Supporting Information

Exploring self-assembly mechanism and effective synergistic antitumor chemophototherapy of biodegradable and glutathione responsive ursolic acid prodrug mediated photosensitive nanodrug

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Experimental section

Materials

Ce6 was purchased from Jinan Daen Pharmaceutical Technology Co., Ltd (Shang Dong, China). Ursolic acid (UA), 3, 3'-dithiodipropionic acid (DTPA), dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP) were purchased from Aladdin Reagent Company. Methylene blue (MB), 1, 3-diphenylisobenzofuran (DPBF), 4,6-diamino-2-phenyl-indole (DAPI), Dichlorofluorescin diacetate (DCFH-DA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheny-ltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemical Company. Fetal bovine serum (FBS), penicillin-streptomycin, phosphate buffered saline (PBS), trypsin and RPMI 1640 Medium and Dulbecco modified Eagle's medium (DMEM) were purchased from Hyclone (America) were purchased from purchased from Sigma Gibco (Grand Island, NY, USA). All organic solvents were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) and used without further purification.

Characterization

The NMR experiments were performed on a Bruker DRX-400 (Rheinstetten, Germany) at 400 MHz. UV–Vis spectra were obtained on a TU-1900 PERSEE spectrometer (Beijing, China). Fluorescence emission spectra were performed with an F-2700 spectrometer. A Quanta 200FEG scanning electron transmission (SEM) at 20 kV (Hillsboro, OR, USA) and a Tecnai G2 F20 S-TWIN transmission electron microscope (TEM) (FEI, America) operating at 200 kV were used to observe the size and morphology of samples. Fourier transform infrared (FT-IR) spectra were acquired using KBr disc samples in Nicolet iS5 (Thermo fisher, USA). Dynamic light scattering (DLS) and Zeta potential were carried out with Zetasizer Nano ZS 90 (Malvern, UK). And irradiation was performed using Solar-500 with a Xenon lamp (NBET, Beijing, China) with central wavelengths at 675±10 nm. Cell imaging were acquired on Fluorescent inverted microscope (UOP, DSY-2000X), and MTT evaluation was performed with a AllSHENG AMR-100 absorbance microplate reader.

Ce6 loading efficiency

UASS-Ce6 NPs were first disassembled by dissolving in DMSO and subsequently analyzed by HPLC (Agilent 1200 series liquid chromatography). The reversed phase TC-C18 column (4.6 mm i.d. × 250 mm, Agilent Technologies, USA) connected to an Agilent G1315B UV-Vis Detector was used at 30 °C. For determination of Ce6, the mobile phase was water (0.2% phosphoric acid) and acetonitrile with a volume ratios of 40:60 at a flow rate of 1.0 mL/min. The detection wavelength was 402 nm. . Concentrations of Ce6 was determined by a known standard curve. The drug loading (DL) was calculated by the following equation: DL (%) = mass of Ce6 in nanoparticles/total mass of UASS-Ce6 NPs× 100%.

In vitro release of Ce6

The in vitro release profiles of UASS-Ce6 NPs were studied by dialyzing the nanoparticle aqueous solutions (1.0 mL) placed in a dialysis bag (Mw cutoff=7 kDa). The bag was dialyzed in 50 mL of PBS solution (pH 7.4 or 6.5) under shaking (100 rpm) at 37 ℃ containing 0.05% (v/v) Tween 80. At predetermined time points, 30 μL of sample solutions were taken out from dialysis bags and dissolved in DMSO for determination of residual Ce6 by Uv-vis spectrophotometer. For investigation of the glutathione responsive properties, an additional 1 or 5 mM of GSH was added to PBS (pH 7.4) solution as a GSH-sensitive group.

DFT calculation

The energy-minimized structures of UASS and Ce6 were simulated using the Gaussian 09 (Revision E.01) package [1] by employing the density functional theory (DFT) calculations with the Becke-3-Lee-Yang-Parr (B3LYP) exchange function. The 6-31G (d, p) basis sets were employed. Meanwhile, the monomolecular aggregates UASS@Ce6 was also optimized by the same method. Besides, the energy difference in their lowest singlet (S_1) and triplet (T_1) excited states were further calculated by time-dependent DFT (TD-DFT) theory.

Measurement $^1O_2$ quantum yield

The $^1O_2$ quantum yield was evaluated by employing 1,3-diphenylisobenzofuran (DPBF) decomposition reaction. Typically, 20 μL Ce6 or UASS-Ce6 NPs (equivalent Ce6: 4.3×10^{-5} mol/L) aqueous solution were separately added to 3 mL DPBF solution (6×10^{-5} mol/L) which had been transferred to a quartz cuvette, and then irradiated by 675±10 nm light and measured the absorption at 418 nm every 10s for a period of 50 s using Uv-vis spectrometer. Methylene blue (Φ=0.52) in DMSO was used as the reference compound. And the $^1O_2$ quantum yield (Φ) was calculated using the following equation:

$$\Phi^S = \Phi^R \frac{m^S F^R P^R}{m^R F^S P^S}$$

Where Φ is $^1O_2$ quantum yield, Superscripts *S* and *R* represent the sample and reference compound, respectively. *m* is the slope obtained by plotting absorption change (ΔOD) of DPBF at 418 nm as a function of irradiation time t. *F* is the absorption correction factor at irradiation wavelength ($F = 1 - 10^{-OD}$). P is absorbed photonic flux.

Cell culture

Breast cancer cells 4T1 (mouse) and MCF-7 (human), and normal human hepatocyte cells LO2 were purchased from Shanghai Cell Bank, Chinese Academy of Sciences. MCF-7 and LO2 cells were cultured in DMEM medium with 10% foetal bovine serum (FBS, Gibco) and 1% penicillin–streptomycin antibiotic (Life technology, USA), while 4T1 cells were cultured in RMPI-1640 medium supplemented with 20% FBS containing 1% antibiotic at 37 °C under a 95% humidified atmosphere

with 5% CO_2.

Cell uptake of UASS-Ce6 NPs

In the cellular uptake experiment, 4T1 cells were seeded into a 6-well plate with a density of 1 × 10^5 cells and incubated for 24 h at 37 °C. Then the cells were incubated with free Ce6 or UASS-Ce6 NPs (equivalent Ce6: 2 μg/mL) for various periods (0.5 h, 1 h). After incubation, fixing and DAPI staining of the cells, then imaged under a fluorescent inverted microscope (FIM). Meanwhile, the mean fluorescence intensity of cellular uptake was performed using flow cytometric analysis (FCS) with the same cell treatments as above, and subsequently, 10^4 cells were analyzed using an Accuri C6 flow cytometer (BD, Ann Arbor, MI).

Cellular ROS generation

For intercellular ROS generation detection, a fluorescence probe DCFH-DA was used. In brief, 10^5 4T1 cells were seeded in 6-well plates and incubated for 24 h. After incubation with free Ce6 or UASS-Ce6 NPs (equivalent Ce6: 1.3 μg/mL) for 4 h, then 25 μM DCFH-DA was added and incubated for another 30 min. After washing twice with PBS, the cells were irradiated for 6 min and incubated for another 10 min, and subsequently imaged by FIM with blue light excitation.

Cell apoptosis assay

For apoptosis analysis, Calcein AM/propidium iodide (PI) live/dead staining was performed to visualize the cytotoxicity of UASS-Ce6 NPs. Briefly, 4T1 cells were seeded in 6-well plates and incubated overnight. Then, the cells were treated with free Ce6 or UASS-Ce6 NPs (Eq. Ce6: 1.0 μg/mL) for 4 h and irradiated for 10 min immediately. After washing, staining with Calcein AM (2 μM)-PI (4.5 μM) solutions for 20 min, washing again, the cells were observed immediately under fluorescent inverted microscope.

Biosafety of UASS-Ce6 NPs

For evaluation of biosafety, normal LO2 cells were seeded in 96-well plates. Then, the cells were treated with various concentrations of UASS-Ce6 NPs for 24 h. And cell viability was determined by MTT assays.

Hemolysis analysis

The blood compatibility of UASS-Ce6 NPs was evaluated by Hemolysis analysis. Typically, 800 μL of various concentrations of UASS-Ce6 NPs PBS dispersion and 200 μL of 10 % (v/v) red blood cells PBS solutions were mixed and incubated for 4 h at room temperature. After centrifugation at 13000 rpm for 10 min, the supernatants of each sample were measured the Uv absorption of 577 nm.

Where, the distilled water and PBS were added as positive and negative groups, respectively. The Hemolytic percent was calculated as follows: $[A_{sample}-A_{PBS}]/[A_{water}-A_{PBS}] \times 100\%$. Where A_{water}, A_{Sample}, and A_{PBS} are separately the absorbance of distilled water, each sample, and PBS and distilled water.

Animal models

Female Balb-c (18-22 g, 6-7 weeks old) were purchased from the Animal Center of the Second Affiliated Hospital of Harbin Medical University. All animal experiments were performed under the guidelines for Animal Care and Use of Laboratory Animals, following protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the Harbin Medical University, China. For tumor inoculation, 100 uL 4T1 cells solution (1640 medium, 2×10^6 cells) was subcutaneously injected to the right-back of each mice.

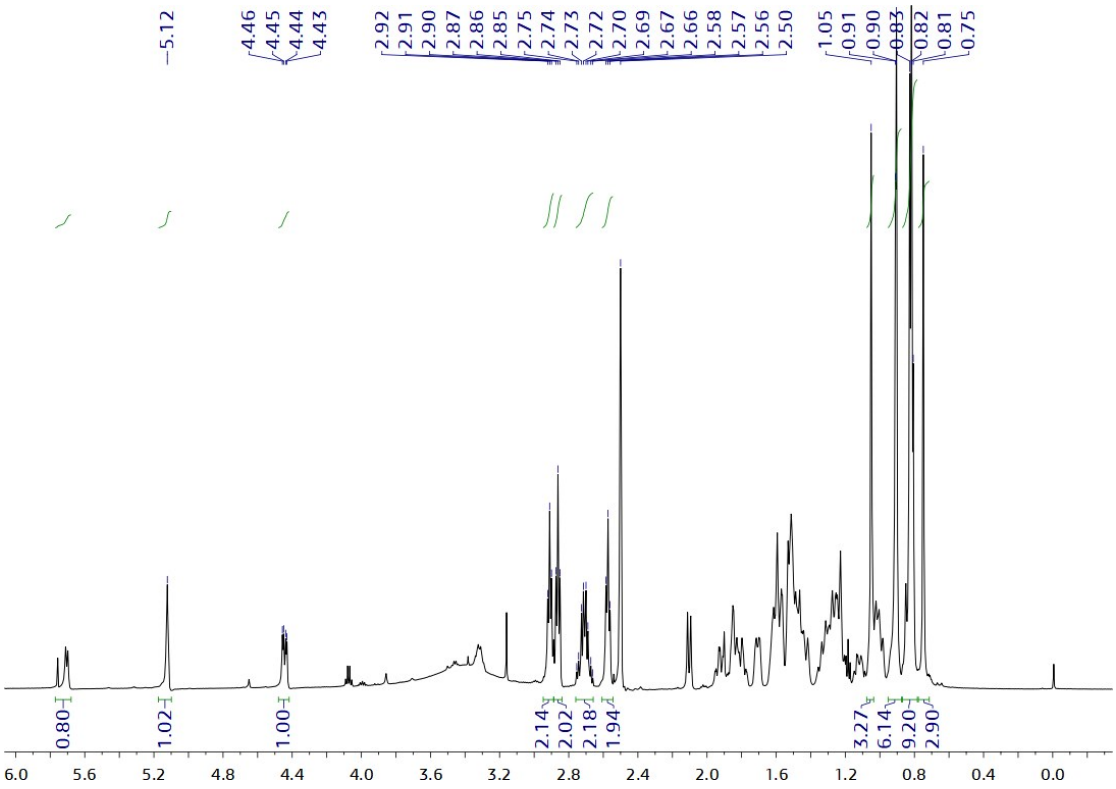

Figure S1. [1]H-NMR spectra of 3-(3, 3'-dithiodipropionic acid) ursolic acid (UASS) in DMSO-D6.

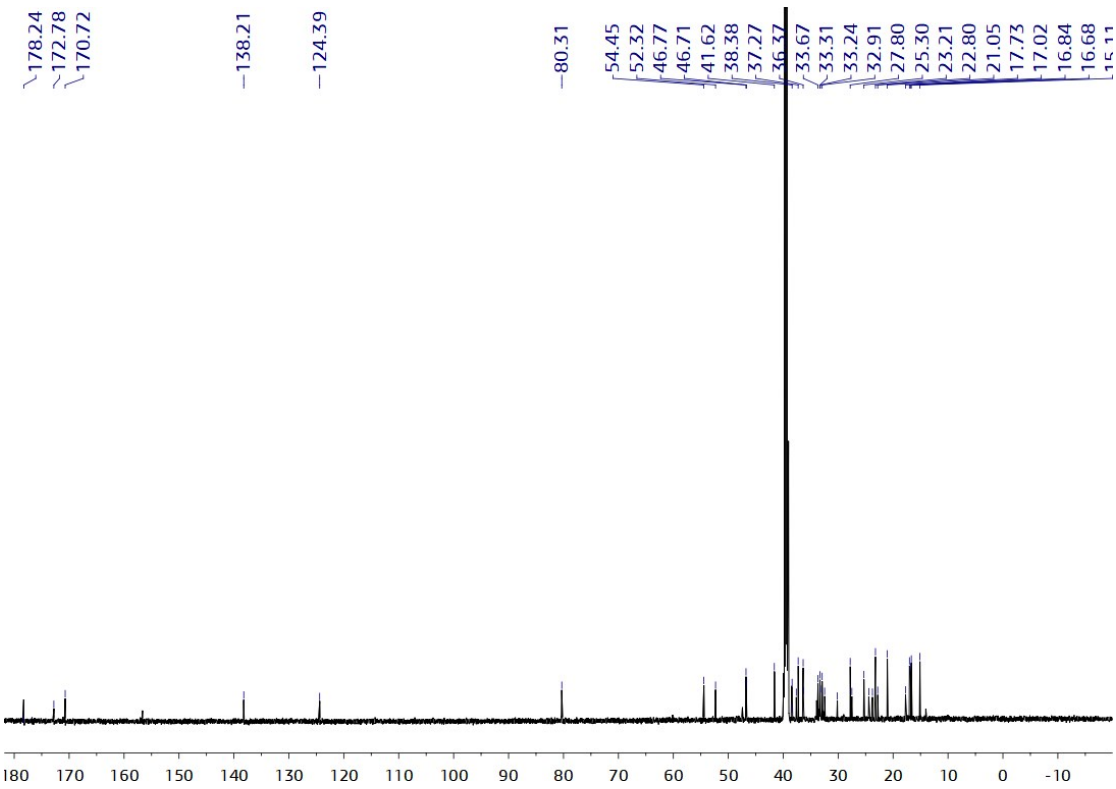

Figure S2. [13]C-NMR spectra of 3-(3, 3'-dithiodipropionic acid) ursolic acid (UASS) in DMSO-D6.

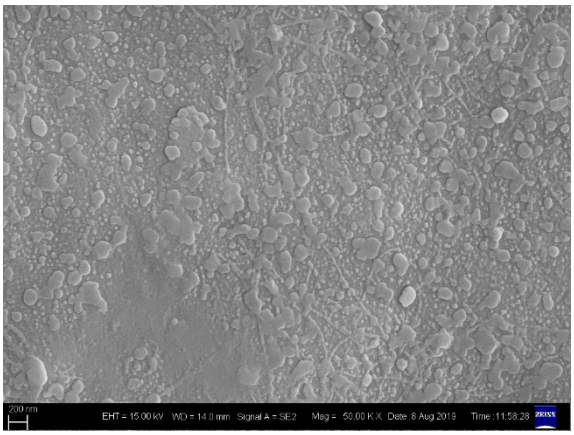

Figure S3. SEM images of free Ce6 assemblies. Where free Ce6 assemblies were prepared via same reprecipitation method as UASS-Ce6 NPs. After centrifugation of the reaction solutions, a large number of free UASS self-assemblies and UASS-Ce6 co-assemblies could be observed, but only a few Ce6 precipitates were obtained for SEM imaging.

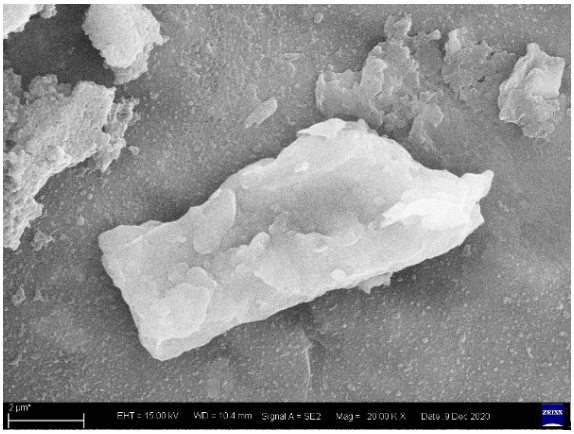

Figure S4. SEM image of UASS-Ce6 NPs using UASS and Ce6 (molar ratio, 4:1) as building blocks, obtained without sonication.

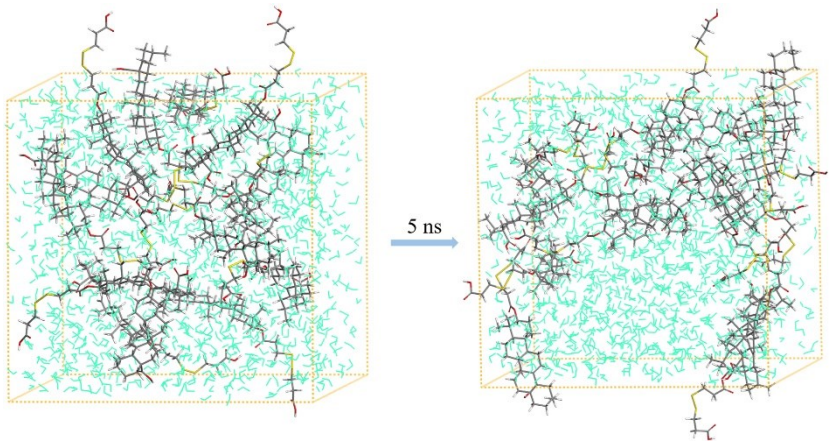

Figure S5. Structure of free UASS self-assemblies before and after MD simulation for a total of 5 ns (5000 ps). The line and ball models were used for H_2O and UASS molecules, respectively.

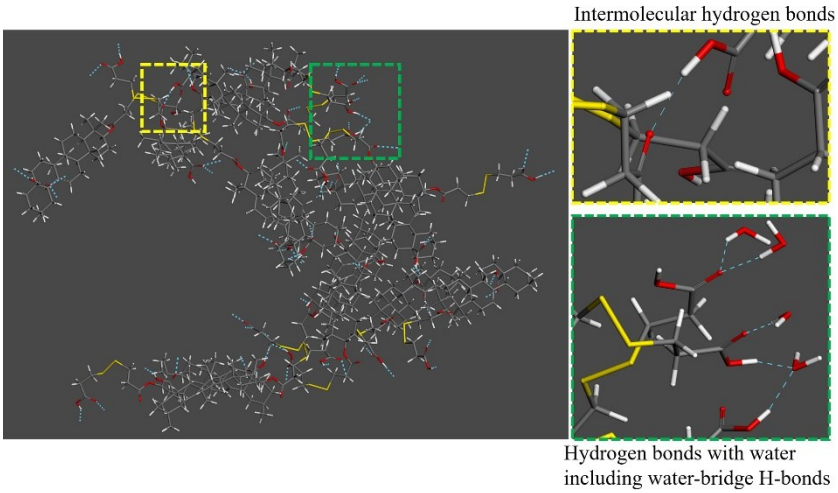

Figure S6. The hydrogen bond information of free UASS self-assemblies after MD simulation and corresponding water molecules are omitted. One pair of intermolecular hydrogen bond between UASS molecules, and numerous hydrogen bonds between UASS and water molecules existing in UASS self-assemblies including water-bridge H-bonds.

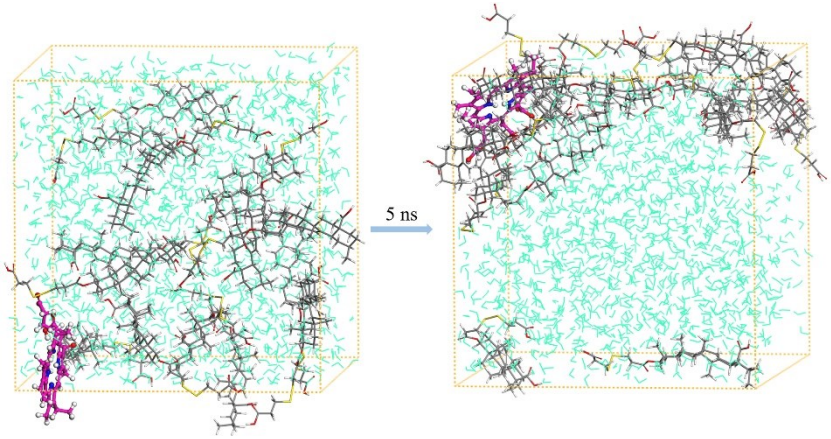

Figure S7. Structure of co-assembled UASS-Ce6 NPs obtained by MD simulation for total 5 ns. Where the C atoms of Ce6 are labeled with purple. The line and ball models were used for H_2O and UASS/Ce6 molecules, respectively.

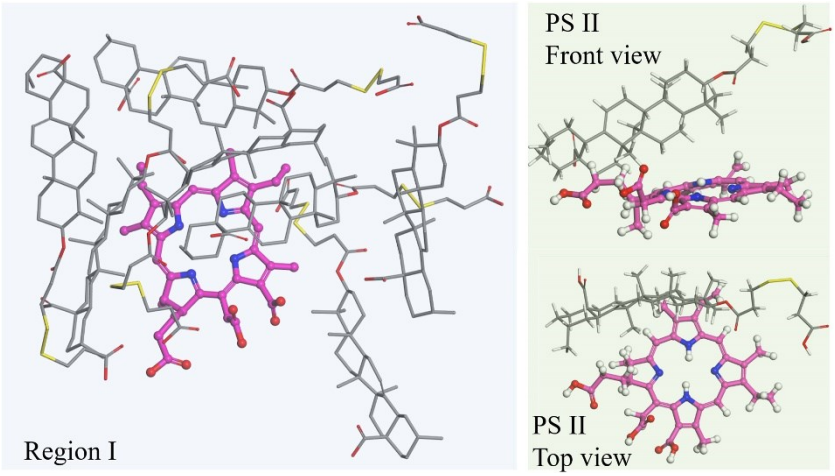

Figure S8. Molecular arrangement structure in the region I of UASS-Ce6 NPs after MD simulation, and corresponding possible single molecule interactions PS-II model between UASS with Ce6, was displayed from the top and front view.

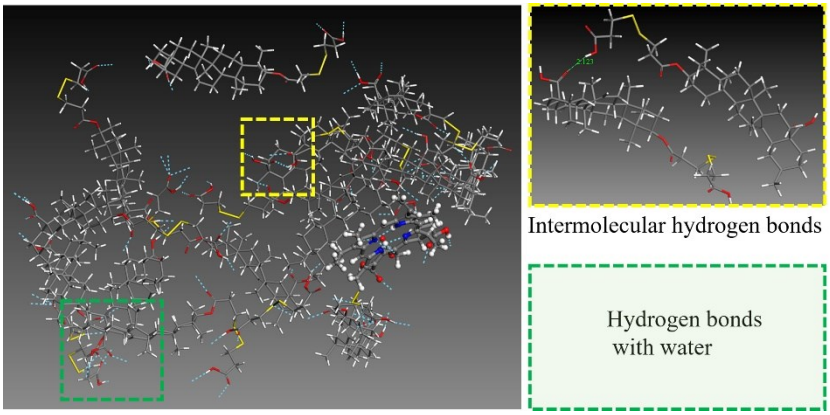

Figure S9. The hydrogen bond information of UASS-Ce6 co-assemblies after MD simulation for 5 ns, and water molecules are omitted. The intermolecular hydrogen bond among UASS molecules and numerous hydrogen bonds between UASS with water also existed in co-assemblies.

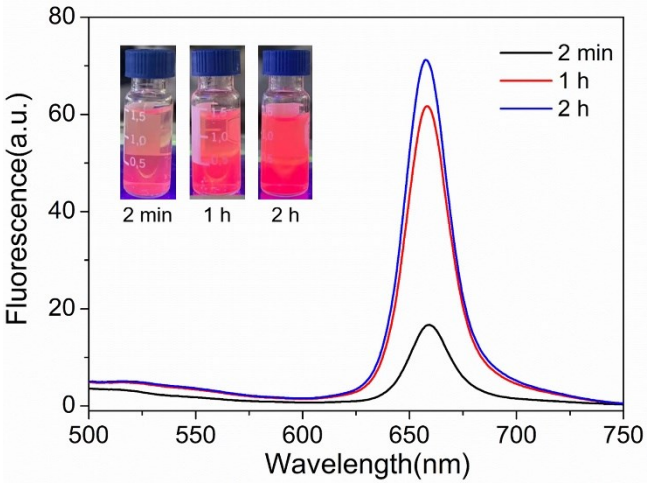

Figure S10. The fluorescence emission spectra of UASS-Ce6 NPs after incubation in GSH (1 mM) PBS (pH 7.4) solution for various times.

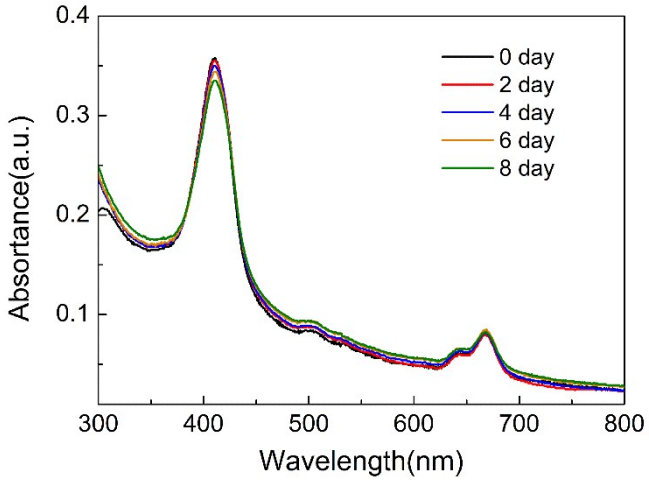

Figure S11. UV absorption changes of UASS-Ce6 NPs after incubation in water for various days.

Table S1. The standard atomic orientation of single-molecule model UASS@Ce6 after optimization by DFT calculation.

Center Number	Atomic Name	Coordinates (Angstroms)		
		X	Y	Z
1	C	2.44831333	6.06182932	-1.19349850
2	C	1.38447120	6.21031174	-0.09455984
3	C	0.45217553	5.03697323	-0.41440970
4	N	1.09535000	4.08965875	-1.15084457
5	C	2.29012018	4.58706559	-1.54883133
6	C	4.12319500	0.74501257	-3.79502923
7	C	4.48912717	2.02743013	-3.41530571
8	C	3.36194307	2.62708077	-2.74461661
9	N	2.36642385	1.69738805	-2.72086035
10	C	2.76493778	0.53343076	-3.33796541
11	C	3.30187891	3.93005476	-2.24185063
12	C	-1.47857250	-1.48253221	-2.76634051
13	C	-0.24426221	-1.82682537	-3.24523273
14	C	0.61203404	-0.66299888	-3.01892362
15	N	-0.06128224	0.34743360	-2.40838179
16	C	-1.33178752	-0.11980086	-2.23813022
17	C	1.95483842	-0.58507325	-3.43594681
18	C	-3.12845611	3.63100531	0.15812314
19	C	-3.53307805	2.41555590	-0.39493978
20	C	-2.39089199	1.84018473	-1.02558103
21	N	-1.35290947	2.74136931	-0.88018631
22	C	-1.73818029	3.85510936	-0.18184363

23	C	-0.88501290	4.98087696	0.02012106
24	C	-2.37120454	0.57928035	-1.62489798
25	C	5.78827505	2.72760937	-3.67397276
26	C	4.96250426	-0.20658569	-4.52347345
27	C	4.55945135	-1.08537330	-5.45153486
28	C	0.18688540	-3.10334923	-3.90258324
29	C	-2.75042624	-2.28359773	-2.80467367
30	C	-3.65900517	-1.92592933	-3.99748465
31	C	-4.88583702	1.77498819	-0.32812479
32	C	2.00733347	6.07593295	1.32067241
33	C	2.66736101	7.38964717	1.79717626
34	C	2.83872576	7.43025655	3.31330889
35	O	3.90186886	7.28196632	3.87065969
36	O	1.70540066	7.62270821	4.02094819
37	C	-1.48486743	6.28281416	0.56424099
38	C	-0.99168460	6.73894553	1.93895674
39	O	-0.43153846	7.80830400	2.11436921
40	O	-1.15646631	5.90091054	2.96650309
41	C	-4.03691717	4.36192346	1.05446751
42	O	-5.20754114	4.60490135	0.85876433
43	O	-3.46287370	4.67645934	2.26756022
44	C	2.19760004	6.96094655	-2.41754488
45	H	3.45375421	6.26318348	-0.80847261
46	H	0.87106688	7.17393552	-0.15482289
47	H	1.43456498	1.78870100	-2.32289714
48	H	4.19404857	4.52213134	-2.41969679
49	H	2.38852779	-1.46432581	-3.89530481
50	H	-0.42085740	2.65133780	-1.27549681
51	H	-3.32023185	0.05863620	-1.57425524
52	H	5.64561326	3.61685339	-4.29957865
53	H	6.49433176	2.07706382	-4.19359421
54	H	6.26057670	3.06077063	-2.74276284
55	H	6.02562904	-0.16088818	-4.29195486
56	H	5.26962817	-1.74902403	-5.93418425
57	H	3.52778614	-1.15114224	-5.78046059
58	H	0.46937162	-2.94539000	-4.95098165
59	H	-0.61351214	-3.84759694	-3.89159125
60	H	1.05542219	-3.54611765	-3.40041590
61	H	-3.30919264	-2.14843827	-1.87191088
62	H	-2.50320999	-3.35060366	-2.85611143
63	H	-4.57311627	-2.52885022	-3.98628700
64	H	-3.14577060	-2.10208246	-4.94808197
65	H	-3.94892044	-0.87085148	-3.97057635
66	H	-5.54648873	2.31057699	0.35324123
67	H	-4.81668239	0.72783451	-0.02046190
68	H	-5.36344050	1.79550404	-1.31507929
69	H	2.74985511	5.26957912	1.31174477
70	H	1.24946618	5.76368742	2.04142301

71	H	2.04008646	8.23570602	1.49474814
72	H	3.65621396	7.51902238	1.35205004
73	H	0.92824581	7.75325528	3.43757436
74	H	-2.57243417	6.21502957	0.60144202
75	H	-1.26287370	7.10797866	-0.11505882
76	H	-1.73224953	5.15647967	2.70391684
77	H	-4.15156625	5.12809860	2.78645584
78	H	2.92303237	6.75911893	-3.21145564
79	H	2.27936315	8.01744491	-2.14189924
80	H	1.19678229	6.79189744	-2.82884003
81	C	4.90487259	-2.46627915	0.79220317
82	C	4.15874426	-3.77270548	0.44814431
83	C	5.14829241	-4.97868288	0.45824907
84	C	6.47691722	-4.72918871	-0.25992104
85	C	7.21282587	-3.49924993	0.31626028
86	C	6.28046726	-2.26045891	0.14817595
87	C	4.33083600	-1.51451077	1.53747987
88	C	2.95914759	-1.58050493	2.14609501
89	C	2.10484103	-2.74257960	1.61395878
90	C	2.98000165	-4.03991548	1.50838064
91	C	0.68724503	-2.86604761	2.32030260
92	C	-0.05394719	-4.07209969	1.64371602
93	C	0.77236519	-5.36050743	1.79571313
94	C	2.09912840	-5.22414778	1.03863046
95	C	-0.11656476	-1.57645601	1.98990856
96	C	-1.60992224	-1.67854464	2.33382146
97	C	-2.25352444	-2.87710540	1.64667350
98	C	-1.58752725	-4.24172808	1.94930604
99	O	-3.65581363	-2.96693555	2.06063141
100	C	0.77501128	-2.98604790	3.86442718
101	C	3.59689420	-4.39696502	2.88815953
102	C	3.62627134	-3.62793989	-1.00775891
103	C	-2.19133812	-5.27241059	0.96521359
104	C	-1.90364398	-4.73016021	3.37948013
105	C	7.56259468	-3.75686314	1.78582365
106	O	7.33614528	-3.03762083	2.73556301
107	O	8.22170526	-4.93753887	1.94729863
108	C	8.54025345	-3.26685753	-0.45498037
109	C	9.23697272	-1.95868098	-0.05911935
110	C	8.32489768	-0.72793612	-0.23690453
111	C	6.98454632	-0.93544429	0.50485686
112	C	8.13425957	-0.35103887	-1.71619349
113	C	-4.58107305	-2.29493330	1.36365924
114	O	-4.35506286	-1.61202090	0.38149932
115	C	-5.95905193	-2.51002063	1.96770050
116	C	-7.04828450	-1.76115850	1.20379962
117	S	-8.67591492	-2.13621403	1.99578260
118	S	-10.00360789	-0.93493449	0.94110166

119	C	-10.47222482	-1.99135731	-0.50120756
120	C	-11.55806923	-1.26815298	-1.29433979
121	C	-11.96630630	-2.04877833	-2.52685860
122	O	-11.47786289	-3.09208237	-2.89918423
123	O	-12.96847641	-1.42651773	-3.19047292
124	H	4.65600527	-5.84961173	0.01141471
125	H	5.38605018	-5.25982485	1.48655463
126	H	6.32131604	-4.55365563	-1.33123850
127	H	7.11090316	-5.61722803	-0.17577095
128	H	6.09943731	-2.22958156	-0.93769347
129	H	4.88173803	-0.60591994	1.76059808
130	H	2.45172371	-0.62516817	1.96316251
131	H	3.07718696	-1.63725065	3.23706172
132	H	1.84420326	-2.47721063	0.58222748
133	H	-0.02962949	-3.83278187	0.56758499
134	H	0.22981435	-6.21746140	1.38510432
135	H	0.94845104	-5.59332483	2.85181440
136	H	2.66559518	-6.15934891	1.11715991
137	H	1.84506335	-5.10533040	-0.01947901
138	H	-0.01924168	-1.35465036	0.91868241
139	H	0.30587625	-0.71736269	2.52150814
140	H	-2.12227795	-0.76891652	2.00296973
141	H	-1.76648417	-1.75170619	3.41520674
142	H	-2.24420173	-2.71034828	0.56500707
143	H	-0.13952001	-2.63323614	4.34424956
144	H	1.58476153	-2.37278227	4.26518433
145	H	0.93539125	-4.00796810	4.21179167
146	H	2.86656024	-4.31172173	3.69035365
147	H	4.43932310	-3.74801438	3.13932063
148	H	3.95467389	-5.43017356	2.90738248
149	H	3.24016440	-4.57438492	-1.39822905
150	H	4.42657972	-3.31499566	-1.68286412
151	H	2.84075680	-2.87323667	-1.08888756
152	H	-3.28336231	-5.25815801	1.02881240
153	H	-1.86913447	-6.29156019	1.19367016
154	H	-1.90919804	-5.05222234	-0.07059649
155	H	-2.96637874	-4.96989034	3.46765018
156	H	-1.67028397	-3.99504917	4.14988181
157	H	-1.33914273	-5.63951941	3.60562424
158	H	8.41725975	-4.99820117	2.89778195
159	H	8.30068936	-3.25565321	-1.52556472
160	H	9.20667817	-4.12068701	-0.29503485
161	H	10.15427544	-1.83718984	-0.64851316
162	H	9.55239300	-2.01852525	0.99070084
163	H	8.82581999	0.12455456	0.24127706
164	H	7.16154833	-0.90714908	1.58318580
165	H	6.31777362	-0.09963033	0.26419358
166	H	7.52490466	0.55480868	-1.80711262

167	H	7.63538581	-1.13883812	-2.29049148
168	H	9.09905055	-0.15532187	-2.19737555
169	H	-5.92199873	-2.19711385	3.01759150
170	H	-6.15277478	-3.58922036	1.98332285
171	H	-7.06844706	-2.07390883	0.15811385
172	H	-6.88114154	-0.68328530	1.23862409
173	H	-10.83333119	-2.95302067	-0.13271495
174	H	-9.59692474	-2.17017696	-1.12830756
175	H	-11.22276289	-0.27664863	-1.62285828
176	H	-12.45315508	-1.09626389	-0.68561936
177	H	-13.16873983	-1.98073717	-3.96389905

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