Electronic Supplementary Information

A phototherapeutic fluorescent β-cyclodextrin branched polymer delivering nitric oxide

Milo Malanga, b Mimimorena Seggio, a Vladimir Kirejev, c Aurore Fraix, a Ivana Di Bari, a Eva Fenyvesi, b Marica B. Ericson c and Salvatore Sortino* a

a Laboratory of Photochemistry, Department of Drug Sciences, University of Catania, I-95125 Catania, Italy. E-mail: ssortino@unict.it

b CycloLab Ltd., H-1097 Budapest, Illatos út 7, Hungary.

C Biomedical Photonics Group, Department of Chemistry and Molecular Biology, m University of Gothenburg, Kemivagen 10, SE 412 96, Sweden.

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1. Materials and methods
All reagents (Sigma-Aldrich) were of high commercial grade and were used without further purification. (±)-Epichlorohydrin (purum, ≥99%) and porphyrin 3 were purchased from Sigma Aldrich and used without further purification. Dialysis tube cellulose membrane molecular weight cut-off= 14.000 was from Sigma.

2. Syntheses and experimental procedures
Monomer 1a, is a fine chemical product of CycloLab. Monomer 1b was prepared according to our previously reported procedure. Briefly, 6-monodeoxy-6-monotosyl-β.CD was gently heated with a large excess of 1,3-diaminopropane. The reaction crude was concentrated under reduced pressure and precipitated with disopropyl ether (DIPE). The solid was filtered-off, extensively wash with DIPE and finally dried until constant weight in a vacuum drying box. The obtained material, 6-monodeoxy-6-mono-(3-aminopropylamino)-βCD, was solubilized in DMSO and heated with an excess of 4-chloro-1-nitro-2-(trifluoromethyl)benzene for two days. The reaction crude was concentrated under reduced pressure and precipitated with chloroform. The solid was filtered-off, extensively washed with chloroform and purified by preparative reverse phase chromatography with water:methanol gradient elution. Polymer 1, β-CD (1 g, 0.88 mmol), 1a (50 mg, 0.03 mmol) and 1b (0.3 g, 0.22 mmol) were suspended in H2O (20 mL); NaOH (0.62 g, 16 mmol) was added to the mixture resulting in complete solubilization then epichlorohydrin (1 mL, 14 mmol) was gradually added and the reaction mixture was heated at 70° C for 5 h. The solution was cooled down to room temperature, neutralized with HCl (5 N), filtered to remove insoluble residue (glass filter porosity 3), dialyzed and freeze drying yielded 1 as a slightly yellow powder (0.8 g).

- Total FITC content based on UV-Vis calibration ca. 0.05 % (w/w).
- Total NOPD content based on UV-Vis calibration ca. 2.4 % (w/w)
- Molecular weight based on SLS: 427 kDa ± 10 (PDI = 0.570)
- Viscosity of a 1% w solution: 1.223 cPoise.
The model NOPD 2 was prepared according to our previously reported procedure.\textsuperscript{25}
Encapsulation of 3 within polymer 1. Solution of 1 was prepared by stirring overnight 2 mg mL\(^{-1}\) of 1 in PBS at pH 7.4. Compound 3 was dissolved in methanol and slowly evaporated to form a thin film. This film was then hydrated with an aqueous solution of 1 in phosphate buffer at a pH of 7.4 0.01 M. The mixture was stirred for 5 hours at 40 °C and then the final solution was left to equilibrate at room temperature and filtered.

Cell mortality experiments
Human squamous carcinoma cells (A431, HPA cultures, Salisbury, UK) were maintained in phenol red-free minimum essential medium (MEM, Invitrogen, Pailsey, UK), supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine EMEM, 1% non essential amino acids (NEAA) at 37°C and in a 5% CO\(_2\) incubator. 2x10\(^4\) cells/well were seeded in a 96-well plate 24 h prior the experiment. 4 h before irradiation full-growth media was substituted with solutions of 1 alone or FBS-free media as a control. After 4 hours of incubation, cells were irradiated with blue light using a 405 nm led. The light doses was ca. 30 J/cm\(^2\) (50 mW/cm\(^2\) for 10 min). Dark control samples were kept in the dark outside incubator during irradiation of tested plates. After irradiation tested solutions were substituted with full growth media and allowed for incubation for 24 h. Afterwards MTT assay was conducted to evaluate phototoxic effect of tested substances on cell viability. Each experiment was repeated in triplicate and the mean SEM for each value was calculated. Statistical analysis of results [Student's t test for paired and unpaired data; variance analysis (ANOVA)] was performed using the statistical software package SYSTAT, version 11 (Systat Inc., Evanston IL, USA). A difference was considered significant at p < 0.05.

3. Instrumentation
\(^1\)H-NMR spectra were recorded on a Varian VXR-600 at 600 MHz. Molecular weight determination was performed using a Malvern Zetasizer Nano ZS instrument running a Static Light Scattering (SLS) method on 1.00 w% aqueous solutions. The viscosities of 1.00 w% solutions were determined by a Cannon-Fenske viscometer at 20.0 °C.

UV-Vis spectra absorption and fluorescence emission spectra were recorded with a JascoV-560 spectrophotometer and a Spex Fluorolog-2 (mod. F-111) spectrofluorimeter, respectively, in air-equilibrated solutions, using either quartz cells with a path length of 1 cm. Fluorescence lifetimes were recorded with the same fluorimeter equipped with a TCSPC Triple Illuminator. The samples were irradiated by a pulsed diode excitation source (Nanoled) at 455 nm. The decay was monitored at 530 nm, each solution was used to register the prompt signal. The system allowed measurement of fluorescence lifetimes from 200 ps. The multiexponential fit of the fluorescence decay was obtained using the following equation:

\[
I(t) = \sum a_i \exp(-t/\tau_i)
\]

Absorption spectral changes were monitored by irradiating the sample in a thermostated quartz cell (1 cm path length, 3 mL capacity) under gentle stirring, using a continuum laser with \(\lambda_{exc} = 405\) nm (ca. 100 mW) having a beam diameter of ca. 1.5 mm.
Direct monitoring of NO release in solution was performed by amperometric detection (World Precision Instruments), with an ISO-NO meter, equipped with a data acquisition system, and based on direct amperometric detection of NO with short response time (< 5 s) and sensitivity range 1 nM – 20 µM. The analogue signal was digitalized with a four-channel recording system and transferred to a PC. The sensor was accurately calibrated by mixing standard solutions of NaNO₂ with 0.1 M H₂SO₄ and 0.1 M KI according to the reaction:

\[ 4H^+ + 2I^- + 2NO_2^- \rightarrow 2H_2O + 2NO + I_2 \]

Irradiation was performed in a thermostated quartz cell (1 cm path length, 3 mL capacity) using the continuum laser with \( \lambda_{\text{exc}} = 405 \) nm. NO measurements were carried out under stirring with the electrode positioned outside the light path in order to avoid NO signal artefacts due to photoelectric interference on the ISO-NO electrode.

Fluorescence microscopy imaging was performed on LSM 710 NLO (Zeiss, Germany) system using confocal microscopy configuration. For imaging 405 and 488 lasers were used. Fluorescence was detected at 500-580 nm and 610-730 nm for both lasers. Additional transmission images were acquired with a detector placed behind the sample. Spectral analysis was performed applying spectral detector and exciting samples with 405 nm and 488 nm lasers.

4. **Fluorescence and NO photorelease quantum yields**

Fluorescence quantum yields were determined using optically-matched solutions at the excitation wavelength of compounds 1 and a solution of Fluorescein in EtOH as standard (\( \Phi_f = 0.79 \)) through the following equation:

\[
\Phi_f = \Phi_{f(s)} \left( \frac{I}{I_{(s)}} \frac{n^2}{n_{(s)}^2} \right)
\]

where \( \Phi_{f(s)} \) is the fluorescence quantum yield of the standard; I and I_{(s)} are the areas of the fluorescence spectra of compounds and standard, respectively; n and n_{(s)} are the refraction index of the solvents used for compounds and standard. Absorbance at the excitation wavelength was less than 0.1 in all cases.

NO photorelease quantum yield was determined at \( \lambda_{\text{exc}} = 405 \) nm within the 20% transformation of 1 by using the following equation

\[
\Phi_p = \frac{[1] \times V \times A}{t \times (1 \times 10^{-8}) \times A}
\]

where \([1]\) is the concentration of phototransformed 1, V is the volume of the sample, t is the irradiation time, A is the absorbance of the sample at the excitation wavelength and I the intensity of the excitation light source.

The concentration of the phototransformed 1 was determined both spectrophotometrically, by taking into account the absorption changes at 400 nm and a \( \Delta \epsilon_{400} = 10,000 \text{ M}^{-1} \text{ cm}^{-1} \), I was calculated by potassium ferrioxalate actinometry.
5. References