Balanced Intersystem Crossing in Iodinated Silicon-Fluoresceins Allows New Class of Red Shifted Theranostic Agents

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1. Materials and instruments

All reagents were commercially available and used without further purification unless otherwise noted. All dry solvents used in reactions were directly obtained from the Mbraun MBSPS5 solvent drying system. The inert atmosphere was obtained by argon. The $^1$H and $^{13}$C-NMR spectra were recorded on a Bruker (500 MHz) spectrometers using CDCl$_3$ as the solvent. The chemical shifts are reported in parts per million (ppm) downfield from an internal TMS (trimethylsilane) reference. Coupling constants ($J$) are reported in hertz (Hz), and the spin multiplicities were specified by the following symbols: s (singlet), d (doublet), t (triplet), and m (multiplet). NMR spectra were processed with MestReNova program. Column chromatography was performed by using thick-walled glass columns and silica Gel 60 (Merck 230-400 mesh). Thin layer chromatography (TLC Merck Silica Gel 60 F254) was performed by using commercially prepared 0.25 mm silica gel plates and visualization was provided by UV lamp. The relative proportions of solvents in chromatography solvent mixtures refer to the volume: volume ratio. Electronic absorption spectra in solution were acquired using a Shimadzu Uv-3600 UV-Vis-NIR spectrophotometer. Fluorescence spectra were determined on Agilent Cary Eclipse fluorescence spectrophotometer. Fluorescence quantum yields of the samples were investigated by using a fluorescence spectrometer (FLS 1000, Edinburgh Instruments) with an integrating sphere accessory. Mass spectra were recorded on Waters Synapt G1 High-Definition mass spectrometer. The purity of the biologically evaluated compound was confirmed by $^1$H and $^{13}$C NMR.

2. Synthesis

![Synthetic pathway diagram](image)

Figure S1. Complete synthetic pathway for SF-I.

Synthesis of SF-I:

SF$_1$ (20 mg, 0.027 mmol) and I$_2$ (15 mg, 0.059 mmol) were dissolved in 20 mL EtOH. Then, Iodic acid solution in 0.5 mL water (10 mg, 0.27 mmol) was added to the reaction mixture. The mixture was heated to reflux for 3 hours. Reaction was cooled down to room temperature and EtOH was removed under reduced pressure. The crude was diluted with 130 mL EtOAc and the
mixture was washed with saturated sodium thiosulfate solution. Organic layer was separated, dried over Na$_2$SO$_4$ and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel (Hex:EtOAc 2:1) and the target compound 20 mg (42% yield) was obtained.

$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.94 (d, $J$ = 7.6 Hz, 1H), 7.60 (s, 2H), 7.55 – 7.51 (m, 1H), 7.48 – 7.45 (m, 1H), 7.00 (dt, $J$ = 7.8, 0.9 Hz, 1H), 6.05 (bs, 2H), 1.23 (s, 3H), 1.01 (s, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 170.37, 156.36, 153.13, 141.46, 139.05, 136.68, 135.24, 129.31, 126.66, 122.25, 122.11, 92.56, 87.82, 84.97, 1.31, 1.02. HR-MS m/z calc. for C$_{22}$H$_{14}$I$_4$O$_4$Si: 877.6840 [M+H]$^+$; found: 878.6923.

3. Photophysical Characterization

**pH Titration:**

To acquire the absorption and fluorescence characteristics of SF-I in aqueous solution, solutions with different pH values (glycine (pH 2.5-3.5), sodium acetate (pH 4.0- 5.5), MES (6.0-6.8), PBS (pH 7.2-8.2) and carbonate (pH 9.2-10.0) in 50 mM concentration) were prepared. In each case concentration of SF-I adjusted to 5 µM and final DMSO amount was %0.5.

**Fluorescence quantum yield calculation:**

Fluorescence quantum yield of SF-I was investigated by using a fluorescence spectrometer (FLS 1000, Edinburgh Instruments) with an integrating sphere accessory. A continuous-wave xenon lamp was used as the excitation source and the emitted fluorescence was detected with a standard photomultiplier (PMT-900) covering a wavelength range of 200-800 nm. During the measurements, the PMT was cooled down to -20 °C by using a built-in housing to reduce the undesired dark current noise. For quantum yield measurement, an integrating sphere (Edinburgh Instruments) was placed inside the sample compartment of the spectrometer. Internal cavity of the sphere was coated with a PTFE-like material to enable a reflectance of approximately >99% (>95%) over the wavelength range between 400 and 1500 nm (250 and 2500 nm). The sphere had two ports which were 90° apart. The excitation beam was sent to the sample through the excitation port and the fluorescence was collected from the emission port. The excitation port of the sphere consisted of a lens to effectively focus the beam on the sample. The emission port was open aperture. Prior to the experiments performed with the samples, the blank spectrum was measured by using the reference solvent (PBS (pH 7.4, 0.5% DMSO)). For both of the measurements (blank and sample), two identical quartz cuvettes with equal volumes were used. First, the reference sample was placed inside the sphere and the emission/excitation slits were adjusted at the excitation wavelength so that the response of the PMT remained linear during the measurements. In order
to cover a scattering range, the emission scans were started from 20 nm below the actual excitation wavelengths (594 nm) and finished at 750 nm. Furthermore, the step size and the integration time of the measurements were set to 1 nm and 0.2 seconds, respectively. After all the emission measurements of the samples and references were complete, the quantum yields of the samples were determined by using the Fluoracle® software. The built-in analysis tool calculates the quantum yield (QY) as

\[ QY = \frac{E_s - E_B}{S_B - S_s} \quad (\text{Eq. 1}) \]

where \( E_s (E_B) \) and \( S_s (S_B) \) are the selected areas for the emitted and scattered signals of the sample (blank).

4. Singlet Oxygen Detection Experiments

a. Chemical detection of singlet oxygen with ADMDA:

Singlet oxygen generation of SF-I and SF was evaluated by using a water-soluble trap molecule 2,2'-(anthracene-9,10-diyl)bis(methylene)dimalonic acid (ADMDA) in aqueous solutions (PBS pH 7.4, 0.5% DMSO). SF-I was tested under 630 nm (24.3 mW/cm²) and 595 nm (9.83 mW/cm²) irradiation, whereas SF was tested only under 595 nm (9.83 mW/cm²) irradiation. Methylene blue (\( \Phi_\Delta = 0.52 \) in PBS buffer) was employed as a reference compound for singlet oxygen quantum yield calculations. SF-I and SF (5 μM) and ADMDA (O.D. = 0.6-1.5) were mixed in oxygen bubbled PBS (pH 7.4, 0.5% DMSO). Initially, several measurements were taken in dark and then the solution was exposed to the LED light (630 or 595 nm) repeatedly from 10 cm of distance in 20 second time interval for 595 nm 30 second time interval for 630 nm. For SF time interval was adjusted as 60 sec. After each irradiation, absorbance of the ADMDA was recorded. For both SF-I, and methylene blue, slope of absorbance maxima of ADMDA at 380 nm versus time graph were drawn. Finally, singlet oxygen quantum yields were calculated according to the equation given below:

\[ \Phi_\Delta(PS) = \Phi_\Delta(ref) \times \frac{m(PS)}{m(ref)} \times \frac{F(ref)}{F(PS)} \times \frac{PF(ref)}{PF(PS)} \quad (\text{Eq. 2}) \]

where PS and ref represent SF-I and methylene blue, respectively. \( m \) is the slope of absorbance maxima of ADMDA at 380 nm versus time graph, \( F \) is the correction factor, which is given by \( F = 1 - 10^{-\text{OD}} \) (OD at the irradiation wavelength, which is 595/630 nm), and PF is absorbed
photonic flux in $\mu$Einstein dm$^{-3}$s$^{-1}$. PF was ignored in the calculations as both SF-I and MB were irradiated with the same light source (595 or 630 nm LED).

Figure S2. Decrease in the absorbance of ADMDA at 380 nm upon 630 nm LED irradiation of SF-I (5 $\mu$M).

Figure S3. Change in the absorbance of ADMDA at 380 nm upon 595 nm LED irradiation of SF (5 $\mu$M).

b. Chemical detection of singlet oxygen with SOSG:

Singlet oxygen generation was evaluated by monitoring the emission change of commercially available SOSG (Singlet Oxygen Sensor Green). To an aqueous solution (pH 7.4, %0.5 DMSO/PBS) of 5 $\mu$M
**SF-I**, SOSG (5 µM) in MeOH was added. The resulting solution was bubbled with O₂ and irradiated with 30 seconds time intervals under both LEDs (630 nm or 595 nm separately). After that, the resulting green emission was recorded at 530 nm by a spectrofluorometer with excitation at 504 nm. As a control, the same experiment was repeated without **SF-I**.

**Figure S4.** Emission spectrum of SOSG upon 630 nm LED irradiation of **SF-I** (5 µM).

**Figure S5.** Emission spectra of SOSG upon 595 nm and 630 nm LED irradiation in the absence of **SF-I**.

5. **Photostability**
Photostability of SF-I was evaluated by exposing aqueous solutions of SF-I (5 µM, PBS pH 7.4, 0.5% DMSO) to LEDs with two different wavelengths (630 nm (24.3 mW/cm²) and 595 nm (9.83 mW/cm²)). SF-I (stock solution: 1 mM in DMSO) were added to oxygen bubbled PBS to obtain 5 µM SF-I (pH 7.4, 0.5% DMSO). Initially, several measurements were taken in dark and then the solution was exposed to the LED light (630 or 595 nm) repeatedly and absorption and emission data were recorded in 30-minute time interval.

![Absorption and emission spectra of SF-I under 630 nm and 595 nm LED irradiation](image)

Figure S6. Absorption (a, c) and emission (b, d) spectra of SF-I under 630 nm and 595 nm LED irradiation respectively.

6. Cell culture

Human colorectal carcinoma (HCT-116) cells and triple negative breast cancer (MDA MB-231) cells were grown in RPMI 1640 and DMEM high glucose supplemented with 10% heat-inactivated FBS, 200 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C, respectively. The cells were sub-cultured at 80–90% confluency in every 2-3 days. Cells were used up to 4-6 passages in all experiments.

7. Dark toxicity and photodynamic effect
To evaluate the dark toxicity, cells were treated with the increasing concentrations (0.5-20 µM) of SF-I (dissolved in DMSO) for 24 h at culture conditions. For the photodynamic effect, cells were treated with same concentrations and irradiated with either 595 ± 5 nm (35.4 J/cm² for 1 h and 70.8 J/cm² for 2 h) or 630 ± 10 nm (87.5 J/cm² for 1 h and 175.0 J/cm² for 2 h) LED light for various time intervals (1 or 2 h), followed by dark incubations up to 24 h at the incubator. Cells viability was calculated by MTT assay.

8. Cell viability assay

The viability of cells was assessed with 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) method. In brief, cells were seeded at a density of 2 × 10⁴ cells/well at 37°C and then incubated with various concentrations of SF-I, as described above. After all incubation periods, 10 µL MTT solution (5 mg/mL in PBS) was added to each well containing 100 µL of fresh medium, and the samples were incubated at 37°C for 4 h. MTT formazan crystals were dissolved with 100 µL 10% SDS in 0.01 M HCl overnight at 37°C. The absorbance of each well was measured at reference wavelengths of 490 nm and 570 nm using a microplate reader (Multiskan Sky, Thermo Scientific, USA). Results were reported as the percentages of DMSO treated vehicle control. LED irradiation has been done at the cell culture conditions (5% CO₂, 37 °C in humidified incubator). The LED light sources (chip leds) were mounted on heat absorbing plates. These plates were cooled with mini fan (low spinning) and cooling jackets to avoid the possible increase in temperature, humidity and air flow. All the experiments were held three times (n=4).

Table S1. Calculated IC₅₀ values of SF-I irradiated with LED light sources (595 nm or 695 nm) at different time intervals (1h or 2h) followed by dark incubation up to 24 h in HCT-116 and MDA MB-231 cancer cell lines.

|                | HCT-116 (IC₅₀) | MDA MB-231 (IC₅₀) |
|----------------|---------------|------------------|
| **LED (λ)**    | **1 h** | **2 h** | **1 h** | **2 h** |
| 595 nm         | 11.34 µM | **5.35 µM** | 7.04 µM | **5.38 µM** |
| 630 nm         | 8.45 µM | 6.72 µM | 13.41 µM | 7.56 µM |

9. Determination of ROS generation

Intracellular formation of ROS was assessed on the basis of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) assay. In brief, cells were seeded at a density of 2 × 10⁴ cells/well. Then cells were treated with SF-I (5 µM) in the presence or absence of 10 mM NaN₃ (singlet oxygen scavenger) and irradiated with 595 ± 5 nm LED light for 2 h at 37°C. After incubation, the medium was removed, and the cells were washed 3 times with PBS. Cells were further incubated with 20 µM DCFH-DA for 30 min at 37°C, shielded from light. Cells were washed with PBS twice and the fluorescence images were captured at 488/535 nm excitation/emission (ex/em) using Zeiss LSM 900 confocal system (Carl Zeiss,
Oberkochen, Germany). H$_2$O$_2$ (500 µM, 2 h) used as a positive control. (20× objective magnification). All the experiments were held in triplicate.

10. Assessment of cellular uptake
Cells were incubated with SF-I (5 µM) for 2 h at 37°C. Then cells were wash with PBS twice and stained with 2 µg/ml Hoechst 33342 in HBSS for additional 10 min at 37°C. After incubations, cells were washed three times with PBS and confocal fluorescence images were captured using Zeiss LSM 900 confocal system (Carl Zeiss, Oberkochen, Germany). Hoechst 33342 and SF-I were visualized at 361/497 nm and 614/630 nm ex/em wavelengths, respectively. 40X oil immersion objective lens was used.

11. Cell apoptosis assay
Cells were treated with SF-I (5 µM) in the presence or absence of 10 mM NaN$_3$ (singlet oxygen scavenger) and irradiated with 595 ± 5 nm LED light for 2 h at 37°C. Then incubated for additional 30 min at dark and stained with Annexin V Alexa Fluor™ 488 (AV)- Propidium Iodide (PI) solution at room temperature in the dark for 15 min, according to the manufacturers protocol. After adding 500 µL of binding buffer, the cells were directly visualized under AV (ex/em: 488/499 nm) and PI (ex/em: 535/617 nm) by Zeiss LSM 900 confocal system (Carl Zeiss, Oberkochen, Germany) (20× objective magnification). All the experiments were held in triplicate.

12. NMR Spectra

Figure S7. $^1$H NMR spectrum of compound SF-I.
Figure S8. $^{13}$C NMR spectrum of compound SF-I.

13. HR-MS

Figure S9. HR-MS spectrum of compound SF-I.

References

1. Grimm, J. B.; Brown, T. A.; Tkachuk, A. N.; Lavis, L. D. General Synthetic Method for Si-Fluoresceins and Si-Rhodamines. *ACS Cent. Sci.* 2017, 3 (9), 975–985.