Electronic Supplementary Information

Monitoring the Release of a NO Photodonor from Polymer Nanoparticles via Förster Resonance Energy Transfer and Two-Photon Fluorescence Imaging†

Claudia Conte,[a§] Aurore Fraix,[b§] Hanna Thomsen,[c] Francesca Ungaro,[a] Venera Cardile,[d] Adriana C. E. Graziano,[d] Marica B. Ericson,[c*] Fabiana Quaglia[a*] and Salvatore Sortino [b*]

[a]*Drug Delivery Laboratory, Department of Pharmacy, University of Napoli Federico II, Via Domenico Montesano 49, I-80131, Napoli, Italy. *Email: quaglia@unina.it*

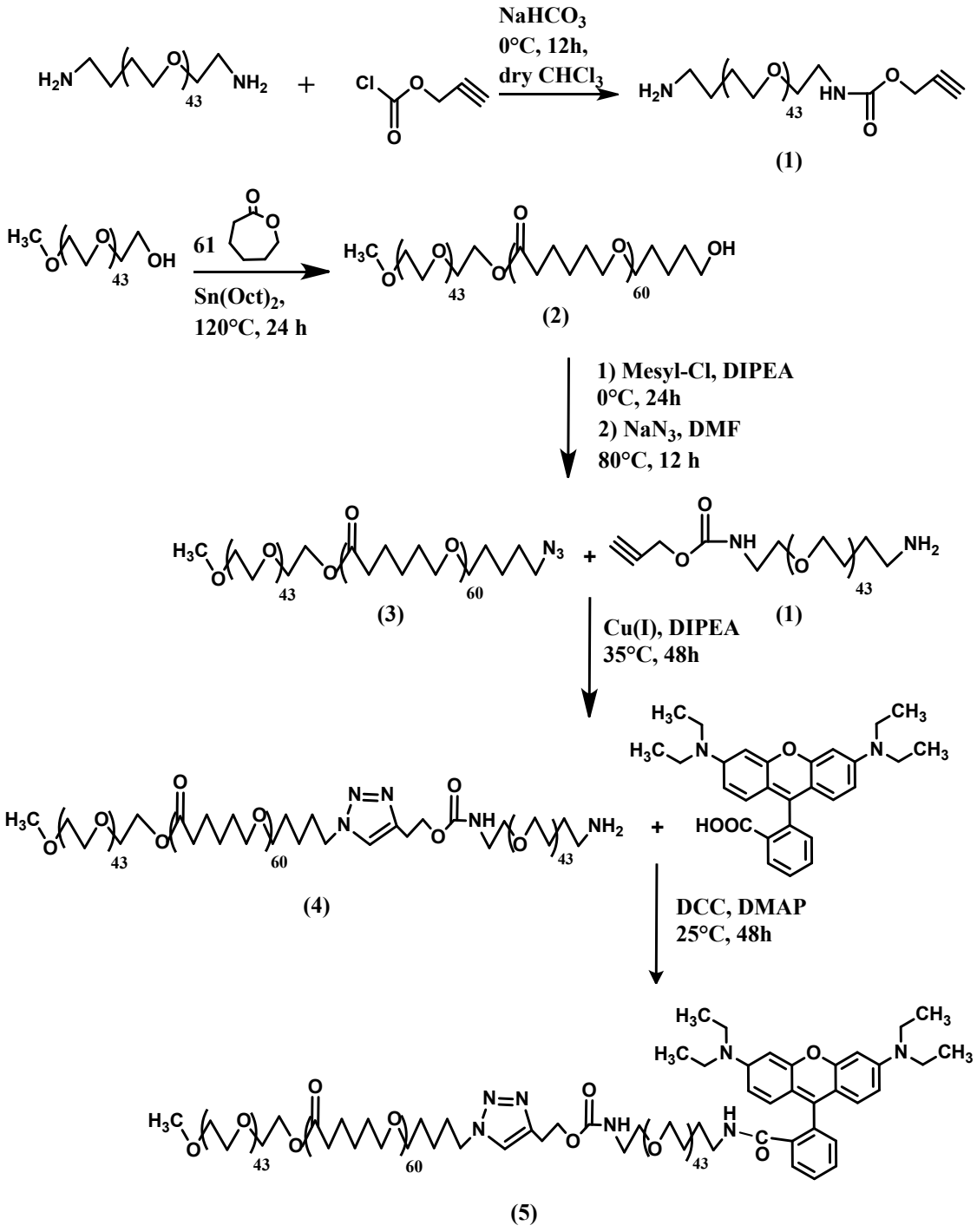
[b]*Laboratory of Photochemistry, Department of Drug Science, Viale Andrea Doria 6, I-95125, Catania, Italy, Catania, Italy. *Email: ssortino@unict.it*

[c]*Biomedical Photonics Group, Department of Chemistry and Molecular Biology, University of Gothenburg, Sweden. *Email: marica.ericson@chem.gu.se*

[d]*Department of Bio-Medical and Biotechnological Sciences, University of Catania, I-95125, Catania, Italy, Catania, Italy*

[§]Equally contributed

S.1 Synthesis of linear triblock mPEG-PCL-PEG-Rhod



Scheme S1. Synthetic steps to obtain linear tri-block $mPEG_{2k}$-PCL_{7k}-PEG_{2k}-Rhod.

S1.1 Synthesis of α-amino-ω-alkyne-PEG_{2k} (1)

Propargyl chloroformate (PCF) (165 mg, 1.4 mmol) was added to a solution of di-amine-PEG_{2k} (3.0 g, 1.5 mmol) and anhydrous Na_2CO_3 (3.0 g, 28.30 mmol) in dry chloroform at 0°C. The mixture was stirred for 4 h. It was allowed to come to room temperature and was stirred for additional 21 h. The reaction mixture was

filtered from bicarbonate, concentrated and purified by silica gel flash chromatography using as eluent methanol in $CHCl_3$ from 5 to 20% in vol. The obtained product, NH_2-PEG_{2k}-alkyne, was precipitated in 300 mL of chilled diethyl ether, filtered on glass porous filter and dried for 24 h under vacuum at 35°C. (2.987 g; 0.49 mmol; yield 97%). **^{1}H-NMR (200 MHz): δ_H** (2.50, 1H, t; 3.10, 2H, t ; 3.64, 180H, s; 4.65, 2H, t; 5.60, 1H, s).

S1.2 Synthesis of m-PEG_{2k}-PCL_{7k}-OH (2)

89 µL of a solution of $Sn(Oct)_2$ in ε-CL (30 mg/mL) were added under inert atmosphere, avoiding water contamination, to *m*-PEG_{2k}-OH (1000 mg, 0.50 mmol) and ε-CL (3.591 g, 31.50 mmol). The mixture was stirred for 24 h at 120°C. The viscous liquid obtained was cooled to room temperature, dissolved in chloroform and precipitated in 300 mL of cold diethyl ether. The obtained solid was filtered on glass porous filter and dried for 24 h under vacuum at 35°C (yield 96%; 4.510 g; 0.51 mmol). **^{1}H-NMR (200 MHz) δ: (PCL:** 1.29-1.78, 184H, m; 2.19-2.43, 122H, m; 3.92-4.21, 122H, t, 4.31,2H, t); (**PEG:** 3.38, 3H, s ; 3.64, 180H, s)

S1.3 Activation of terminal hydroxyl group of m-PEG_{2k}-PCL_{7k}-OH

Methanesulfonyl-chloride (mes-Cl) (346 mg, 3.04 mmol) was added to a solution of (2) (3.0 g, 0.33 mmol) and DIPEA (392 mg, 3.04 mmol) in dry chloroform at 0°C. The mixture was stirred for 4 h. After cooling at room temperature, the mixture was stirred for additional 21 h, concentrated and precipitated in 300 mL of cold diethyl ether/petroleum ether 3/2 (v/v). The obtained solid, m-PEG_{2k}-PCL_{4k}-mes, was filtered on a glass porous filter and dried for 24 h under vacuum at 35°C (2.987 g; 0.32 mmol; yield 97%). **^{1}H-NMR (200 MHz) δ: (PCL:** 1.29-1.78, 184H, m; 2.19-2.43, 122H, m; 3.00, 3H, s; 3.92-4.21, 122H, t, 4.31,2H, t); (**PEG:** 3.38, 3H, s ; 3.64, 180H, s)

S1.4 Nucleophilic substitution of m-PEG_{2k}-PCL_{7k}-mes with sodium azide

Sodium azide (800 mg, 12.30 mmol) was added to a solution of m-PEG_{2k}-PCL_{7k}-mes (1.645 g; 0.183 mmol) in DMF (25 mL). The mixture was heated at 80°C for 12 h. The mixture was dried and solid residue was solved in chloroform. Organic solution was filtered, concentrated and precipitated in 300 mL of a diethyl ether/petroleum ether 3/2 (v/v) cold mixture. The obtained solid (3) was filtered on a glass porous filter and dried for 24 h under vacuum at 35°C (1.481 g, 0.164 mmol yield 90%). **^{1}H-NMR (200 MHz) δ: (PCL:** 1.29-1.78, 184H, m; 2.19-2.43, 122H, m; 3.20, 2H, t; 3.92-4.21, 122H, t, 4.31,2H, t); (**PEG:** 3.38, 3H, s ; 3.64, 180H, s).

S1.5 1-3 Huisgen cycloaddition between m-PEG_{2k}-PCL_{7k}-N_3 (3) and NH_2-PEG_{2k}-alkyne (1)

50 mL of DIPEA (65.52 mg, 0.52mmol) were added to a solution of (3) (1.560 g, 0.173 mmol) and (1) (373.3 mg, 0.18 mmol) in dry THF (18 mL) under inert atmos phere. This mixture was frozen in liquid nitrogen and thawed under vacuum for three times and then transferred under argon in a bottle containing bromo tris(triphenylphosphine)Cu(I) (48.37 mg, 0.052 mmol). After catalyst dissolution, mixture was stirred for 48 h

at 35°C. The mixture was filtered through a neutral alumina column to remove copper, then concentrated and precipitated in 300 mL of cold diethyl ether. The obtained solid was filtered on glass porous filter and dried for 24 h under vacuum at 35°C. To remove unreacted PEG, the solid product (4) was washed for 12 h under stirring with methanol at room temperature. The dispersion was centrifuged and the solid dried for 24 h under vacuum at 35°C (956 mg, yield 70%). **^{1}H-NMR (200 MHz) δ: (PCL:** 1.29-1.78, 184H, m; 2.19-2.43, 122H, m; 3.20, 2H, t; 3.92-4.21, 122H, t, 4.31,2H, t; 7.37, 1H, s); (**PEG**: 3.10, 2H, t ; 3.64, 360H, s; 4.65, 2H, t; 5.60, 1H, s).

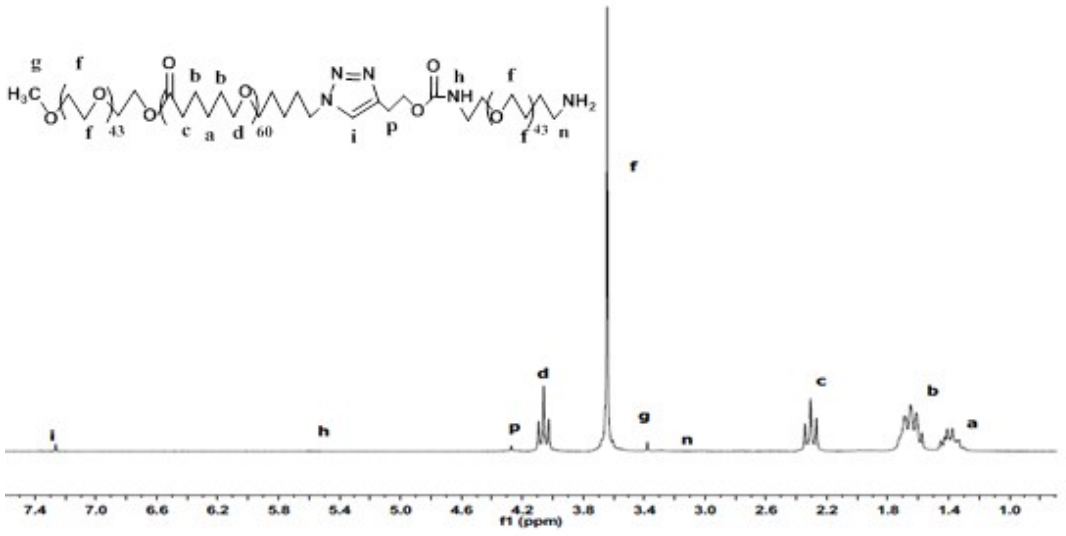


Fig. S1. ^{1}H-NMR of m-PEG_{2k}- PCL_{7k}-PEG_{2k}-NH_2 with proton assignment.

S1.6 Coupling between m-PEG_{2k}-PCL_{7k}-PEG_{2k}-NH_2 and NHS-activated Rhodamine B

A 1M DCC solution (4.5 mL, 4.5 mmol) was added to a solution of Rhodamine B (Rho) (500 mg, 1.04 mmol), DMAP (73.2 mg, 0.6 mmol) and N-hydroxysuccinimide (441,5 mg, 3.83 mmol) in dry chloroform (30 mL) under inert atmosphere. The mixture was stirred at 25°C for 24 h. Thereafter, m-PEG_{2k}-PCL_{7k}-PEG_{2k}-NH_2 (2.0 g, 0.181 mmol) and DIPEA (65.52 mg, 0.52 mmol) were added under nitrogen. After 48 h, the mixture was filtered to eliminate solid dicyclohexylurea, concentrated and precipitated in 300 mL of cold diethyl ether/petroleum ether 3/2 (v/v). The obtained pink solid, m-PEG_{2k}-PCL_{7k}-PEG_{2k}-NH-Rho was filtered on glass porous filter and dried for 24 h under vacuum at 35°C (yield 89%; 2.932 g; 2.67 mmol). **^{1}H-NMR (400 MHz) δ: (PCL:** 1.29-1.78, 184H, m; 2.19-2.43, 122H, m; 3.20, 2H, t; 3.92-4.21, 122H, t, 4.31,2H, t; 7.37, 1H, s); (**PEG**: 3.10, 2H, t ; 3.64, 360H, s; 4.65, 2H, t; 5.60, 1H, s) (**Rhod**: 1.20, 12H, t; 3.50, 8H, t; 6.20-6.50 4H, m; 7.10, 2H, d).

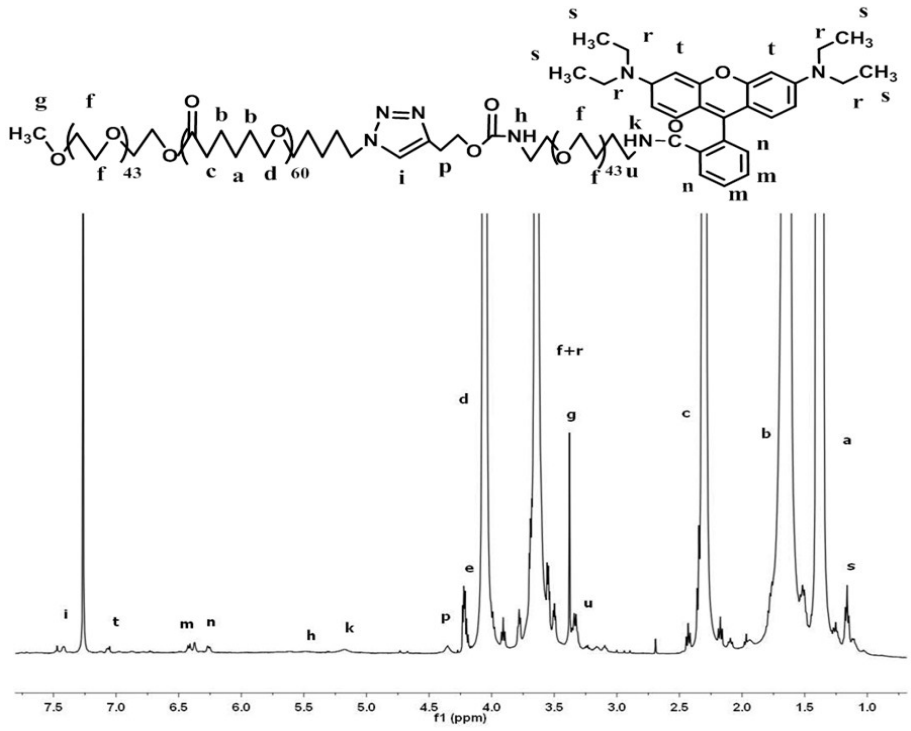


Fig. S2. ^{1}H-NMR of m-PEG$_{2k}$- PCL$_{7k}$-PEG$_{2k}$- Rhod with proton assignment.

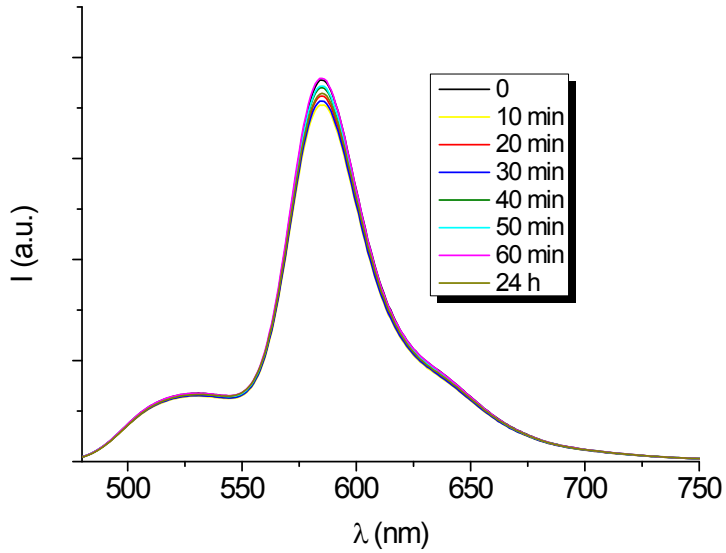


Fig. S3 Fluorescence emission spectra of di-block **Rhod-DBL** NPs loaded with NOPD **1** in HSA aqueous dispersions at different incubation time (T = 25°C). The negligible spectral changes observed clearly indicates that not release of **1** occurs in the protein medium even after 24 h. In fact, any displacement of NOPD **1** from NPs by HSA would have resulted in the suppression of FRET with consequent revival of the fluorescence of the energy donor NOPD **1** at 525 nm and decreasing of the emission of the energy acceptor Rhod at 585 nm.

Supplemental Video. Representative z-stack aquired using TPE fluorescence microscopy (l_{exc} = 900 nm) of human skin after exposure to Rhod-DBL nanoparticles loaded with NO-photodonor (24 hours passive diffusion). Red rhodamine signal is observed in the upper cell layers, while intense green emission suggest release of NO-photodonor in the microscopic skin furrows. Field of view is 424 x 424 mm, and depth of z-stack is 18 mm. Sectioning starts from 18 mm, stepping upward towards the skin surface.