (SEM, JEOL JSM-6701F, Japan). Then, Gd@C_{82} (50 mg) was suspended in 14% sodium hydroxide solution (50 mL) with β-alanine (3.6 g) for 2 h at 80℃ with a stirring speed of 1500 r/min. After reaction, the solutions were quenched and centrifuged to remove unreacted particles. The concentration of gadolinium ions in solution was detected by UV-Visible/NIR absorption spectroscopy (Hitachi UH4150, Japan) with arsenazo Ⅲ as chromogenic agent (Fig. S1a). The total concentration including GF-Ala and Gd^{3+} was determined by inductively coupled plasma mass spectroscopy (ICP-MS, Agilent

7700X, USA). The percentage of GF-Ala was expressed as the following equation:

$$\text{yield of GF - Ala} = \frac{\text{CICP} - \text{CUV}}{\text{CT}} \times 100\%$$

where C_{ICP} represents total concentration including GF-Ala and Gd^{3+} detected by ICP-MS, C_{UV} represents the concentration of Gd^{3+} detected by UV-Vis, and C_T represents theoretical concentration of GF-Ala.

Before characterization and cell experiments, the reaction solution was dialyzed against deionized Milli-Q water (Millipore Milli-Q Integral 5 system, USA) to remove Gd^{3+} and saline ingredient and then lyophilized. The chemical composition of GF-Ala was determined by elemental analysis (Thermo Finnigan, Flash EA 1112, USA), thermogravimetric analysis (TGA, Shimadzu DTG-60H, Japan), X-ray photoelectron spectroscopy (XPS, Thermo Scientific ESCALAB 250Xi, USA) as previously described. The size of GF-Ala was determined by dynamic light scattering (DLS, Malvern Zetasizer Nano ZS90, England).

1.3. Measurement of Hydroxyl Radical Scavenging Activity. The hydroxyl radical scavenging activity of GF-Ala was determined by electron paramagnetic resonance (EPR, Bruker ESP 300, USA) spin-trapping technique. Under irradiation of ultraviolet light (500 W), reaction solution containing fresh 1 mM DMPO (40 μL), 100 mM H_2O_2(20 μL) and ultrapure water (20 μL) could generate hydroxyl radical and produce DMPO-OH with EPR signal. Taking place of ultrapure water, 120 μM GF-Ala (20 μL) could compete with DMPO to capture hydroxyl radical. After 4 min of irradiation, the X-band EPR spectrum of DMPO-OH was recorded in the dark.

1.4. Analysis of Cell Viability. The cytotoxic activity of GF-Ala was assessed by Cell Counting Kit-8 assay as described previously. L02 cells were seeded onto 96-well plates with 1.0×10^4 cells in 200 μL medium per well in the dark for 24 h. Then cells were incubated with different concentration of GF-Ala in complete medium for 24 h. Thereafter, the solutions were discarded, and the cells were washed twice with phosphate buffer solution (PBS). Next, CCK-8 solution was added to each well and incubated at 37 ℃ for 1 h. Finally, the absorbance of each well was determined at 450 nm using the microplate reader (iMark microplate reader, Bio-RAD, USA). The viabilities of treated groups were expressed as percentages of control group, which was assumed to be 100%.

H_2O_2 induced oxidative damage on L02 cells was used for evaluating the protective activity of GF-Ala. L02 cells were seeded onto 96-well plates by the method described above. After 24 h, cells were incubated with different concentration of GF-Ala for 3 h, and then exposed to 200 μM H_2O_2 for 1 h. After that, the cell viabilities were determined by CCK-8 assay.

1.5. Measurement of Intracellular ROS levels. The intracellular ROS levers were determined using CM-H_2DCFDA as oxidation-sensitive fluorescent probe. L02 cells were cultured into 6-well plates with 2.0×10^5 cells in 2 mL medium per well at 37℃ for 24 h. The cells were preincubated with GF-Ala prepared in complete culture

medium at 25 μM, 50 μM and 100μM concentration for 3 h. Then the medium was exchanged for 500 μM H_2O_2 solution in culture medium for 1 h. Then L02 cells were washed twice with PBS and loaded with 10 μM CM-H_2DCFDA in serum-free media (1 mL) for 30 min at 37℃. Subsequently, treated cells were rinsed with PBS for three times, collected by centrifugation and resuspended in 500 μL PBS. Finally, CM-H_2DCFDA fluorescence was measured by flow cytometry (Life, USA) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Besides, L02 cells were seeded on a 35 mm confocal dish with 1.0×10^5 cells in 1 mL medium and treated with the same methods of cell culture and stain as above. Finally, stained cells were rinsed and visualized with laser scanning confocal microscopy (Olympus FV1000-IX81, Japan).

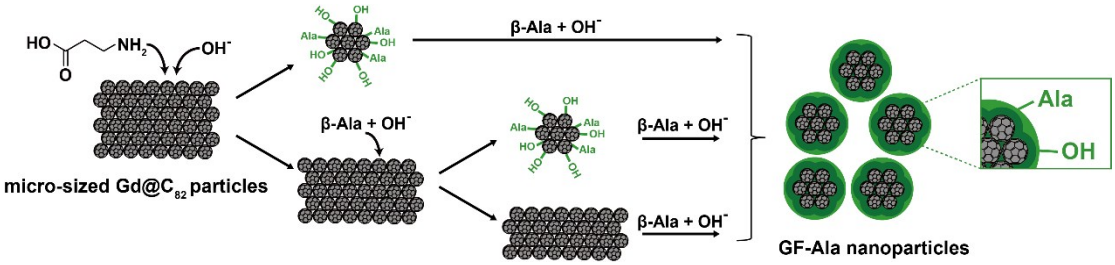
1.6. Apoptosis Analysis. Cell apoptotic rates were analyzed by flow cytometry using Annexin V-FITC/PI Apoptosis Detection Kit. In brief, after seeded into 6-well plates at a density of 2.0×10^5 cells per well for 24 h, L02 cells were incubated with GF-Ala at 25 μM, 50 μM and 100μM concentration for 3 h and exposed to 500 μM H_2O_2 for at least 1 h. Then the treated cells were harvested by trypsinization without EDTA, rinsed twice with Binding buffer provided with the assay kit and resuspended in Binding buffer at a density of 1.0×10^6 cells/mL. Every 100 μL cell suspension was mixed with 5μL Annexin V-FITC and incubated at room temperature in the dark for 10 min. Then 5 μL PI was added to the stained cells and incubated at room temperature in the dark for 5 min. Finally, the stained cells were diluted to 500 μL with PBS and examined by flow cytometry at an excitation wavelength of 488 nm and emission wavelength of 530 nm.

1.7. Determination of Mitochondrial Membrane Potential (MMP, ΔΨm). The mitochondrial membrane potential was measured by flow cytometry using Mitochondrial Membrane Potential Assay Kit with JC-1. Briefly, L02 cells (2.0×10^5 cells per 2 mL per well) were cultured into 6-well plates at 37℃ for 24 h. After incubation with GF-Ala at 25 μM, 50 μM and 100μM concentration for 3 h, the cells were exposed to 500 μM H_2O_2 for at least 1 h. Then treated cells were rinsed and incubated in complete medium containing 5.0 μg/mL JC-1probe at 37℃ for 20 min. Thereafter, the cells were collected, rinsed and resuspended in 500 μL PBS for the flow cytometric analysis at an excitation wavelength of 488 nm and emission wavelengths of 530 and 590 nm as previously described.

1.8. Western Blotting. Expression of Bax, Bcl-2, Caspase 3 and PARP was examined by Western blotting. After the same treatment as above, cells were rinsed, harvested and extracted with protein lysis buffer. Then the lysates were centrifuged at 12,000 rpm for 10 min at 4℃. Protein extractions was separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, USA). After blocking for 1 h by 5% skimmed milk, membranes were incubated with primary antibodies, followed by incubation with secondary antibodies. Protein bands were detected using ECL by chemiluminescence detector (LIUYI WD-9413B, China).

2. Scheme of the synthesis of GF-Ala via a solid-liquid reaction.

As shown in Scheme R1, the GF-Ala nanoparticles were synthesized by Top-Down approach from micro-sized Gd@C_{82} particles. At first, β-alanine and hydroxy groups were directly bonded to Gd@C_{82} molecules at the particle surface via nucleophilic attack of amino group and hydroxyl ions. After grafted with a few of hydrophilic groups, Gd@C_{82} molecules were stripped from the particle and formed molecular clusters in solution, followed by further reaction with β-alanine and hydroxy groups. At the same time, Gd@C_{82} molecules at the newly-exposed surface began to react with β-alanine and hydroxy groups. Finally, Gd@C_{82} molecule clusters were grafted with lots of β-alanine and hydroxy groups. Because of hydrogen bond formation between hydroxyls and amino groups, Gd@C_{82} molecule clusters aggregated into GF-Ala nanoparticles.



Scheme S1. The schematic diagram of the synthesis of GF-Ala nanoparticles via a solid-liquid reaction.

3. Measurement of concentration of Gd^{3+} in reaction solution.

Arsenazo Ⅲ was used to determine the concentration of gadolinium ions in water as chromogenic agent by spectrophotometry. At first, gradient of concentration of gadolinium standard solution (200 μl) were added to five tubes containing 0.06wt% arsenazo Ⅲ solution (200 μl) and sodium chloroacetate buffer solution (500 μl, pH 2.9) respectively. Then by diluting to 2ml with water, 0ug/ml, 0.5 ug/ml, 1.0 ug/ml, 1.5 ug/ml and 2.0 ug/ml gadolinium mixture solution were obtained for UV-Visible/NIR absorption spectroscopy.As shown in Fig. S1a, the UV-Vis spectra of the complex of arsenazoⅢand gadolinium ion was significantly different from the one of arsenazo Ⅲ. Arsenazo Ⅲ exhibited a marked absorption band with a maximum at 545 nm, while the complex showed two absorption peaks between 600 and 700 nm. According to the absorbency at 669 nm to concentration of gadolinium ions, the standard curve and linear regression equation were established as $Abs_{(669\ nm)} = 0.37[Gd^{3+}]_{(\mu g/ml)} + 0.23$ with r = 0.999 (Fig. S1b). Then the concentration of Gd^{3+}in GF-Ala solution was determined by the same method.

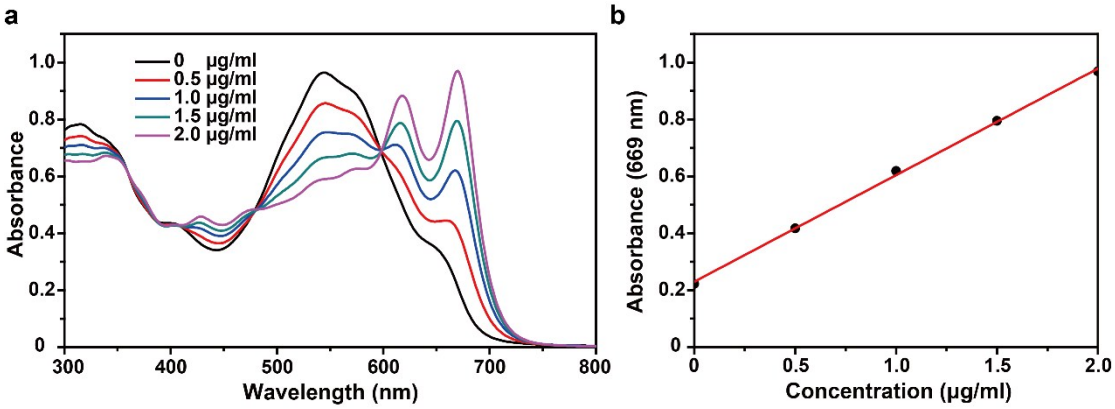


Fig. S1. (a) UV-Vis absorption spectrum of arsenazo Ⅲ after complexion with Gd^{3+} of different concentration. (b) The linear relationship between the absorbance at 669 nm and the concentration of Gd^{3+}.

4. Characterizations of GF-Ala.

Solid GF-AlaNPs were obtained by freeze drying, then characterized by elemental analysis (EA), TGA and XPS. Firstly, according to atom ratio of carbon and nitrogen from EA (Table S1), the average number of amino acid group was calculated to be five per $Gd@C_{82}$. Secondly, TGA was conducted to estimate water content of the sample. According to the weight loss before 200 ℃, physically absorbed water accounted for 8.1 percent (Fig. S2). Next, according to atom ratio of carbon and hydrogen after deducting water, the average number of hydroxyl group was calculated to be nine per $Gd@C_{82}$. Then, XPS was carried out to confirm the chemical composition of GF-Ala. The C_{1s} XPS band at 284.8 eV was assigned to C-C, C=C, C-H and C-N. And the C_{1s} peaks centered at binding energies of 286.5 and 288.8 eV were assigned to C-O and C=O separately. The relative atomic percent contents of three peaks were 82.5%, 9.6% and 7.9% (Table S2). According to the relative atomic percent contents of different C_{1s}, the average number of carbonyl group was calculated to be eight. Five of them belonged to grafted β-alanine and three belonged to $Gd@C_{82}$. Taken together, the average chemical structure of GF-Ala was deduced as $Gd@C_{82}(O)_3(OH)_9(NHCH_2CH_2COOH)_5$.

Moreover, we supplied Fourier transform infrared (FT-IR) spectroscopy and atomic force microscope (AFM) to characterize the structure and size, respectively (Fig. S4). The stretching vibration peaks of C-H (ca. 2750-3000 cm^{-1}) and C-N (ca.1380 cm^{-1}) demonstrated that β-alanine was bonded to $Gd@C_{82}$. And O-H stretching vibration bands at 3430 cm^{-1} proved the existence of hydroxy group. AFM image showed that GF-Ala nanoparticles seemed quite uniform in morphology with a measured average diameter of ~60 nm.

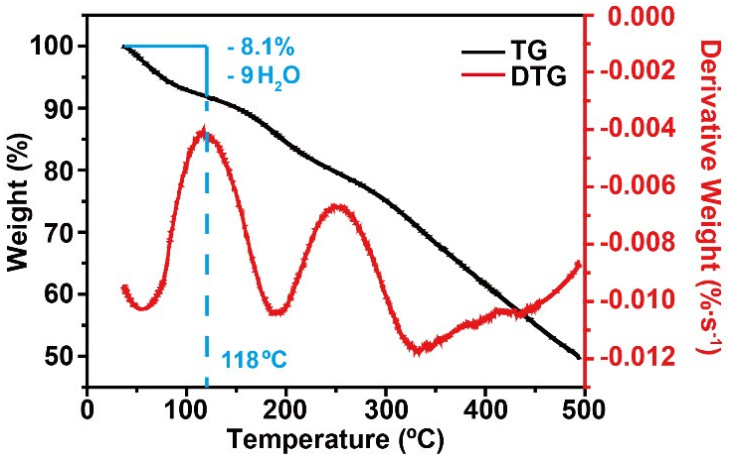


Fig. S2. TGA (black curve) and DTA (red curve) of GF-Ala NPs (under N2, 5 °C/min, 20-500 °C)

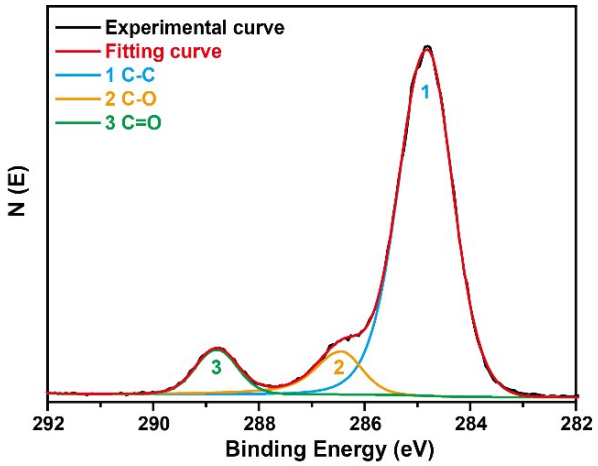


Fig. S3. XPS spectra of C1s of total C in GF-Ala (black line), the Gaussian analysis of C1s of C-C, C=C, C-H, C-N (blue line), C-O (yellow line) and C=O (green line), respectively. Fitting curve is red line.

Table S1. Elemental analysis of carbon, hydrogen and nitrogen contents of GF-Ala NPs

	C (%)	H (%)	N (%)
GF-Ala	56.26	2.80	3.36

Table S2. Fitting of C_{1s} binding energy.

Bond (C1s)	C-C, C=C, C-H, C-N (284.8 eV)	C-O (286.5 eV)	C=O (288.8 eV)
GF-Ala	88747	10362	8502

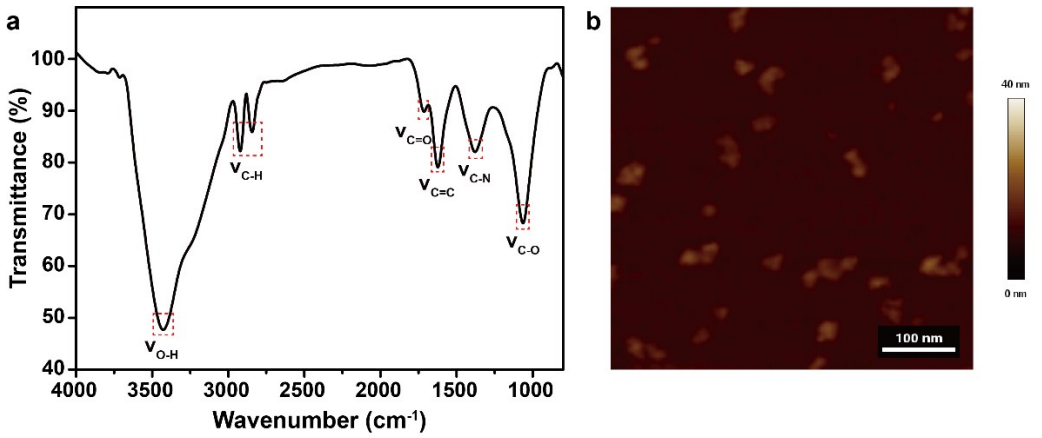


Fig. S4. (a) FT-TR spectra and (b) AFM image of GF-Ala.

5. Intracellular ROS scavenging capability of GF-Ala

We conducted the experiment mentioned in the revision. L02 cells were incubated with 100 μM GF-Ala for 3 h and then stained by CM-H_2DCFDA for analysis with flow cytometry. As shown in Fig. S5, due to the redox homeostasis inside the normal L02 cells without H_2O_2, treatment with GF-Ala alone had no significant effect on intracellular ROS.

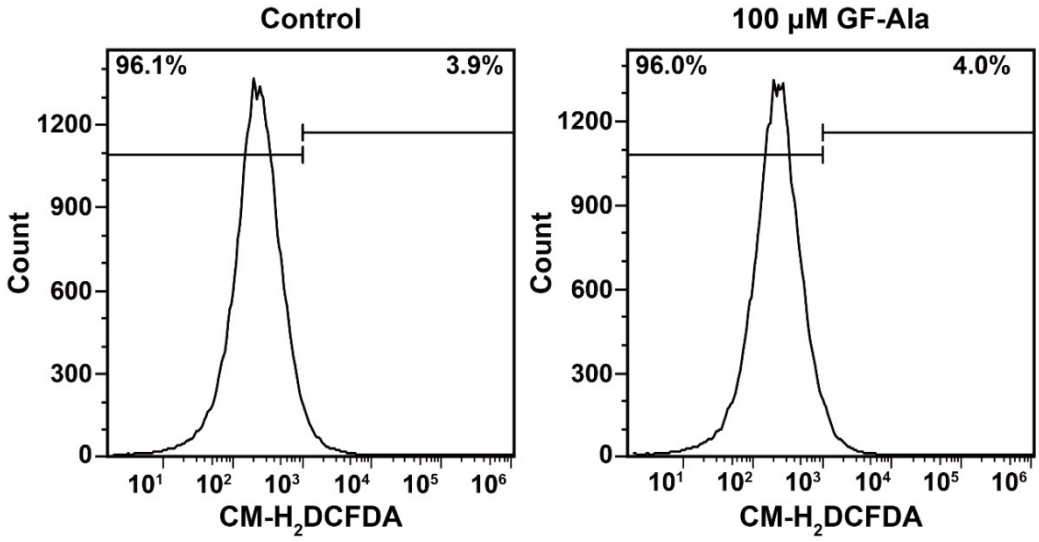


Fig. S5. Flow cytometry results of L02 cells incubated with 100 μM GF-Ala alone for 3 h.