

Supplementary Information

Two-Photon Photoexcited Photodynamic Therapy with Water-Soluble Fullereneol Serving as the Highly Effective Two-Photon Photosensitizer Against Multidrug-Resistant Bacteria

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To subtract the approximately m/z value (720) of C_{60} from that (1228) and then divide by approximately 17 (m/z value of hydroxyl group), resulting in approximately 30. Consequentially, the number of hydroxyl groups was confirmed to be 30 based on the results provided by the field desorption mass spectrometer (Figure S1).

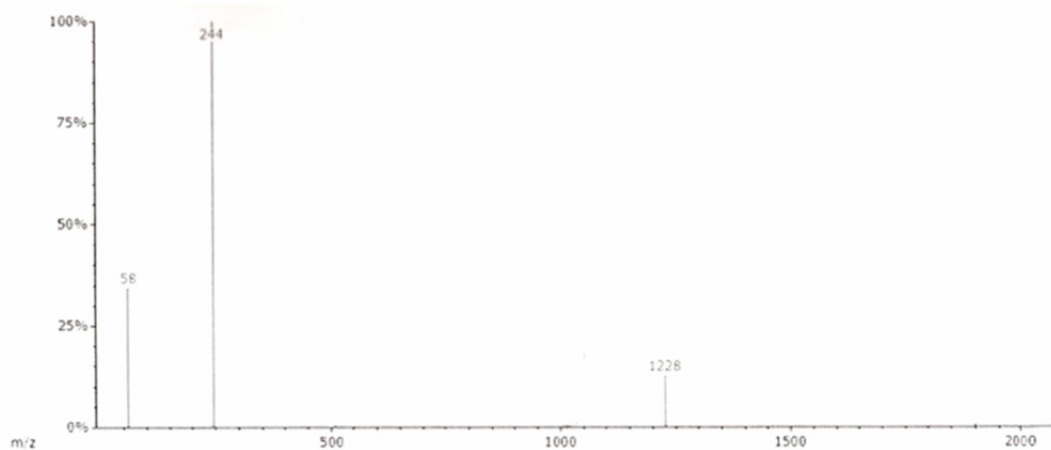


Figure S1 Field desorption mass spectrometry spectra of fulleranol.

Table S1 Stability of well-prepared water-soluble $C_{60}(OH)_{30}$ fullereneol in physiological environments.

	pH 7 aqueous solution	1X PBS	Culture medium of MRSA
Newly prepared	130	129	131
Prepared and stayed for 3 months	130	130	130

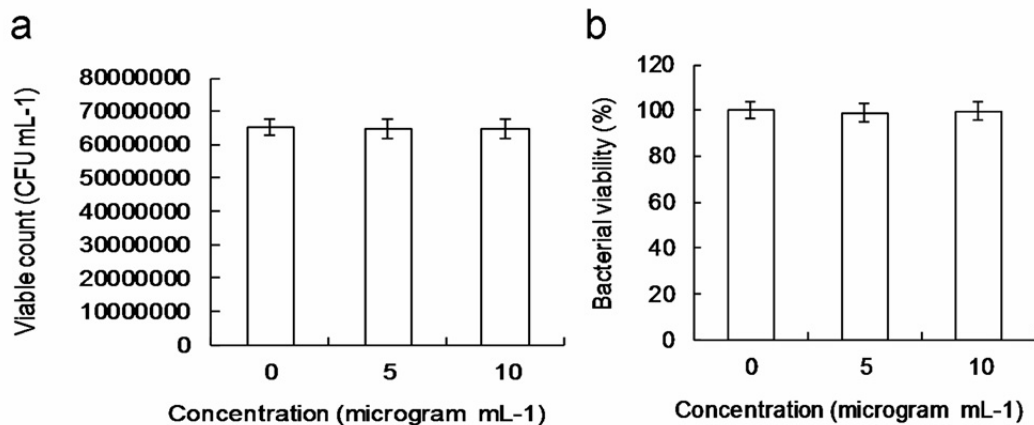


Figure S2 The number of surviving (A) material-treated bacteria was determined using the CFU counting assay and was expressed as a percentage (%) for (B) bacteria that corresponded to the unit of CFU mL⁻¹. Delivered dose: OD₆₀₀ of 0.05 of MRSA and 0–10 μg mL⁻¹ of water-soluble C₆₀(OH)₃₀ fullereneol. Data are means ± SD (*n*=6).

Table S2 Amount of ROS generated¹⁻⁹ by conducting TPE (228.80 nJ pixel⁻¹, 600 or 900 scans; Ex: 760 nm) and by using water-soluble C₆₀(OH)₃₀ fullereneol (5 µg mL⁻¹) was monitored in the dark and monitored. Data are means ± SD (*n*=6).

¹ O ₂ (by SOSG) ^c					
	Negative control ^{ac}	ROS neutralization ^{abc}	Positive control ^{cd}	C ₆₀ (OH) ₃₀	ROS neutralization ^{bc}
600 scans	233±11	233±12	2703±127	2465±119	230±11
900 scans	231±10	232±11	2896±140	2637±123	232±10
¹ O ₂ (by <i>t</i> -MVP) ^e					
	Negative control ^{ac}	ROS neutralization ^{abc}	Positive control ^{de}	C ₆₀ (OH) ₃₀	ROS neutralization ^{bc}
600 scans	339±22	340±23	9364±236	8904±202	341±21
900 scans	340±22	341±24	9453±245	9151±218	340±20
O ₂ ^{•-} (by XTT) ^f					
	Negative control ^{af}	ROS neutralization ^{abf}	Positive control ^{df}	C ₆₀ (OH) ₃₀	ROS neutralization ^{bf}
600 scans	0	0	1.93±0.15	1.88±0.12	0.02±0.01
900 scans	0	0	2.06±0.21	1.95±0.19	0.03±0.01
O ₂ ^{•-} (by GSH) ^g					
	Negative control ^{ag}	ROS neutralization ^{abg}	Positive control ^{dg}	C ₆₀ (OH) ₃₀	ROS neutralization ^{bg}
600 scans	0	0	95.1±4.3%	84.9±3.8%	0.2±0.1%
900 scans	0	0	99.3±4.6%	89.8±4.0%	0.3±0.1%

^aNegative control: only treat reagent and laser radiation without material (0 µg mL⁻¹).

^bROS neutralization: with the treatments of nanomaterial, the laser irradiation and 30 ppm of antioxidant α -Tocopherol/methyl linoleate.

^cSOSG reagent (Ex/Em: 488/525 nm) has a specific reactivity to generate fluorescence recorded by a PL spectrometer.

^dPositive control: the treatment of 50 µM *tert*-butyl hydroperoxide (TBHP) and laser irradiation.

^e*t*-MVP (Ex/Em: 352/465 nm) can react with ¹O₂, forming a dioxetane intermediate that generates fluorescence upon decomposition to 1-pyrenecarboxaldehyde, and monitored by a PL spectrometer.

^fXTT would interact with O₂^{•-} and produce the XTT-formazan generating strong absorption (470 nm in wavelength).

^gGSH containing a thiol-tripeptide can prevent damages to cellular or bacterial components caused by stress of oxidation. Thiol group from GSH can be oxidized to disulfide bond converting GSH to glutathione disulfide. GSH oxidation was used to determine the generated O₂^{•-}. Loss of GSH (%) = (absorbance difference between of sample and negative control / absorbance of negative control) × 100 %.

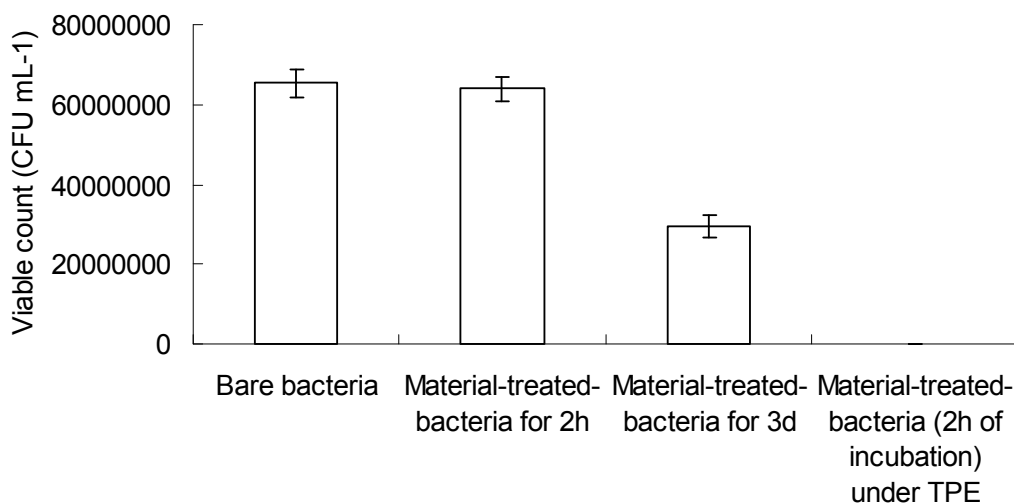
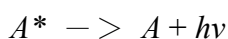


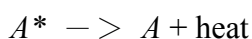
Figure S3 The number of surviving nanomaterial-treated-bacteria was determined by CFU counting assay, corresponding to **Figure 5f**, which is expressed as the percentage (%) for bacteria that corresponds to the unit of CFU mL⁻¹. Delivered dose: OD₆₀₀ ~ 0.05 of MRSA and 5 μg mL⁻¹ water-soluble C₆₀(OH)₃₀ fulleranol. Data are means ± SD (*n*=6).

Calculation of radiative and non-radiative decay rates¹⁰

Upon the absorption of a photon, one of the weakly bound electrons of the fluorescent molecule—a fluorophore—is promoted to a higher energy level. The fluorophore is then in an excited state, A^* . This state is metastable; therefore the fluorophore will return to its stable ground state, A . It can do so either radiatively by emitting a fluorescence photon $h\nu$,



or nonradiatively by dissipating the excited state energy as heat



The depopulation of the excited state depends on the de-excitation pathways available. Fluorescence is the radiative deactivation of the lowest vibrational energy level of the first electronically excited singlet state, S_1 , back to the electronic ground state, S_0 . The singlet states are the energy levels that can be populated by the weakly bound electron without a spin flip. The absorption and emission processes are illustrated by an energy level diagram named after Aleksander Jablonski.

The fluorescence lifetime, τ , is the average time a fluorophore remains in the electronically excited state S_1 after excitation. τ is defined as the inverse of the sum of the rate parameters for all excited state depopulation processes: Eq. (3), where the nonradiative rate constant k is the sum of the rate constant for internal conversion, k_{ic} , and the rate constant for intersystem crossing to the triplet state, k_{isc} , such that $k = k_{ic} + k_{isc}$. Fluorescence emission always occurs from the lowest vibrational level of S_1 , a rule known as Kasha's rule, indicating that the fluorophore has no memory of its excitation pathway, for example, one-photon excitation and two-photon excitation yield the same fluorescence spectrum, quantum yield and lifetime.

References

1. Kuo WS, Hsu CLL, Chen HH, et al. Graphene quantum dots conjugated with polymers for two-photon properties under two-photon excitation. *Nanoscale*. 2016;8:16874–16880.
2. Wu PC, Wang JY, Wang WL, et al. Efficient two-photon luminescence for cellular imaging using biocompatible nitrogen-doped graphene quantum dots conjugated with polymers. *Nanoscale*. 2018;10:109–117.
3. Chang WT, Chen SJ, Chang CY, et al. Effect of size-dependence photodestructive efficacy by gold nanomaterials with multiphoton Laser. *ACS Appl Mater Interfaces*. 2015;7:17318–17329.
4. Kinen MM, Kamal-Eldin A, Lampi AM, et al. Effects of α - and γ -tocopherols on formation of hydroperoxides and two decomposition products from methyl linoleate. *J Am Oil Chem Soc*. 2000;77:801–806.
5. Sharma P, Jha AB, Dubey RS, et al. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *J Bot*. 2012;2012:1–26.
6. Possel H, Noack H, Augustin W, et al. An oxidant, *tert*-butyl hydroperoxide (TBHP), to serve as a positive control. *FEBS Lett*. 1997;416:175–178.
7. Thompson A, Lever JR, Canella KA, et al. Chemiluminescence mechanism and quantum yield of synthetic vinylpyrene analogues of benzo[*a*]pyrene-7,8-dihydrodiol. *J Am Chem Soc*. 1986;108:4498–4504.
8. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys*. 1959;82:70–77.
9. Carmel-Hare O, Storz G. Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. *Annu Rev Microbiol*. 2000;54:439–461.
10. Suhling K, Hirvonen LM, Levitt JA, Chung PH, Tregido, C, Marois Al, Rusakov DA, Zheng K, Ameer-Beg S, Poland S, Coelho S, Dimble R. Fluorescence lifetime imaging (FLIM): basic concepts and recent applications. In: Becker W, editor. *Advanced time-correlated single photon counting applications*. Germany: Springer; 2015:119–188.