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Supporting Information

Oxyhemoglobin Nano-recruiter Preparation and its Application for Biomimetic Red Blood cell to Relieve Tumor Hypoxia and Enhance Photodynamic Therapy Activity

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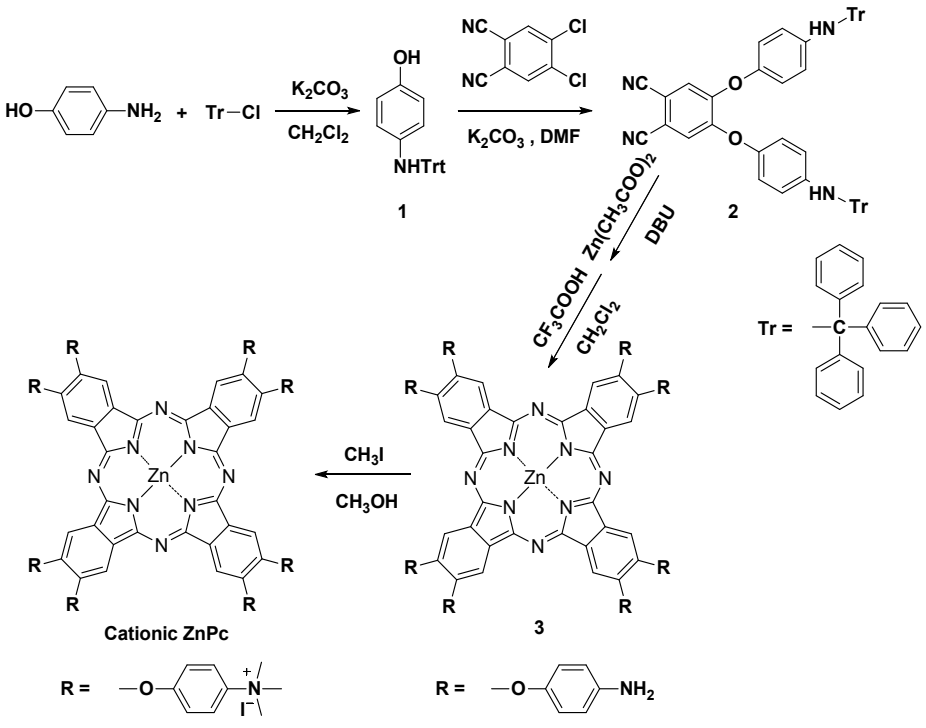
1. Experimental Section

Materials. Iron chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), copper nitrate trihydrate ($Cu(NO_3)_2 \cdot 3H_2O$), sodium acetate (C_2H_5ONa), ethylene glycol, Sephadex-25, sodium dithionite, 4-aminophenol, 4,5-dichlorophthalonitrile, triphenylchloromethane, iodomethane, trifluoroacetic, 1,8-diazabicyclo-(5.4.0) undec-7-ene (DBU) and agar obtained from Sinopharm Chemical Reagent Co. Ltd. OxyHb was obtained from Shanghai YuanMu Biological Technology Co. Ltd. tris (4,7-diphenyl-1,10-phenanthroline) ruthenium (II) dichloride [$Ru(dpp)_3Cl_2$] received from Alfa Aesar. Haematoxylin and eosin (H&E), 4,6-diamidino-2-phenylindole (DAPI), OCT embedding medium, pimonidazole hydrochloride (PH), horseradish peroxidase-connected anti-rabbit IgG and anti-mouse IgG1 obtained from Shanghai Yisheng Biological Technology Co. Ltd. MC-plastic embedding kit obtained from Nanjing Musai Biological Technology Co. Ltd. Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959), 9,10-anthracenedip-ropionic acid (ADPA), hyaluronic acid (HA) and hyaluronidase (HAase) were obtained from Sigma. Phosphate buffer (PBS), Tyrisin, dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) were from GIBCO. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was from Amosco. 2'7'-dichlorofluorescin diacetate (DCFH-DA), singlet oxygen sensor green (SOSG), Annexin V-FITC/PI apoptosis detection kit and were from Beyotime. Blue lysosome tracker (lysotracker blue) was from thermo fisher.

Instruments. FTIR spectra were obtained by Nicolet Nexus 670 FTIR-Spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker Advance 400 MHz NMR spectrometer (^{1}H NMR spectra of the HA-MA derivatives dissolved in deuterium oxide of 6 mg mL^{-1} and performed at 80 °C). Mass spectra were obtained on UltraflexXreme MALDI-TOF-MS spectrometer. Elemental analysis was carried out by Vario MICRO Elementar. Ultraviolet-Visible (UV-Vis) absorption and fluorescence spectra were measured with a Varian Cary 50 spectrophotometer and a Cary Eclipse fluorometer. The fluorescence signal inside cells were observed under Nikon Ti-E-A1R laser scanning confocal microscope and Nikon Ti Observer fluorescence microscope. TEM images were obtained from a HITACHI H7650 transmission electron microscope. Flow cytometry (FCM) experiments were carried out using Beckman XL Flow Cytometer. The electrochemical property were obtained by a Chenhua CHI 660 E electrochemical analyzer. A 665 nm LED (5 W) was used as light source. Relaxivity and MRI was got by NIUMAG MesoMR23-060H-I magnetic resonance imaging system. The absorbance or florescence intensity value inside cells detection was obtained using a Tecan Spark 10M Microplate Reader. Zeta potential of samples and DLS were detected using MALVERN NANO-ZS90 laser particle size analyzer. In vivo fluorescence images were obtained by IVIS Lumina K (Series III).

Synthetic of cationic ZnPc. Brief synthetic routes of cationic ZnPc were shown in Scheme S1.



Scheme S1. Brief synthetic route of cationic ZnPc.

4-(tritylamino) phenol (compound 1). 4-(amino) phenol (436 mg, 4.00 mmol) was dissolved in DMF (8 mL) under N_2 protection at 25 °C by string. After that, triphenylmethyl chloride (1.67 g, 6.00 mmol) and trimethylamine (2 mL) in CH_2Cl_2 (25 mL) was added to the solution dropwise over a period of 6 h and the formed solid material was filtered off and washed with CH_2Cl_2, saturated salt solution and H_2O for 3 times. After been drying with anhydrous Na_2SO_4 for 4 h, the product was obtained by filtrating. Compound 1 (1.07 g, 76%) was purified by column chromatography (eluent: ethyl acetate/petroleum ether, 1:4, v/v). M. P. 151 °C. IR (KBr, cm^{-1}): 3543 (OH), 3429 (NH), 3026 (Ar-H), 2824, 1597, 1512, 1248, 762. ^1H NMR (400 MHz, $CDCl_3$): *δ* (ppm) 7.65 (t, 6H, *J*=4.4 Hz, Ar-H), 7.43-7.29 (m, 8H, Ar-H), 7.25-7.18 (m, 5H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6): *δ* (ppm) 157.2, 146.8, 132.4, 129.6, 128.3, 127.0, 115.1, 70.6, 48.4. Anal. calcd. for $C_{26}H_{23}NO$: C, 85.44; H, 6.02; N, 3.99. Found: C, 85.40; H, 6.07; N, 4.07.

4, 5-bis (4-(tritylamino) phenoxy) phthalonitrile (compound 2). 250 mg 4, 5-dichlorophthalonitrile (1.27 mmol), 1.11 g compound 1 (3.16 mmol) and 561.2 mg K_2CO_3 (4.06 mmol) were mixed in DMF by string under N_2 protection at 75 °C. After 24 h, the product was cooling to 25 °C. The product was obtained by filtering and washed by CH_2Cl_2, saturated salt solution and H_2O for 3 times. After dried by anhydrous Na_2SO_4, compound 2 (white solid, 0.89 g, 84.8%) was purified by column chromatography (eluent: ethyl acetate/petroleum ether, 1:3 v/v, ethyl acetate/petroleum ether, 1:1, v/v, ethyl acetate/petroleum ether, 2:1 v/v). M.P. > 200 °C. IR (KBr, cm^{-1}): 3438 (NH), 3059 (Ar-H), 2229 (CN), 1587, 1489, 1214 (C-O-C), 899, 712. ^1H NMR (400 MHz, $CDCl_3$): *δ* (ppm) 7.62-7.60 (m, 13H, Ar-H), 7.59-7.52 (m, 4H, Ar-H), 7.37-7.35 (m, 13H, Ar-H), 7.34-7.31 (m, 6H, Ar-H), 7.14-7.08 (m, 4H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6): *δ* (ppm) 152.3, 151.4, 146.2, 139.3, 131.1, 127.7, 128.3, 125.8, 121.0, 113.3, 72.1, 47.6. Anal. calcd. for $C_{58}H_{42}N_4O_2$: C, 84.24; H, 5.22; N, 6.49. Found: C, 84.47; H, 5.30; N, 6.54.

2, 3, 9, 10, 16, 17, 23, 24-8 ((amino) phenoxy) zinc phthalocyanine (compound 3). In 8 mL n-pentanol, 410 mg compound 2 (0.50 mmol), 55.2 mg $Zn(CH_3COO)_2$ (0.30 mmol) and 0.38 mL DBU (2.82 mmol) were mixed and heated (140 °C). After 12 h, the product was cooled to 25 °C. Then, 50 mL CH_3OH was poured into above solution to precipitate green solid (0.53 g, 50.6%). Then, 500 mg above product (0.148 mmol) was dissolved in 20 mL CH_2Cl_2 and 2 mL trifluoroacetic acid (TFA) and the mixture was stirred at 25 °C (3 h). After that, the green solid precipitate compound 3 (0.16 g, 77.1%) was obtained by filtration and washed with CH_2Cl_2. M.P. > 200 °C. IR (KBr, cm^{-1}): 3444 (NH_2), 1649 (NH_2), 1385, 1206 (C-O-C), 1092, 812. ^1H NMR (400 MHz, DMSO-d_6): *δ* (ppm) 8.09 (d, 16H, *J* = 7.6 Hz, Ar-H),

7.42 (s, 16H, Ar-H), 7.30 (s, 8H, Pc-H). MS (MALDI-TOF, $[M + H]^+$) m/z: calcd. for $C_{80}H_{56}N_{16}O_8Zn$: 1432.4. Found: $[M+H]^+$ 1433.1. Anal. calcd. for $C_{80}H_{56}N_{16}O_8Zn$: C, 66.97; H, 3.93; N, 15.62. Found: C, 67.04; H, 3.97; N, 15.69.

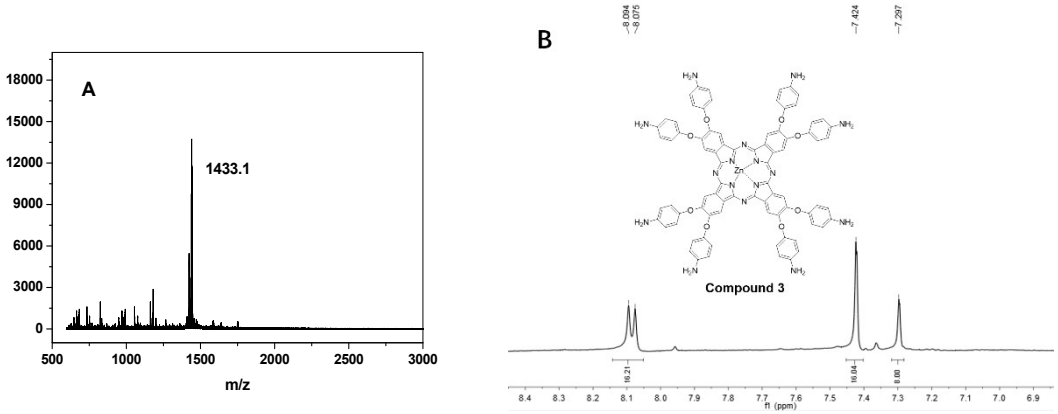


Figure S1. The UltraflexXreme MALDI-TOF-MS spectrometer (A) and ^1H NMR spectrum (B) of compound 3.

2, 3, 9, 10, 16, 17, 23, 24-8- (((amino) methyl)) phenoxy) zinc phthalocyanine (cationic ZnPc). 300 mg compound 3 (0.20 mmol) was dissolved with 25 mL methanol and heated to reflux and stirring for 2 h under N_2 protection. Excess iodomethane was gradually added to above solution and reacted for another 24 h. After cooled to 25 °C, cationic ZnPc (0.30 g, 53.3%) was collected by filtration and washed with ethyl acetate and dichloromethane for 3 times. M. P. > 200 °C. IR (KBr, cm^{-1}): 3436, 3054, 1609, 1499, 1396, 1269 (C-O-C), 1215, 1088, 889; ^1H NMR (400 MHz, DMSO-d_6): δ (ppm) 7.88-7.19 (br, 40H, Ar-H, Pc-H), 2.92 (s, 72H, CH_3). Anal. calcd. for $C_{104}H_{112}I_8N_{16}O_8Zn$: C, 44.70; H, 4.04; N, 8.02. Found: C, 44.92; H, 4.45; N, 7.93.

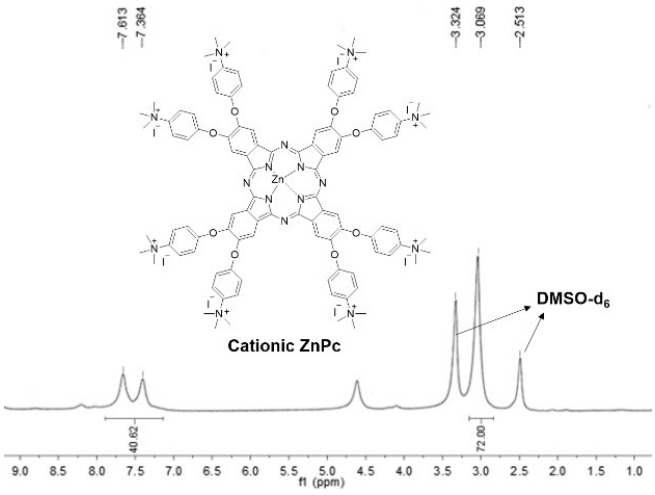


Figure S2. The ^1H NMR spectrum of cationic ZnPc.

***Synthesis of methacrylic anhydride modified HA (m-HA)*:** m-HA was synthesized according to previous reports by our research group. Briefly, HA (0.20 g) and methacrylic anhydride (0.04 g) were dissolved in 10 mL distilled water (pH=8~9) and the mixture was kept under 4 °C for 48 h stirring. After that, the product can form precipitate by adding acetone. m-HA was purified by dialyzing against water for 48 h (dialysis tube: 12-14 KD). ^{1}H NMR (400 MHz, D_2O, δ): 1.846 (s, 3H, CH_2 = $C(CH_3)CO$), 1.920 (s, 3H, $NHCOCH_3$), 5.621 (s, 1H, CH_1H_2 = $C(CH_3)CO$), 6.117 (s, 1H, CH_1H_2 = $C(CH_3)CO$). Methacrylic anhydride modification degree of our m-HA was about 17%.

O_2 release behavior of BRBC using chemical method. $Ru(dpp)_3Cl_2$, a O_2 sensitive fluorescence probe, was used to analyzed the extracellular and intracellular O_2 release behavior of BRBC. [1] To detecting the extracellular O_2 release behavior of BRBC, 500 μL drugs, including ZnPc, BRBC and BRBC+HAase, were added into 2.5 mL N_2 saturated $Ru(dpp)_3Cl_2$ (10 μg) solution. After that, the three solutions were irradiated by 665 nm LED for 2 min at intervals of 10 min and their fluorescence intensity from $Ru(dpp)_3Cl_2$ was acquired by fluorescence spectroscopy at predetermined time points. The above solutions were also measured at intervals of 10 min without irradiation as control.

2. Result and discussions

The DLS results proved the well and stable dispersing statuses of particles in water and different culture medium, including saline and DMEM (Figure S3).

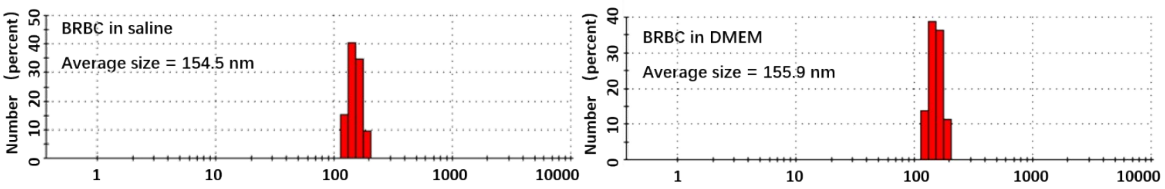


Figure S3. DLS pattern of BRBC in saline and BRBC in DMEM.

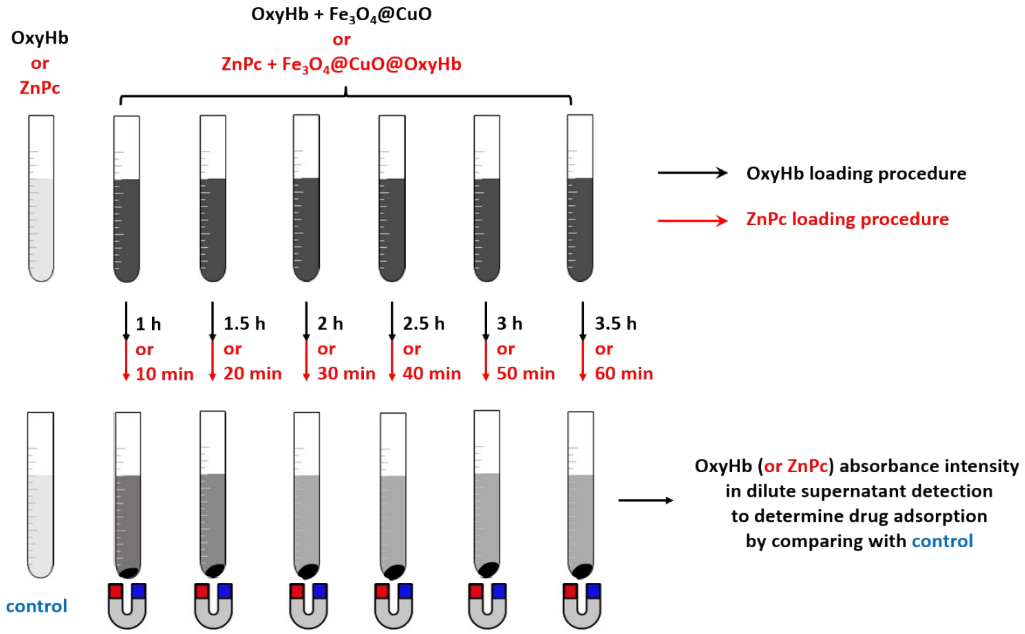


Figure S4. The schematic presentation of drug loading procedure.

$Ru(dpp)_3Cl_2$ showed strong fluorescent signal under hypoxic condition and weak fluorescent signal under O_2 rich environment. As shown in Figure S5, there were no obvious fluorecence changes of BRBC and ZnPc in the dark condition. The fluorescence intensity of BRBC+HAase group showed gradually decreasing, which indicated O_2 releasing from BRBC after HA shell degradation. After 665 nm light irradiation, the fluorescence intensity of the ZnPc was increased rapidly in 10 minutes, indicating the O_2 consumption during PDT process. On the contrary, the fluorescence intensity of BRBC and BRBC+HAase groups, tended to stable during 10 min irradiation, which indicated that there was a balance between O_2 supply by BRBC and consumption by PDT process.

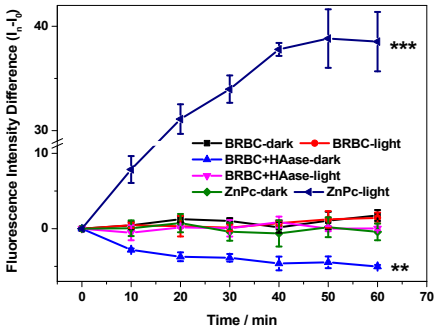


Figure S5. The $Ru(dpp)_3Cl_2$ fluorescence changing in hypoxic solution with ZnPc, BRBC and BRBC+HAase during 10 min 665 nm under light irradiation or dark condition. The fluorescence intensity difference value was obtained from $Ru(dpp)_3Cl_2$ before irradiation (I_0) and after irradiation (I_n). (**p<0.01, ***P<0.001, ZnPc-light or BRBC+HAase-dark vs. ZnPc-dark)

Similar to catalase, Fe_3O_4@CuO nanoplates can catalyze the reaction of the substrate 3,3',5,5'-tetramethylbenzidine (TMB) by H_2O_2 to produce a blue color product, with maximum absorbance at 652 nm, to verify O_2 generation (Figure S6). [2]

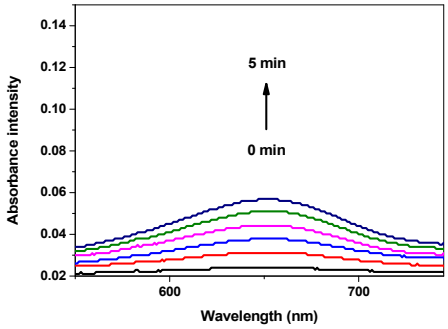


Figure S6. Absorbance spectra changing from the aqueous mixture of TMB (0.33 μM), H_2O_2 (100 μM) and Fe_3O_4@CuO (7.2 μg mL^{-1}) in 0 to 5 min.

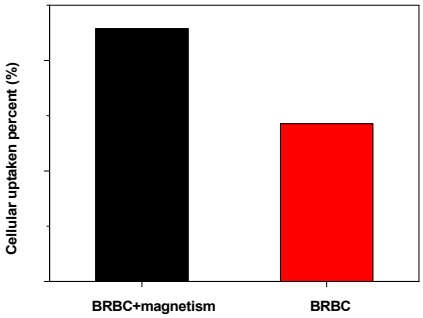


Figure S7. Cellular uptake percent comparing of BRBC with or without magnetism during 4 h incubation time.

To verify the delivery capacity of oxygen by BRBC, a Fe_3O_4@CuO@MetHb@ZnPc@HA was prepared. As showed in Figure S8, in vitro anticancer activity of the new particle was obviously lower than that of BRBC under hypoxia condition, which prove that BRBC have effective O_2 delivery capacity.

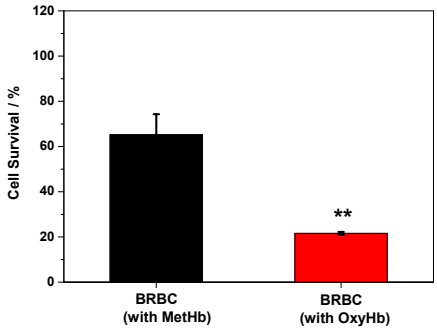


Figure S8. Light toxicity comparison of Fe_3O_4@CuO@MetHb@ZnPc@HA and BRBC (Fe_3O_4@CuO@OxyHb@ZnPc@HA) under hypoxia condition.

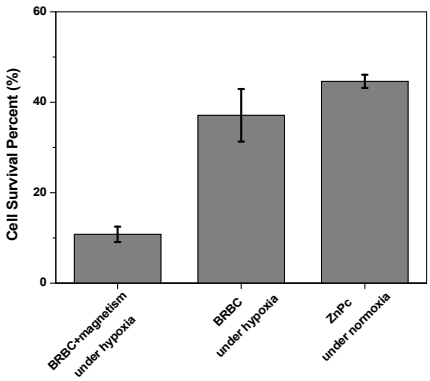


Figure S9. The in vitro photodynamic anticancer activity comparison of BRBC, BRBC+maganatism under hypoxia with ZnPc under normoxia condition.

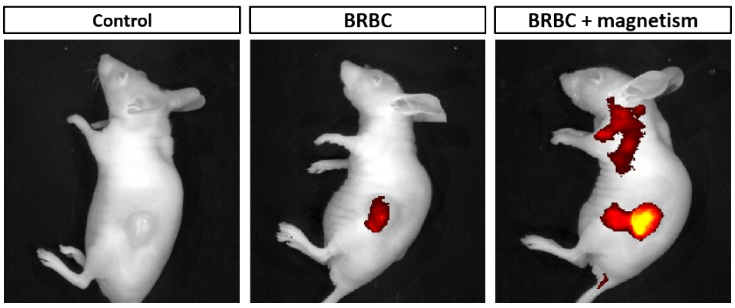


Figure S10. In vivo animal fluorescence images of BRBC in tumors of tumor bearing nude mice with or without magnetic field.

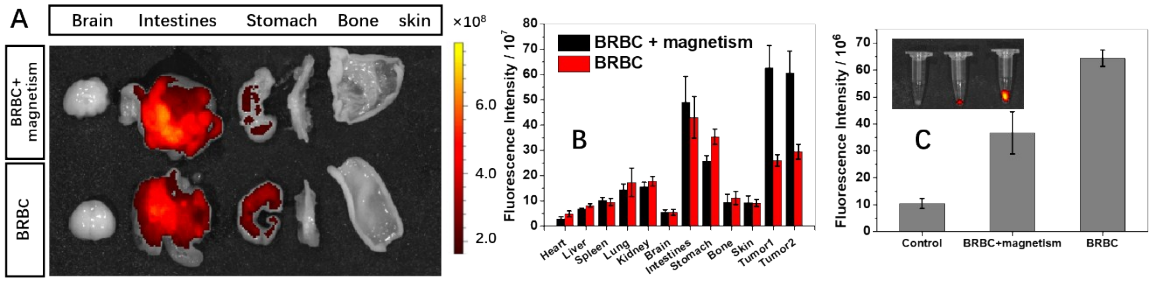


Figure S11. (A) The drug distribution in brain, intestines, stomach, bone and skin for BRBC alone and BRBC+magnetism mice. (B) Fluorescence intensity comparison for all organs. (C) Fluorescence signal and intensity comparison in blood for BRBC alone and BRBC+magnetism mice.

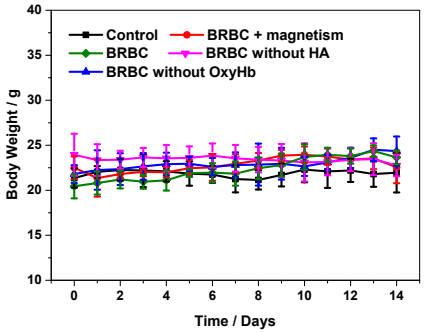


Figure S12. Weight changing of mice during 14 days treatments by various drugs.

Reference

1. P. Wang, X. Li, C. Yao, W. Wang, M. Zhao, A. M. El-Toni and F. Zhang, *Biomaterials*, 2017, **125**, 90-100.

2. W. He, Y. Liu, J. Yuan, J.J. Yin, X. Wu, X. Hu, K. Zhang, J. Liu, C. Chen, Y. Ji and Y. Guo, *Biomaterials*, 2011, **32**, 1139-1147.