Supporting Information

Multihydroxylated $[Gd@C_{82}(OH)_{22}]_n$ Nanoparticles: Antineoplastic Activity of High-Efficiency and Low-Toxicity

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S1, Preparation and characterization of $Gd@C_{82}$

The metallofullerenes were synthesized using arc discharge method and extracted using a high-temperature and high-pressure method. Separation and purification of $Gd@C_{82}$ were performed using the high performance liquid chromatography (HPLC, LC908-C60, Japan Analytical Industry Co) coupling with 5PBB (Figure S1-A) and then Buckyprep (Figure S1-B) columns (Nacalai Co. Japan). The isolated $Gd@C_{82}$ species were identified by the matrix-assisted laser desorption time-of-flight mass spectrometer (MADLI-TOF-MS, AutoFlex, Bruker Co., Germany), Figure S1-C. The purity of the final $Gd@C_{82}$ product was greater than 99.5%.

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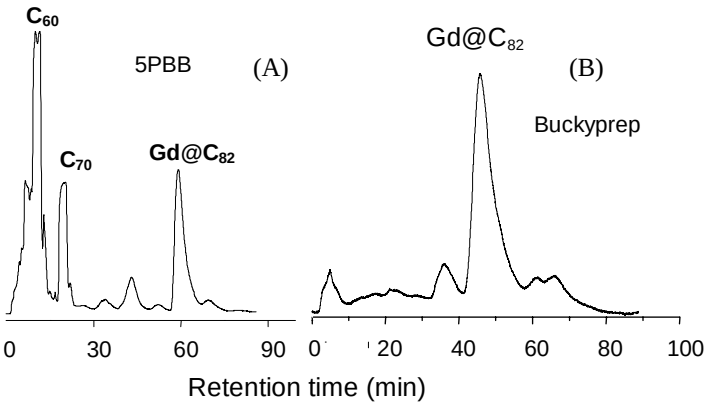


Figure S1, The HPLC chromatogram for $Gd@C_{82}$ in 5PBB columns (A) and Buckyprep columns (B).

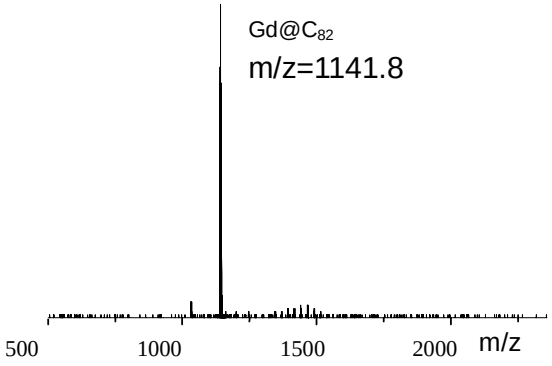


Figure S1-C, Maldi-TOF-mass spectrum of the isolated $Gd@C_{82}$

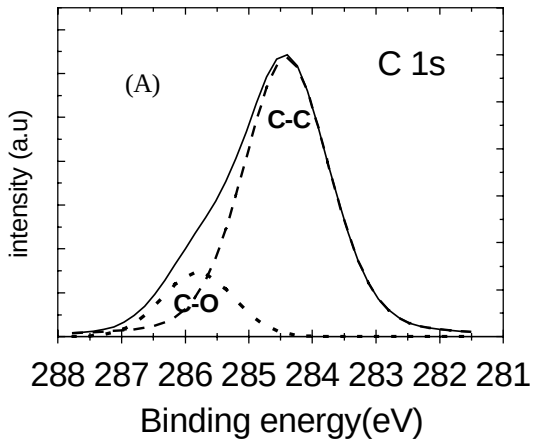
S2, Preparation and characterization of $Gd@C_{82}(OH)_x$

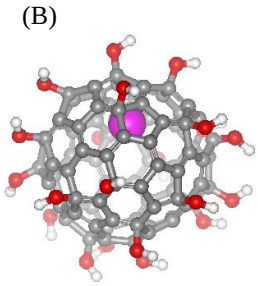
The water-soluble Gd-fullerenol was synthesized by the alkaline reaction. The $Gd@C_{82}$ toluene solution was first mixed with aqueous solution containing 50 % NaOH,

and then several drops of catalyst of 40 % TBAH (tetrabutylammonium hydroxide) were added into the reaction system. The mixture of solutions was vigorously stirred at room temperature, the color of the solution in beaker was changed from the originally deep violet into colorless, meanwhile a brown sludge precipitated onto bottom of the beaker. After adding more water into the brown sludge, it was stirring over night. The brown precipitate was washed using MeOH which was then removed by the vacuum-evaporation system. This washing manipulation was repeated several times for a complete removal of the remnant TBAH and NaOH. Finally, the brown precipitate was dissolved into deionized water with continuous stirring for 24 hrs until the solution colour became a clear reddish brown. Then it was purified by a Sephadex G-25 column chromatography (5x50 cm^2) with an eluent of neutralized water. The remained trace catalyst and Na^+ ions were completely removed in this process. To obtain a final Gd-metallofullerenol product of a narrow region of distribution of the hydroxyl number, the fraction (eluate) was collected in a time interval of only several minutes.

The elemental analysis method was first used to measure the number of hydroxyl groups, giving the hydroxyl number of n=24~27 in $Gd@C_{82}(OH)_n$. Because this is not a precise method, we hence tried to analyze $Gd@C_{82}(OH)_n$ using MALDI-TOF-MS technique, but it is quite difficult to observe the mass peak of molecular ions. Comparing with $Gd@C_{82}$ molecule, $Gd@C_{82}(OH)_n$ showed a tendency of more easy fragmentation. This indicates that the stability of hydroxylated metallofullerene $Gd@C_{82}(OH)_n$ is somewhat declined as compared with $Gd@C_{82}$ of non-hydroxylation. A further measurement for the hydroxyl number was performed using X-ray photoemission spectroscopy (XPS). The samples used in XPS experiment were deposited onto the high purity golden substrates to obtain thin films for the XPS measurements which were carried out at ultra vacuum chamber with background pressure of ~$8x10^{-10}$ Torr, and ~$1x10^{-9}$ Torr during the measurement. The photon with energy hν=400.0 eV from synchrotron radiation was used as the excitation source. The experimental energy resolution was estimated to be ~0.5 eV. To inspect the contamination, XPS survey scans on the surface were performed before and after measurements. Figure S2 show the binding energy spectra of C1s electrons for C-C and C-O bonds in the $Gd@C_{82}(OH)_n$

molecule. For the pure component of C1s electrons, the XPS spectrum should be symmetric and well described by a true Voigt function with a Gaussian dispersion. The Gaussian analysis of the measured XPS data for $Gd@C_{82}(OH)_n$ is also shown in Figure S2. The binding energy spectra of C1s electrons exhibits at least two components: one centred around 284.9 eV is the C1s binding energies of sp^2 non-functionalized carbons (C-C), in good agreement with the value observed from C_{60}, the other centred around 286.1 eV is for hydroxylated carbons (C-OH). The XPS spectra can differentiate the different carbons in the $Gd@C_{82}(OH)_n$ molecule, this provide us with a more precise method to determine the hydroxyl number in $Gd@C_{82}(OH)_n$ based on the intensities for the non-functionalized and hydroxylated carbons. The intensities of C1s components for non-functionalized and hydroxylated carbons in $Gd@C_{82}(OH)_n$ were estimated from integration of the corresponding peak areas under the dot line (hydroxylated carbons, C-O) and the broken-line (non-functionalized carbons, C-C) in Figure S2, respectively. As the total number of carbons is known to be 82, hence from the intensity ratio of sp^2 non-functionalized and hydroxylated carbons (by normalizing them to the total area under the solid curve), we can calculate the number of hydroxylated carbons that are just the number of n. It is ~21, smaller than the value obtained by EA. Taking into account of the ambiguity in both analysis methods, n was finally determined to be 22±2. Thus, the chemical form of Gd-metallofullerenol used for the experiment in vivo is $Gd@C_{82}(OH)_{22}$.





$Gd@C_{82}(OH)_{22}$

Figure S2(A), synchrotron radiation x-ray photoemission spectra for $Gd@C_{82}(OH)_n$. The binding energy spectra of C1s electrons for C-C and C-O bonds in the $Gd@C_{82}(OH)_n$ molecule are shown by thin and bold dotted lines, respectively, which were obtained from Gaussian analysis. (B) The schematic draw of the $Gd@C_{82}(OH)_{22}$ molecule.

S3, The size of $[Gd@C_{82}(OH)_{22}]_n$ nanoparticles.

Figure S3, high resolution atomic force microscopic image of $[Gd@C_{82}(OH)_{22}]_n$ nanoparticles in saline. It was measured by a high resolution Atomic Force Microscopy (Digital Instruments Nanoscope). The average size of the particles was estimated to be about 22.4 nm.

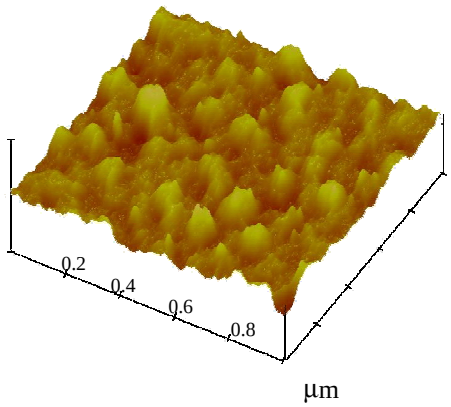


Figure S3, The high resolution atomic force microscopic image of $[Gd@C_{82}(OH)_{22}]_n$ nanoparticles.

S4, Antitumor experiment. Antitumor studies of $[Gd@C_{82}(OH)_{22}]_n$ were performed on female Kunming mice. Animals were housed in a ventilated, temperature-controlled standardized sterile animal room of Chinese Academy of Medical Science. All the animal experiments were conducted under approved protocols of the Institutional Animal Care and Use Committee at the Institute of Tumor in Chinese Academy of Medical Science. The mice were subcutaneously implanted with 1×10^6 cells of H22

hepatoma (in 100 μl of saline) in the right lag leg of each mouse. Primary tumors (before administration of antitumor agents) were measured with calipers. The end point of the experiment was determined by the diameter of the mice's leg loaded with tumor up to 2 or 2.2 centimeter. The growth of tumor size was monitored through measuring the diameter of the tumor every 24 hours. Tumor volumes were then calculated according to the formula: $V=4\pi r^3/3$. Tumor growth curve was obtained by the diameter of the tumor as a function of the time.

The 40 mice of weight ranging from 20 to 22 g (adult and female) were randomly and averagely divided into 6 groups. The tumor-bearing mice were then systemically treated with $[Gd@C_{82}(OH)_{22}]_n$ saline solution by intraperitoneal injections once a day. Two doses of $Gd@C_{82}(OH)_{22}$, 114 and 228 μg/kg corresponding to $1x10^{-7}$ and $2x10^{-7}$ mol/kg, respectively, were used in the experiment. The widely used antineoplastic agent for patients, CTX was used for the positive control, with a dose 30 mg/kg ($1x10^{-4}$ mol/kg, MW 279.1) of the currently clinic use for cancer therapy. Because of its side effects, treatments by 0.1 and 0.05 mmol/kg of CTX continued for the first 7 days. Antitumor agents of $[Gd@C_{82}(OH)_{22}]_n$ or CTX was prepared by dissolving it into 0.9% saline prior to use.

Each mouse was administrated intraperitoneally (i.p.) a single dose of 0.2 ml per day from the second day of inoculation and continued to the day before sacrifice. The change of the tumor size was precisely measured every 24 hours. At the end point of the day, blood was taken out from eyeball of mice for biochemical analyses. As long as the tested mice were killed at the endpoint of experiment, tumors were rapidly removed from mice and weighed immediately.