Electronic supplementary materials *Mendeleev Commun.*, 2020, **30**, 747–749

Microflow synthesis of fluorescent markers based on 1,8-naphthalimide for polylactide nanoparticles and bioimaging

Maxim S. Oshchepkov, Aleksey S. Semyonkin, Alexey O. Menkov, Pavel A. Melnikov, Marat P. Valikhov, Inna N. Solovieva, Sergey V. Tkachenko and Julia A. Malinowskaya

Contents

1. Materials

Reagents such as 4-chloro-1,8-naphtalic acid anhydride, hexamethylenediamine, morpholine, sodium hypochlorite, hydrochloric acid, metallic sodium, ethanol, 2-ethoxyethanol, DMF of AR grade were obtained from Aldrich, and EKOS-1 (RF). Coumarin 153 for fluorescence quantum yields measurements was obtained from Aldrich. Distilled water was used for all the studies.

2. Instruments

The molecular structures of intermediate Fluorescent markers were proved by ^{1}H and ^{13}C NMR spectroscopy (Bruker AVANCE II 400 spectrometer, 400.13 MHz) at ambient temperature for reagent aqueous solutions in the 5 mm diameter sample tubes. The external standard solutions of TMS (^{1}H, ^{13}C) were placed in a 1 mm inner coaxial tube.

Synthesis was conducted in continuous flow microreactor Qmix (CETONI, Germany). A metal T-shaped mixer and a coil of 860 mm length were used for the reaction.

Quantitative analysis was conducted using Millichrom A-02 equipped with UV-detector and ProntoSOL-120-5-C18 column (2x75 mm, 5 μm grains); gradient elution (eluent A: 0.2 M $LiClO_4$ aqueous solution – 0.05 M $HClO_4$ aqueous solution, eluent B: acetonitrile).

Electronic absorption spectra were recorded operating UNICO UV-Vis 2804 spectrophotometer and Shimadzu UV-1800 Spectrophotometer. Fluorescence measurements were carried out with luminescence spectrometer Shimadzu RF-6000 operating with a xenon lamp as a light source. All spectral measurements were carried out in quartz sample cells (pathlength l = 1 cm) at 20 ± 1 °C operating the air-saturated solutions. The wavelengths of fluorescence excitation were matched to values of its absorption maxima for all samples.

Nikon A1R MP inverted microscope (Nikon, Japan) with 405-nm laser (emission 410-470 nm) were used for confocal laser scanning microscopy imaging.

3. Synthesis and characterization of target 1,8- naphtalimide derivatives

Synthesis of compounds 2, 5 by *N*-acylation of hexamethylenediamine

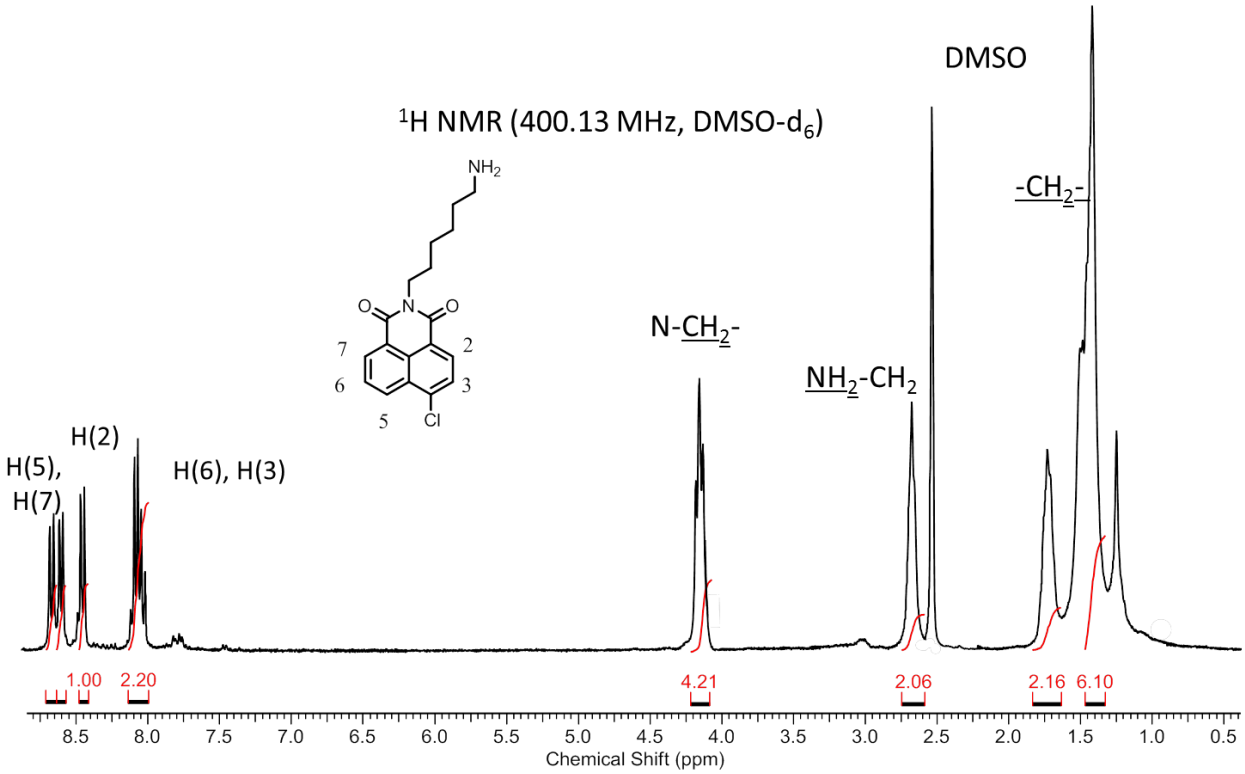
Method 1. *Common synthetic procedure*. Hexamethylenediamine (1.08 g, 9.31 mmol), the corresponding 4-substituted 1,8-naphtalic anhydride **1** or **4** (7.76 mmol), and ethanol (50 ml) were placed in a round-bottom flask. The mixture was stirred at 70 °C for 12 h. Then the formed precipitate was filtered off and discarded, and the filtrate was evaporated *in vacuo*. The remaining brown powder was purified by flash-chromatography. Adapted from [S1].

Method 2. *Common synthetic procedure*. 4-Substituted-1,8-naphthalic acid **1** or **4** (0.429 mmol) was dissolved in ethanol (100 ml) while stirring, making the *solution A* (concentration 1 mg ml^{-1}). *Solution B* is prepared by dissolving the excess diamine excess with respect to the molar concentration of reagent **1** or **4**. The solutions were poured into the corresponding containers **A** and **B** and connected to the microreactor feeding tubes. T-Shaped mixer and 860 cm coil-pipe with inner channel diameter of 1 mm were set on microfluidic reactor. The reaction zone volume was 3 ml. The reactor and coil-pipe were heated evenly along their entire length. Then, pumps were turned on and the solutions were transferred into dosing syringes. Flow rates for solutions A and B were set in 1:1 ratio using computer software. At the first stage the temperature was 20 °C. Then, flow rates for each reagent were set taking into account their ratio. Selection of optimal diamine excess was conducted at the following flow rates: A – 0.25 ml min^{-1}; B – 0.25 ml min^{-1}, temperature – 70 °C. Each sample was collected for every 3 ml (dead volume of the reactor) of solution passed through the microflow reactor. Each sample was evaporated *in vacuo* and analyzed by HPLC using a previously developed method. After the optimal diamine excess is selected, flow rates and temperature were optimized. Reagents flow rates were varied from 0.1 to 1 ml min^{-1}. The temperature was varied from 25 to 78 °C. The obtained under optimal conditions compounds **2** and **5** were isolated via dry-column flash chromatography.

2-(6-Aminohexyl)-6-chloro-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (2)

R_f 0.19 (ethyl acetate as eluent), m.p. 70–73°C. (lit.[S2] 69–71°C). The yield in a flask was 65%, in a microreactor – 62%.

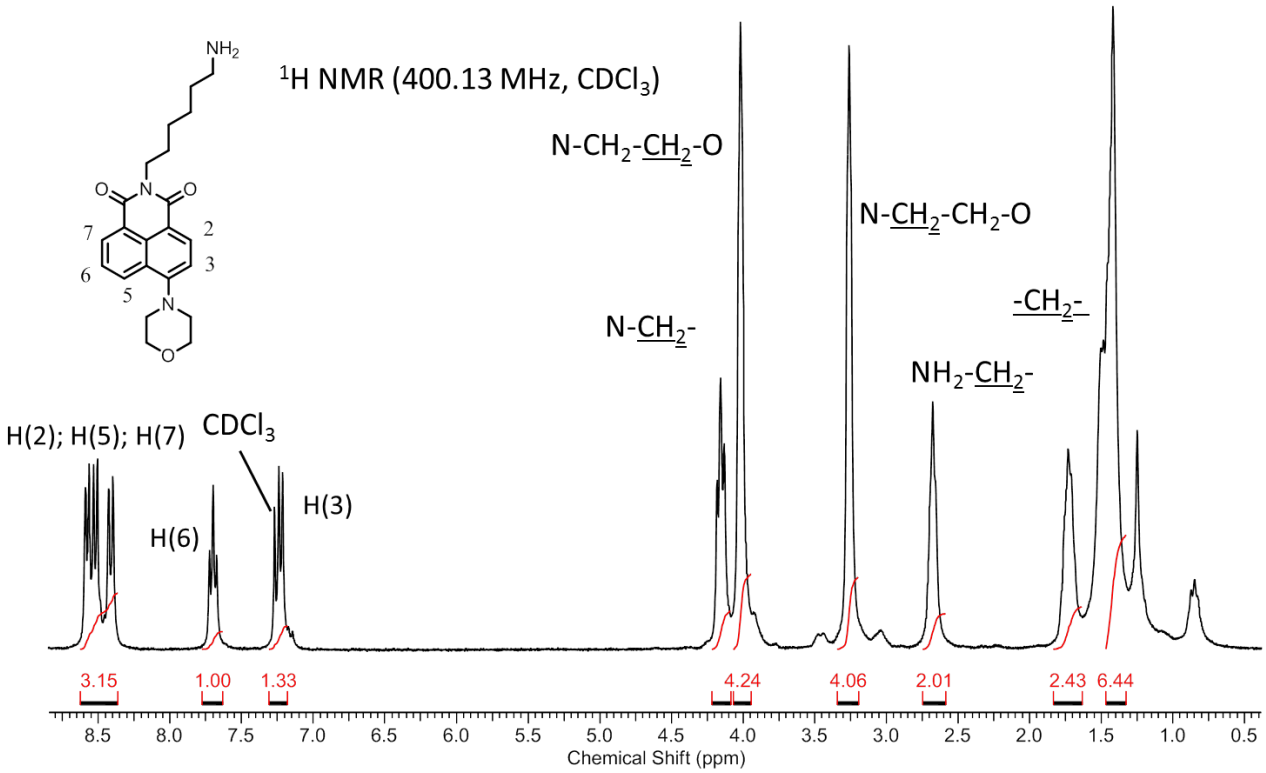
^{1}H NMR (400.13 MHz, DMSO-d_6, 23.0 ºC, δ / ppm, *J* / Hz): 8.67-8.57 (m, 2H, H(5); H(7), J_1 = 8.07, J_2 = 7.34,), 8.48-8.32 (d, 1H, H(2), J_1 = 7.87), 8.11-8.01 (t, 2H, H(6), H(3), *J* = 7.87), 4.09-4.02 (t, 2H, N-$\underline{CH_2}$-CH_2-), 2.65-2.86 (m, 2H, $\underline{NH_2}$-CH_2-CH_2-), 1.71-1.60 (t, 2H, NH_2-$\underline{CH_2}$-CH_2-1.47-1.18 (m, 6H, $\underline{CH_2}$-$\underline{CH_2}$- $\underline{CH_2}$-).



2-(6-Aminohexyl)-6-morpholino-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (5)

B.p. 118–119°C (lit. 117–119°C [S3]). The yield in a flask was 67%, in a microreactor – 68%.

^{1}H NMR (400.13 MHz, $CDCl_3$, 23.0 ºC, δ / ppm, *J* / Hz): 8.62-8,43 (m, 3H, H(2); H(5); H(7), J_1 = 8.07, J_2 =7 .15, J_3 = 8.25), 7.75-7.70 (t, 1H, H(6), J_1 = 7.34, J_1 = 8.25), 7.3-7.2 (d, 1H, H(3), *J* = 7.89), 4.21-4.16 (t, 2H, N-$\underline{CH_2}$-CH_2-), 4.05-3.95 (d, 4H, N-CH_2-$\underline{CH_2}$-O), 3.37-3.20 (d, 4H, N-$\underline{CH_2}$-CH_2-O), 2.77-2.63 (m, 2H, NH_2-$\underline{CH_2}$-), 1.84-1.68 (m, 2H, N-CH_2-$\underline{CH_2}$-) 1.66-1.36 (m, 6H, $\underline{CH_2}$-$\underline{CH_2}$- $\underline{CH_2}$).



Synthesis of 3 by nucleophilic aromatic substitution

Method 1. Common synthetic procedure. Compound **2** (3.6 mmol), K_2CO_3 (5 g) and 2-ethoxy-ethanol or DMF (25 g) were placed in a round-bottom flask. The mixture was heated at 135–155 °C for 12 h. Next, the mixture was decomposed with water, and the precipitated product was filtered off and purified via dry-column flash chromatography.

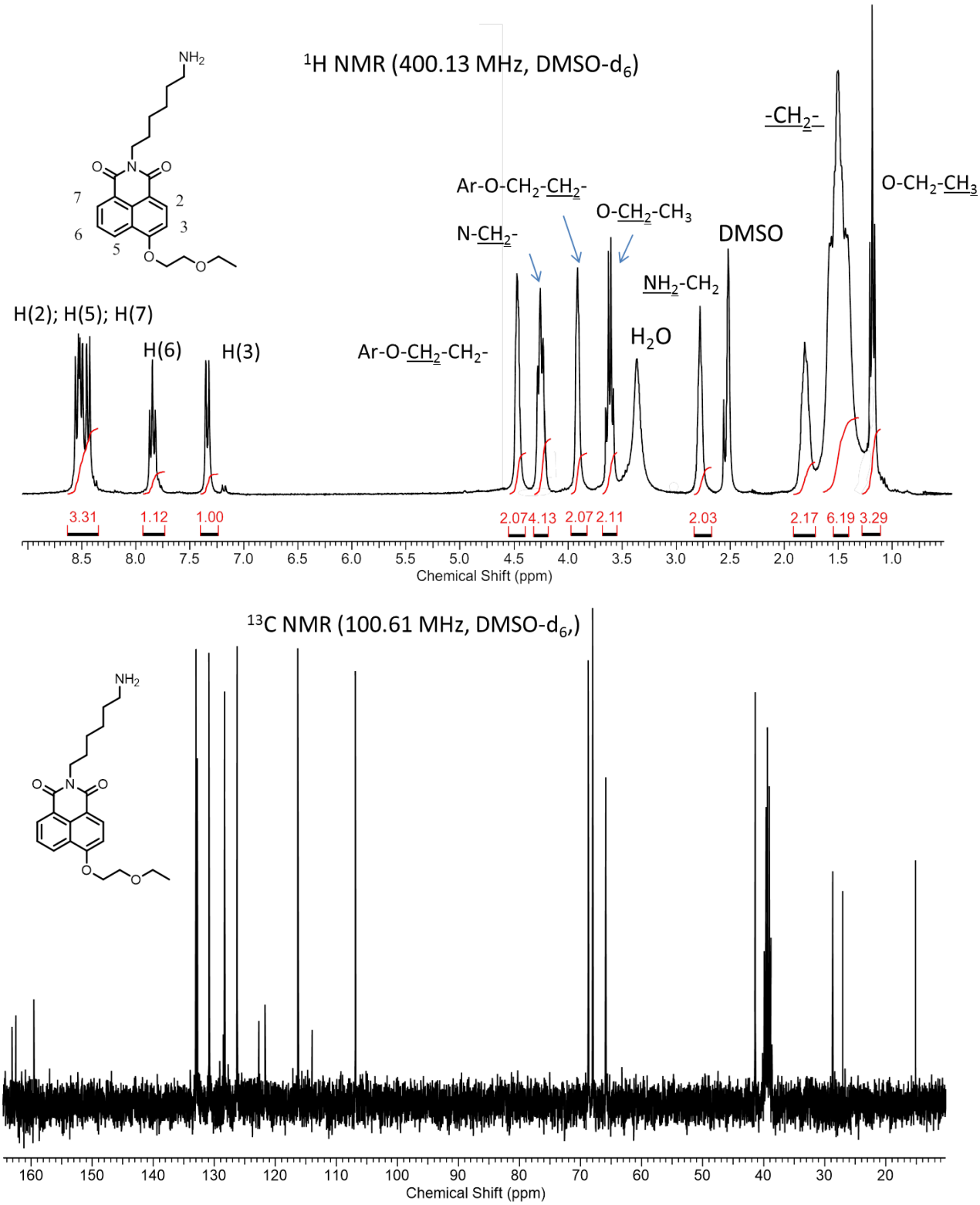
2-(6-Aminohexyl)-6-(2-ethoxyethoxy)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (3)

Light-brown crystals, poorly soluble in organic solvents. Rf = 0.31 (eluent – ethyl acetate). M.p. = 140–143 °C. After the column purification the yield is 53% (no significant differences when conducting the reaction in DMF or 2-ethoxyethanol).

^{1}H NMR (400.13 MHz, DMSO-d_6, 23.0 ºC, δ / ppm, *J* / Hz): 8.51-8.39 (m, 3H , H(2); H(5); H(7), J_1 = 8.07, J_2 = 8.8), 7.84-7.78 (t, 1H, H(6), J_1 = 7.34; J_2 = 7.71), 7.32-7.39(d, 1H, H(3), 4.47-4.44 (t, 2H, -O-$\underline{CH_2}$-CH_2-O-CH_2-CH_3), 4.09-4.02 (t, 2H, N-$\underline{CH_2}$-CH_2-),3.92-3.90 (t, 2H, -O-CH_2-$\underline{CH_2}$-O-CH_2-CH_3), 3.63-3.58 (m, 2H, -O-CH_2-CH_2-O-$\underline{CH_2}$-CH_3), 2.65-2.86 (m, 2H, $\underline{NH_2}$-CH_2-CH_2-), 1.71-1.60 (t, 2H, NH_2-$\underline{CH_2}$-CH_2-1.47-1.18 (m, 6H, $\underline{CH_2}$-$\underline{CH_2}$- $\underline{CH_2}$-).1.21-1.16 (t, 2H, -O-CH_2-$\underline{CH_3}$).

^{13}C NMR (100.61 MHz, DMSO-d_6, 23.0ºC, δ/ppm): 15.8, 26.4, 26.6, 30.3, 32.4, 41.7, 42.0, 65.2, 70.1, 70.8, 102.3, 129.5, 129.8, 130.9, 133.2, 134.6, 138.1, 152.5, 157.4, 159.9, 161.91, 168.8.

ESI-MS m/z: 385.5 $[M+H]^+$. Elemental Analysis for $C_{22}H_{28}N_2O_4$ calculated (%): C, 68.73; H, 7.34; N, 7.29; Found (%): C, 68.81; H, 7.29; N, 7.34.



Method 2. Common synthetic procedure. The two solutions were prepared for the synthesis. Solution **A** contained compound **2** with concentration of 1 mg ml^{-1} in DMF or 2-ethoxyethanol. Solution B is prepared by dissolving sodium 2-ethoxyethanolate in 20-fold excess with respect to molar concentration of the reagent **A** in DMF or 2-ethoxyethanol. Synthesis sequence was as follows: the solutions were transferred to syringe pumps by 10 ml for dosing. Flow rates of solutions **A** and **B** were equal. Reagents entered the T-shaped mixer and, subsequently, 3 ml

reaction column. Synthesis temperature is raised by 10°C at each step and spans from 80°C to the solvent boiling point. At the exit point the reaction mixture was collected into 1 ml test tubes. Each sample was neutralized with 10% HCl and evaporated *in vacuo*. Resultant samples were analyzed by HPLC-UV method using a previously developed procedure. The product was purified via dry-column flash chromatography to determine the compound conversion.

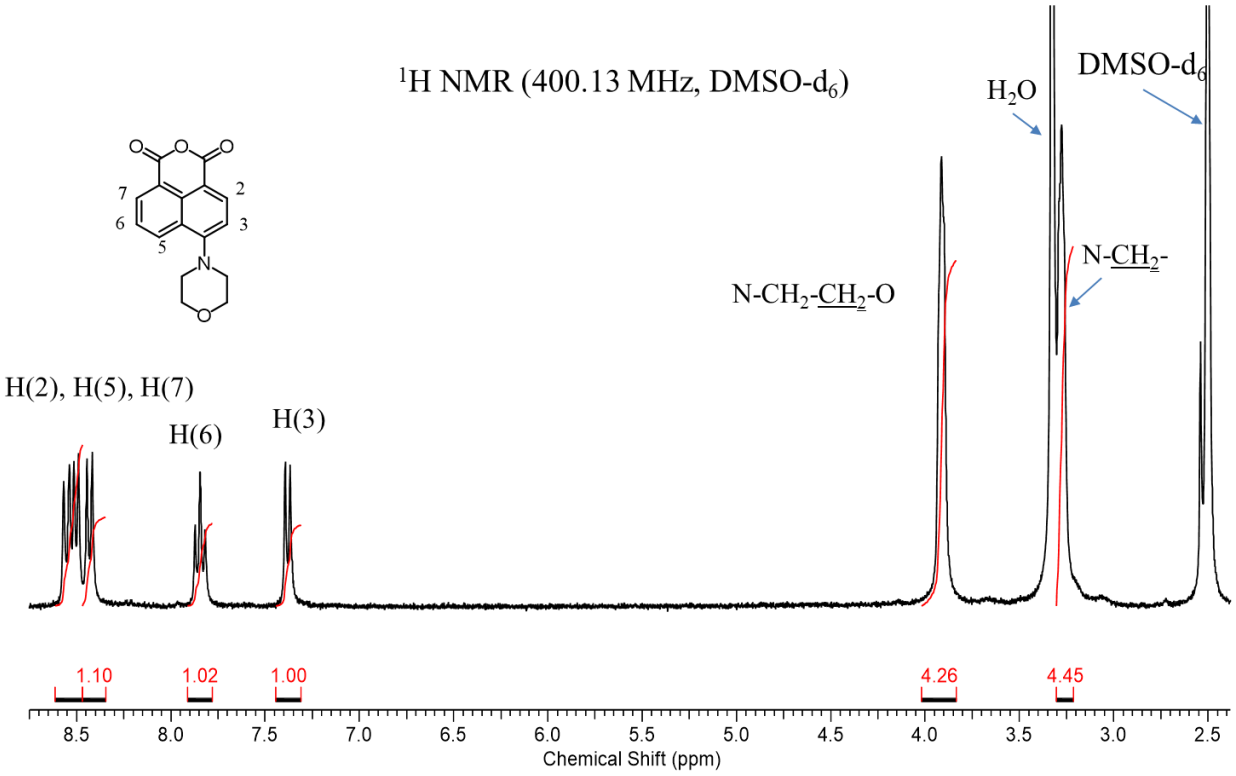
The yield in microreactor was 63% and 40% using DMF and 2-ethoxyethanol, respectively.

Synthesis of 4-morpholino-1,8-naphthalic anhydride (4)

Method 1. A mixture of 4-chloro-1,8-naphthalic anhydride **1**)(10 g, 36.1 mmol) and morpholine (6.29 g, 72.2 mmol) was refluxed in 2-ethoxyethanol or DMF under nitrogen atmosphere for 8 h. Precipitated burgundy crystals were filtered off and recrystallized from 1,2-dichloroethane. The obtained orange crystals of 4-morpholino-1,8-naphthalic anhydride **4** were then purified via dry-column flash chromatography (gradient elution: ethyl acetate/hexane, 1:2, v/v). R_f = 0.36 (ethyl acetate:hexane, 1:3), m.p. 226–229°C (lit. [S4] 228–229°C). The yield in 2-ethoxyethanol was 6 g (49%), in DMF – 8 g (65 %).

Method 2. 4-Chloro-1,8-naphtalic acid anhydride **1** (0.1 g, 0.429 mmol) was dissolved in DMF or 2-ethoxyethanol (100 ml) under stirring, giving solution **A** with concentration 1 mg ml^{-1}. Solution **B** is prepared by mixing morpholine in excess with respect to molar concentration of compound **1** in DMF (100 ml). The solutions were poured into the corresponding containers **A** and **B** and connected to the microreactor feeding tubes. T-Shaped mixer and 860 cm coil-pipe with inner channel diameter of 1 mm were set on the microfluidic reactor. Reaction zone volume was 3 ml. The reactor and coil-pipe were heated evenly along their entire length. Then, pumps were turned on and the solutions were transferred into dosing syringes. Flow rates for solutions **A** and **B** were set in 1:1 ratio using computer software. At the first stage the temperature was 20 °C. Then flow rates for each reagent were set taking into account their ratio. Selection of optimal morpholine excess was conducted at the following flow rates: **A** – 0.25 ml min^{-1}; **B** – 0.25 ml min^{-1}, temperature – 135 °C. The flow was sampled every 3 ml of solution passed through microfluid reactor (3 ml is the dead volume of the reactor). Each sample was evaporated *in vacuo* and analyzed by HPLC using a previously developed method. After the optimal morpholine excess was selected, flow rates and temperature were optimized. Reagents flow rates were varied from 0.1 to 1 ml min^{-1}. The temperature was varied from 80 to 155°C. The obtained in optimal conditions 4-morpholin-1,8-naphtalic anhydride (**4**) was isolated via dry-column flash chromatography (eluent – ethyl acetate:hexane 1:3). R_f = 0.36. m.p. 226–229°C (lit. [S4] 228–229°C) The yield was 53% and 68% in 2-ethoxyethanol and DMF, respectively.

^{1}H NMR (400.13 MHz, DMSO-d_6, 23.0 ºC, δ / ppm, *J* / Hz): 8.62-8,42 (m, 3H, H(2); H(5); H(7), J_1 = 8.07, J_2 = 7.15, J_3 = 8.44), 7.75-7.66 (t, 1H, H(3), J_1 = 7.89), 7.3-7.2 (d, 1H, H(6), *J* = 8.07, 4.11-3.95 (d, 4H, N-CH_2-$\underline{CH_2}$-O), 3.36-3.17 (d, 4H, N-$\underline{CH_2}$-CH_2-O).



Quantitative analysis for all synthesized compounds was performed on Millichrom A-02 with a UV detector. The ProntoSIL-120-5-C18 column, 2x75 mm in size, with a grain size of 5 μm was used. Eluent A was a solution of 0.2 M $LiClO_4$, 0.05 M $HClO_4$. Eluent B was acetonitrile.

Quantitative analysis of the compounds was carried out by the absolute calibration method using the MultiChrome chromatogram processing program. Calibration solutions of standard substances were prepared in a concentration range from 0.05 to 1 mg ml^{-1}. Calibration graphs were constructed for the quantitative determination of substances using peak areas averaged for three measurements. At least 5 calibration points were used for each calibration graph. Detailed description of the method and calibration curves are presented in the next section of the electronic supplementary information.

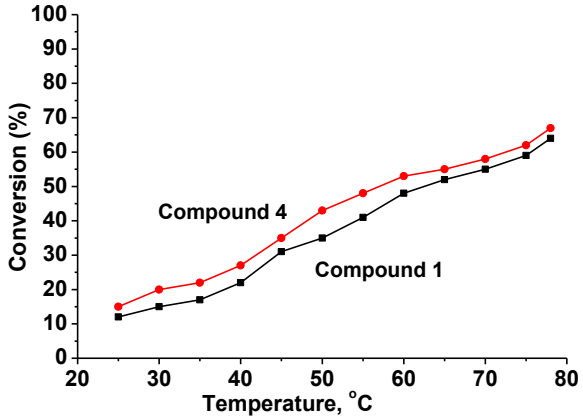


Figure S1. The dependence of the conversion of compounds **1** and **4** on the reaction temperature, reaction in ethanol.

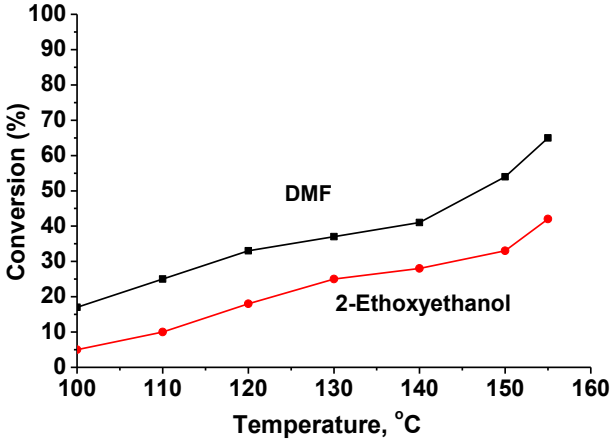


Figure S2. The dependence of the conversion of compound **1** on the reaction temperature during the reaction in DMF and 2-ethoxyethanol.

4. Calibration curves for the determination of derivatives of 1,8-naphtalic acid by reverse-phase HPLC

Five calibration solutions with concentrations ranging from 0.05 to 1 mg ml^{-1} were prepared for each compound **1-5**; all of them were previously purified with column chromatography. Acetonitrile was used as a solvent for all the solutions. Samples of compounds **1-5** were dissolved using an ultrasonic bath and then in a centrifuge. Vials with solutions were placed in the sampler of the device. The analysis was carried out on a ProntoSIL-120-5-C18 AQ chromatographic column at 35 °C at an eluent flow rate of 150 µl/min. Eluent **A**: 0.2 M $LiClO_4$ solution and 0.05 M $HClO_4$ solution. Eluent **B**: acetonitrile. The elution mode was gradient (from 0% of eluent **B** to 100% of eluent **B**), with the volume of eluent passing through the column in one analysis from 2000 to 2500 µl (depending on the compound). The used wavelength interval of the UV detector was λ = 220 – 240 nm. The volume of the sample introduced into the chromatograph was 2 µl of the solution. After a series of analyzes, the obtained chromatographic peaks were processed in the

MultiChrom program. Using peak areas averaged after three measurements, calibration graphs were constructed for the quantitative determination of substances. Calibration plots are shown below in figures S3-7.

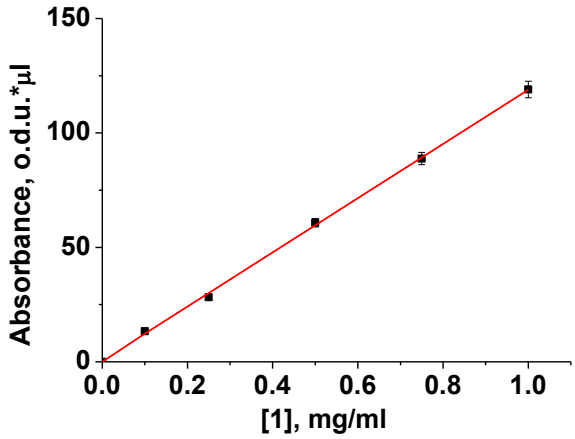


Figure S3. Calibration curve for compound **1** (R_{corr} = 0.9997)

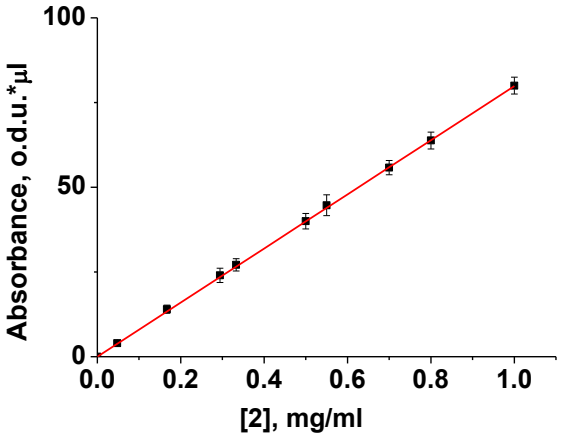


Figure S4. Calibration curve for compound **2** (R_{corr} = 0.9997)

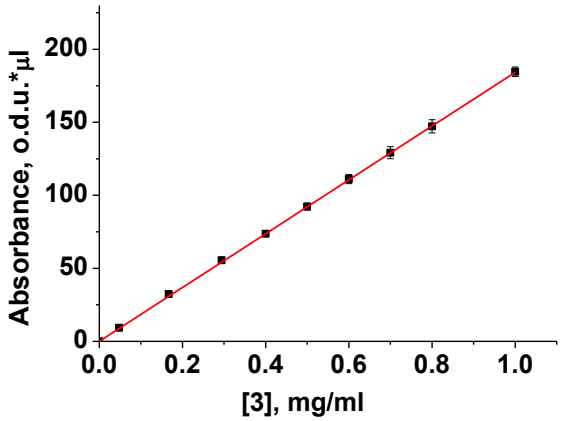


Figure S5 Calibration curve for compound **3** ($R_{corr.}$ = 0,9996)

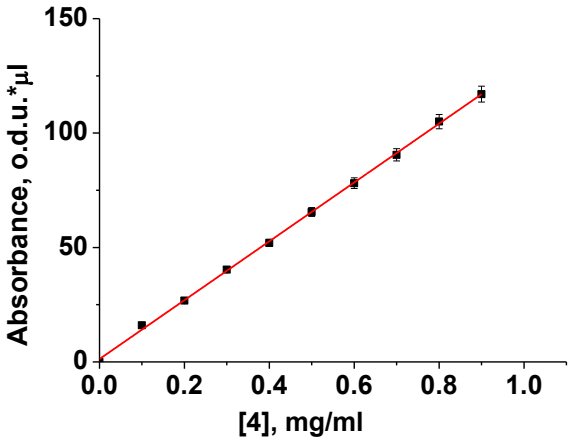


Figure S6. Calibration curve for compound **4** (R_{corr} = 0.9997)

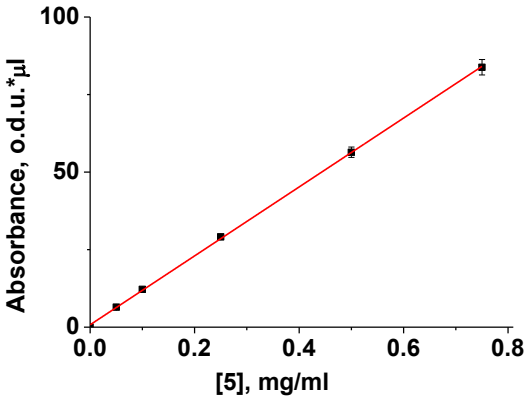


Figure S7. Calibration curve for compound **5** (R_{corr} = 0.9992)

5. Synthesis and the study of the properties of PLGA nanoparticles with covalently bound fluorescent dye

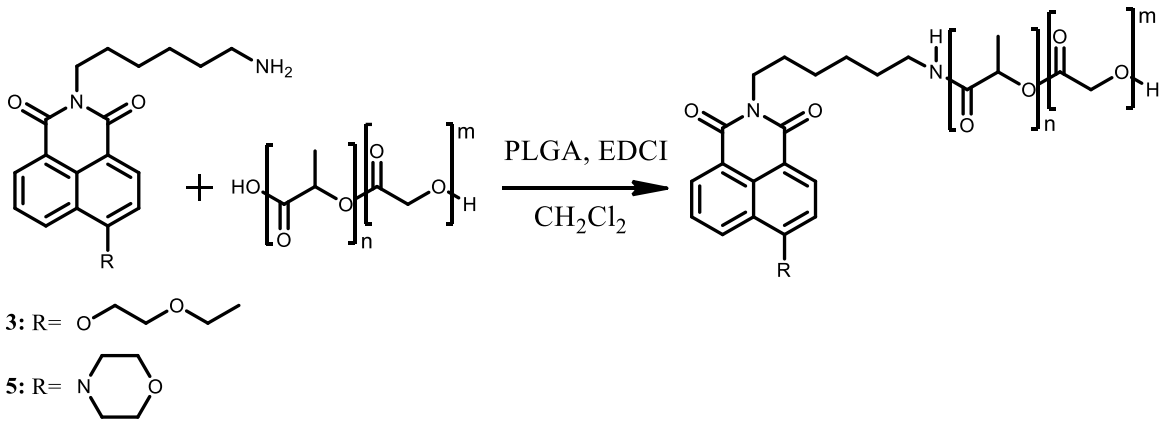


Figure S8. Scheme of conjugation of compounds **3** and **5** with polylactide.

Synthesis of the PLGA with covalently bound 3 or 5

Common synthetic procedure. Compound **3** or **5** (13 μmol), PLGA (Resomer® RG 502 H, 0.50 g), 1-ethyl-3-(3-dimethylaminopropil)carbodiimide (EDCI, 24.9 mg, 130 μmol) and diisopropylamine (2 mg) were dissolved in dichloromethane (5 ml). The mixture was stirred at room temperature in the darkness for 48 h. After that it was washed with a mixture of water and methanol (3×20 ml, 1:1, v/v) to remove byproducts and starting reagents. The organic phase was separated, dried over anhydrous sodium sulfate and evaporated *in vacuo*. The residue was dissolved in ethyl acetate (5 ml), and the solution was added dropwise to *n*-hexane (50 ml) to precipitate the polymer. The precipitate was filtered off and dried *in vacuo* yielding light yellow powder.

The yield of the modified PLGA polymer PLGA-**3** is 0.41 g (72%), PLGA-**5** – 0.45 g (78%).

Absorbance and emission spectra for the free dye **3** and its PLGA conjugate are shown in Figures S9 and S10 respectively.

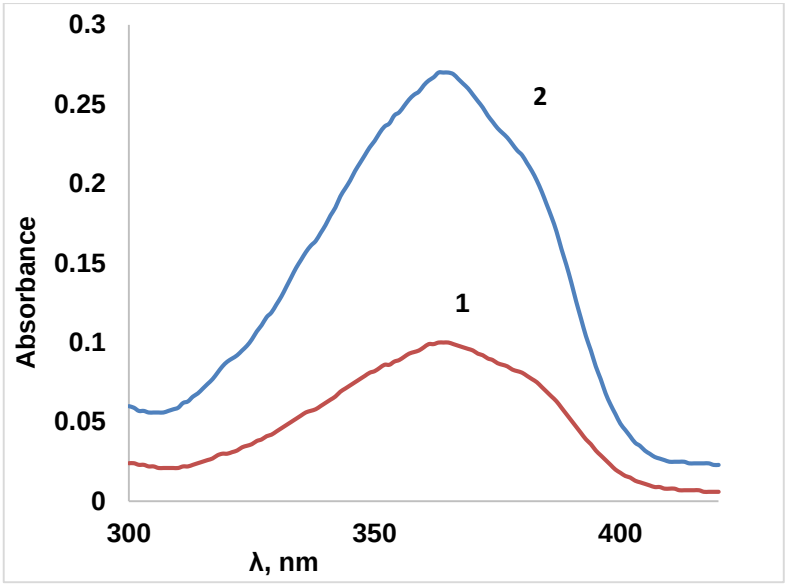


Figure S9. Absorbance spectra of the dye **3** (9.1 μM) (1) and PLGA-**3** with a theoretical mass ratio of dye: polymer 1: 100 (0.5 mg ml^{-1}) (2)

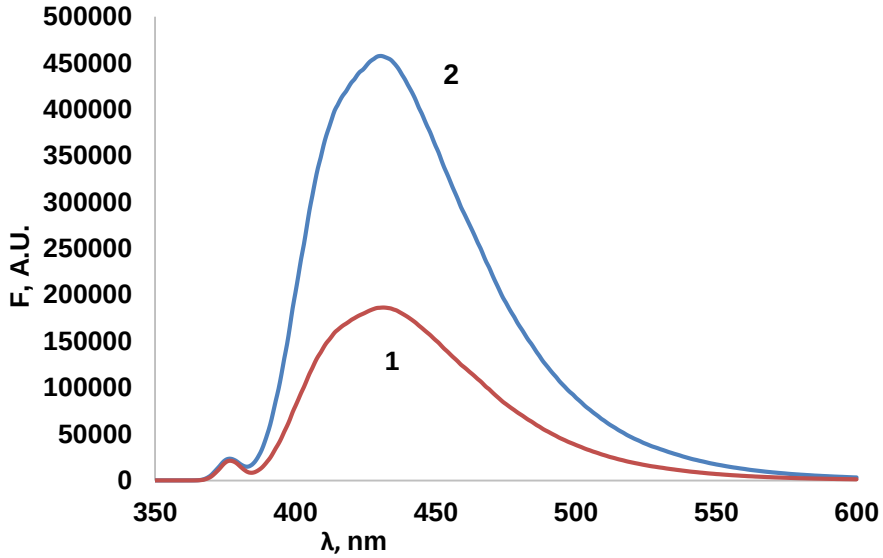


Figure S10. Emission spectra of the dye **3** (9.1 μM) (1) and PLGA-**3** with a theoretical mass ratio of dye: polymer 1: 100 (0.5 mg ml^{-1}) (2)

TLC was used to identify the modified PLGA-**3** or PLGA-**5**. IR and NMR spectroscopy are not informative in this case as the signals from fluorescent fragment which is about 1,0 mass % of the polymer are not visible against the background signals from polymer chain.

Nanoparticles preparation

General procedure. Nanoparticles were produced by emulsion solvent evaporation technique. Equal weights (100 mg) of the original and modified polymers were dissolved in 4 ml of dichloromethane. The solution was added to 20 ml of 1% (w/v) PVA aqueous solution with subsequent homogenization for 1 min on UltraTurrax at 23,600 RPM with ice bath, followed by high-pressure homogenization on Avestin C-3 Emulsiflex at 20,000 psi. Dichloromethane was

removed using rotary evaporator and the formed suspension was filtered through sintered glass filter Por1 (pore size ~150 μm) to remove larger fractions. An amount of cryoprotector D-mannitol, sufficient to produce 2.5% concentration, was added to the filtrate. The suspension was dispensed into vials, frozen and freeze-dried. The average yield of the nanoparticles with modified PLGA polymer is 0.146 g (73%, by PLGA).

The study of the properties of the obtained nanoparticles

Table S1. Optical properties of the 1,8-naphthalimide dyes and dye-labeled nanoparticles

Compound	ε, $l \cdot mol^{-1} \cdot cm^{-1}$	Quantum yield, %	
		In solution	In nanoparticles
Dye 3	10955	83	88
Dye 5	10500	39	41

Table S2. Sizes and zeta potentials of the obtained nanoparticles

	Z-average diam., nm	PDI	Diam. by volume, nm	Zeta potential, mV
PLGA Dye 3 NPs	125±1	0.140±0.014	122±35	−22.90±3.60 (50-fold dilution) −11.62±1.62 (no dilution)
PLGA Dye 5 NPs	135±2	0,164±0,026	124±7	−30.04±0.79 (50-fold dilution) −3.42±7.42 (no dilution)

The optical properties of nanoparticles in media with different pH values were investigated. For this, the contents of one bottle was resuspended in 1 ml of distilled water, 200 μl aliquots were taken, the sample volume was brought to 2 ml with water, phthalate buffer solution (pH 5.5) and phosphate buffer solutions (pH 7.4 and 9.75). Absorption and fluorescence spectra were taken for the resulting suspensions, Figure S11.

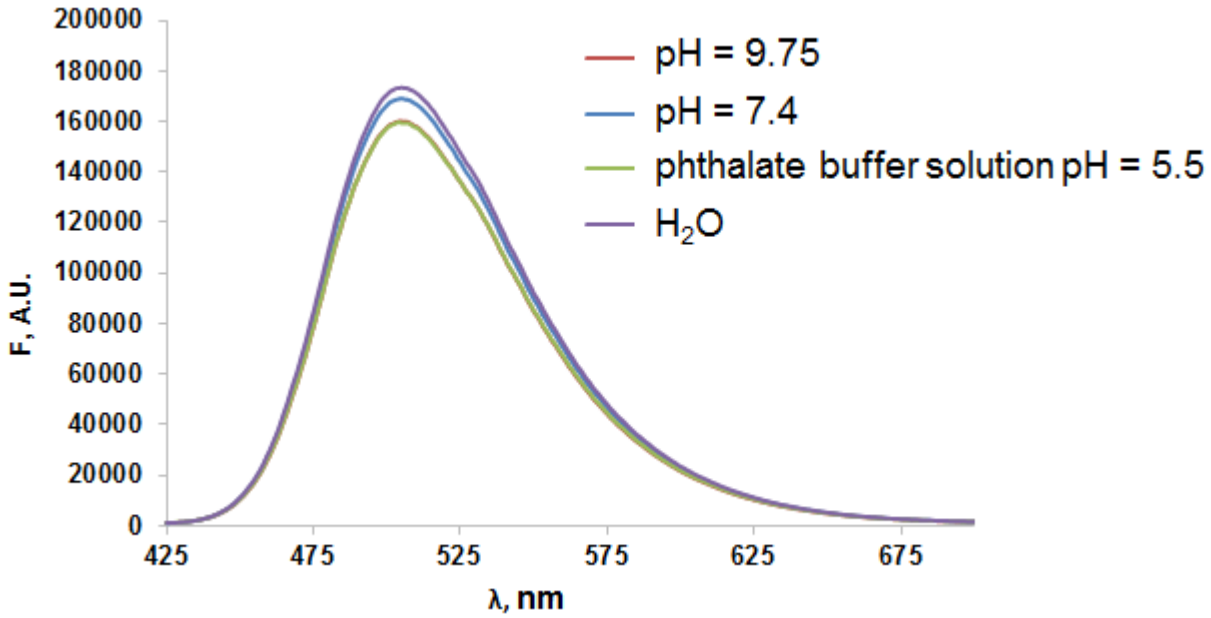


Figure S11. Emission spectra for nanoparticles with covalently bound **5** at different pH values

Fluorescence quantum yields measurements

Spectrophotometer Shimadzu UV-1800 and spectrofluorimeter Shimadzu RF-6000 were used to measure the optical properties of the dyes and nanoparticles labelled by them. In order to calculate quantum yields we have used the procedure described in [S5] with certain modifications. A series of solutions of the free dyes and the chosen standard coumarin 153 with varying concentrations were prepared. For each sample absorbance at 375 nm and integral fluorescence intensity excited by the light of the same wavelength were recorded and obtained. For nanoparticles, series of suspensions with varying dilution were prepared. Using this data, graphs in coordinates Fluorescence Intensity F – Absorption A were plotted and the slope ratios for the straight lines were determined, Figures S12-14.

For nanoparticles, a similar procedure was followed except that since colloid objects scatter light significantly, absorbance spectra were recorded and for each spectra baseline corresponding to scattering component in the absorbing region was created using OriginLab software. The difference between the OD value and baseline was taken as the absorbance. The rest of the procedure was as described for dyes solutions. The relative quantum yield was calculated as

$$QY_R = QY_{st} \cdot \frac{tg\alpha_x}{tg\alpha_{st}} \cdot \frac{n_x^2}{n_{st}^2},$$

Where QY_{st} is the standard's absolute quantum yield, $tg\alpha_x/tg\alpha_{st}$ is the slope ratio for the sample and standard, n_x, n_{st} are the refractive indexes of the mediums, 1.3330 for water and 1.3636 for 96% ethanol.

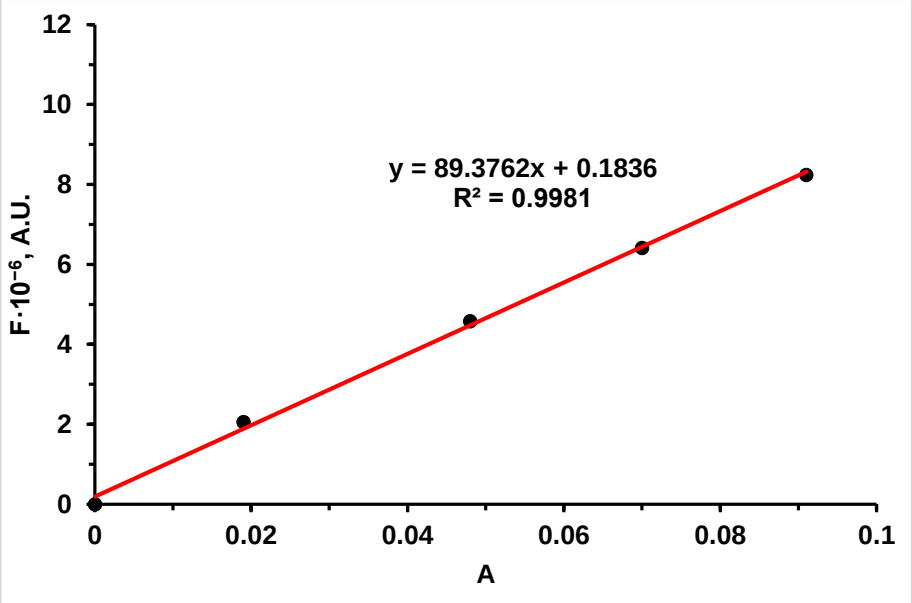


Figure S12. Dependence of integrated fluorescence intensity on absorbance for a series of coumarin 153 standard solutions

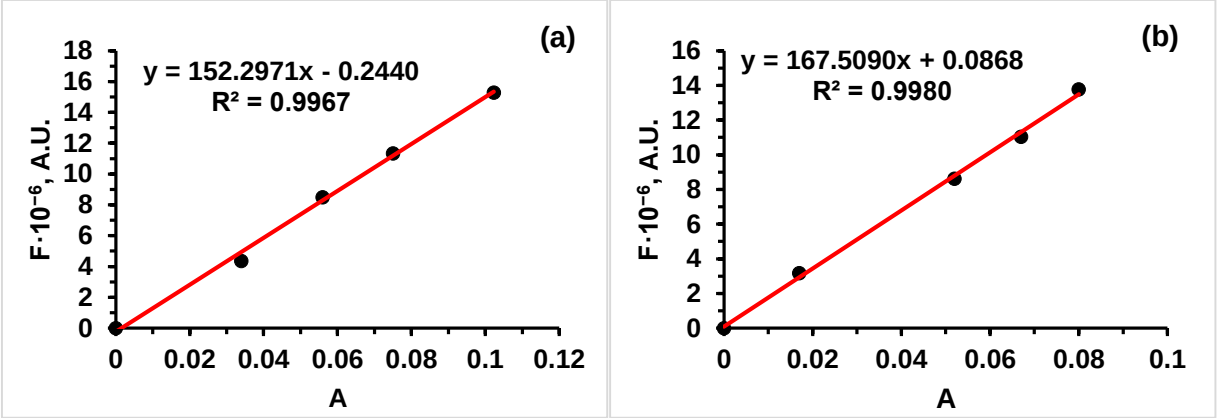


Figure S13. Dependence of integrated fluorescence intensity on absorbance for a series of solutions of **3** (a) and suspensions of nanoparticles PLGA-**3** (b)

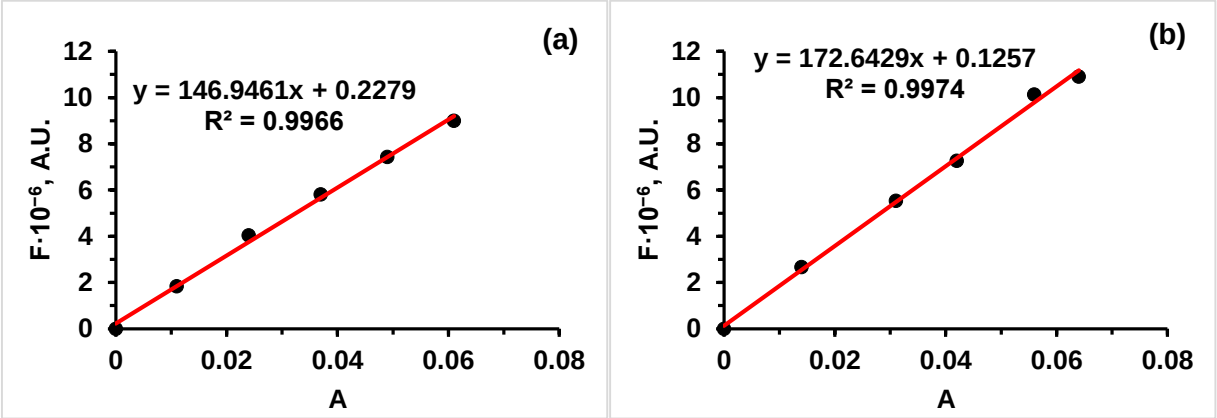


Figure S14. Dependence of integrated fluorescence intensity on absorbance for a series of solutions of **5** (a) and suspensions of nanoparticles PLGA-**5** (b)
* - optical density units

6. Cell culture experiments

Cell line

4T1 breast adenocarcinoma cell line was obtained from ATCC® (Cat. No.CRL-2539). 4T1 Cells were cultured at 37 °C in humidified atmosphere with 5% CO_2 using RPMI-1640 medium with 10% fetal bovine serum, 2 mM of L-glutamine and 1% antibiotic-antimycotic mixture (10,000 units per ml penicillin, 10,000 units per ml streptomycin).

The 4T1 cells were cultured on coverglass-bottom confocal dishes (3,5 SPL Life Sciences Co., Ltd.) upon reaching 70% confluence 24 h prior to the study.

Confocal microscopy

Confocal laser scanning microscopy (CLSM) was used to estimate the uptake of fluorescently labelled nanoparticles (NP) by 4T1 cells and to investigate their intracellular distribution.

Cell lysosomes were stained with LysoTracker Red DND-99 (50 nM, Life technology, USA) according to a manufacturer's protocol. Cells were incubated with LysoTracker for 10 min and washed with PBS.

PLGA-**5** NPs were added to the cell culture medium to a final concentration of 100 μg/ml and the cells were incubated for 1 h. The concentration of 200 μg/ml was used for PLGA-**3** NPs. The cells were washed 3 times with PBS after incubation.

Real time imaging was performed using Nikon A1R MP inverted microscope (Nikon, Japan). 405-nm laser (emission 410-470 nm) was used for excitation of **3** and **5** dyes. Maximum projections along Z-axis were made using NIS-elements AR software.

All experiments were performed in triplicates. The results on the localization distribution of fluorescently labelled nanoparticles are presented in Figures S15-19.

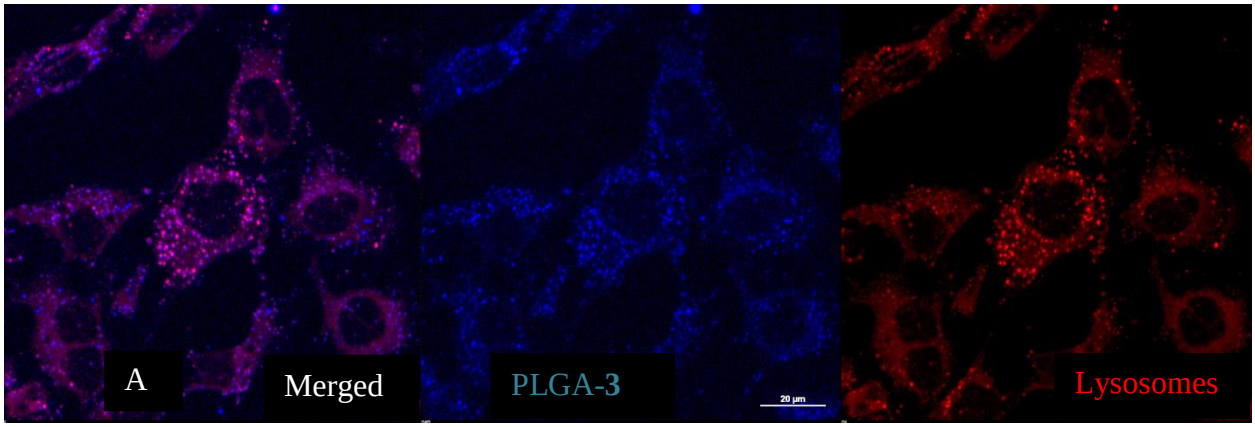


Figure S15. Partial co-localization of PLGA-**3** NPs (blue) with lysosomes (red) and merged image (A).

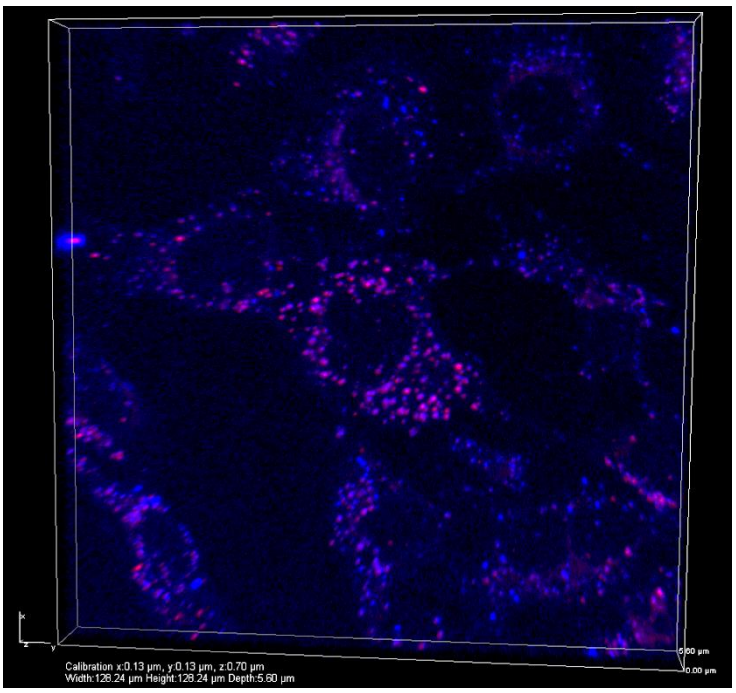


Figure S16. Confocal z-stack 3D reconstruction of PLGA-**3** NPs (blue) internalization in cells with stained lysosomes (red). Scale bar is 10 µm.

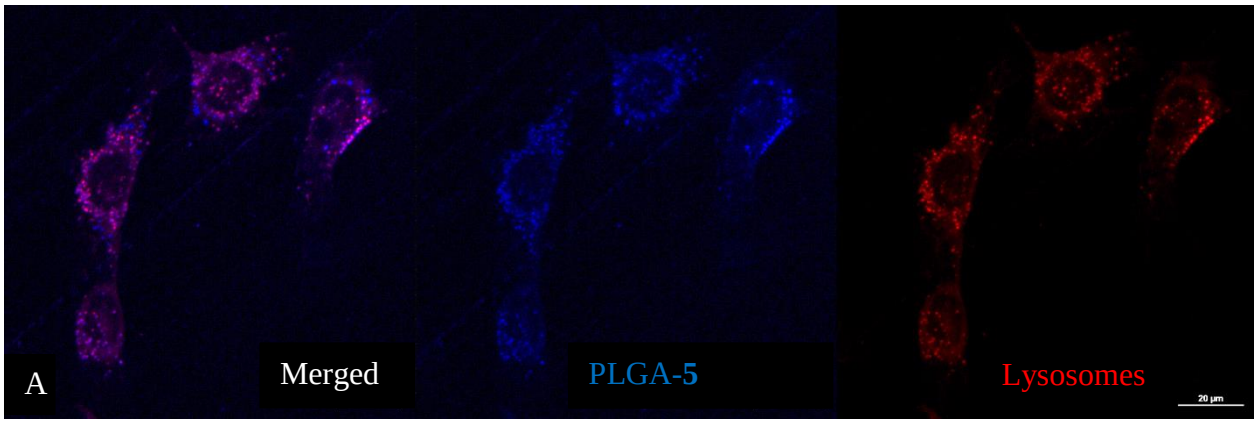


Figure S17. Partial co-localization of PLGA-**5** NPs (blue) with lysosomes (red) and merged image (A).

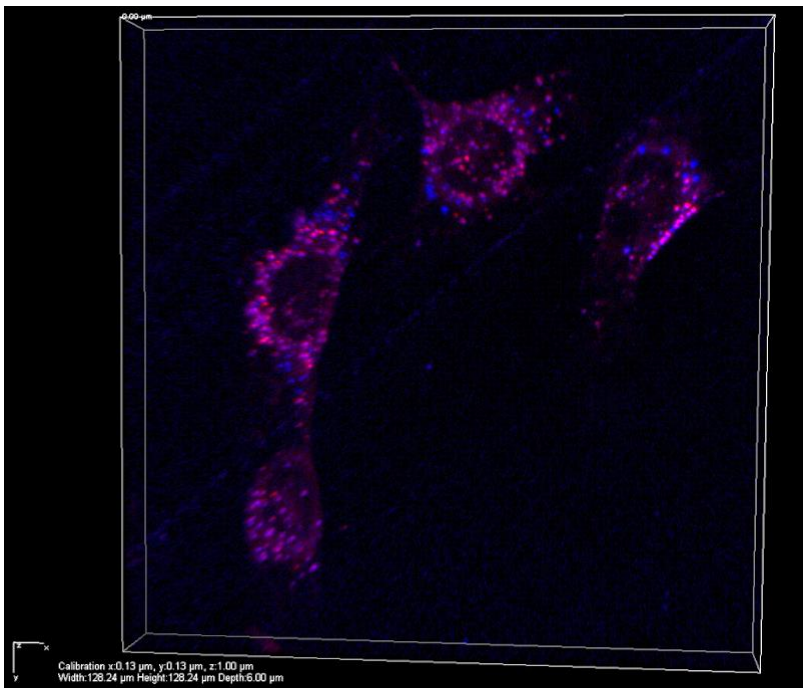


Figure S18. Confocal z-stack 3D reconstruction of PLGA-**5** NPs (blue) internalization in cells with stained lysosomes (red).

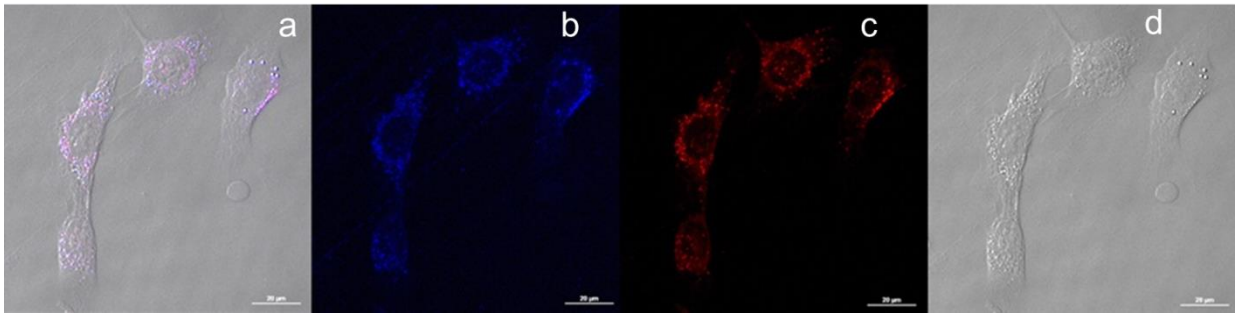


Figure S19. Confocal imaging with differential interference contrast (DIC) of 4T1 cells: a) combined image; b) 1 h after incubation with PLGA-5; c) cell lysosomes stained with LysoTrackerRedDND-99 (red); d) confocal visualization. The scale is 20 microns.

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