Supplementary Information

Effects of fullerene derivatives on bioluminescence and application for protease detection

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Materials and methods

Materials

The plasmids containing the gene of hGluc: pcDNA3.1-zipper-hGluc (1) and pcDNA3.1-zipper-hGluc (2) were kindly provided by Prof. Stephen W Michnick (Université de Montréal, Canada).[1] *Gaussia* luciferase assay kit was purchased from New England Biolabs (UK). α-Thrombin was obtained from Haematologic Technologies Inc. (Essex Junction, VT, USA). The restriction endonucleases were purchased from TaKaRa (Japan). Oligonucleotides were synthesized by Invitrogen (Invitrogen Biotechnology Co., Ltd., Shanghai, China). All other chemicals used in the experiments were of analytical grade unless stated otherwise. Deionized water (18.2 MΩ, 0.22-μm filter, Merck Millipore, Germany) was used for all experiments.

Preparation and characterization of the water-soluble fullerene derivatives

Malonic acid C_{60} carboxyl derivative ($C_{60}[C(COOH)_2]_3$, C_{60}-COOH for short) was prepared according to a previously reported method.[2] In brief, C_{60} was converted into C_{60} malonic ester following treatment with diethyl bromomalonate (Sigma-Aldrich) and 1,8-diazobicyclo[5,40]undec-7-ene (Alfa-Aesar) in dry toluene under nitrogen. The malonate derivative of C_{60} was separated by silica gel column chromatography. MALDI-TOF mass spectra (AXIMA Assurance) verified the targeting product (shown in Fig. S1). Hydrolysis of the purified product with NaH and CH_3OH led to the formation of the corresponding acids, as confirmed by Fourier transform infrared spectroscopy (FTIR, iN10 MX IR, Nicolet) (shown in Fig. S2A). The size distribution of C_{60}-COOH was characterized by dynamic light scattering (Nano-ZS ZEN3600, Malvern Instruments, Germany) (shown in Fig. S2C).

Fullerene C_{60} amino hydroxyl derivative ($C_{60}(NH_2)_x(OH)_y$, C_{60}-NH_2 for short) was synthesized according to the reference. [3] Briefly, the suspension of C_{60} in the mixture of 30% aqueous H_2O_2 and 28% aqueous NH_3 was obtained and vigorously stirred at 60 ^oC under air for 12 hours, till the black suspension was turned into a dark yellow suspension. After careful centrifugation and decantation, ethanol was added to the clear solution to afford a precipitation. After another careful centrifugation and decantation, the residual solid was treated with water and then ethanol for re-precipitation. The precipitated residual solid was washed twice with ethanol under ultrasonic irradiation and dried under vacuum at room temperature for 18 hours. Finally, the water-soluble fullerene derivative was obtained as a yellowish powder. Due to the complexity and high ionization potential of $C_{60}(NH_2)_x(OH)_y$, it is quite difficult to observe the molecular ion peak through Mass Spectrum. Alternatively, X-ray photoelectron spectroscopy (ESCALab220i-XL electron spectrometer from VG Scientific using 300W AlKα radiation) is used to evaluate the chemical structure of final product according to area integral for the characteristic binding energies of carbon, nitrogen and oxygen elements under different chemical environment (as shown in Fig. S3 and Table 1 and 2). Fourier transform infrared spectroscopy (FTIR, iN10 MX IR, Nicolet) verified the structure and the functional groups of amino and hydroxyl on the carbon cage (data are shown in Fig. S2B). The size distribution of C_{60}-NH_2 was characterized by dynamic light scattering (Nano-ZS ZEN3600, Malvern Instruments, Germany) (shown in Fig. S2D).

Expression and purification of proteins

The two plasmids, pET28a-His_6-TM-SL-hGluc (short linker) and pET28a-His_6-TM-LL-hGluc (long linker) (TM symbolized the α-thrombin cleavage site containing aa: LVPRGS; Short linker (SL) is HMGGGGS, and long linker (LL) contains 21 aa with HMSNK TRIDE ANQRA TKMLE F.), were constructed as the reference,[4] using the forward primers, 5'- TGCTAACATA TGTCCAACAA AACCAGAATT GATGA-3', and 5'-TGCTAACATA TGGGTGGAGG TGGATCGAAG CCCACCGAGA ACAACGAAG-3', respectively, with the same reverse primer: 5'- ATATAAGCTT TTAGTCACCA CCGGCCCCCT TG-3', from the pET28a-hGluc plasmid we constructed previously. [4]

All constructed plasmids were confirmed by DNA sequencing (Invitrogen Biotechnology Co., Ltd., Shanghai, China).

Protein expression and purification

The constructed plasmids were transformed in *Escherichia coli* BL21(DE3) bacteria to express the proteins. When bacteria OD at 600 nm arrived around 0.5, Isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.5 mM final concentration) was used to induce the expression of the fusion proteins overnight at 18 ^{o}C. Then the bacterial suspensions in the binding buffer (20 mM tris-HCl pH 8.0, 20 mM imidazole, 0.5 M NaCl) were ultrasonicated using an ultrasonication system (Sonics vibra-cell, Ultrasonic processor VCX 750 Watt; Sonics & Materials, Newtown, CT, USA). After centrifuging to remove bacteria fragments, the lysate was subsequently filtrated by a 0.44 μm syringe filter before purification. The fused proteins were purified by nickel affinity chromatography using a HisTrap FF column (GE Healthcare, USA) on the AKTA purifier (GE Healthcare, USA). All the purified proteins were concentrated using a Amicon Ultra-15 centrifugal filter device (10,000 kDa, Merck KGaA, Darmstadt, Germany) to remove excess imidazole. The protein sizes were analyzed using SDS-PAGE and the protein concentrations were assessed by the bicinchoninic acid (BCA) assay (Pierce Rockford, IL, USA).

Solution preparations

The powder of correspondingly divalent chloride (Sinopharm Chemicals, Shanghai, China) was dissolved in water to prepare 10 mM stocked solutions: Ni^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}. hGluc buffer consisted of 20 mM Tris-HCl buffer, pH 7.4 and 200 mM NaCl. α-Thrombin buffer was comprised of 50 mM Tris-HCl buffer, pH 8.0, 150 mL NaCl, 5 mM $MgCl_2$, and 2.5 mM $CaCl_2$. 0.25 M EDTA and 1 M imidazole (Sigma-Aldrich) were prepared in deionized water.

Bioluminescence and UV-Vis spectra measurement

The bioluminescence and UV-Vis/bioluminescence spectra were measured on a plate reader with automatic injection system (BioTek Synergy H1 Hybrid Reader, BioTek Instruments, Inc., VT, USA). Spectra of hGluc bioluminescence and UV-Vis were scanned from 400-600 nm by step of 5 nm. The same concentration (250 μM) of C_{60}-COOH and C_{60}-NH_2 was used for UV-Vis scanning. hGluc with final concentration of 0.5 μM was adopted in all experiments. Bioluminescence of all samples in the experiments was obtained by the relative luminescence values. The substrate (*Gaussia* luciferase assay kit) was prepared following the instruction

provided by the manufacturer, and the bioluminescence was detected by injecting 10 µl of a 10-fold substrate dilution to a total volume of 100 µl BRET solution using the Synergy H1 system.

α-Thrombin cleaving experiments

As indicated in the instruction of α-thrombin, the cleaving reaction was performed at 20 ^{o}C for 16 hrs. C_{60} derivatives, Ni^{2+} and fused hGluc luciferases were diluted in the α-thrombin buffer in the cleaving experiments and bioluminescence was tested in the hGluc buffer (20 mM Tris-HCl buffer, pH 7.4 and 200 mM NaCl).

Fig. S1. MALDI-TOF mass spectra of C_{60}-COOH ($C_{60}[C(COOH)_2]_3$).

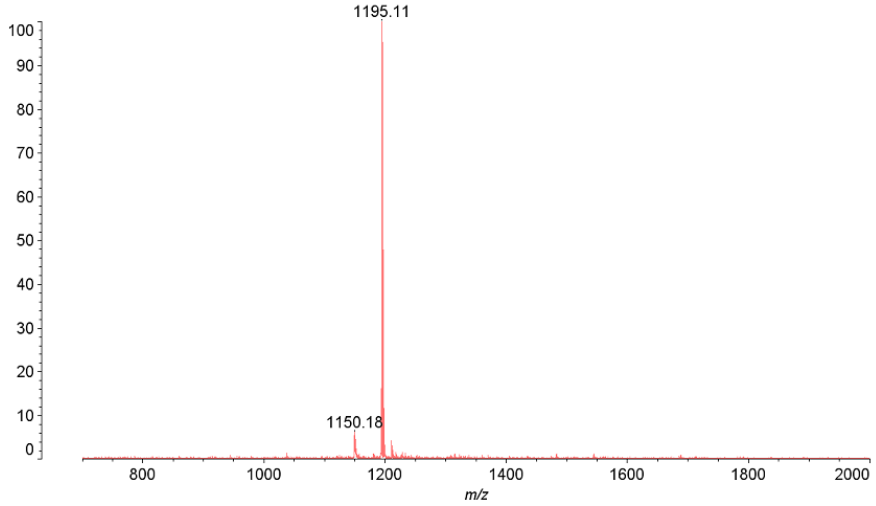


Fig. S2. FTIR spectra of C_{60}-COOH (A) and C_{60}-NH_2 (B) and DLS spectra of C_{60}-COOH (C) and C_{60}-NH_2 (D). The FTIR data show that the C_{60} have been successfully modified by –COOH and –NH_2. From the DLS data, the C_{60}-COOH in the aqueous solution appeared homogeneous with the average size 72 nm and C_{60}-NH_2 with the average size 40 nm. Therefore, they are monodispersed and suitable for biosensors.

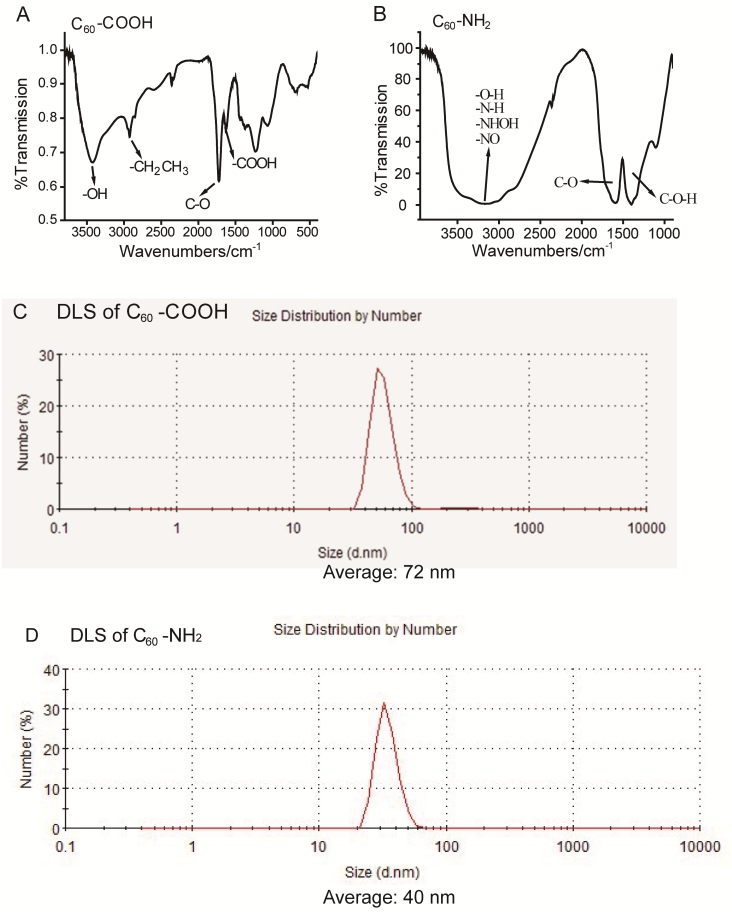


Fig. S3. XPS spectra of DF C1s (A) and N1s (B)binding energy.

XPS spectra of C_{60}-NH_2 The C_{1s} peaks centered at binding energy of 284.8, 286.2 and 288.2 eV should assign for C-C, C-O (C-N) and C=O, respectively. The fitting N_{1s} peaks centered at ca. 399.2 eV and 401.1 eV should correspond to the free amine group (-NH_2) and partially protonated amine (-NH_3^+), indicating that amino groups have been successfully introduced on the carbon cages, which is in well agreement with the FTIR result (see Fig. S1). Considering the C:O:N atomic ratio and the relative contents of different chemical states for carbon, oxygen, and nitrogen elements, the average molecular formula can be estimated as $C_{60}O_{\sim 14}(OH)_{\sim 12}(NH_2)_{\sim 7}$ (see Table S1 and S2, the protonated amine was combined with the free amine groups for clarity).

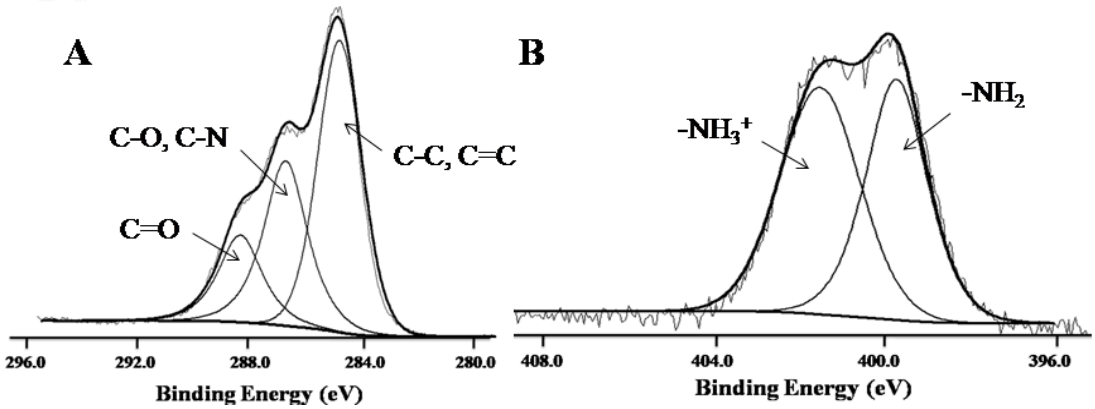


Table S1. Fitting of C1s binding energy

Bond (C1s)	Binding Energy (eV)	Area	FWHM (eV)	%GL (%)
C-C,C=C	284.8	70907	1.8	40
C-O,C-N	286.7	43758.43	1.8	80
C=O	288.3	23153.93	1.7	80

Table S2. Fitting of N1s binding energy

Bond (N1s)	Binding Energy (eV)	Area	FWHM (eV)	%GL (%)
-NH2	399.7	11665.23	1.7	60
-NH3+	401.5	13664.86	2.2	30

Combined with C1s/N1s integral area and their sensitive factor (C1s = 1, N1s = 1.8), the average molecular formula can be designed as $C_{60}O$~14(OH)~12 (NH_2)~7.

Fig. S4. UV-Vis spectra of the two C_{60} derivatives at 500 μM and 250 μM. The figures have shown that the spectra of 250 μM and 500 μM C_{60}-COOH have similar characteristics, but different absorption intensity, and after normalization by the absorption value at 470 nm, the two spectra almost overlapped, as shown in (A). The spectra of 250 μM and 500 μM C_{60}-NH_2 are similar as shown in (B). These figures showed that there were no insoluble C_{60}-derivative particles in the solutions, even at concentration as high as 500 μM, which is 100 times higher than the

concentration used in the BRET assay.

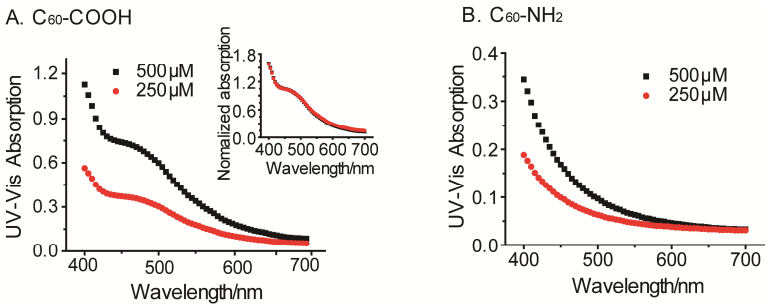
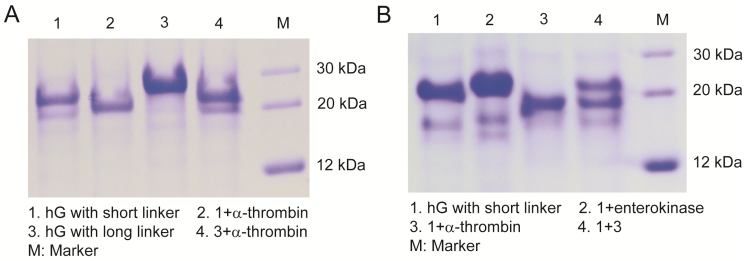


Fig. S5. SDS-PAGE of the two fusion proteins His_6-TM-SL-hGLuc and His_6-TM-SL-hGLuc before and after cleavage by α-thrombin (A) and the specificity of His_6-TM-Sl-hGluc to α-thrombin (B). A. SDS-PAGE experiments of the samples before and after cleavage of α-thrombin for the two fusion hGluc proteins (molecular weight of hGluc with short linker: 21KDa, 19 KDa after cleavage; molecular weight of hGluc with short linker: 23 KDa, 21 KDa after cleavage.). Lane 1 and lane 3 are the proteins before cleavage. Lane 2 and lane4 are the cleaving products. B. SDS-PAGE results on the fused hGluc with short linker. Lane 1 is the fused hGluc. Lane 2 is the fused hGluc reacted with an irrelevant protease enterokinase, which could not cleave the site, indicating the similar band as that in lane 1. Lane 3 is fused hGluc cleaved by α-thrombin, which cleaved the site efficiently. Lane 4 is the mixture of lane 1 and lane 3.



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3. K. Matsubayashi, K. Kokubo, H. Tategaki, S. Kawahama and T. Oshima, *Fuller Nanotub Car N*, 2009, **17**, 440-456.
4. F. Li, J. Yu, Z. Zhang, Z. Cui, D. Wang, H. Wei and X. E. Zhang, *Analytica chimica acta*, 2012, **724**, 104-110.