Nanoparticles Conjugated with Photo-Cleavable Linkers for the Intracellular Delivery of Biomolecules

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1- Materials

All solvents were dry and synthesis grade, unless specified. Reactions were performed under Argon, unless specified. External bath thermometers were used to record all reaction temperatures. Thin-layer chromatography (TLC) was performed on F254 250-μm silica gel plates, and components were visualized by observation under UV light, or by treating the plates with ninhidrine or p-anisaldehyde followed by heating. Final products were purified by column chromatography using silica gel as stationary phase. Conditions for purification consisted on different gradients of both mixtures Hexane/AcOEt or CH_2Cl_2/MeOH. The fractions containing the product were dried using anhydrous Na_2SO_4, concentrated under reduced pressure, and the identity of the final products was confirmed by NMR and MS.

2- Synthesis and characterization of PCLs

2.1- ***(9H-fluoren-9-yl)methyl (1-(2-nitrophenyl)-3-oxo-3-(prop-2-yn-1-ylamino)propyl)carbamate*** **(4).** A solution of propargylamine (42.0 mg, 0.763 mmol, Aldrich) and N,N-Diisopropylethylamine (DIPEA, 244.0 μL, 1.4 mmol, Aldrich) in 2.0 mL of CH_2Cl_2 was dropwise added to a previously prepared solution of 3-(9-Fluorenylmethyloxycarbonyl)amino-3-(2-nitrophenyl)propionic acid (Fmoc-ANP, 300 mg, 0.694 mmol, Iris biotech), 1-Hydroxybenzotriazole (HOBt, 103.1 mg, 0.763 mmol, Fluka), (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HBTU, 289.4 mg, 0.763 mmol, Fluka) and DIPEA (244.0 μL, 1.4 mmol) in 10.0 mL of CH_2Cl_2. The mixture was stirred at room temperature and in the dark for 1.5 h, and a precipitate was formed. The precipitate was filtered, dried with Na_2SO_4 and analyzed by TLC showing a ≥99.9% purity. The obtained white solid (322.0 mg, 98.9% yield) was used without any further purification in the next step.

^{1}H-NMR (400 MHz, d^6-DMSO) δ: 8.28-7.29 (m, 12H), 5.42 (td, 1H), 4.20 (d, 2H), 3.88 (s, 2H), 3.11 (t, 1H), 2.70 (d, 2H), 2.55 (s, 1H).

^{13}C-NMR (101 MHz, d^6-DMSO) δ: 168.27 (1, CO), 155.17 (1, CONH), 148.01 (1, C_{Ar}), 143.83 (1, C_{Ar}), 143.53 (1, C_{Ar}), 140.64 (2, C_{Ar}), 138.01 (1, C_{Ar}), 133.51 (1, CH_{Ar}), 128.27 (2, CH_{Ar}), 127.58 (2, CH_{Ar}), 127.05 (1, CH_{Ar}), 127.00 (1, CH_{Ar}), 125.10 (1, CH_{Ar}), 125.03 (1, CH_{Ar}), 123.89 (1, CH_{Ar}), 120.07 (2, CH_{Ar}), 80.95 (1, C), 73.09 (1, CH), 65.41 (1, CH2), 47.12 (1, CH), 46.58 (1, CH), 40.44 (1, CH2), 27.89 (1, CH2).

HRMS (ESI Q-TOF): [MH]+ calcd. for $C_{27}H_{24}N_3O_5$ = 470.16, found: m/z 470.1735

2.2- ***3-amino-3-(2-nitrophenyl)-N-(prop-2-yn-1-yl)propanamide*** **(PCL-1).** 500 μL (5.1 mmol) of piperidine were added to a suspension of **4** (305.0 mg, 0.65 mmol) in 5.0 mL of Dimethylformamide (DMF) at room temperature and in the dark. Complete solubility was achieved after three minutes of stirring. The mixture was then stirred at room temperature for 1 h, the resulting crude concentrated under reduced pressure to eliminate the excess of piperidine and finally purified by silica column chromatography (CH_2Cl_2:MeOH, 0 → 15% MeOH). Organic fractions corresponding to the desired product were then collected and solvent was eliminated under reduced pressure. A yellow oil (142.1 mg, 88.4% yield) was identified as the desired product.

^{1}H-NMR (400 MHz, d^6-DMSO) δ: 8.40 (t, 1H), 7.81 (m, 2H), 7.69 (td, 1H), 7.46 (m, 1H), 4.58 (t, 1H), 3.83 (d, 2H), 3.09 (s, 1H), 2.48 (m, 2H).

^{13}C-NMR (101 MHz, d^6-DMSO) δ: 169.67 (1, CO), 148.48 (1, C_{Ar}), 139.99 (1, C_{Ar}), 132.82 (1, CH_{Ar}), 128.40 (1, CH_{Ar}), 127.68 (1, CH_{Ar}), 123.45 (1, CH_{Ar}), 80.97 (1, C), 72.90 (1, CH), 47.37 (1, CH), 43.50 (1, CH2), 27.65 (1, CH2).

HRMS (ESI Q-TOF): [MH]+ calcd. for $C_{12}H_{14}N_3O_3$ = 248.10, found: m/z 248.0433

2.3- ***3-isothiocyanato-3-(2-nitrophenyl)-N-(prop-2-yn-1-yl)propanamide*** (**PCL-2**). A solution of carbon disulfice (CS_2, 12.0 μL, 0.2 mmol, Aldrich) in tetrahydrofuran (THF, 0.75 mL, thus 16 μL/mL) was dropwise added to a previously cooled (0 ºC, ice-water bath) solution of **PCL-1** (45.0 mg, 0.18 mmol) and Et_3N (76.2 μL, 0.546 mmol, Sigma) in THF (1.5 mL) under inert atmosphere (N_2). Once addition has finished, reaction was stirred at room temperature in the dark for 1.5 h. The resulting bright-yellow solution was then cooled down (0 ºC, ice-water bath) and 4-toluenesulfonyl

chloride (Ts-Cl, 40.0 mg, 0.209 mmol, Aldrich) was added. The resulting pale yellowish solution was stirred at room temperature in the dark for 30 min. Then, 0.2 M HCl was added and the resulting crude was extracted with CH_2Cl_2. Organic fractions were collected, dried with Na_2SO_4 and solvent was evaporated under reduced pressure. The resulting solid was purified by silica column chromatography (AcOEt:Hex, 0 → 80%) yielding to the desired product as a pale yellowish solid in a 80.0% yield (42.0 mg). Note: when THF that is not dry (distilled) is used, the yield was 51.0%.

^{1}H-NMR (400 MHz, *d*^{6}-DMSO) δ: 10.21 (m, 1H), 8.02 (d, 1H), 7.73 (t, 1H), 7.57 (t, 1H), 7.44 (d, 1H), 5.22 (m, 1H), 4.95 (dd, 1H), 4.82 (dd, 1H), 3.35 (s, 1H), 3.09 (m, 1H), 2.88 (m, 1H).

^{13}C-NMR (101 MHz, *d*^{6}-DMSO) δ: 180.41 (1, CONH), 164.63(1, CO), 147.54 (1, C_{Ar}), 134.18 (1, CH_{Ar}), 133.66 (1, C_{Ar}), 129.74 (1, CH_{Ar}), 127.85 (1, CH_{Ar}), 125.32 (1, CH_{Ar}), 79.25 (1, C), 73.48 (1, CH), 48.03 (1, CH), 36.58 (1, CH2), 34.53 (1, CH2).

HRMS (ESI Q-TOF): [MH]+ calcd. for $C_{13}H_{12}N_3O_3S$ = 290.06, found: m/z 290.0588

2.4- ***4-((1-(2-nitrophenyl)-3-oxo-3-(prop-2-yn-1-ylamino)propyl)amino)-4-oxobutanoic acid*** **(PCL-3).** To a previously prepared solution of **PCL-1** (40.0 mg, 0.162 mmol) and 4-(Dimethylamino)pyridine (DMAP, 29.6 mg, 0.243 mmol, Fluka) in 4.0 mL of CH_2Cl_2 was added the succinic anhydride (17.8 mg, 0.178 mmol, Aldrich) at room temperature. Resulting mixture was stirred at room temperature in the dark overnight. The resulting precipitate was then filtered and a white solid was obtained after drying under reduced pressure in a 83% yield (46.8 mg). After the corresponding characterization the compound was finally identified as the desired product, and it was used without further purification.

^{1}H-NMR (400 MHz, *d*^{6}-DMSO) δ: 8.57 (d, 1H), 8.30 (t, 1H), 7.88 (d, 1H), 7.69 (t, 2H), 7.62 (d, 1H), 5.53 (t, 2H), 4.58 (dd, 1H), 3.84 (m, 2H), 3.09 (d, 2H), 2.58 (d, 2H), 2.32 (s, 1H).

^{13}C-NMR (101 MHz, *d*^{6}-DMSO) δ: 173.75 (1, COOH), 169.77 (1, CONH), 168.34 (1, CONH), 148.12 (1, C_{Ar}), 137.78 (1, C_{Ar}), 133.30 (1, CH_{Ar}), 128.38 (1, CH_{Ar}), 128.08 (1, CH_{Ar}), 123.87(1, CH_{Ar}), 81.05 (1, C), 73.02 (1, CH), 45.54 (1, CH), 40.42 (1, CH2), 30.06 (1, CH2), 29.25 (1, CH2), 27.84 (1, CH2).

HRMS (ESI Q-TOF): [MH]+ calcd. for $C_{16}H_{18}N_3O_6$ = 348.12, found: m/z 348.1133

2.5- Photocleavage of the PCLs. Solutions of PCLs, both in water or in DMSO (350 μM for absorbance analyses and PCL-1 for photocleavage ^{1}H-NMR analyses, and between 63 and 89 mM for ^{1}H-NMR analyses), were exposed to a UV lamp (365 nm, 100 mW/cm^2, 5 cm distance between the light and the sample) or a blue laser (405 nm, 80 mW/cm^2, 5 cm distance between the light and the sample; ThorLabs) for different times. The percentage of PCL photocleavage was monitored by absorbance (Synergy spectrophotometer) or ^{1}H-NMR. ^{1}H-NMR analyses at concentrations between 63 and 89 mM (in DMSO-d_6) were performed for the calculation of photocleavage constants and the values were calculated as elsewhere [1].

3. Synthesis of an azide-sensitive fluorescent dye. Propargylamine (17.3 μL, 0.257 mmol, Aldrich) was added to a solution of fluorescein isothiocyanate isomer (FITC, 100 mg, 0.257 mmol, Aldrich) in 15 mL of a mixture of EtOH with THF (3:2). Then Et_3N (36 μL, 0.257 mmol) was also added to the mixture and reaction was stirred at room temperature for 4 h. The resulting crude was purified by silica column chromatography (8/2 CH_2Cl_2/$CHCl_3$:MeOH, 0 → 10% MeOH) yielding to a bright orangish product (110 mg, 96% yield).

^{1}H-NMR (400 MHz, *d*^{6}-DMSO) δ: 10.14 (s, 3H), 8.41 (m, 1H), 7.73 (m, 1H), 7.16 (d, 1H), 6.68 (d, 2H), 6.57 (m, 4H), 5.28 (d, 2H), 4.86 (s, 1H), 1.06 (t, 1H).

^{13}C-NMR (101 MHz, *d*^{6}-DMSO) δ: 168.75 (1, CO), 159.37 (2, CS & CO), 151.86 (2, C_{Ar}), 145.31 (1, C_{Ar}), 129.01 (2, CH_{Ar}), 127.05 (1, C_{Ar}), 124.25 (2, CH_{Ar}), 112.50 (2, CH_{Ar}), 109.82 (3, C_{Ar}), 102.15 (3, CH_{Ar}), 82.97 (2, C), 55.99 (1, CH2), 40.07 (1, C), 18.50 (1, CH).

HRMS (ESI Q-TOF): [MH]+ calcd. for $C_{24}H_{17}N_2O_5S$ = 445.08, found: m/z 445.0936

4. Preparation and characterization of Lyso-NPs

4.1- Conjugation of PCL-2 to lysozyme. A solution of PCL-2 (10 μL, 10 mg/mL) in DMSO was added to a fresh Lysozyme solution (300 μL, 0.7 mg/mL) in 100mM carbonate buffer pH 9.0. The mixture was stirred at 4ºC in the dark for 2 h. Then, 1 M NH_4Cl (10 μL) was added to the reaction crude and resulting mixture was stirred for 10 min. Lyso-PCL was purified from free PCL-2 by

ultracentrifugation (0.5 mL, Amicon® Ultra MWcutoff 3kDa). Recovery rate was evaluated by UV-Vis absorption (280 nm) showing values higher than 80%. Samples were analysed by size-exclusion HPLC (Shimadzu LC systems i-Series, Superdex® 200 5/150 GL column [150 × 5 mm, 13 μm], PBS eluent in isocratic mode).

4.2- Preparation of PEI:DS NPs (6). NPs were prepared by the electrostatic interaction of polyethylenimine (PEI, polycation) with dextran sulfate (DS, polyanion) in water, at room temperature, as previously described by us [2]. Briefly, an aqueous DS solution (1 mL, 10 mg/mL) was added drop-by-drop to an aqueous solution of PEI (5 mL, 10 mg/mL) and stirred for 5 min. Then, an aqueous solution of $ZnSO_4$ (0.6 mL; 1 M) was added and stirred for 30 min. The NP suspension was then dialyzed (Spectra/Por® 1 regenerated cellulose dialysis membrane, MWcutoff 6000-8000 Da, Spectrum) for 24 h, in the dark, against water before use.

4.3- Conjugation of PEI:DS NPs with NHS-Peg$_4$-azide (7). N_3-Peg_4-COOH (84.5 mg, 0.29 mmol, Broadpharm US) was added to an 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 127 mg, 0.73 mmol, Fluka) and N-hydroxysulfosuccinimide (sulfo-NHS, 287 mg, 1.45 mmol, Aldrich) solution (1 ml, 100 mM MES buffer pH 6.0) and reacted for 15 min at room temperature, followed by the addition of 100 mM PBS buffer 7.6 (2.4 mL) to adjust the pH. The solution (40 μL, 1 mg/mL) was then added to a suspension of PEI:DS NPs (2 mg/mL, 250 μL) in 100 mM carbonate buffer pH 9.0. The reaction mixture was stirred at room temperature for 2 h. Afterwards, 50 mM TRIS buffer pH 7.7 (50 μL) was added to the NP suspension and the resulting mixture was centrifuged (14.000 g, 3 min) and finally dialyzed (MWcutoff of 50,000 Da) against PBS overnight at room temperature.

4.4- Quantification of azide functional groups in PEI:DS NPs-Peg4-azide (7). The *azide-*

sensitive fluorescent dye (0.3 mM, 25 μL, in EtOH:DMSO) was reacted with PEI:DS NPs-Peg4-azide (125 μg, 90 μL) for 1 h. The NPs were then centrifuged (14.000 g, 3 min, 3 times) and resuspended, each time, in PBS (150 μL). The fluorescence of the NPs was evaluated in a plate-reader (λ_{ex} = 492 nm; λ_{em} = 518 nm). Concentration was determined against a calibration curve of the azide-sensitive fluorescent dye.

4.5- Conjugation of Lyso-PCL to PEI:DS NPs-Peg4-azide (8). NPs (5 mg/mL, 400 μL) were suspended in PBS buffer pH 7.4 and reacted with Lyso-PCL (195 μg in 250 μL PBS, 55 μM) for 2 h in the presence of copper sulfate (20 μM in PBS), tris(3-hydroxypropyltriazolyl-methyl)amine (THPTA, 50 μM in PBS) and sodium ascorbate (100 μM in PBS): The modified NPs were then centrifuged (14.000 g, 10 min), the pellet resuspended in buffer (400 μL) followed by a dialysis step (MWcutoff of 50,000 Da) against PBS overnight at room temperature. The NPs were collected, centrifuged (14.000 g, 10 min) and resuspended in buffer (400 μL). The activity of immobilized lysozyme was determined using *Micrococcus lysodeikticus* bacterial cells as a substrate. NPs (30 μL) were added to a *M. lysodeikticus* suspension (270 μL in PBS 6.3) and the decrease in turbidity was measured at 450 nm. The amount of lysozyme immobilized was collected based in a calibration curve with soluble lysozyme.

4.6- Release studies of lysozyme from NPs. NPs (100 μL, 5 mg/mL) were irradiated by a UV lamp at 365 nm for 5 min. The NPs were then centrifuged (14.000 g, 10 min) and the pellet resuspended in PBS (100 μL). This procedure was repeated twice. The supernatants in each run were joined and freeze-dried. The powder was solubilized in PBS buffer 7.4 (30 μL) and an enzymatic assay was performed (see above).

5. Preparation and characterization of PEI-atRA NPs

5.1- ***(2E,4E,6E,8E)-3,7-dimethyl-N-(1-(2-nitrophenyl)-3-oxo-3-(prop-2-yn-1-ylamino)propyl)-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenamide*** **(atRA-PCL, 9).** A solution of **PCL-1** (150 mg, 0.607 mol) and DIPEA (128.0 μL, 0.735 mmol) in 1.5 mL of DMF was dropwise added to a previously cooled (0 ºC, ice-water bath) solution of all-trans retinoic Acid (atRA, 227.8 mg, 0.7563 mmol, ACROS), HOBt (114.8 mg, 0.849 mmol), HBTU (322.1 mg, 0.849 mmol) and DIPEA (400.0 μL, 2.298 mmol) in 6.0 mL of DMF. Reaction mixture was stirred at room temperature and in the dark overnight. Then 100 mM HCl (7.5 mL) was added to the reaction crude, and the resulting mixture was extracted with CH_2Cl_2 (3 × 20 mL). Organic fractions were collected, dried with Na_2SO_4 and solvent was evaporated under reduced pressure. The resulting DMF solution was purified by silica column chromatography (AcOEt:Hex, 0 → 20%) obtaining a bright-yellow product (171 mg, 53.2% yield) identified as the desired compound.

^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.98 (s, 1H), 7.93 (d, 1H), 7.68 (d, 1H), 7.57 (t, 1H), 7.38 (d, 1H), 6.88 (m, 1H), 6.24 (t, 2H), 6.13 (m, 2H), 5.91 (t, 1H), 5.81 (s, 1H), 5.16 (m, 1H), 3.95 (m, 2H), 2.86 (m, 2H), 2.22 (s, 3H), 2.16 (t, 1H), 2.03 (t, 2H), 1.97 (s, 3H), 1.61 (m, 3H), 1.47 (m, 2H), 1.26 (s, 2H), 1.03 (s, 6H).

^{13}C-NMR (101 MHz, $CDCl_3$) δ: 170.53 (1, CO), 166.63 (1, CO), 149.56 (1, C), 147.95 (1, C), 138.87 (1, C), 137.70 (1, C), 137.42 (1, C), 137.31 (1, CH), 135.48 (1, CH), 133.81 (1, CH), 130.06 (1, CH), 129.84 (1, C), 129.62 (1, CH), 128.83 (1, CH), 128.28 (1, CH), 128.18 (1, CH), 124.89 (1, CH), 120.86 (1, CH), 79.10 (1, C), 71.48 (1, CH), 47.22 (1, CH), 40.57 (1, CH2), 39.61 (1, CH2), 34.25 (1, C), 33.10 (1, CH2), 29.13 (1, CH2), 28.96 (2, CH3), 21.75 (1, CH3), 19.23 (1, CH2), 13.61 (1, CH3), 12.85 (1, CH3).

HRMS (ESI Q-TOF): [MH]+ calcd. for $C_{32}H_{40}N_3O_4$ = 530.30, found: m/z 530.2887

5.2- Conjugation of PEI with NHS-Peg$_4$-azide (PEI-Peg$_4$N$_3$, 10). N_3-Peg_4-COOH (84.5 mg, 0.29 mmol) was added to an EDC (127 mg, 0.73 mmol) and sulfo-NHS (287 mg, 1.45 mmol) solution (1 ml, 100 mM MES buffer pH 6.0) and reacted for 15 min at room temperature, followed by the addition of 100 mM PBS buffer 7.6 (2.4 mL) to adjust the pH. The solution was then added to a solution of branched-PEI (50 mg, 25 kDa, 2.4 mL) in 100 mM phosphate buffer pH 7.6. The

reaction mixture was stirred at room temperature for 1 h. Afterwards, 50 mM TRIS buffer pH 7.7 (1.0 mL) was added to the reaction crude and the resulting mixture was dialyzed, against water (regenerated-cellulose membrane, 2 kDa MWcutoff) for 18 h, at 4ºC and in the dark. Resulting aqueous solution was freeze-dried obtaining a yellowish powder (137.2 mg, 99.9% yield) as the desired product.

5.3- Conjugation of PEI-Peg$_4$N$_3$ with atRA-PCL (PEI-atRA, 11) and preparation of PEI-atRA NPs. PEI-Peg4N$_3$ (16.5 mg, 275 μL) was solubilized in PBS buffer pH 7.4 and reacted with atRA-PCL (10 mg in 1 mL DMSO) for 2 h in the presence of copper sulfate (20 μM in PBS), tris(3-hydroxypropyltriazolyl-methyl)amine (THPTA, 50 μM in PBS) and sodium ascorbate (100 μM in PBS). The reaction solution was then dialyzed (regenerated-cellulose membrane, 50 kDa MWcutoff) against DMSO:water (1:1, v/v) for 18 h at room temperature in the dark, followed by a dialysis against water, in the same conditions. The NPs were formed during the dialysis procedure and collected by centrifugation (14.000 g, 10 min) and finally resuspended in PBS buffer.

5.4- Release studies of RA from NPs. The release of RA from NPs was evaluated by a biological assay, using a reporter cell line (NB4-RARE cells) that expresses luciferase after the activation of RA receptor. NB4-RARE cells[3] (2.5×10^4 cells/condition) were plated in V-shaped 96-well plates and cultured with soluble RA (10 μM), PEI-atRA (10 μg/mL) or PEI-atRA (10 μg/mL) for 4 h in RPMI-1640 medium supplemented with 1% fetal bovine serum. The cells were then washed by centrifugation (300 g, 5 min) to remove non-internalized stimulus. The PEI-atRA was irradiated outside the cells, prior to cell transfection with either 1 min or 3 min UV light (365 nm, 100 mW/cm^2, 5 cm distance between the light and the sample) while soluble RA and PEI-atRA NPs were irradiated after cell transfection with UV light (100 mW/cm^2). Following transfection and irradiation, cells were cultured for 24 h in RPMI-1640 medium supplemented with 10 % fetal

bovine serum and 100 U/mL PenStrep. After this time, cells were washed with PBS (200 μL) and then resuspended in cell lysis buffer (60 μL; the buffer was formed by 2.5 mM of magnesium chloride, 33 mM DL-dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid, 20 mM tricine, 2.5 mM magnesium sulfate and 1 % Triton X-100). The plate was kept on ice, under agitation for 15 min to allow complete lysis and then placed on -80 ºC for the amount of time necessary for the samples to freeze. Finally, the plate was thawed at slow rate on ice. The luciferase luminescence was quantified, in a well-by-well mode, in a microplate luminometer reader LumiStar Galaxy (BMG Labtech), at 37ºC with constant agitation, by adding, with an injector, the reading buffer (100 μL; the reading buffer was formed by 0.5 mM luciferin, 0.5 mM ATP, 2.5 mM of magnesium chloride, 33 mM DL-dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid, 20 mM tricine and 2.5 mM magnesium sulfate) to the sample (50 μL). Protein was quantified using Pierce BCA Protein Assay Kit (Thermo Scientific) in order to normalize the luminescence signal to the mass of protein in each condition. All conditions were performed in triplicate.

6. Characterization of the NPs

The diameter of the NPs was measured by photon correlation spectroscopy (PCS) using quasi-elastic light scattering equipment (Zeta-Pals™ Zeta Potential Analyzer, Brookhaven Instruments Corp., Holtsville, NY) and ZetaPlus™ Particle Sizing Software (version 4.03). To measure NP diameter, the NP suspension (2 mL, 50 μg/mL in water of molecular biology) was added to a cuvette and allowed to stabilize for 10 min and then analysed (3 times) at room temperature. In some experiments, the cuvette was then exposed to UV light (365 nm) or blue light (405 nm) for 5 min. The values of NP diameter and NP counts (Kcps) were recorded. The average diameters described in this work are number-weighted average diameters. The zeta potential of NPs was determined in a 1 mM KCl pH 6 solution, at 25 °C (2 mL, 50 μg/mL). All data were recorded with at least 5 runs (in triplicate) with a relative residual value (measure of data fit quality) of 0.03. The

diameter of NPs was also confirmed by ultra-high-resolution analytical FE-SEM SU-70 with a dedicated detector of STEM. Diluted NP suspensions (in H_2O) were placed on a 400-mesh 3 mm copper grid coated with a carbon support film (Taab Labs Ltd.) and dried overnight.

7. Internalization studies of PEI-atRA NPs by NB4 cells

7.1. Labelling of PEI-atRA NPs with TRITC. A suspension of NPs (80 µL, 1 mg/mL) was incubated with TRITC (0.4 mM) in 0.1 M carbonate buffer at pH 9.2 for 1 h at room temperature. Unreacted TRITC was removed with four steps of centrifugation (14000 g, 3 min) in PBS.

7.2. Incubation of NB4 cells with PEI-atRA NPs labelled with TRITC. NB4 cells (2.5×10^4) were seeded in a v-shaped 96-well plate and incubated with TRITC labelled PEI-atRA NPs (10 µg/mL) for 4 h in RPMI medium supplemented with 1% of FBS. After incubation, cells were centrifuged and washed with PBS to remove non-internalized NPs. To label cell membrane, cells were incubated with PKH67 (2 µM) for 3 min and centrifuged to remove excess of fluorophore. Then cells were deposited onto a glass slide using cytospin centrifugation followed by fixation with 4% PFA for 20 min. After washing with PBS, cell nuclei were stained with DAPI for further visualization under confocal microscope.

7.3. Confocal microscopy. Images were acquired using a Zeiss LSM 710 confocal microscope (Carl Zeiss, Jena, Germany) with a 40× objective/ 1.4 numerical aperture oil PlanApochromat immersion lens. PKH67 Green fluorescence was detected using the 488 nm laser line of an Ar laser (25 mW nominal output) and an LP 505 filter. TRITC fluorescence was detected using a 561 nm HeNe laser (1 mW) and an LP 560 filter. Z-stacks were acquired to confirm the intracellular

localization of TRITC labelled NPs. The thickness of the slices and the interval between slices were set to 0.7 μm.

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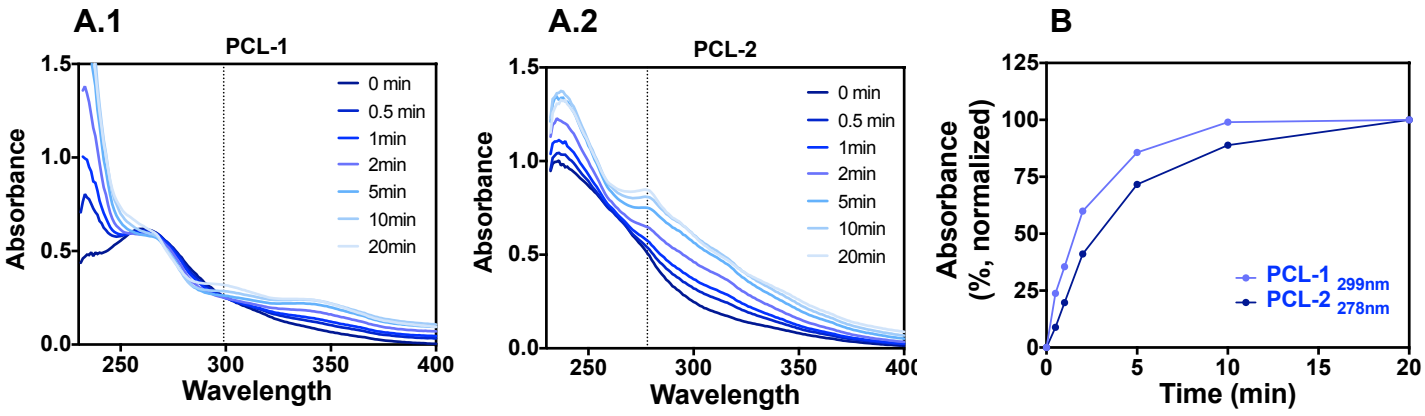

Figure S1- Time-course for the photocleavage of PCL-1 and PCL-2 by UV radiation in aqueous solution. (A) Absorbance spectra of PCL-1 (A.1) and PCL-2 (A.2) solutions (350 μM in PBS) after exposure to a UV lamp for different period of times (indicated in the graph). (B) Absorbance monitored overtime at 299 (PCL-1) and 278 nm (PCL-2) was normalised by the maximum absorbance at those peaks (approximately at 20 min).

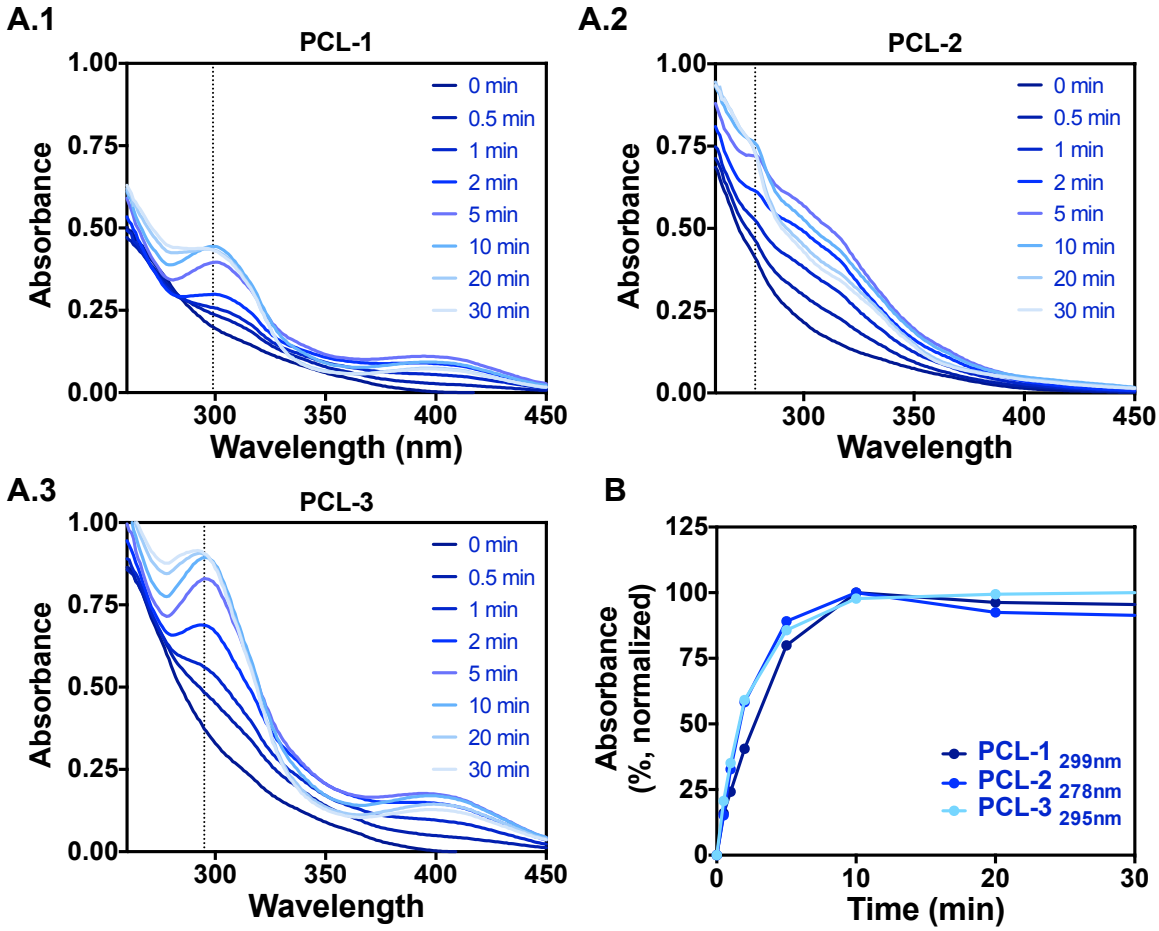

Figure S2- Time-course for the photocleavage of PCL-1, PCL-2 and PCL-3 by UV radiation in DMSO. (A) Absorbance spectra of PCL-1 (A.1), PCL-2 (A.2) and PCL-3 (A.3) solutions (350 μM in DMSO) after exposure to a UV lamp for different period of times (indicated in the graph). (B) Absorbance monitored overtime at 299 (PCL-1), 278 (PCL-2) and 295 nm (PCL-3) was normalised by the maximum absorbance at those peaks.

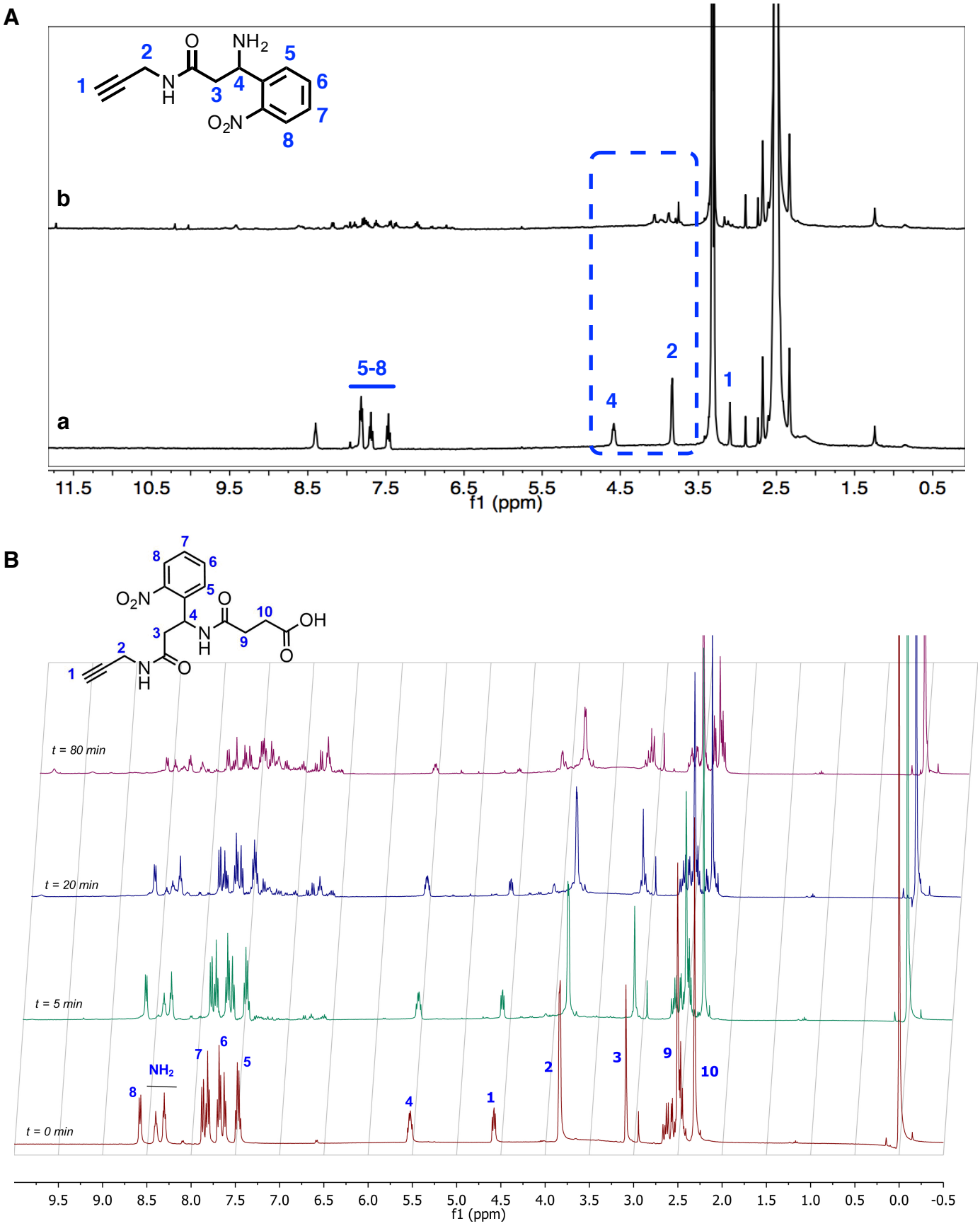

Figure S3- Photocleavage of PCL-1 and PCL-3 by UV radiation (365 nm, 100 mWcm^{-2}) in DMSO-d6. (A) ^{1}H-NMR spectra of PCL-1 (0.35 mM) before (a) and after (b) exposure the NMR tube to an UV lamp at a distance of approximately 5 cm during 10 min. (B) ^{1}H-NMR spectra of PCL-3 (63 mM) exposing the same solution to UV radiation (5 cm distance to light) for incremental times (0, 5, 20 and 80 minutes). NMR spectra were referenced for TMS. Figure shows also the NMR assignments to the chemical structure.

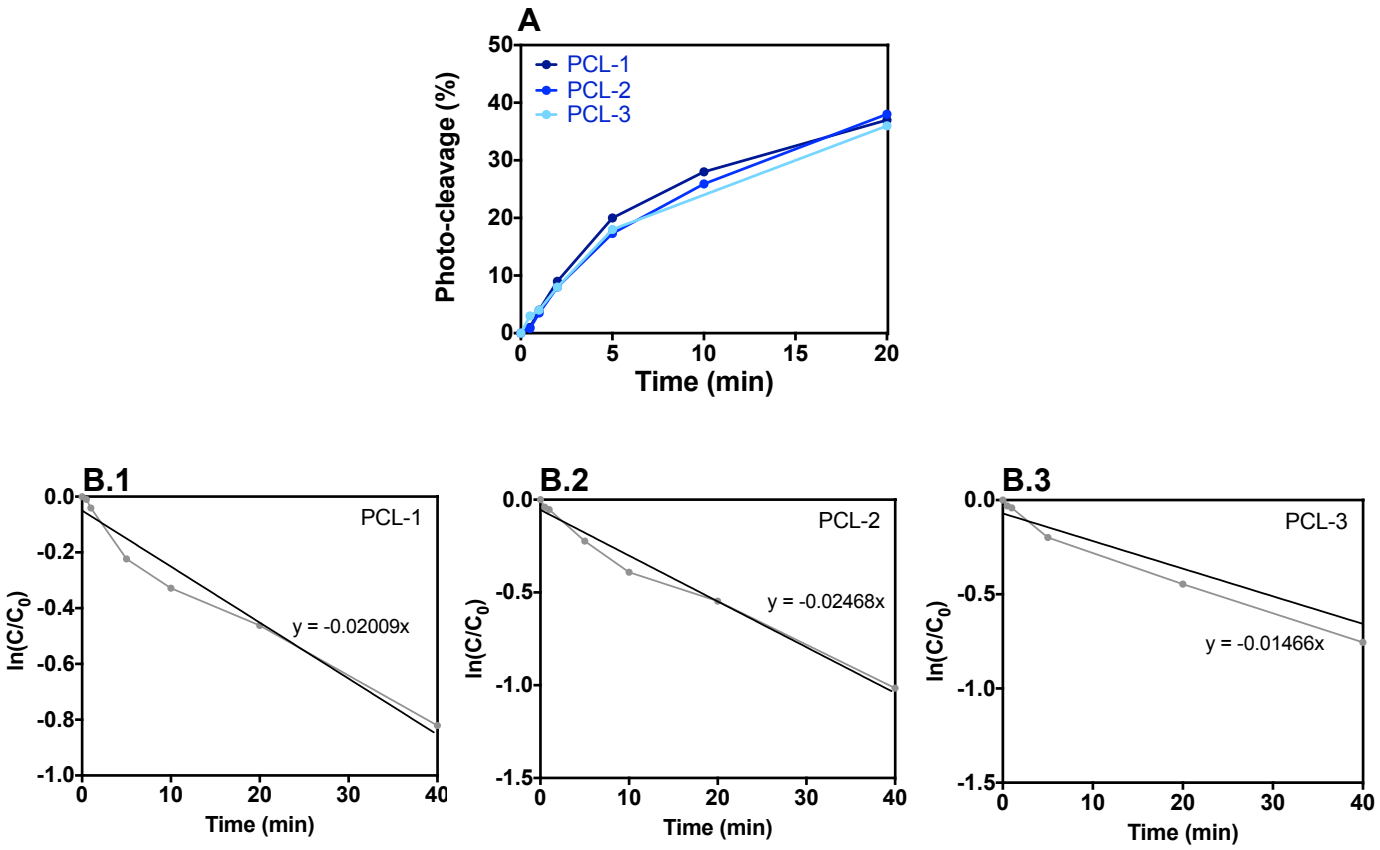

Figure S4- Time-course for the photocleavage of PCL-1, PCL-2 and PCL-3 by UV radiation (365 nm, 100 mWcm^{-2}) in DMSO as evaluated by ^{1}H-NMR. ^{1}H-NMR spectra were obtained of the same solution exposed to UV radiation for incremental times. The solution (89 mM for PCL-1, 76 mM for PCL-2, 63 mM for PCL-3 in DMSO) in a 5 mm NMR tube was exposed to UV lamp at a distance of approximately 5 cm. The photocleavage was calculated from the ratio of peak area at 4.6 ppm for PCL-1, 5.2 ppm for PCL-2, 5.5 for PCL-3 and 0 ppm (TMS). (B) Calculation of photocleavage constants (see experimental section).

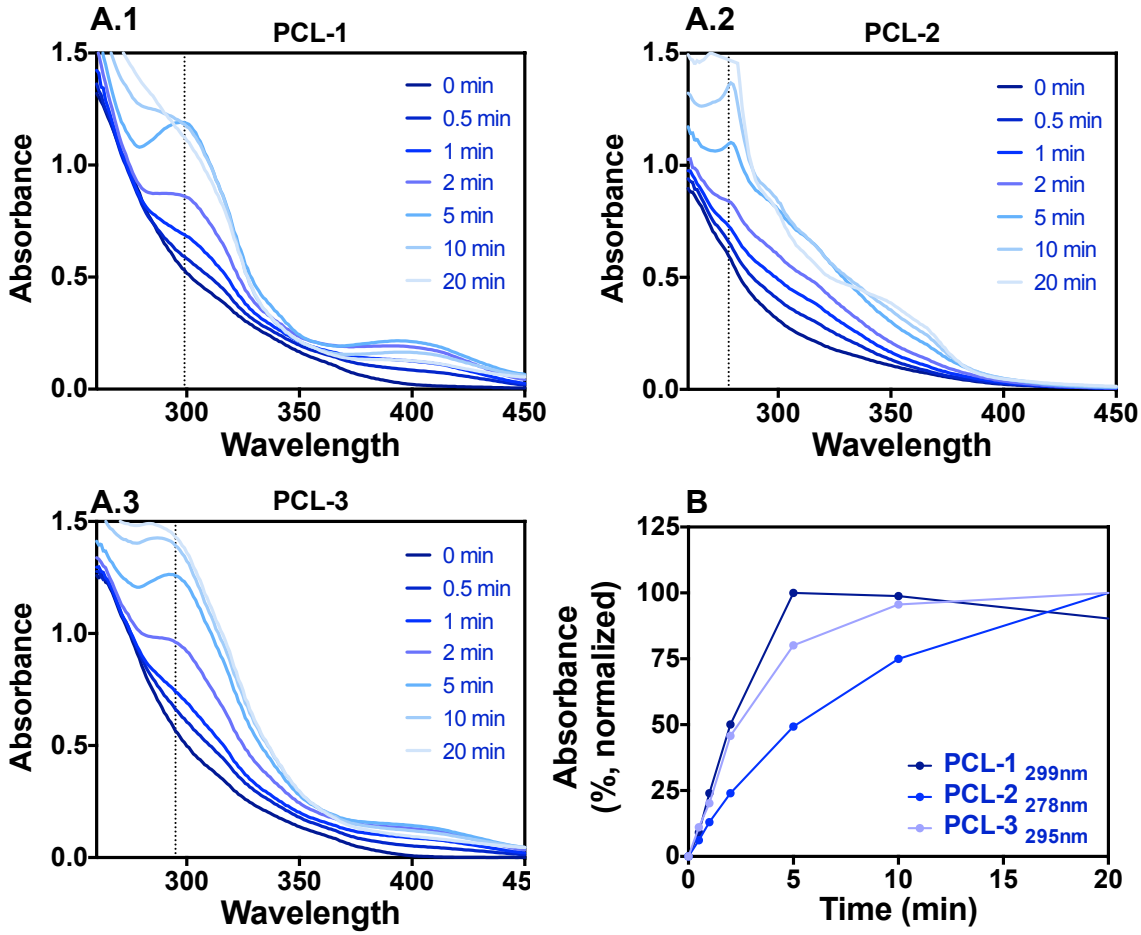

Figure S5- Time-course for the photocleavage of PCL-1, PCL-2 and PCL-3 by a blue laser (405 nm, 80 mWcm^{-2}) in DMSO. (A) Absorbance spectra of PCL-1 (A.1), PCL-2 (A.2) and PCL-3 (A.3) solutions (350 μM in DMSO) after exposure to a UV lamp for different period of times (indicated in the graph). (B) Absorbance monitored overtime at 299 (PCL-1), 278 (PCL-2) and 295 nm (PCL-3) was normalised by the maximum absorbance at those peaks.

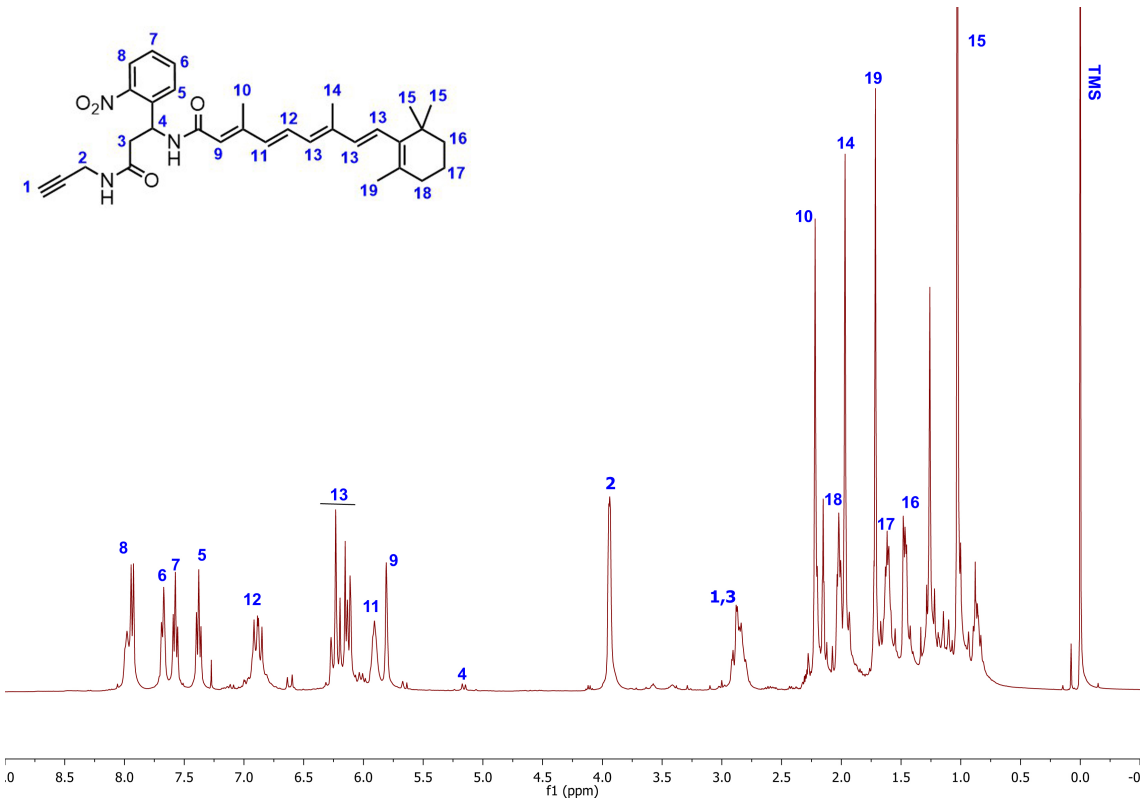

Figure S6- ^{1}H-NMR spectrum of atRAPCL in DMSO-d6. Figure shows also the NMR assignments to the chemical structure.

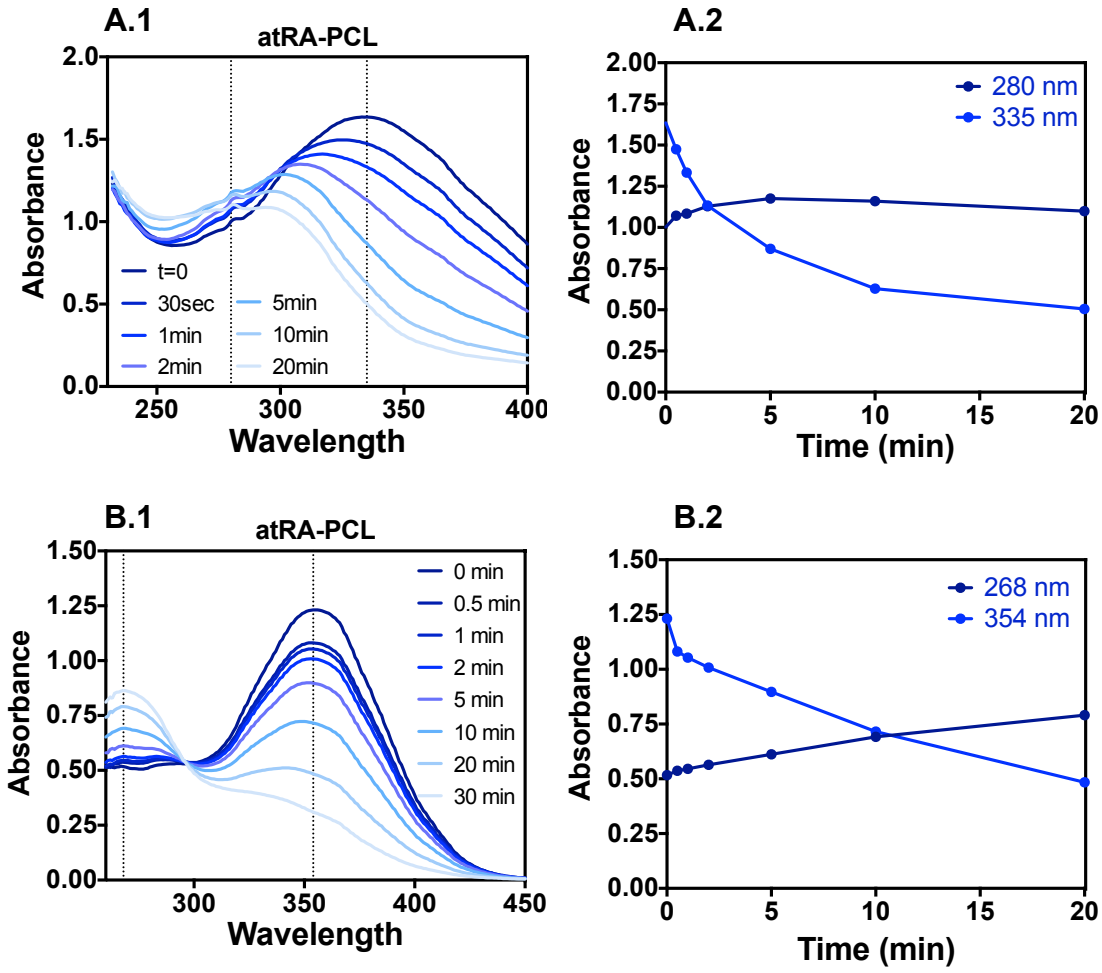

Figure S7- Photocleavage of atRA-PCL by UV radiation in aqueous solution (A) or DMSO (B). (A.1) Absorbance spectra of an aqueous solution of atRA-PCL (100 μM in PBS) after exposure to UV light (365 nm, 100 mW/cm^2, 5 cm distance) for different period of times (indicated in the graph). (A.2) Absorbance monitored overtime at 280 (PCL-1) and 335 nm (atRA). (B.1) Absorbance spectra of atRA-PCL (175 μM in DMSO) after exposure to a UV light. (B.2) Absorbance monitored overtime at 268 (PCL-1) and 354 nm (atRA).

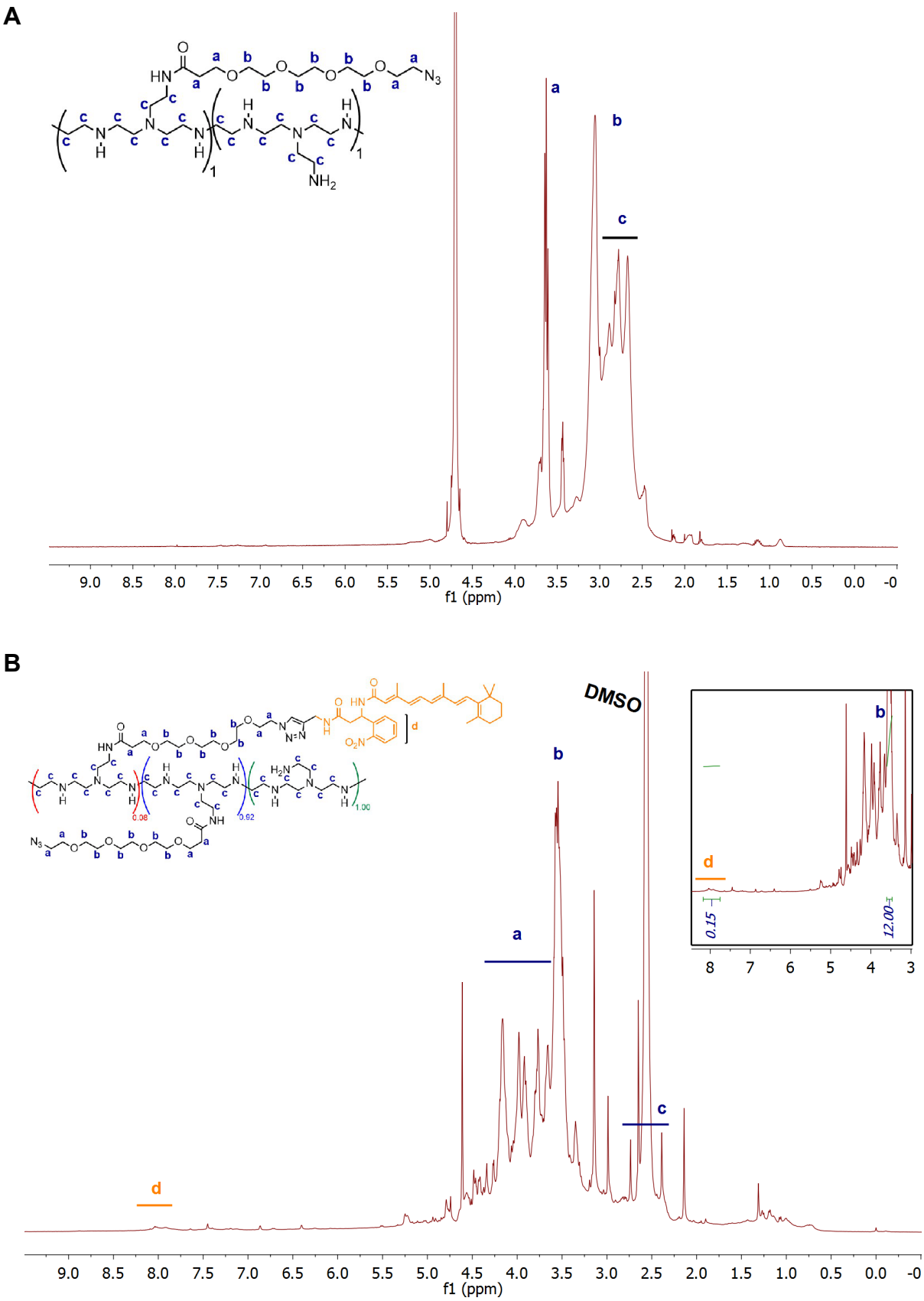

Figure S8- ^{1}H-NMR spectrum of PEI-Peg4N3 (compound **10**) in D_2O and PEI-atRA (compound **11**) in DMSO-d_6. Figure shows also the NMR assignments to the chemical structure.

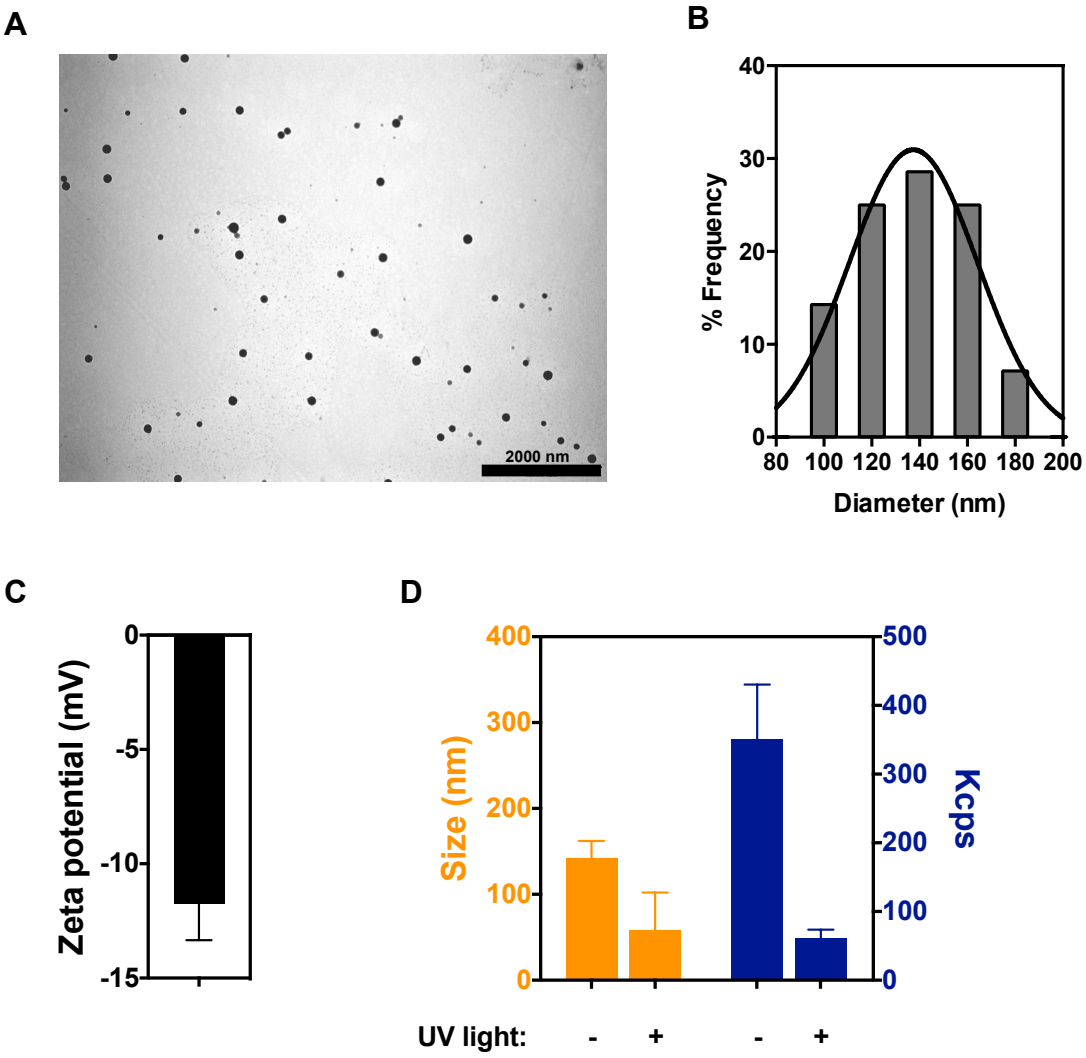

Figure S9 - Characterization of NPs conjugated with atRA. (A) Representative TEM images of PEI-atRA NPs. TEM images were obtained by a FEI-Tecnai G2 Spirit Bio Twin TEM microscope, at 100 kV. NP suspensions (in H_2O) were placed on a 200-mesh copper grid coated with a carbon support film (Taab Labs Ltd.) and dried overnight. (B) NPs size distribution obtained from TEM images. (C) Zeta potential of PEI-atRA NPs in PBS. (D) Size (orange) and number of NPs (as assessed by Kcps; blue) of PEI-atRA NPs before and after UV light irradiation determined by DLS. In D, results are average ± SEM, n=3.

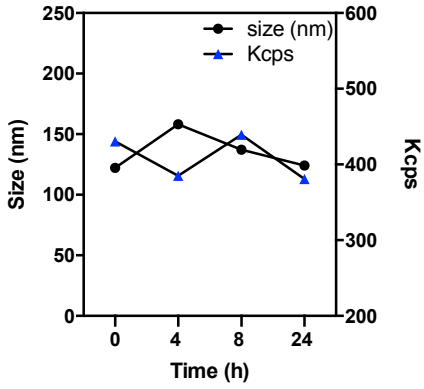

Figure S10 - Stability of PEI-atRA NPs in cell culture medium at 37 ºC. Diameter and counts (kcps) of PEI-atRA NPs suspended in RPMI-1640 medium supplemented with 1% fetal bovine serum (medium to culture NB4-RARE reporter cell line). The average diameter of NPs was determined by a dynamic light scattering method (DLS) using a Zeta Plus analyzer (Brookhaven).

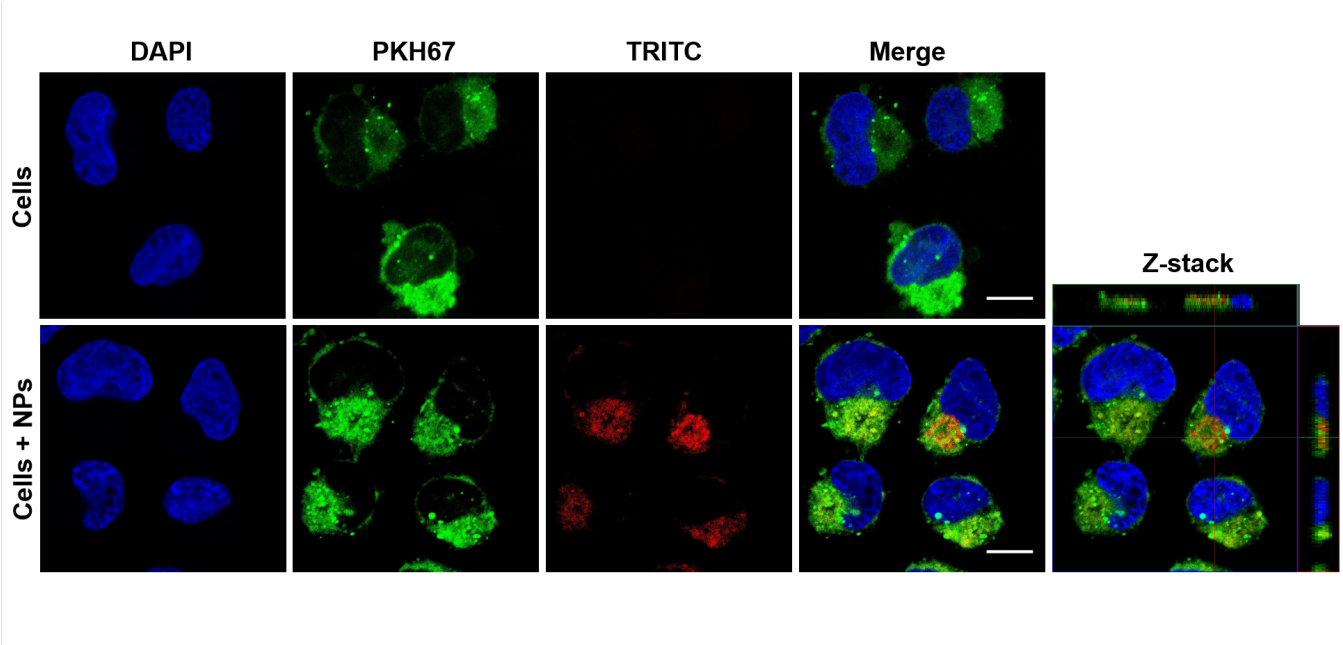

Figure S11 - Internalization of TRITC-labelled PEI-atRA NPs by NB4-RARE cells. Confocal images of NB4 cells incubated for 4 h with PEI-atRA NPs labelled with TRITC (10 μg/mL). Cell membrane was labelled with PKH67. Intracellular localization of PEI-atRA NPs was confirmed by a Z-stack scan. Scale bar corresponds to 10 μm.