Supplementary Information

Preventing Radiobleaching of Cyanine Fluorophores Enhances Stability of Nuclear/NIRF Multimodality Imaging Agents

Reinier Hernandez[1,2], Sandra Heskamp[1], Mark Rijpkema[1], Desirée L. Bos[1], David M. Goldenberg[3], William J. McBride[3], Alfred Morgenstern[4], Frank Bruchertseifer[4], Weibo Cai[2,5,6], Otto C. Boerman[1,*]

[1]Department of Radiology and Nuclear Medicine, Radboud University Medical Center, Nijmegen, The Netherlands

[2]Department of Medical Physics, University of Wisconsin, Madison, Wisconsin, USA

[3]Immunomedics, Morris Plains, NJ, United States

[4]European Commission, Joint Research Centre, Institute for Transuranium Elements, Karlsruhe, Germany

[5]Department of Radiology, University of Wisconsin, Madison, WI, USA

[6]University of Wisconsin Carbone Cancer Center, Madison, WI, USA

*Corresponding author; email: Otto.Boerman@radboudumc.nl

Materials and Methods

Fluorescent Compounds and Radioisotopes

IRDye 800CW was purchased from LI-COR Biotechnology (Bad Homburg, Germany) and Cy7, Cy5.5, and Cy3 were acquired from GE Healthcare (Eindhoven, The Netherlands). RDC018, a pretargeting peptide conjugated to 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) chelator and the NIRF dye Dylight 800 was prepared and provided by Immunomedics Inc. (Morris Plain, NJ). $^{111}InCl_3$ (Mallinckrodt, Petten, The Netherlands) was purchased as HCl (0.01 M) solution with a specific activity (SA) of 60.5 MBq/nmol. ^{68}Ga was eluted in 3mL of 0.1 M Ultrapure HCl (J.T. Baker, The Netherlands) from a TiO_2-based $^{68}Ge/^{68}Ga$ generator (IGG-100, Eckert & Ziegler, Berlin, Germany); at least 6 h were allowed between elutions. ^{213}Bi was eluted from a $^{225}Ac/^{213}Bi$ generator in 600 µL of 1:1 0.1 M HCl: 0.1M NaI. Radionuclides were used without further purification/concentration.

RDC018 Spectra

Fluorescence readings were performed in an Infinite 200 Pro (Tecan, Männedorf, Switzerland) plate reader using Costar flat bottom 96 well plates (Fisher Scientific, Waltham, MA). To record RDC018 absorption spectrum, 50 µL of an aqueous solution containing 75 pmol of RDC018 were added to transparent 96 well plates, and the absorption of the sample was determined

between 600 nm and 900 nm, in 5 nm increments. Fluorescence emission spectrum was acquired using 740 nm excitation. To black 96 well plates, 15 pmol of RDC018 in 200 µL of water were added, and fluorescence emission was recorded between 760 nm and 850 nm, in 2 nm wavelength increments. The excitation wavelength was selected 50 nm short of the expected fluorescence maximum to minimize background signal from the excitation laser. Spectra were presented as normalized fluorescence vs. wavelength. For the rest of the studies using 800CW or RDC018, the excitation and emission wavelength were set to 740 nm and 796 nm, respectively.

Buffer Selection

The influence of the pH and type of buffer in the fluorescence of 800CW was determined. Four buffer solutions were prepared by dissolving NaAc, NH_4Ac, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), and 2-(N-morpholino)ethanesulfonic acid (MES) at a concentration of 0.1 M, and the pH adjusted to 4.5. Solutions of 800CW (5µg/mL) were prepared using each buffer and fluorescence intensity determined. NaAc buffer, which displayed the strongest fluorescence signal output, was selected for the rest of the experiments. Following, several 0.1 M NaAc solutions were adjusted to pH between 3 and 5.5 and 800CW was added to a final concentration of 5 µg/mL. Additionally, another 800CW solution was prepared in PBS (pH 7.4). 800CW fluorescence under different pH was determined as described above.

Temperature

To investigate the effect of heating on the fluorescence of 800CW, 5 µg/mL solutions of the dye in NaAc (0.1 M, pH 4.5) were placed in a heating block at 95°C for 5, 15, 30, or 60 min. Following, samples were cooled down to room temperature, measured in the plate reader, and the fluorescence of each sample was normalized to a non-heated control.

IRDye800CW Irradiations and Mass Spectrometry

To assess the radiosensitivity of 800CW, 5 µg/mL aqueous solution of the dye in 0.1 M NaAc buffer pH 4.5 were prepared and mixed with increasing activities of ^{68}Ga, ^{111}In, and ^{213}Bi (1-20 MBq) for a 310 µL final volume. Radioactive solutions were incubated in 1.5 mL Eppendorf tubes at 4°C in the dark, and 100 µL aliquots taken at 0.5, 6, and 24 h time points for fluorescence measurement. The effect of volume on 800CW's radiosensitivity was determined by incubating 150, 300, 550,800, or 1050 µL of 5 µg/mL buffered dye solution with 20 MBq of ^{68}Ga. All fluorescence readings were normalized to non-irradiated controls.

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry analysis was performed on 800CW samples irradiated with 20 MBq of ^{68}Ga. Before analysis, samples were kept at 4 ºC for, at least, ten ^{68}Ga half-lives. 0.01M NH_4Ac was employed as incubation buffer to avoid compatibility issues with MALDI-TOF.

Radioprotection with Radical Scavengers

The radioprotective effect of three common hydroxyl radical scavengers, ethanol, gentisic acid (GA) and ascorbic acid (AA) was tested. Buffered 800CW solutions were prepared as described in the previous section, spiked with different concentrations of each scavenger, and incubated with 20 MBq of ^{68}Ga for 0.5, 6, and 24 h at 4°C and protected from light. Radioprotection was assessed by measuring sample's fluorescence. ^{68}Ga, ^{111}In, and ^{213}Bi irradiations of 800CW, as described in "*IRDye800CW Irradiation*" section, were repeated with each sample containing 0.1 % (w/v) AA.

Imaging

PET/CT imaging was performed in an Inveon microPET/microCT scanner (Siemens Medical Solutions USA, Knoxville, TN). Four samples were prepared containing 5 µg/mL of 800CW in 0.1 M NaAc, and either 0.1 % (w/v) AA, ^{68}Ga (20 MBq), or both. The samples were placed in the scanner and sequential CT, then PET, images were acquired. Image co-registration and analysis was perform using the Inveon Research Workplace software (Siemens Medical Solutions USA, Knoxville, TN). Near-infrared fluoresce images of the samples were acquired after 0.5, 3, and 6 h of incubation, in an IVIS Spectrum Preclinical In Vivo Imaging System (Perkin Elmer, Waltham, MA) using 745 nm and 800 nm excitation and emission filters, respectively. Region-of-interest (ROI) analysis of the fluorescent images was carried on the system dedicated software, and quantitative data were expressed as radiant efficiency ([p/sec/cm$^{2/}$sr]/[µW/cm^2]), mean ± SD.

Radiosensitivity of Cyanine Dyes

To determine their radiosensitivity, three other cyanine dyes, Cy7, Cy5.5, and Cy3 were incubated with 10 MBq of ^{68}Ga in NaAc (0.1 M; pH 4.5). Two hours after irradiation, the normalized fluorescence was determined for each dye using the recommended excitation/emission wavelength pair: Cy7 (λ_{ex}:740 nm; λ_{em}: 774 nm), Cy5.5 (λ_{ex}:640 nm; λ_{em}: 695 nm), and Cy3 (λ_{ex}:520 nm; λ_{em}: 570 nm). We also corroborated the radioprotective effects of the addition of AA (0.1%w/v) to the samples prior irradiation. The two-hour data point for Cy5.5 was obtained by interpolation of the 0.5 h and 15 h time points using an exponential decay fit.

RDC018 Irradiation and Radioprotection

To assess RDC018 (MW: 2541.9 g/mol) radiosensitivity, 70 ng/MBq of the peptide in NaAc (0.1 M; pH 4.5) were mixed with 350 MBq of ^{68}Ga, 116 MBq of ^{111}In, or 30 MBq of ^{213}Bi, and incubated for 10 min at 95ºC in Eppendorf vials. Instant thin-layer chromatography (ITLC) using 0.1 NH_4Ac/0.1 M EDTA as mobile phase was employed to confirm radionuclide incorporation into RDC018. Vials were stored at 4°C in the dark and 100 µL samples taken at 0.5, 6, and 24 h post-incubation for fluorescence measurement. The experiments were repeated in solutions containing 0.1% AA. Finally, buffer solutions containing 2.5 µg of RDC018 were prepared with or without adding 0.1% AA. Different ^{213}Bi activities were spiked and samples incubated for 0.5,

6, and 24 h. The extent of radiobleaching was determined at each incubation time point by measuring normalized fluorescence.

Calculations and Statistical Analysis

All measurement were performed in triplicate and result expressed as mean ± SD. Unless otherwise stated, all fluorescence measurement were normalized to the appropriate controls. Estimation of the dose imparted by each radionuclide to a water sphere was performed using OLINDA/EXM software. Since ^{213}Po was considered in secular equilibrium with ^{213}Bi, the integral decays of both isotopes were considered equal.

Figures

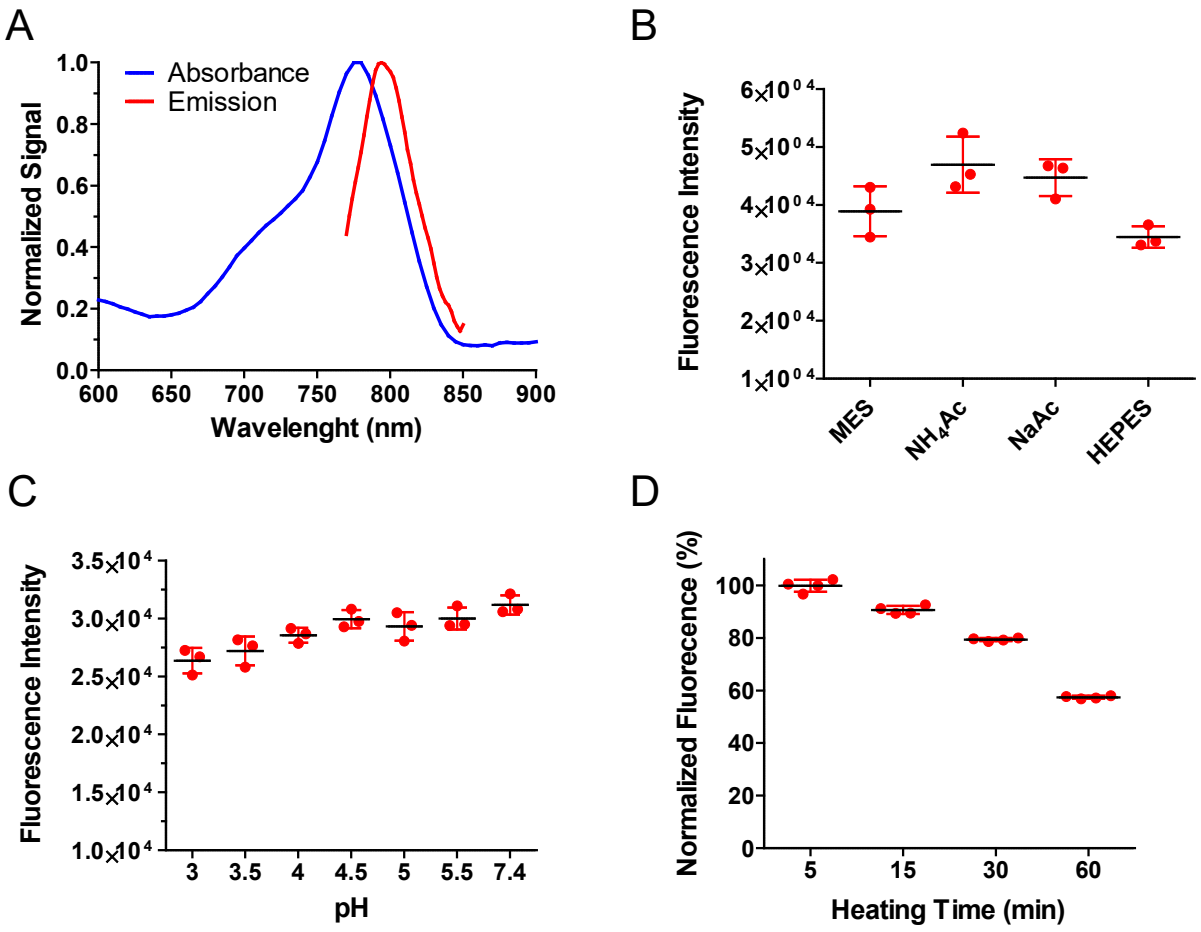

Figure S1. Characterization of the experimental conditions employed for radiobleaching studies. **A)** Absorption and emission spectra of RDC018. Similar to 800CW spectra, RDC018 shows an emission maximum at 796 nm. **B)** Effect of buffer selection on 800CW fluorescence signal under the same pH = 5.5. NaAc and NH_4Ac had the least influence. **C)** Influence of the pH of 0.1 M NaAc buffer on 800CW fluorescence signal. Fluorescent intensity slightly decreased with lowering pH. **D)** Effect of prolonged heating on the photostability of 800CW solutions. A linear decrease in fluorescence down to ~60% of the initial values was observed after 1h incubation at 95°C.

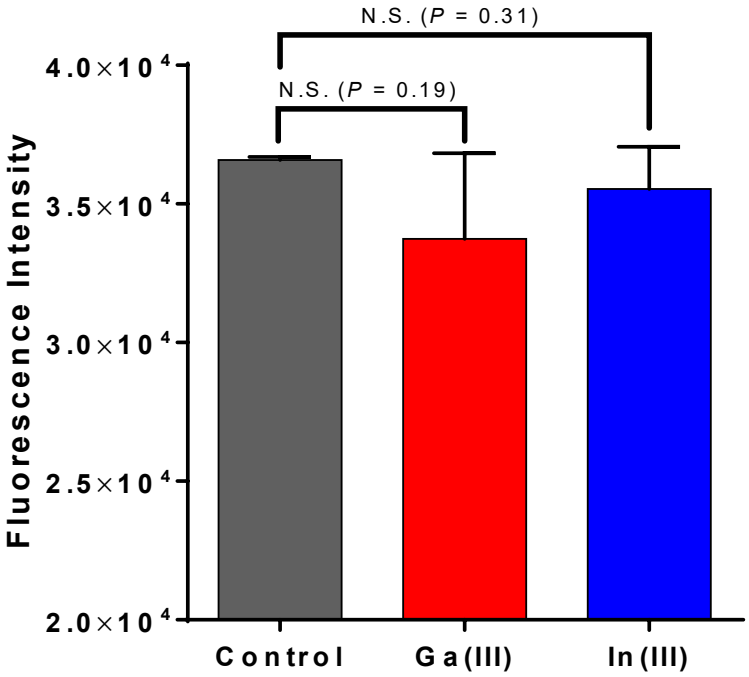

Figure S2 Effect of non-radioactive metals on the fluorescence of 800CW. No significant (N.S.) effects on the fluorescence signal were noted after incubation with trace amounts of non-radiactive Ga(III) and In(III).

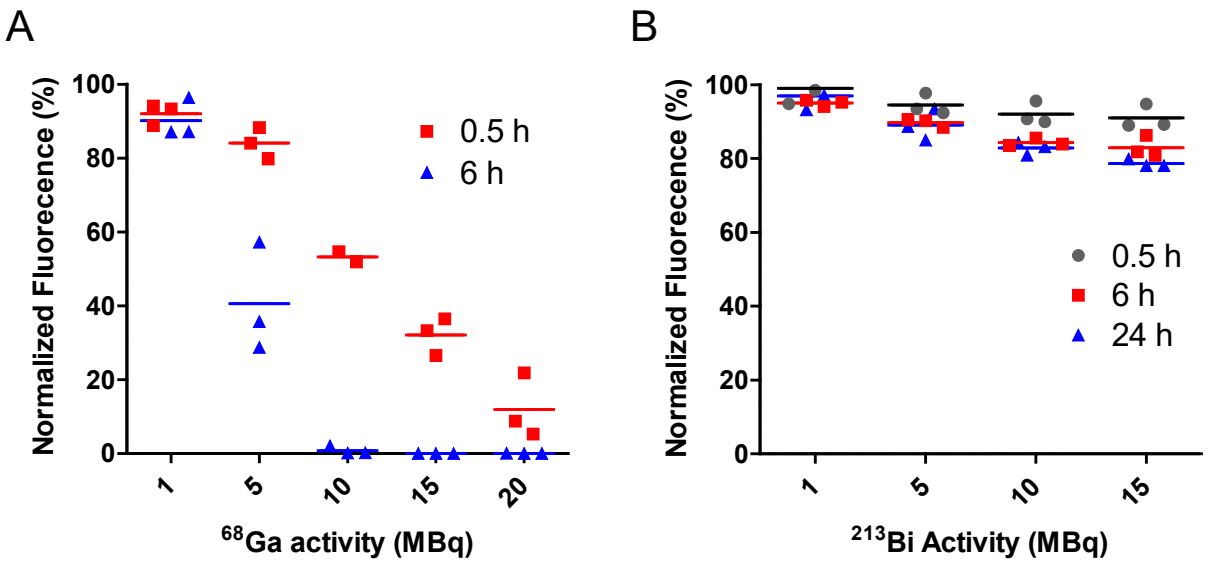

Figure S3 Time and activity-dependent radiobleaching of irradiated IRDye800CW solutions. Normalized 800CW fluorescence 0.5, 6, and 24 h after exposure to increasing activities of ^{68}Ga **(A)** and ^{213}Bi **(B)**.

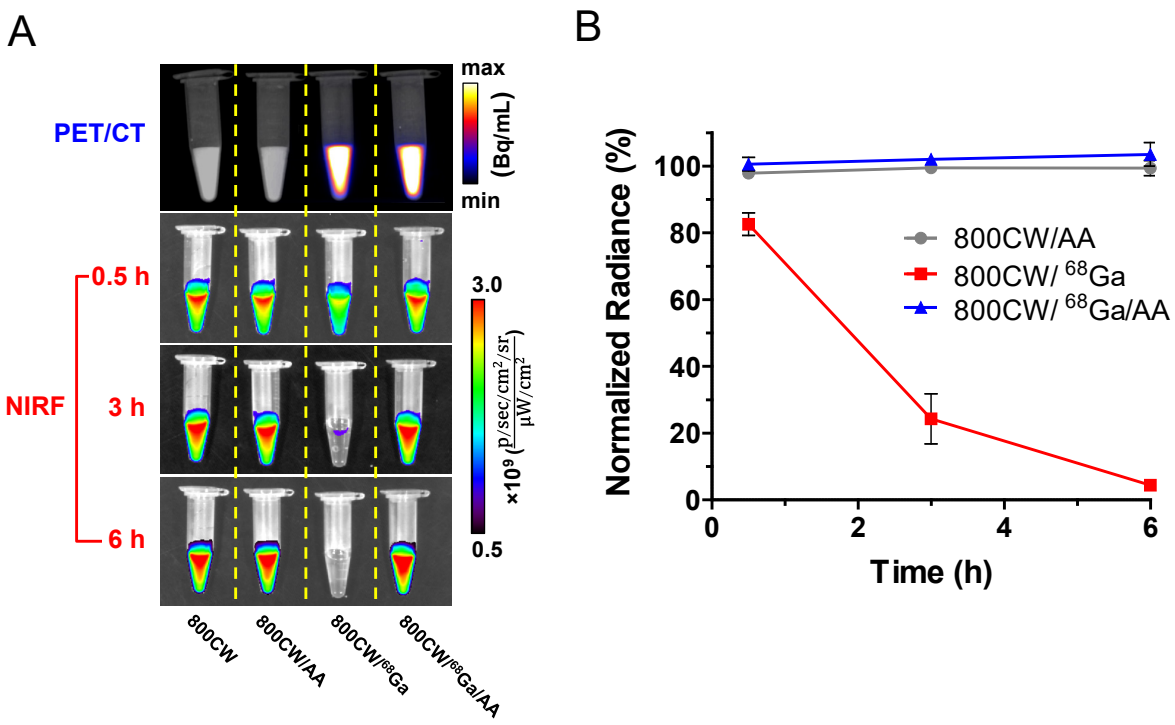

Figure S4 Fluorescent imaging of irradiated 800CW solutions. **A)** PET/CT and NIRF images reveal that 800CW solution incubated with 20 MBq of ^{68}Ga experienced a marked radiobleaching. 800CW fluorescence was conserved in solution containing 0.1 (%w/v) AA. **B)** Quantitative ROI analysis of NIRF images shows a drastic decline in normalized radiance of ^{68}Ga-irradiated 800CW solutions, effect that was counteracted by the addition of AA.

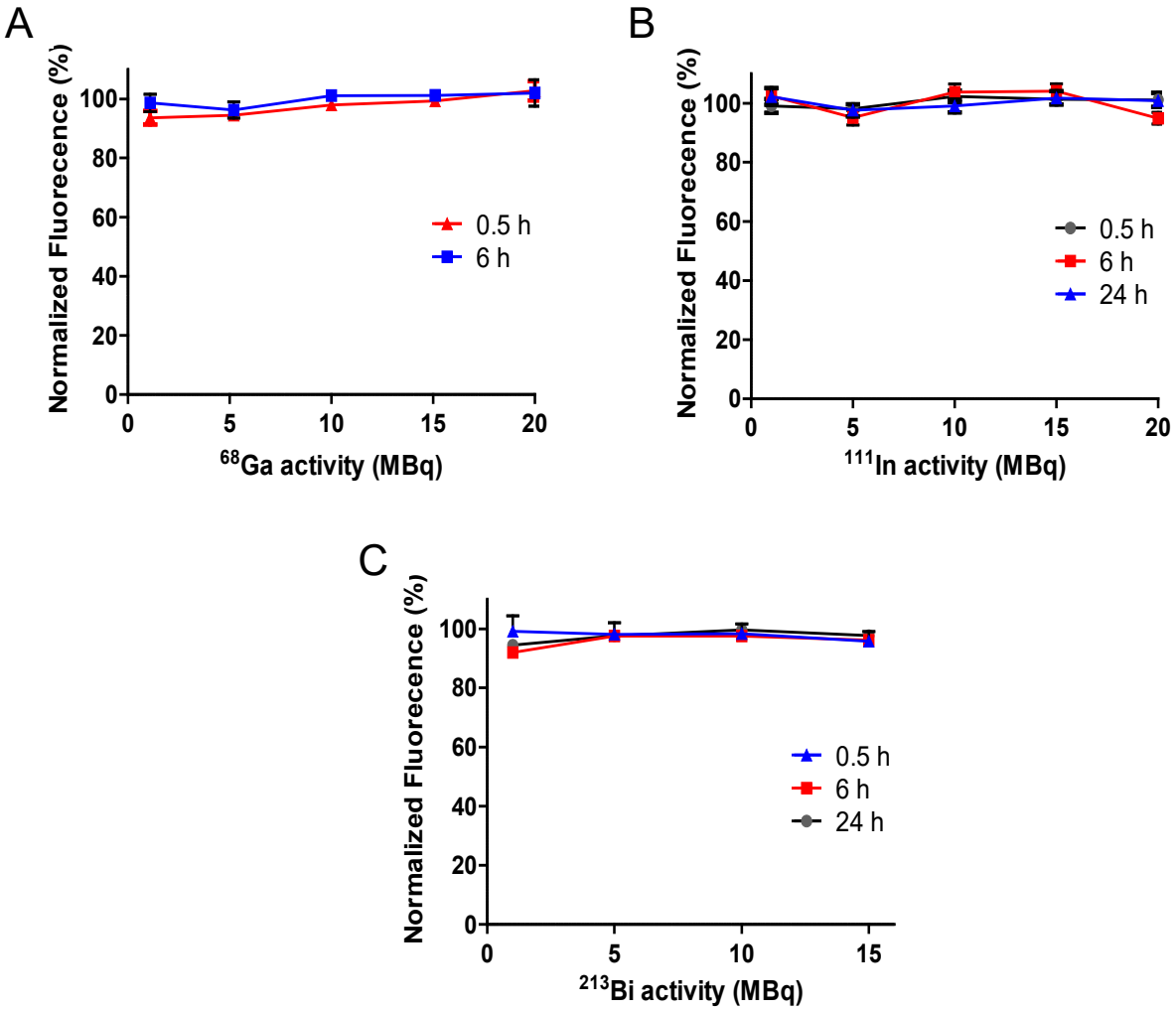

Figure S5 Radioprotection of 800CW by the addition of 0.1 % AA. AA preserves the fluorescence of 800CW at all tested activity levels of ^{68}Ga **(A)**, ^{111}In **(B)**, and ^{213}Bi **(C)**.

Table S1. Estimated integral dose imparted to water per mCi of ^{68}Ga, ^{111}In, ^{213}Bi, and ^{213}Po.

Volume (mL)	Calculated integral dose to water (mGy/mCi)			
	^{68}Ga	^{111}In	^{213}Bi	^{213}Po*
0.01	1.92E+04	1.62E+05	2.45E+04	5.28E+05
0.1	4.03E+03	1.72E+04	3.03E+03	5.28E+04
0.5	1.03E+03	3.50E+03	6.47E+02	1.05E+04
1.0	5.63E+02	2.23E+03	3.33E+02	5.28E+03
2.0	2.97E+02	1.17E+03	1.69E+02	2.65E+03
4.0	1.56E+02	6.26E+02	8.57E+01	1.32E+03
6.0	1.06E+02	4.34E+02	5.76E+01	8.84E+02
8.0	8.08E+01	3.34E+02	4.34E+01	6.63E+02
10.0	6.56E+01	2.74E+02	3.48E+01	5.28E+02
20.0	3.37E+01	1.49E+02	1.75E+01	2.65E+02
40.0	1.73E+01	8.18E+01	8.84E00	1.32E+02
60.0	1.18E+01	5.77E+01	5.92E00	8.84E+01
80.0	8.96E00	4.51E+01	4.46E00	6.63E+01
100.0	7.20E00	3.74E+01	3.58E00	5.28E+01
300.0	2.53E00	1.53E+01	1.20E00	1.76E+01
400.0	1.93E00	1.21E+01	9.08E-01	1.32E+01
500.0	1.56E00	1.01E+01	7.26E-01	1.05E+01
600.0	1.31E00	8.74E00	6.08E-01	8.84E+00
1000.0	8.08E-01	5.84E00	3.67E-01	5.28E+00
2000.0	4.29E-01	3.39E00	1.87E-01	2.65E+00
3000.0	2.94E-01	2.48E00	1.25E-01	1.76E+00
4000.0	2.25E-01	1.99E00	9.43E-02	1.32E+00
5000.0	1.83E-01	1.67E00	7.58E-02	1.05E+00
6000.0	1.55E-01	1.46E00	6.32E-02	8.84E-01

* ^{213}Po activity was estimated assuming secular equilibrium with ^{213}Bi
1 mCi = 37 MBq
Radiation doses were calculated using a water sphere model implemented in OLINDA/EXM software.