Supplementary Information for

Photostable far-red emitting pluronic silicate nanoparticles: perfect blood pool fluorophores for biphotonic in-vivo imaging of the leaky tumour vasculature

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Synthesis

2,7-dibromo-9,9-dimethyl-9H-fluorene

2,7-dibromofluorene (0.44 g, 1.35 mmol) and benzyltriethylammonium chloride (0.15 g, 0.68 mmol) were dissolved in DMSO (8 mL) under argon atmosphere. NaOH (1.25 mL, 50 wt%) was added to this mixture portion-wise over 5 min. Bromoethane (0.44 g, 4.04 mmol) was slowly dropped to the reaction mixture. The mixture was stirred at room temperature for 2 days. Ethyl acetate (50 mL) was added to the reaction mixture and then this mixture was washed by HCl (35 wt%) and water. The organic layer was dried over Na$_2$SO$_4$ and filtered. The solution was evaporated to afford a white solid (0.49 g, yield: 95%). $^1$H NMR (300 MHz, CDCl$_3$, ppm): $\delta$ 7.49 (m, 6H), 2.00 (q, $J$ = 7.4 Hz, 4H), 0.31 (t, $J$ = 7.3 Hz, 6H); $^{13}$C NMR (75 MHz, CDCl$_3$, ppm): $\delta$ 151.7, 139.4, 130.2, 126.2, 121.5, 121.1, 56.7, 32.6, 8.4.

7-bromo-9,9-diethylfluorene-2-carbaldehyde

A solution of 2,7-dibromo-9,9-dimethyl-9H-fluorene (1 g, 2.63 mmol) in anhydrous THF (8 mL) was cooled to $-78^\circ$C under argon using an acetone dry-ice bath. n-BuLi (1.64 mL, 2.62 mmol., 1.6 M in hexanes) was added dropwise over 20 min. Dry DMF (0.3 mL) was added slowly to the reaction solution after 1 h at $-78^\circ$C. After stirring for 2 h at $-78^\circ$C, the mixture was brought back to room temperature and the reaction was quenched by carefully adding HCl (2 N). The solution was extracted with dichloromethane. The organic layer was dried over Na$_2$SO$_4$, filtered, and concentrated. The crude product was purified by chromatography on silica gel eluting with ethyl acetate/petroleum ether (1:20 by volume) to afford a white solid (0.8 g, yield: 92 %). $^1$H NMR (300 MHz, CDCl$_3$, ppm): $\delta$ 10.02 (s, 1H), 7.82 (m, 2H), 7.78 (d, $J$ = 8.2, 1H), 7.62 (d, $J$ = 8.6, 1H), 7.48 (m, 2H), 2.03 (m, 4H), 0.26 (t, $J$ = 7.3 Hz, 6H); $^{13}$C NMR (75 MHz, CDCl$_3$, ppm): $\delta$ 192.1, 153.4, 150.3, 146.7, 138.9, 135.6, 130.6, 130.5, 126.5, 123.2, 123.1, 122.2, 120.0, 56.6, 32.5, 8.4.

Optimization of Nanoparticles Preparation and Characterization

F127-SiO2 NPs

In a 5mL round-bottom flask, Pluronic® F127 (200mg) was dissolved in 2-3mL of dichloromethane. A predetermined amount $m$ of dye was added that gave a dye to pluronic weight fraction between 0.05 and 0.30wt% (see entry 1-5, table S-1 below). The solution was subsequently stirred at room temperature for 40min to obtain a homogeneous red solution. Dichloromethane was then evaporated
with a gentle flow of argon to give a film. 0.85N hydrochloric acid (3.12mL) was added and the mixture was stirred until a stable and optically transparent solution was obtained. TEOS (360μL) was then added to the solution and the stirring continued for 105min. DEDMS (30μL) was finally added to terminate the particle growth. The final suspension was kept stirring at room temperature for 24h. The final suspension was kept stirring at room temperature for 24h. The solution was dialyzed for two days against distilled water to remove hydrochloride as well as unreacted low-molecular weight components, using a membrane bag with a 14,000 cut-off molecular weight. The suspension was then filtered through a 0.2μm syringe filter to remove large aggregates. The final volume is 3.6mL.

**Estimation of the encapsulation efficiency**

0.1mL of the above solution was added to DMF (2.5mL) in which complete dissolution of the dye occurred (solution A). The same amount m of dye was dissolved in DMF (3.6mL). 0.1mL of this solution was added to 2.5mL DMF to give the same dilution as above (solution B). The quantity of dye encapsulated into the F127-SiO$_2$ NPs was estimated by measuring the absorption spectra after dissolution of the dye in DMF with reference to a standard in DMF. The encapsulation efficiency (Table S-1, Figure S-1) is defined as the ratio of the amount of the dye loaded in the NPs to the total amount of the dye in the feed mixture.[2]

$$\text{Encapsulation efficiency (in %)} = \frac{\text{Abs}_{\text{solution A}}}{\text{Abs}_{\text{solution B}}} \times 100 \quad (1)$$

<table>
<thead>
<tr>
<th>Entry</th>
<th>Dye 1 feeding ratio</th>
<th>Dye 1 loading ratio</th>
<th>Encapsulation efficiency [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>0.048</td>
<td>96</td>
</tr>
<tr>
<td>2</td>
<td>0.10</td>
<td>0.093</td>
<td>93</td>
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<td>3</td>
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<tr>
<td>5</td>
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<td>0.15</td>
<td>51</td>
</tr>
<tr>
<td>6</td>
<td>0.40</td>
<td>0.17</td>
<td>43</td>
</tr>
</tbody>
</table>

* weight ratio of dye 1 to that of F127 in the feed mixture; * weight of loaded 1 to that of the F127 matrix in the NPs.
**Fig. S1.** Encapsulation efficiency as a function of dye feeding ratio.

**Fig. S2.** TEM image and B) DLS distribution of 1@F127-SiO\textsubscript{2} NPs. C)

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**Optical measurements**

**Absorption spectra** (UV-Vis) were recorded on a dual beam Jasco 670 spectrometer. **Fluorescence spectra** were performed on a Horiba Jobin-Yvon Fluorolog-3® spectrofluorimeter equipped with a red-sensitive Hamamatsu R928 photomultiplier tube. Spectra were reference corrected for both the excitation source light intensity variation (lamp and grating) and the emission spectral response (detector and grating).

**Fluorescence quantum yields** \(\Phi_F\) were measured in diluted solutions. Rubrene in methanol (\(\Phi_F=27\%)\) [3] was used as reference. Sample and reference are excited at the same wavelength (\(\lambda_{exc}\)). The quantum yield relative to the reference is given by equation (2):

\[
\Phi_S = \Phi_{Ref} \left( \frac{S}{S_{Ref}} \right)^2 \left( \frac{n_{Ref}}{n_d} \right)^2 \tag{2}
\]

where \(S\) is the slope obtained by plotting the integrated area under the fluorescence emission spectrum vs the absorbance at \(\lambda_{exc}\) and \(n_d\) the refractive index of the solvents. Superscript \(Ref\) and \(s\) correspond to the reference and the sample respectively. For each experiment 5 points were recorded, all corresponding to an absorbance at \(\lambda_{exc}\) (and for wavelengths above) below 0.1.

**Absolute photoluminescence quantum yield for solid** were measured as described by de Mello et al. [4] and Porrès et al. [5] using an integrating sphere collecting all the emission (2\(\pi\) steradians covered with spectalon®), model F-3018 from Horiba Jobin Yvon. The sphere was calibrated with known standard (Coumarin 153, Rhodamine 6G, DMANS, Erythrosine B and Tetraphenylporphyrin).[6]

**Two-photon absorption spectra** were obtained by two-photon excited fluorescence measurements using a femtosecond Ti:sapphire laser in the range 730–960nm (80MHz, 140fs) as the light source. The excitation beam (5mm diameter) is focalized with a lens (focal length 10cm) at the middle of the fluorescence cell (10mm). The fluorescence signal, collected at 90° from the
excitation beam, was focused into an optical fiber (diameter 600µm) connected to an Ocean Optics S2000 spectrometer. The incident beam intensity was adjusted to 50mW in order to ensure an intensity-square dependence of the fluorescence over the entire spectral range of excitation. Detector integration time was fixed to 1s. Calibration of the spectra was performed by comparison with published 700-900nm Coumarin 307, Coumarin 510, Coumarin 540 and Fluorescein two-photon absorption spectra.[7] Measurements were performed in chloroform (for 1) or PBS (for 1@F127-SiO2 NPs) with concentrations in the range of 1.0×10⁻⁴ to 1.5×10⁻⁴ mol.L⁻¹. Two-photon cross sections $\sigma$ are given by equation (3):

$$\sigma = \frac{\Phi_{\text{Ref}} \cdot \epsilon_{\text{Ref}} \cdot n_d^{S} \cdot F_{\text{Ref}}}{\epsilon_{\text{S}} \cdot n_d^{S} \cdot F_{\text{S}}}$$

(3)

where $c$ is the concentration of the solution, $n_d$ is the refractive index of the solution, $F$ is the TPEF integral intensities of the solution emitted at the exciting wavelength, and $\Phi$ is the fluorescence quantum yield. Superscript $\text{Ref}$ and $\text{S}$ stand for reference and sample respectively.

## Stability measurements

**Photostability** was monitored by recording the fluorescence intensity $I$ over time (60min) under continuous excitation. *Figure S-3* shows the fluorescence intensity over time measured for 1 in dilute chloroform solution, for 1@F127-SiO2 NPs in water and DPBS (1x) and for Fluorescein in water (0.1M NaOH). Fluorescence intensity is expressed as $I/I_0$, where $I_0$ is the fluorescence intensity at maximum emission for time 0.

![Normalized intensity vs. time](image_url)

*Fig. S3*. Photostability of 1 and 1@F127-SiO2 NPs compared with Fluorescein. Samples were excited at 500nm and monitored at their maximum emission peaks.

**Colloidal stability** was checked recording the evolution of the DLS size distribution (*Figure S-4*) and the fluorescence intensity and profile (*Figure S-5*) of 1@F127-SiO2 NPs in 1× DPBS over a two-weeks period of time.
Fig. S4. Evolution of the DLS size distribution evolution for 1@F127-SiO₂ NPs in 1× DPBS over two weeks.

Fig. S5. Evolution of the fluorescence intensity (I/I₀) of 1@F127-SiO₂ NPs in 1× DPBS over two weeks.

Cytotoxicity Cells Assays

**Cell culture protocol:** HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen Corp.) supplemented with 10 % (v/v) fetal bovine serum (Invitrogen Corp.), 50 U.mL⁻¹ penicillin, and 50 μg.mL⁻¹ streptomycin (Invitrogen Corp.) in a humidified incubator containing 5 % CO₂ in air at 37°C.

**Viability tests:** 10.10³ HepG2 cells were seeded in 100μL of supplemented DMEM in a clear 96 well plate (Corning Costar). After 24h incubation, the medium was removed. Cells were washed with PBS and medium was replaced by an appropriate volume of DMEM (Invitrogen Corp.). The NPs solution from the synthesis, after dialysis and filtration, was diluted (final volume of 150µL) to create a range of concentration (dilution 1:100 to 1:10000). Cells were then incubated for 24h. The supernatant was subsequently removed and cells were washed with PBS. 100μL of DMEM and 20μL of the MTS reagent (CellTiter 96R AQueous NonRadioactive Cell Proliferation Assay, Promega) were added. Cells were incubated for another 4h period and the absorbance at 490nm was measured with a microplate.
reader (Multiscan GO Microplate Spectrophotometer, Thermo Scientific). The percentage of viable cells was calculated by dividing the absorbance at a given dilution by the absorbance of the blank. The results given (Fig. S-6) are the average of 2 independent experiments performed in triplicate.

![Fig. S6. MTT assay of HepG2 cells treated with 1@F127-SiO₂ NPs suspensions at different dilutions for 24 h.](image)
**In vivo two-photon microscopy**

**Image processing** was performed with NIH ImageJ software.[8] Vasculature and tumour segmentation were performed using supervised classification with Ilastik software.[9] A 3D model of segmented objects was generated using ImageJ 3D viewer.[10] Texture projection and 3D rendering were performed with Blender (GNU General Public License, Blender Foundation). See legends of figures for details.

**Fig. S7.** Two-photon microscopy image of the maximum intensity projection after 1 min circulation of blue NPs in a mouse ear. The longest arrow indicates the heterogeneous circulation of the NP in a capillary: areas with the slowest perfusion are associated with higher maximum intensity projections. The smallest arrow shows endogenous fluorescence signals from sebaceous glands. These glands are at the surface of the skin, indicating that the imaging depth is less than 100 µm. Two-photon excitation was at 750 nm, scale bar = 100µm.
Fig. S8. 3D two-photon microscopy image of the functional vasculature (red signal of circulating $\text{I}@\text{F127-SiO}_2$ NPs) at the surface of a human glioma growing subcutaneously in a mouse ear. The image is a 3D reconstruction using free software (Blender) after a 3D mosaic acquisition (x-y plane: 6x6 images of 512² pixels with 91 slices in the z-direction, step-size 2µm, imaging depth 360 µm, total acquisition time 5600 s).
**Fig. S9.** @F127-SiO₂ NPs: time lapse acquisition over 1 hour (1 image per minute) showing the vasculature and the lack of diffusion in the tumour (2hv excitation 1000nm).
Fig. S10. RhB-dextran 70 kDa: time lapse acquisition over 1 hour (1 image per minute) showing the vasculature and the diffusion of RhB-dextran 70 kDa in tumour (2hv excitation 800nm).
References


Additional Data

**Fig. S11.** Absorption spectra of 1 in various solvents.

**Fig. S12.** Absorption spectrum and two-photon absorption cross-sections of 1 in CHCl₃ solution in the 740–960 nm range (1@F127-SiO₂ NPs in PBS is given for comparison).
**Fig. S13.** Variation of the absorption spectra of 1 in acetone/water mixture with increasing water fraction (wt %).

**Fig. S14.** Variation of the emission spectra of 1 in acetone/water mixture with increasing water fraction (wt %). Inset: increase of the emission intensity with increasing water fraction showing the AIE behaviour of 1.
Fig. S15. Emission spectra of 1 in crystal, in aggregate in acetone/water mixture (80% wt water), in 1/1 (v/v) toluene/CHCl$_3$ solution and of 1@F127-SiO$_2$ NPs in PBS.
Fig. S16. $^1$H NMR spectrum of 2,7-dibromo-9,9-diethylfluorene in CDCl₃.

Fig. S17. $^{13}$C NMR spectrum of 2,7-dibromo-9,9-diethylfluorene in CDCl₃.
Fig. S18. $^1$H NMR spectrum of 7-bromo-9,9-diethylfluorene-2-carbaldehyde in CDCl$_3$.

Fig. S19. $^{13}$C NMR spectrum of 7-bromo-9,9-diethylfluorene-2-carbaldehyde in CDCl$_3$. 
Fig. S20. $^1$H NMR spectrum of 7-(diphenylamino)-9,9-diethylfluorene-2-carbaldehyde 2 in CDCl$_3$.

Fig. S21. $^{13}$C NMR spectrum of 7-(diphenylamino)-9,9-diethylfluorene-2-carbaldehyde 2 in CDCl$_3$. 


**Fig. S22.** $^1$H NMR spectrum of 1 in CDCl$_3$.

**Fig. S23.** $^{13}$C NMR spectrum of 1 in CDCl$_3$. 
**Fig. S24.** HRMS spectrum of 1.