A light-triggerable nanoparticle library for the controlled release of non-coding RNAs

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SUPPORTING INFORMATION

MATERIALS and METHODS

Synthesis of the photocleavable linker P1. The photocleavable linker P1 (2-nitro-1,3-phenylene)bis(methylene) diacrylate was synthesized and purified according to a previously reported procedure (Biomaterials 2014, 35, 5006-5015). Briefly, to a solution of (2-nitro-1,3-phenylene)dimethanol (5.5 g, 30 mmol, ARCH Bioscience) in anhydrous dichloromethane (38 mL, Fisher Chemical), was added dropwise trimethylamine (10.5 mL, 75 mmol, Sigma-Aldrich) under argon atmosphere. Then, acryloyl chloride (9.8 mL, 120 mmol, Merck) was added dropwise into the reaction mixture using a dropping funnel over 15 min at 0 °C. The mixture was stirred for additional 18 h at room temperature. After removing the formed solid by filtration, the filtrate was dried under vacuum and then resuspended in ethyl acetate (Fisher Chemical). The resulting solution was washed with saturated sodium chloride solution and then dried overnight with sodium sulfate. The final product was obtained after purification by silica gel chromatography (hexane: ethyl acetate as eluent, 1:1, v/v) as a white crystal.

Optimization of the photocleavable linker ratio in poly(amido amine)s. To optimize the amount of the photocleavable linker in the poly(amido amine), ratios of 25:75 and 50:50 of P1 to A (methylenebisacrylamide) were used in the synthesis of some polymers. To calculate the ratio of incorporation of P1 into the polymer, polymers were precipitated in water, lyophilized, resuspended in DMSO-d_6 and analyzed by ^{1}H-NMR (Bruker Avance III 400 MHz) relative to TMS. Size and count decrease of the nanoparticles after UV exposure were measured by DLS.

Optimization of the ratio siRNA:NP, transfection time and irradiation time for efficient gene knockdown. Several parameters were optimized for the high throughput screening of NP@siRNA mediated gene silencing capacity of the polymeric NP library. Firstly, siRNA:NP ratio was optimized to maximize GFP knockdown. Therefore, NPs (200 μg/mL) were complexed with siRNA against GFP in ratios of 1:12.5 and 1:50 for 2 h in nuclease free sterile water shaking on an orbital shaker (250 rpm) at room temperature. To evaluate bioactivity of the complexes, HeLa-GFP cells were seeded at a density of 40.000 cells/mL for 24 h prior the experiment. Cells were transfected for 4 h with NP@siRNA complexes (20 μg/mL) in starvation (DMEM). Cells were then washed, fresh medium with reduced serum (DMEM, 5% FBS, 0.5% PenStrep) added and cells were cultured for 48 h. At the end, cells were stained with H33342 and PI (both 0.25 μg/mL) and analyzed by fluorescence microscopy on a high-content microscope (In Cell Analyzer 2200). Cell viability and GFP knockdown were quantified as described in high content imaging section below.

In a separate experiment, the transfection time was optimized. The motivation here was to identify a time relatively short that could lead to significant gene knockdown. Cells were transfected with NP@siRNA complexes (20 μg/mL; 1:50 siRNA:NP) from 10 min to 4 h. In a separate experiment, different UV light sources were evaluated for NP activation within cells. Cells were placed (right after transfection and medium replacement) on a 20 cm support (distance from top 15 cm) in a transilluminator (UVP BioSpectrum 500) and irradiated with 365 nm light (1 mW/cm^2) from the top for 10 min. In both experiments cells were cultured in medium with reduced serum (DMEM, 5% FBS, 0.5 % PenStrep) until 48 h. Cells were stained with H33342 and PI (both 0.25 μg/mL) and analyzed by fluorescence microscopy on a high-content microscope (In Cell Analyzer 2200) for GFP knockdown (described in high content imaging section below).

Synthesis of polymers with photocleavable moieties. Prior to synthesis, diamines (1-32), bisacrylamides (A-E) and photocleavable linker P1, were diluted to 1.6 M in DMSO. Specifications

of all monomers can be found in Supplementary Table 1. The monomers (25 µL of P1, 25 µL of bisacrylamides A-E, 50 µL of amines 1-32) were added to a polypropylene 96 well plate, the plate sealed with aluminum foil and then incubated at 60ºC under agitation (orbital shaker, 250 rpm) for 5 days. Polymer synthesis was performed at final monomer concentration of 0.8 M. Polymers were finally end capped with 20% molar excess (10 µL to 100 µL reaction volume) of the respective diamine 1-32 for 2 h (60ºC, 250 rpm) and stored at 4ºC until usage.

Gel permeation chromatography (GPC) analyses. Number average molecular weights (Mn) and molecular weight distributions (Mw/Mn) were measured by GPC on a HPLC Agilent 1260 system equipped with a guard column (Agilent, Aquagel, 10 mm, 10 µm) followed by three columns: (i) Agilent, Aquagel-OH 40, 300 × 7.5 mm, 8 µm, (ii) Agilent, Aquagel-OH 50, 300 × 7.5 mm, 8 µm and (iii) Phenomenex, Polysep-GFC-P2000, 300 × 7.8 mm, range 100 – 10 k Da, connected to a UV (254 and 280 nm) and RI detector (Agilent). An acetate buffer (0.5 mol/L, pH = 4.5) was used as an eluent, at a flow rate of 0.7 mL/min and 35 °C. Polyethylene oxide standards (EasyVial PEG/PEO, range 194 – 1000 k Da) were used to calibrate the SEC, since it has been demonstrated that such eluent composition allows PEO to be a suitable calibration standard for poly(amido-amines)[1].

NP preparation and activation. For the high-throughput screening of NPs, NPs were prepared in sterile conditions using sterilized 96-deepwell polypropylene plates (VWR). Therefore, each polymer solution (15 µL; in DMSO) was precipitated into sterile nuclease free molecular grade water (960 µL) with subsequent addition of sterile zinc sulfate (25 µL, 1M). Plates were sealed with PP adhesive seals and incubated shaking (250 rpm) on an orbital shaker at room temperature overnight. NPs were purified by centrifugation at 4 ºC, 8000 g for 8 min. The mass concentration of each purified formulation was determined after lyophilizing samples. The efficiency of NP formation was up to 47%, calculated according to equation:

$$NP\ formation\ efficiency\ (\%) = \frac{M_{NP}}{M_{polymer}} \times 100$$

where M_{NP} denotes the weight of material recovered after NPs purification and freeze-drying and $M_{polymer}$ is the theoretical polymer weight.

NP size and zeta potential analyses. The size and zeta potential of NPs was measured by a ZetaPALS analyzer (Brookhaven Instruments Corp.). NPs were resuspended in 1 mM KCl and diluted to achieve average count rates about 200 kcps to perform the DLS measurement. Values were expressed as the mean of 5 measurement runs, each with a duration of 1 min. To determine light sensitivity of the NPs, a duplicate of the sample was used. NP disassembly was triggered by a UV lamp (365 nm, 100 Watt, 5 cm distance, 10 min). Samples NP size and average count rates were determined as for the non-irradiated sample. Light sensitivity is expressed as percent count decrease respective to the initial average count rate, which is an indicator for NP concentration**.**

TEM analyses. A suspension of P1C7@siRNA NPs (500 µg/mL) was prepared in molecular grade water. A droplet of the suspension was added to the surface of an ultrathin carbon coated 400 mesh copper grid, irradiated or not with UV light (10 min, 365 nm, 1 mW/cm^2) and left air-dry for 5 h at room temperature in a closed petri dish. NPs were viewed with a JEOL-2100-HT microscope. Digital images were acquired with a fast-readout "OneView" 4k x 4k CCD camera that operates at 25 fps (300 fps with 512 x 512 pixel) and features drift correction. The diameter of NPs was analysed with the Particle Tool from ImageJ.

High-throughput complexation of NPs with siRNAs. NPs were suspended in sterile nuclease free molecular grade water to a concentration of 400 µg/mL. Complexation with siRNA against eGFP (GFP Duplex I, GE Dharmacon) was then done at a ratio of 1:50 (siRNA:NP, w:w) in a 96-deepwell polypropylene plate (VWR). From each NP formulation, an aliquot (50 µL) was added to a deep well

plate, following the plate layout for subsequent cell transfection. A solution of siRNA containing 4 μg/mL siRNA and 4 μg/mL Cy5-tagged siRNA was prepared in sterile molecular grade, nuclease free water. The siRNA solution (50 μL) was added to the NPs in the same volume (50 μL) using a multichannel pipette. As control for siRNA activity and transfection, the same procedure was followed for lipofectamine RNAiMAX (15 μl/mL; Invitrogen). The plates were sealed with adhesive polypropylene seals and allowed to incubate shaking at room temperature for 2 h on an orbital shaker (250 rpm, room temperature). Samples were then diluted 1:10 with DMEM to 20 μg/mL NP concentration and directly used for cell transfection or determination of complexation efficacy. Complexation efficacy was determined indirectly from Cy5 tagged-siRNA after separating NPs and non-complexed siRNA by centrifugation (4ºC, 14000g, 15 min), quantifying Cy5 fluorescence in three replicates of the supernatant. Concentration of siRNA was determined relative to a standard curve.

High-throughput siRNA transfections. HeLa-GFP (CellBiolabs Inc.) cells were cultured DMEM (without phenol red) containing FBS (10%, v/v), PenStrep (0.5%, v/v, 50 μg/mL) and blasticidin (10 μg/mL). HeLa-GFP cells were seeded 24 h prior to experiment in 96 well plates (Costar) with a density of 4.000 cells per well. Cells were transfected with NP@siRNA complexes (20 μg/mL) or lipofectamine RNAiMAX (1.5 μL/ml) in DMEM as described above. Transfections were performed with three technical replicates and each plate in duplicate. After 10 min of transfection, the medium was replaced by DMEM containing 5% FBS (v/v, to slow down cell proliferation), PenStrep (0.5%, v/v, 50 μg/ml) and blasticidin (10 μg/ml). In one plate, NPs were activated for 10 min (using 365 nm light from top on a transilluminator UVP BioSpectrum 500 at 15 cm distance), while with the second plate (with the same NP formulations as the first plate), no activation of NPs was performed. This experiment allowed us to compare the bioactivity of released siRNA with and without application of the stimulus. At 48 h, cells were stained and placed in an automated incubator (Cytomat 2, Thermo)

for further incubation and analyses by high-content imaging with an automated fluorescence microscope (In Cell 2200, GE Healthcare).

High-content imaging analyses. Cell nuclei were stained at 48 h with Hoechst H33342 (Sigma-Aldrich, 0.25 µg/mL) and propidium iodide (PI, Sigma-Aldrich, 0.25 µg/mL). Dead cells stained for both Hoechst H33342 and PI, while live cells stained only for Hoechst H33342. At 48 h and 72 h, four random fields per well were imaged on a high-content microscope (In Cell 2200, GE Healthcare) with a 20× objective. Automated image analyses were performed using the In Cell Developer software from GE Healthcare. GFP knockdown was accessed from the mean GFP fluorescence intensity in the cytoplasm of live cells. Hoechst 33342 was used to define a nuclear mask, excluding dead cells (with 10% overlap of PI and H33342 stain), which was then dilated to cover as much of the cytoplasmic region as possible. Removal of the original nuclear region from the dilated mask creates a ring mask that covers the cytoplasmic region outside the nuclear envelope. GFP knockdown was expressed as percentage of fluorescence on non-treated HeLa-GFP cells (after subtracting fluorescence background of HeLa cells). Cell viability was calculated from the total number of cells (quantified by cell nuclei) after subtraction of dead cells (cells presenting >10% overlap of PI and Hoechst stain). Internalization of NPs was quantified by the fluorescence signal of NPs in cells.

siRNA release from NPs after light exposure. The release of siRNA from NPs after UV-irradiation (10 min, 365 nm, 1 mW/cm^2) was investigated by 1% agarose gel electrophoresis (containing orange G (1X) in TBE buffer (1X)). siRNA (15 µL, 2 µg/mL) and NP@siRNA (100 µg/mL) solution/suspension was applied to separate gel lanes, and electrophoresis was performed for 15 min at 100 V. The gel was visualized under a UV transilluminator (Gel Doc XR+, BioRad) at an excitation wavelength of 302 nm. The obtained bands were analyzed using ImageJ software 1.48 v (National institutes of Health, USA).

Cellular internalization of NPs. NP@siRNA complexes were exposed for 1 h to human dermal keratinocytes (HaCaT cells; CLS Cell Lines Service GmbH, Eppelheim, Germany), human normal dermal fibroblast (NHDF) or human umbilical vein endothelial cells (HUVEC, Lonza) and characterized by flow cytometry or confocal microscopy. For flow cytometry, HaCaT and NHDF cells were cultured in DMEM medium while HUVECs were cultured in EGM-2 medium (Lonza). All media was supplemented with FBS (10%, v/v) and PenStrep (0.5%, v/v, 50 µg/mL). Cells were seeded in 24 well plates (HaCaT and HUVECs at 25.000 cells/well while NHDF cells at 12.000 cells/well) and allowed to adhere for 24 h. Cells were transfected for 1 h with NP@siRNA-Cy5 or lipofectamine@siRNA-Cy5 complexes in DMEM or EGM-2 media. Complexes were removed and cells were washed with PBS.

For confocal microscopy, HaCaT and NHDF cells were cultured in DMEM medium while HUVECs were cultured in EGM-2 medium. All media was supplemented with FBS (10%, v/v) and PenStrep (0.5%, v/v, 50 µg/ml). Cells (HaCaT and HUVECs: 20000 cells/well; NHDF cells: 10.000 cells/well) were seeded in black glass bottom 96 well plates (IBIDI, Germany) coated with 0.1% gelatin (Sigma) and allowed to adhere for 24 h. Prior to transfection cells were stained with CellTrace™ CFSE 488 (5 µM; Molecular Probes, Life Technologies) according to manufacturer's instructions. Cells were transfected for 1 h with NP@siRNA-Cy5 complexes in DMEM (for HaCaT or NHDF cells) or EBM-2 (for HUVECs). Cells were stained with Lysotracker Red (100 nM; Molecular Probes, Life Technologies) for 30 min during cell transfection. Complexes were removed and cells were washed twice with PBS and fixated with 4% (v/v) paraformaldehyde (Alfa Aesar) in PBS for 10 min at room temperature. Nuclei were stained with H33342 (2 µg/mL) for 10 min. Cells were then washed 3 times with PBS and analysed by confocal microscopy (Zeiss LSM710) using a 40× immersion oil objective. Each condition is represented by two technical replicates and four or

more representative images per field were acquired. Colocalization of NP@siRNA-Cy5 with Lysotracker red was performed using JaCoP on Image J.

Uptake of P1C7@siRNA-Cy5 in the presence of chemical inhibitors. HeLa cells were plated in a 24 well plate at a density of 5×10^4 cells/well and left to adhere overnight. Cells were pre-incubated with endocytosis inhibitors for 30 min followed by 1 h incubation with P1C7@siRNA-Cy5 (20 μg/mL). The following inhibitors were tested: filipin III (80 μM), nocodazole (3 μM), polyinosinic acid (100 μg/mL), dansylcadaverine (25 μM) cytochalasin D (1 μM), dynasore (30 μM) and EIPA (80 μM). As controls, we used cells without NPs and cells incubated with NPs without inhibitor at 37 °C and at 4 °C. At the end of each point, cells were centrifuged, washed three times with PBS and then resuspended for flow cytometry analysis.

Intracellular trafficking and siRNA release. Endosomal escape can be determined by galectin 8 recruitment [1]. A7r5-Gal8YFP[2] reporter cells (kindly donated by Craig Duvall's lab) were used to study the colocalization of the Cy5 signal from the NP@siRNA-Cy5 complexes with YFP-Gal8 spots from releasing endosomes [1]. A7r5-Gal8YFP cells were cultured in DMEM supplemented with FBS (10%, v/v), PenStrep (0.5%, v/v, 50 μg/mL) and blasticidin (10 μg/mL). For the experiment 4.000 cells were seeded in each well of a black 96 well plate with glass bottom (IBIDI), suitable for confocal microscopy and allowed to adhere overnight. Cells were transfected for 10 min with NPs (20 μg/mL) or L2000 complexes with siRNA-Cy5 in DMEM. Cells were then washed and cell culture medium was added to the cells. UV light (365 nm, 1 mW/cm^2) activation was performed for 10 min followed by cell culture. Cells were fixated (4% PFA for 10 min) at different times (t=-10 immediately after transfection, t=0 after light activation and t=+15, 30. 45, 60 min post light activation), washed with PBS, cell nuclei stained with H33342 (1 μg/mL, Sigma) and analysed by a confocal microscope (Zeiss LSM710, 40x immersion oil objective). Each condition is represented by two technical

replicates and 4 or more images per field were acquired representing the total cell population. The area of YFP-Gal8 and Cy5-NPs spots were analysed with the Particle Plugin from ImageJ. Colocalization of Cy5-labelled NPs with YFP-Gal8 spots was performed using JaCoP on Image J.

Complexation of miR150 to the NPs. The complexation of miR150 (GE Dharmacon) to P1C7 NPs followed the same procedure previously described for siRNA. Briefly, miR150 and P1C7 NPs were mixed in molecular grade nuclease free, sterile water (Fisher Bioreagents) in a ratio of 1:50 (w/w, miRNA to NPs), and the suspension agitated on an orbital shaker for 2 h at room temperature. After complexation, the NP suspension was diluted in cell culture medium before use.

Bioactivity of P1C7@miRNA150 formulation. Human keratinocytes were cultured in DMEM supplemented with 10% FBS and 0.5% PenStrep, harvested and then seeded in a 96 well plate at a density of 25.000 cells/well to grow to a monolayer in approximately 48 h. Cells were then inhibited by mitomycin (5 µg/mL, in cell culture medium, Tocris Bioscience) for 2 h, transfected for 4 h with P1C7 NPs (40 µg/mL in DMEM) or P1C7 NPs@miRNA150 complexes (40 µg/mL in DMEM), washed with PBS to remove non-internalized NPs, exposed or not to UV light (10 min, 365 nm, 1 mW/cm^2). The cell monolayer was then scratched with a pipette tip and then cultured in cell culture medium for 48 h. As controls, cells were transfected for 4 and 24 h with the commercial agent lipofectamine RNAiMAX or RNAiMAX-miR150 complexes. In both cases (NPs and lipofectamine), wound closure was monitored by an automated fluorescence microscope (In Cell 2000, GE Healthcare, 4× objective) every 12 h. Wound closure was analyzed from the image field in the center of the well, measuring the wound area with Image J. The percentage of wound closure was calculated by well considering the initial wound area and then normalized to the control of the respective group.

Quantitative analyses of miR150 transfection. To demonstrate that HaCaT cells were successfully transfected with P1C7 NPs@miRNA150 complexes, cells were transfected with P1C7 NPs or P1C7 @miRNA150 NPs for 4 h, washed with PBS to remove non-internalized NPs, light activated, and finally cultured for 48 h. Next, cells were harvested, lysed and RNA isolated by a miRCURY™ RNA isolation kit (Exiqon) following manufacturer´s instructions. The cDNA was then synthesized using the Mir-X™ miRNA First Strand Synthesis (Exiqon). Expression of miRNA was quantified by quantitative RT-PCR (7500 Fast Real-Time PCR System, Applied Biosystems, Carlsbad, CA, USA) using Mir-X™ SYBR qRT-PCR kit (Clontech, California, USA) and NZYSpeedy qPCR Green Master Mix (NZYTech, Portugal). For normalization of microRNA expression levels, RNU6 was used as (housekeeping) control (Supplementary Table 3). Results were analyzed using the $\Delta\Delta C_T$ method to indicate relative miR150 expression from light activated miR-carrying to non-carrying P1C7 NPs.

Quantitative analysis of target gene knockdown. HaCaT cells transfected with P1C7 NPs or P1C7@miRNA150 NPs (see section before) were analyzed for the expression of *cMYB*, a target gene of miR150. Therefore, cDNA was synthesized from 1 µg total RNA using TaqMan™ reverse transcription reagents (Applied Biosystems, CA, USA). Quantitative RT-PCR was performed using NZYSpeedy qPCR Green Master Mix (NZYTech, Portugal) on a RT-PCR (7500 Fast Real-Time PCR System, Applied Biosystems, Carlsbad, CA, USA). Quantification of the target gene was analyzed relative to GAPDH as housekeeping gene: $relative\ expression = 2^{[-(C_T\ Sample - C_T\ GAPDH)]}$. Minimal cycle threshold values (C_T) were calculated from at least 3 independent reactions. $\Delta\Delta C_T$ was calculated to determine relative *cMYB* expression from light activated miR-carrying to non-carrying P1C7 NPs.

***In vivo* wound healing experiments.** Animal protocol was approved by the Ethics Committee of the Faculty of Medicine of the University of Coimbra (ORBEA_159_2017/05052017). Male C57BL/6 mice (8 weeks) were purchased from Charles River (Wilmington, MA, USA). Mice were separated on individual cages 24 h prior the induction of the skin wounds. They were anesthetized with xylazine/ketamine (xylazine hydrochloride - Rompun, 10 mg/kg of body weight; ketamine hydrochloride – Imalgene 1000, 80 mg/kg of body weight), shaved with an electric clipper on the back and remaining hair removed with depilatory cream (Dove), the skin was disinfected with betadine and two 6 mm-diameter dorsal full-thickness excisional wounds were created with a sterile biopsy punch in each animal. The treatments (P1C7@miRNA150, P1C7@scramble, PBS; n=8) were administered as intradermal injection at 4 locations around the wound. Light (5 min; 5 sec on/off; 405 nm blue laser; Thorlabs, Dachau, Germany) activation of the NPs was performed 30 min post-injection, to allow the NPs to be internalized by the skin cells. During the first 2 days, the mice received every 8 h buprenorphine (0.05 mg/Kg of body weight, Bupaq) to relief the animals from any pain or distress caused by the procedure. The animals were observed daily and wound area was measured. At day 3 and 10 mice were sacrificed by cervical dislocation after an overdose of anesthesia. Skin biopsies were taken for histological and gene expression analyses.

Histological analyses of skin wounds. Skin wounds were excised with a margin of epidermis outside the wound (approx. 2 mm), and processed for routine histology. Therefore, freshly excised wounds were placed onto a small piece of cardboard, with the subcutaneous tissue facing down, and immersed in 10% neutral buffered formalin for 24 h. After fixation, trimming was performed longitudinally, in the direction of the hair flow and centered on the wound. Samples were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. Histological analysis was performed by a pathologist blinded to experimental groups and measurements were performed using NDP.view2 software coupled to Nanozoomer SQ slide scanner (Hamamatsu).

Quantitative analysis of miRNA150 target gene in skin wounds. To quantify downregulation of miR150 gene targets in the skin wounds, samples at day 3 post-surgery were analyzed. RNA was isolated using TRIzol (500 µL, Invitrogen) reagent from 15-50 mg of tissue. Samples on ice were homogenized by a TissueLyser (Qiagen) operated at 30 Hz in three cycles of 2 min. Samples were then processed according to manufacturer´s instructions for RNA isolation and RNA was quantified on a NanoDrop™ (Thermo Scientific). cDNA was prepared from 1 µg total RNA using TaqMan™ reverse transcription reagents (Applied Biosystems, CA, USA). Quantitative RT-PCR (qRT-PCR) of murine cMYB was performed using NZYSpeedy qPCR Green Master Mix (NZYTech, Portugal) and detection on a RT-PCR (7500 Fast Real-Time PCR System, Applied Biosystems, Carlsbad, CA, USA) equipment. Quantification of the target gene was analyzed relative to mouse GAPDH as housekeeping gene: $relative\ expression = 2^{[-(C_T\ Sample - C_T\ GAPDH)]}$. Minimal cycle threshold values (C_T) were calculated from at least 3 independent reactions. $\Delta\Delta C_T$ was calculated to determine downregulation of *cMYB* relative to control skin (tissue day 0).

Light activation of P1C7 NPs through a skin barrier. To demonstrate that NPs can be dissociated by a 405 nm laser through the skin barrier, back skin from C57BL/6 mice was used as barrier between the light source and the NPs. Briefly, hair was removed from mouse skin with depilation cream and washed several times with PBS and mounted on a cardboard plate with a 1 cm^2 hole with the geometry of DLS cuvettes. A 405 nm laser (Thorlabs, Germany) was used for the experiment. Laser power intensity by cm^2 was measured with a digital optical power and energy meter (Thorlabs, Germany) with and without the skin barrier (skin thickness 0.26-0.29 mm). P1C7 NPs (50 µg/mL) were activated for 10 min with the laser with or without the skin barrier and then measured by DLS. Laser power transmission and NP count decrease were analyzed for 3 individual samples.

REFERENCES

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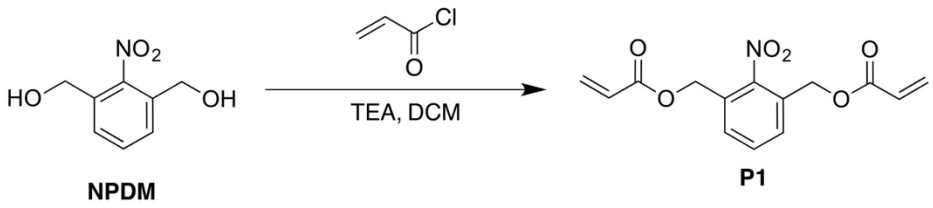
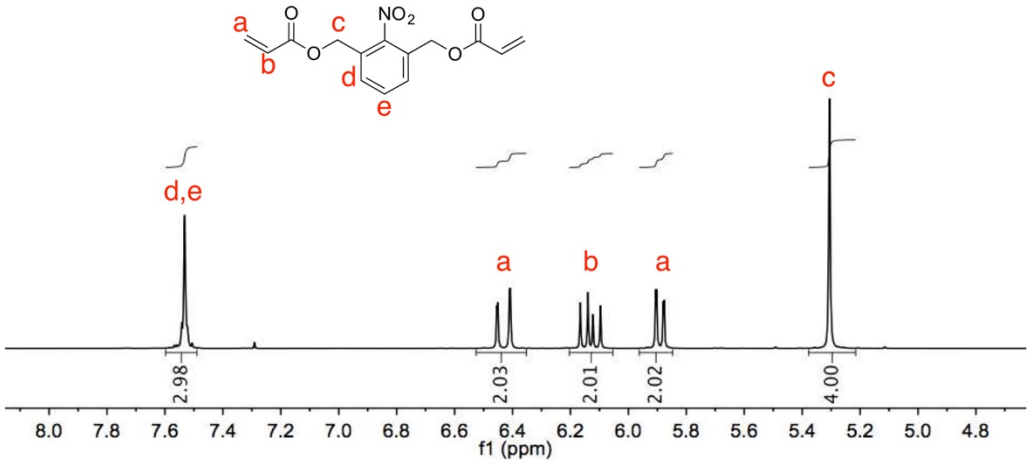
2. Kilchrist, K.V., et al., *Mechanism of Enhanced Cellular Uptake and Cytosolic Retention of MK2 Inhibitory Peptide Nano-polyplexes.* Cell Mol Bioeng, 2016. **9**(3): p. 368-381.

A



NPDM P1

B



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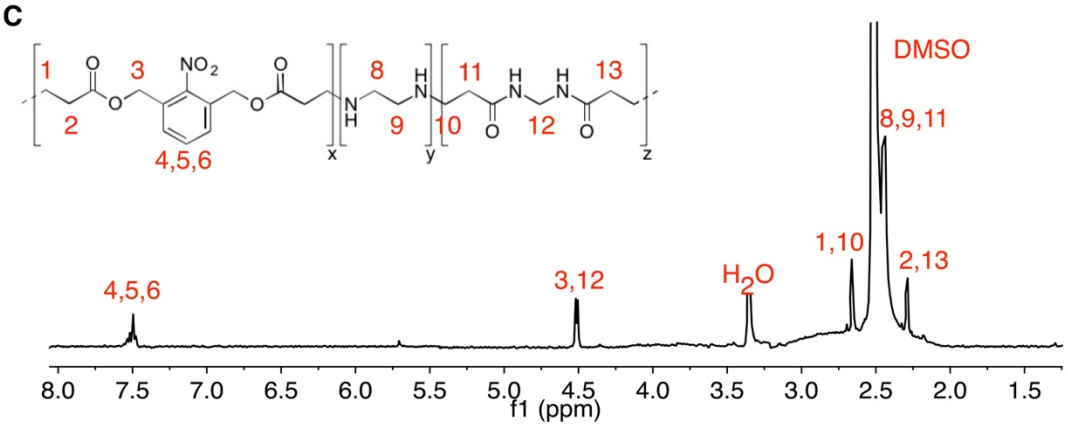

D

	X:Y:Z ratio	UV	Diameter (nm)	Counts (kcps)	Counts decrease (%)
P1A1	12.5:50:37.5	No	246.9	135.2	40.7
		Yes	188.5	80.2	
	25:50:25	No	211.1	248.3	81.6
		Yes	197.6	45.8	
P1A7	12.5:50:37.5	No	298.8	359.4	55.1
		Yes	213.4	161.2	
	25:50:25	No	168.6	215.0	77.8
		Yes	252.7	47.7	

Figure S1. Synthesis of a photo-cleavable linker and its incorporation in polymers. (A) Reaction scheme for the synthesis of the photo-cleavable linker P1. (B) 1H spectrum of P1 in $CDCl_3$. (C) 1H spectrum of P1A1 in DMSO-d_6 with P1 (x): amine (y): bisacrylamide (z) ratio of 25:50:25. (D) Optimisation of the ratio between P1, bisacrylamide and amine in P1A1 and P1A7 NPs, to obtain NPs that are highly light responsive, and thus photo-cleavable.

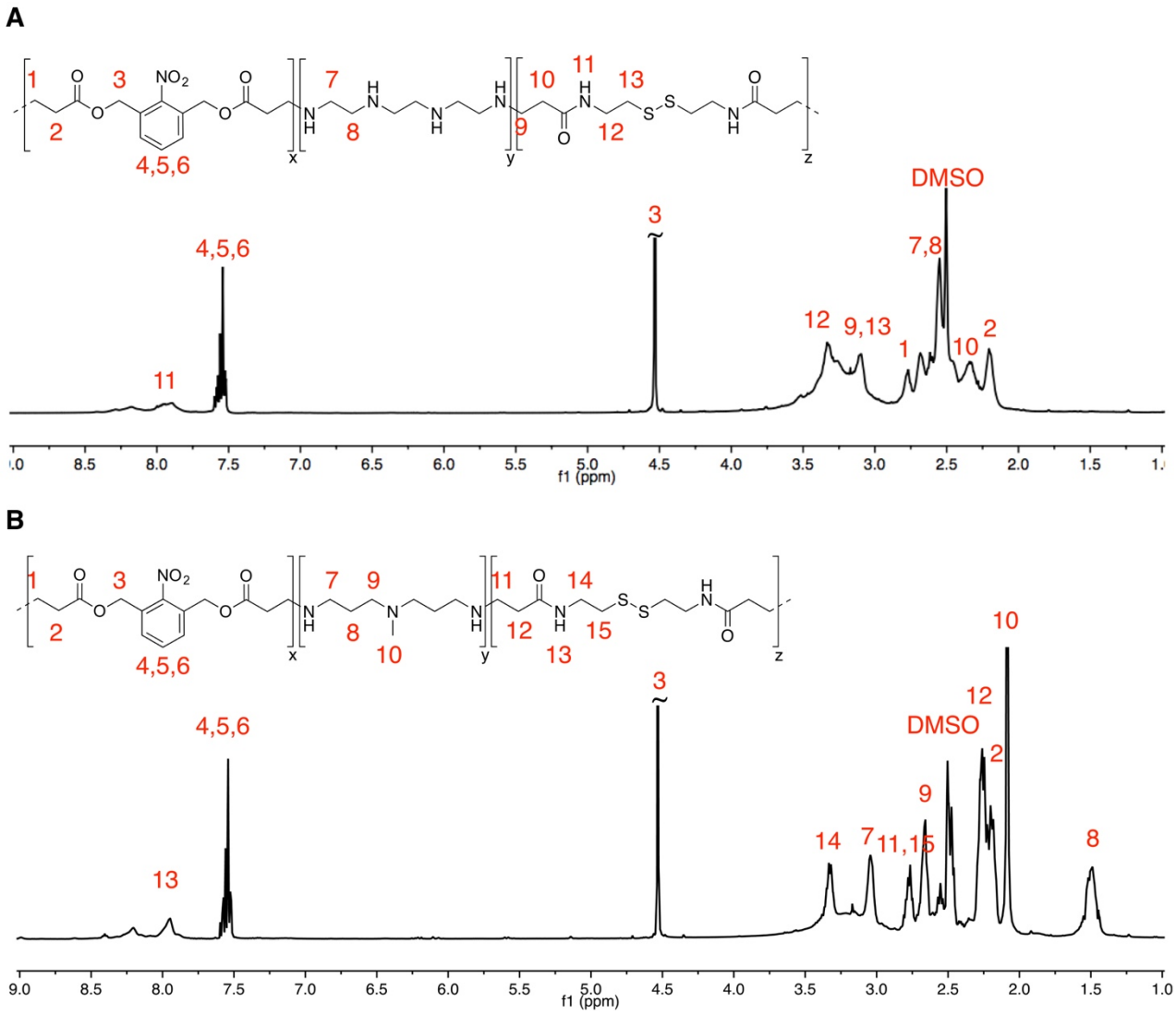

Figure S2. ^{1}H spectra of P1C5 (A) and P1C7 (B) polymers in DMSO-d_6 with P1 (x): amine(y): bisacrylamide (z) ratio of 25:50:25.

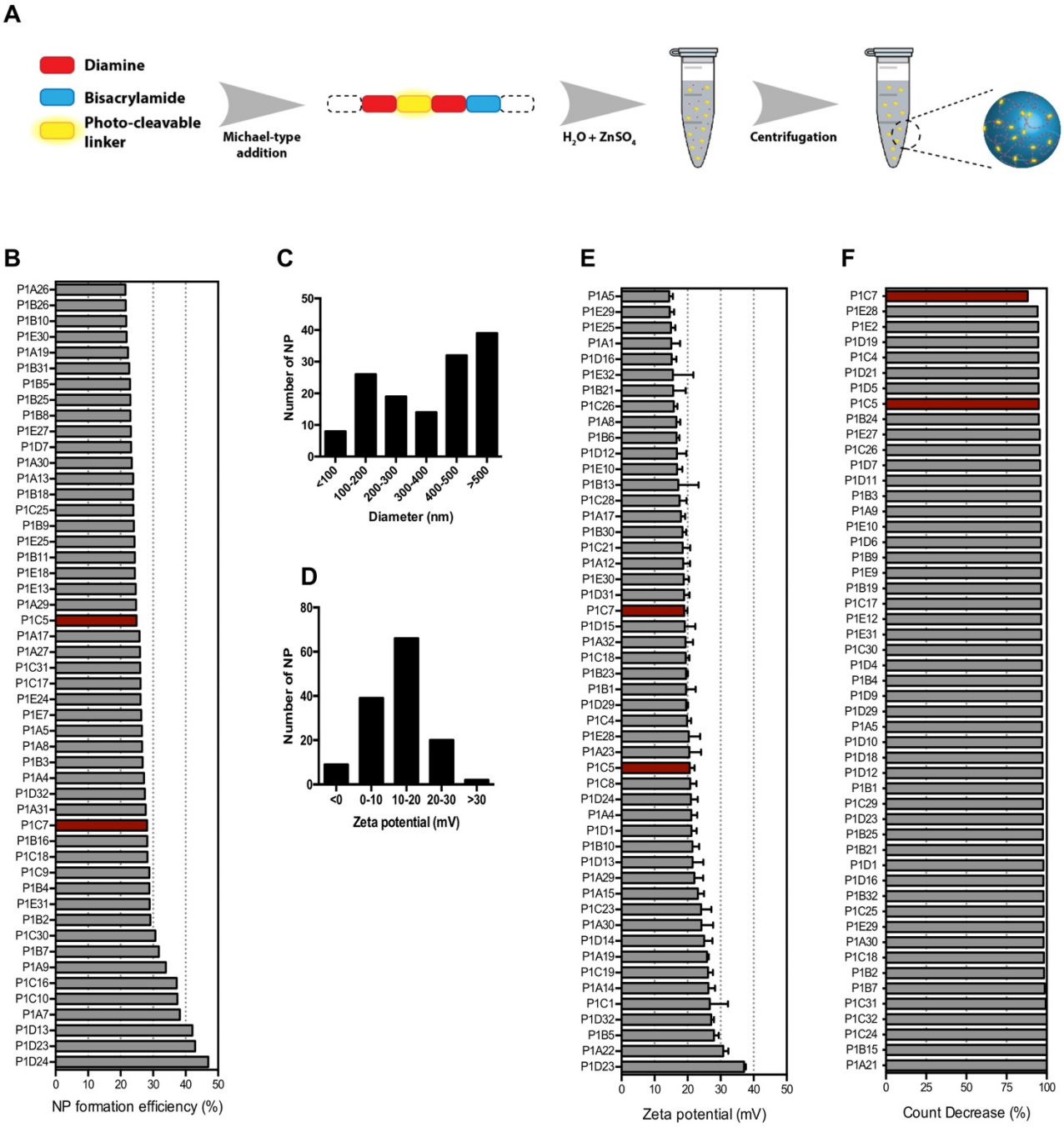

Figure S3. Physicochemical properties of the NP library. (A) Scheme illustrating the synthesis of the polymers and formation of NPs. (B) NP formation efficiency of the top 50 formulations. NP formation efficiency was calculated by the ratio between the weight of NPs after purification and the theoretical molecular weight of the polymer. (C-D) Frequency distribution of NP diameter (C) and zeta potential (D). (E) Zeta potential of the top 50 positive NPs. The results are expressed as Mean ± SEM (n = 3). (F) NP count decrease of the top 50 after 10 min UV irradiation (365 nm). The light dissociation of the NPs was calculated by the ratio between the counts (as determined by DLS) before and after light exposure.

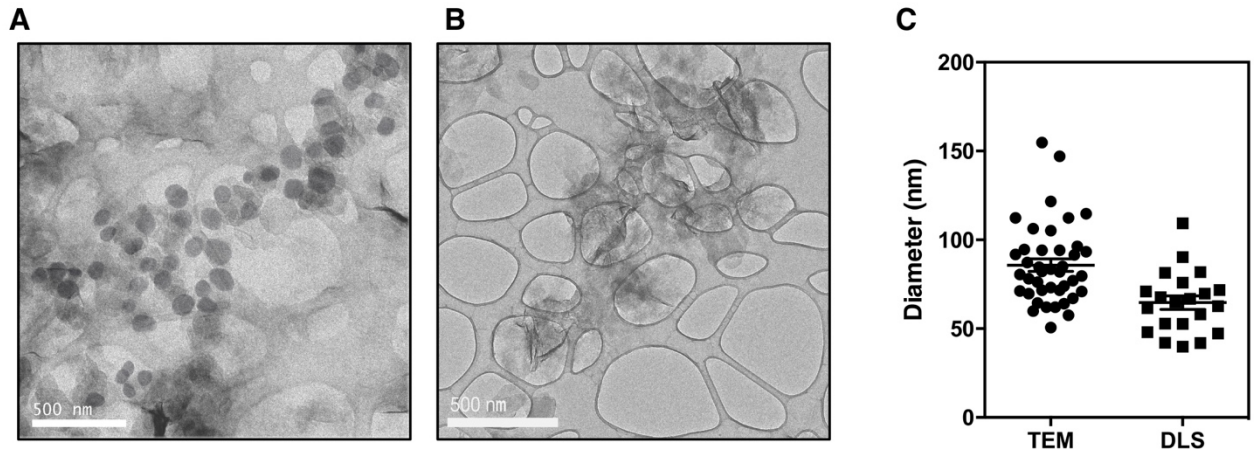

Figure S4. Characterization of P1C7 NPs. Representative TEM images of P1C7@siRNA NPs before (A) and after irradiation (B) with UV light (10 min, 365 nm, 1 mW/cm^2). (C) NP diameter distribution as determined by TEM and DLS analyses. For DLS (Brookhaven ZetaPALS), data from five NP (50 μg/mL) samples was collected with five measurement runs (1 min) on each sample. Results are Mean ± SEM (n=5). In case of TEM analyses, NP suspensions (500 μg/mL) were applied on carbon coated 400 mesh copper grids, left to air dry and analyzed (JEOL-2100-HT microscope). Up to 3 images were acquired and analyzed on ImageJ. Results are Mean ± SEM (*n* = 3, up to 20 nanoparticles per image).

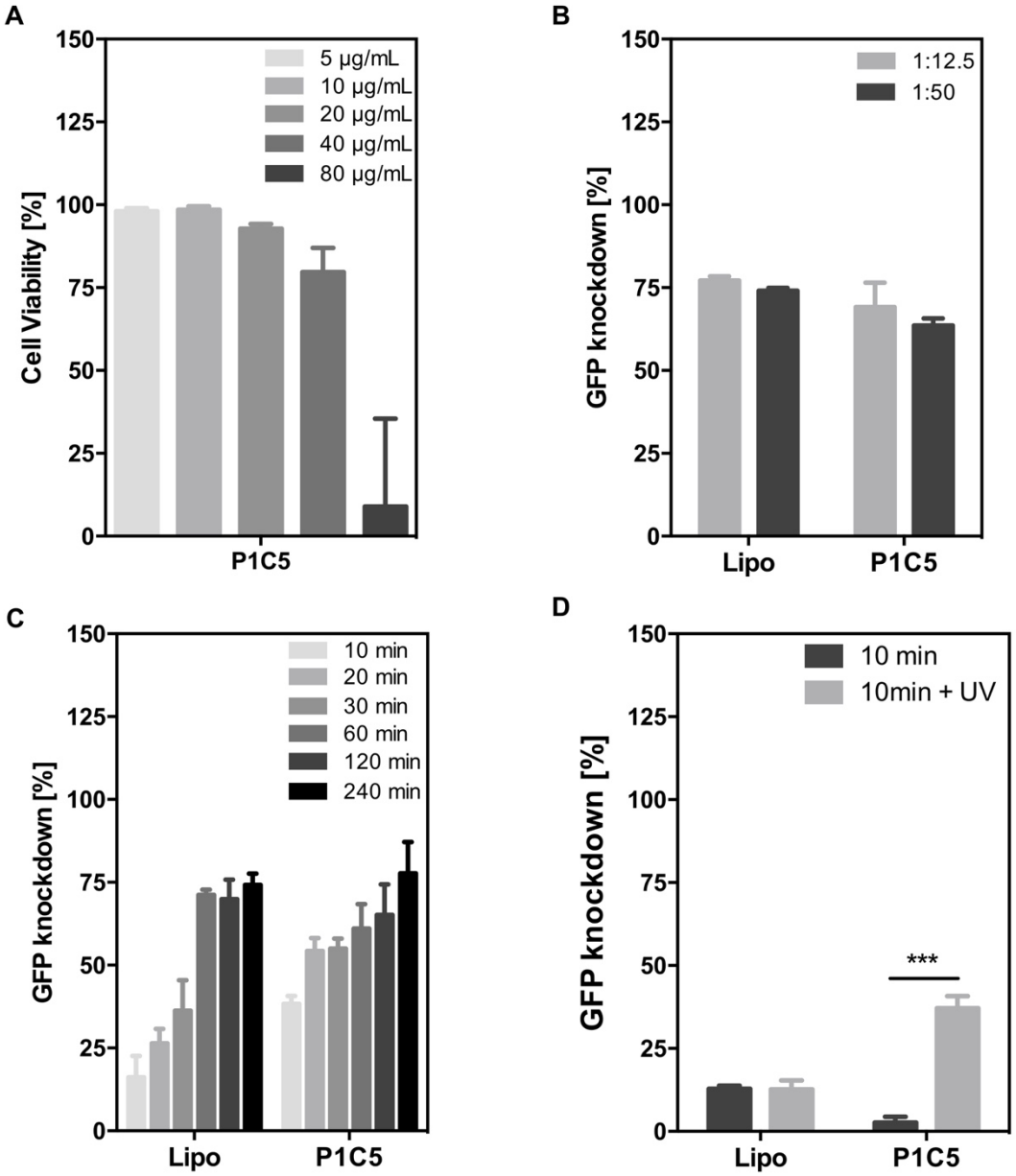

Figure S5. Determination of the siRNA@NP complex concentration, siRNA: NP ratio, kinetics and effect of light activation in gene knockdown. RNAiMAX complexed with siRNA was used as control. Viability and gene knockdown studies was performed in HeLa cells expressing GFP. GFP knockdown was monitored by high content microscopy. (A) HeLa cell viability after transfection with NPs (siRNA:NP ratio = 1:50) at different concentrations for 4 h. Cell nuclei were stained with Hoechst H33342 and propidium iodide after 48 h. Cell viability was calculated as the % of dead nuclei from the total count of nuclei. (B) Effect of siRNA:NP (w/w) ratio keeping NPs concentration constant at 20 µg/mL in terms of GFP knockdown analysed after 48 h. (C) Effect of transfection time of NP@siRNA complexes (siRNA:NP ratio = 1:50; 20 µg/mL) in GFP knockdown analysed after 48 h. After transfection, cells were irradiated with UV-light (10 min, 365 nm, 1 mW/cm^2). (D) Influence of UV light irradiation (365 nm, 1 mW/cm^2) on NP@siRNA complex and consequently GFP knockdown. Cell transfection was performed for 10 min followed by 10 min of UV irradiation. GFP knockdown was analysed at 48 h. Results are Mean ± SEM (n = 4). Statistical analysis was performed using an unpaired student t-test; ***P<0.001.

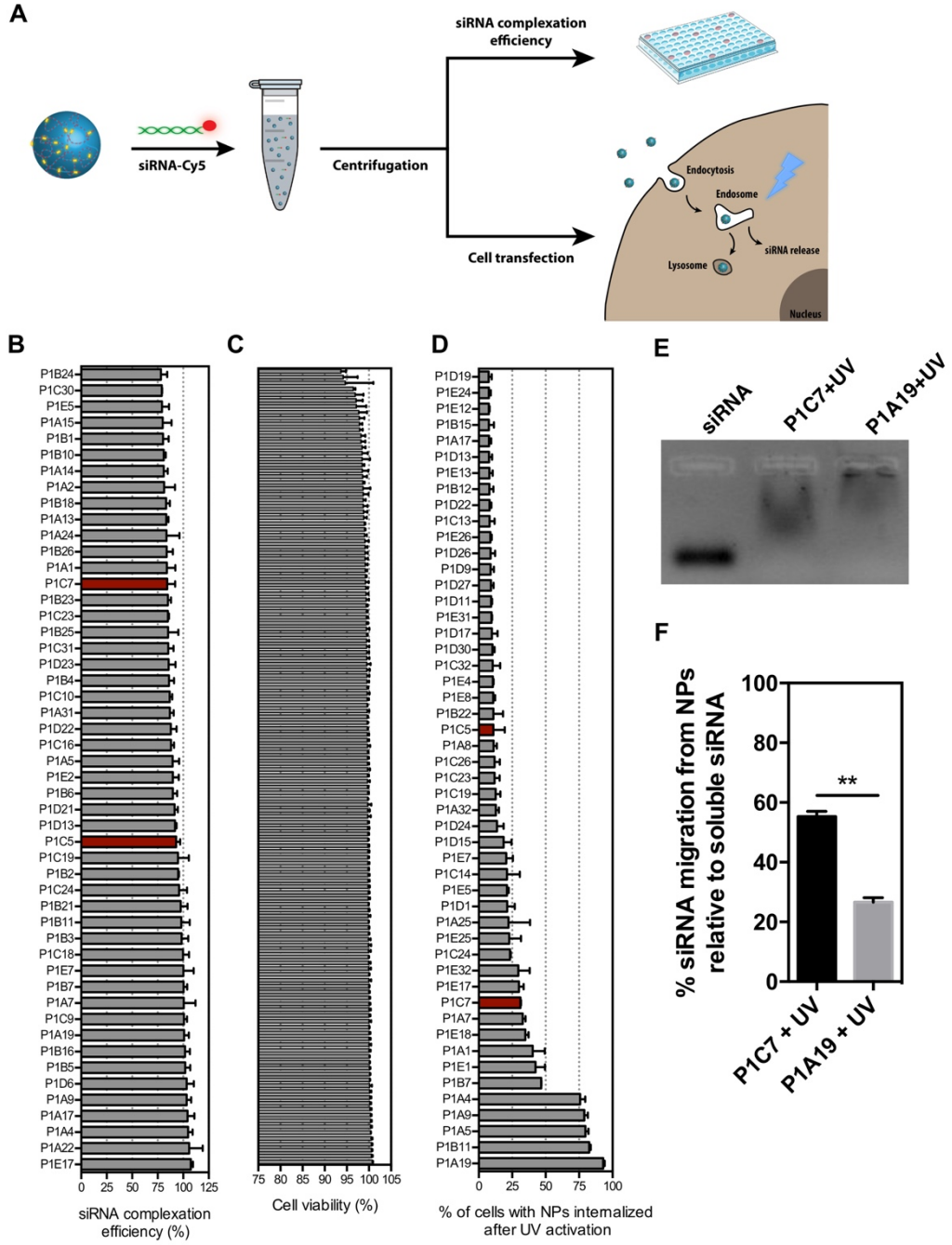

Figure S6. Complexation capacity of the NPs for siRNA as well as cytotoxicity and cellular internalisation of NP@siRNA complexes. (A) Schematic representation of siRNA complexation with NPs (siRNA:NP ratio = 1:50) and cellular internalisation studies of NP@siRNA complexes (20 µg/mL) with transfection time of 10 min. (B) siRNA complexation efficiency of the top 50 formulations determined in NPs@siRNA-Cy5. (C) HeLa cell viability at 48 h post transfection without UV irradiation. Cell nuclei were stained with Hoechst H33342 and propidium iodide at 48 h, and cell viability calculated as the % of dead nuclei from the total count of nuclei. (D) Percentage of cells stained for NPs@siRNA-Cy5 at 48 h post-transfection. The top 50 conditions with higher NP internalisation in HeLa cells are displayed. Results are Mean ± SEM (n = 3). (E) Gel electrophoresis of soluble siRNA or NPs@siRNA (100 µg/mL) after UV irradiation (10 min, 365 nm, 1 mW/cm^2). (F) Percentage of siRNA migration from P1C7 and P1A19 NP formulations relative to soluble siRNA (control) after UV irradiation. The calculation was done by considering the mean of each gel band. Results are Mean ± SEM (n = 2). Statistical analysis was performed using an unpaired student t-test. ** P<0.01.

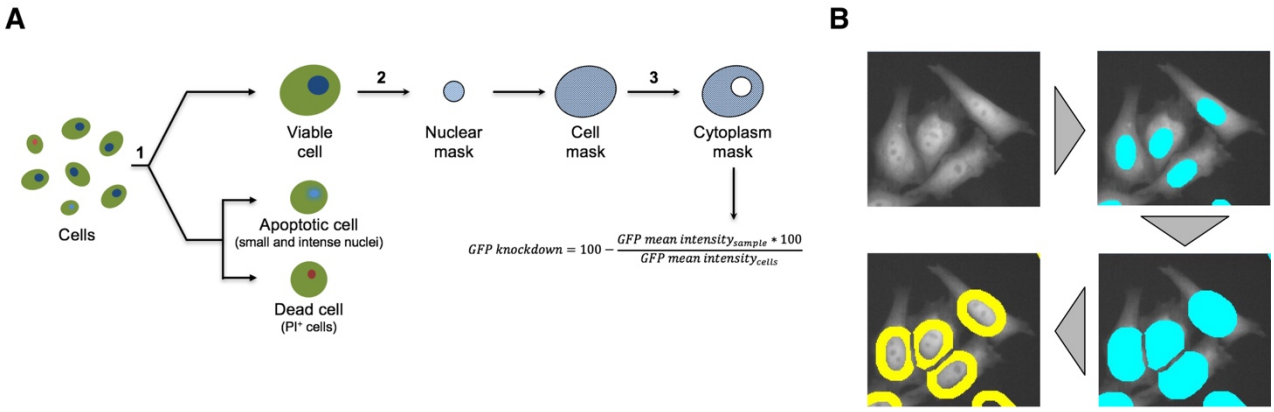

Figure S7. **Schematic representation of image analysis.** (A) Image analysis steps: (1) definition of healthy cell population to analyse; (2) definition of area to measure GFP levels and (3) creation of cytoplasm mask by subtracting the nuclear mask from the cell mask. (B) Image analysis was conducted using In Cell Developer Software which implements machine learning techniques. Cell viability was analyzed calculating the percentage of nuclei with form factor >0.95 and at least 10% overlap with propidium iodide staining, in the total nucleus population. By subtraction of the dead masked nuclei from the total nuclei population, a healthy nucleus mask can be defined. Consecutively the healthy nucleus mask was dilated to cells and the nucleus mask was subtracted to achieve a cytoplasm mask, where GFP fluorescence can be measured minimizing artefacts from other stains (i.e. H33342) and flattening the detection pane. GFP knockdown can then be calculated as percentage decrease of GFP fluorescence signal relative to 0% GFP knockdown control from untreated HeLa-GFP cells and 100% GFP knockdown of HeLa background control cells.

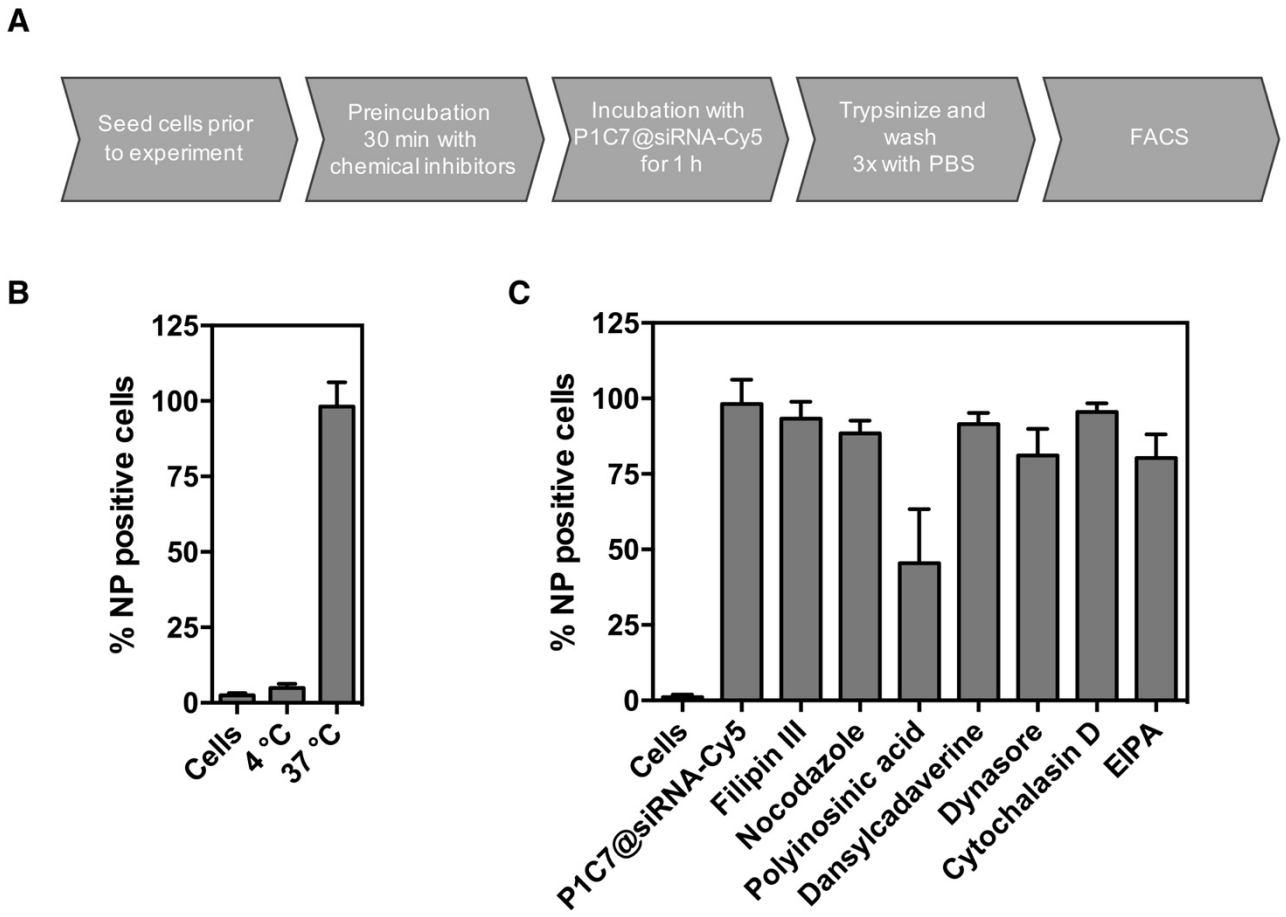

Figure S8. Internalization mechanism of P1C7@siRNA-Cy5 NPs. (A) Schematic representation of the experimental protocol. (B) Effect of temperature in the cellular uptake of P1C7@siRNA-Cy5 NPs. (C) Uptake of P1C7@siRNA-Cy5 in the presence of several endocytosis inhibitors: filipin III inhibits cholesterol dependent internalization mechanisms, nocodazole inhibits microtubule dependent pathways, polyinosinic acid inhibits scavenger receptors, dansylcadaverine and dynasore inhibits clathrin-mediated endocytosis, cytochalasin D inhibits all pathways dependent on actin (including macropinocytosis) and ethylisopropylamiloride (EIPA) inhibits macropinocytosis. The concentrations tested for each inhibitor were confirmed before the experiment to be non-cytotoxic. Cellular internalisation of P1C7@siRNA-Cy5 NPs was evaluated by flow cytometry. Results are expressed as Mean ± SEM (n = 3).

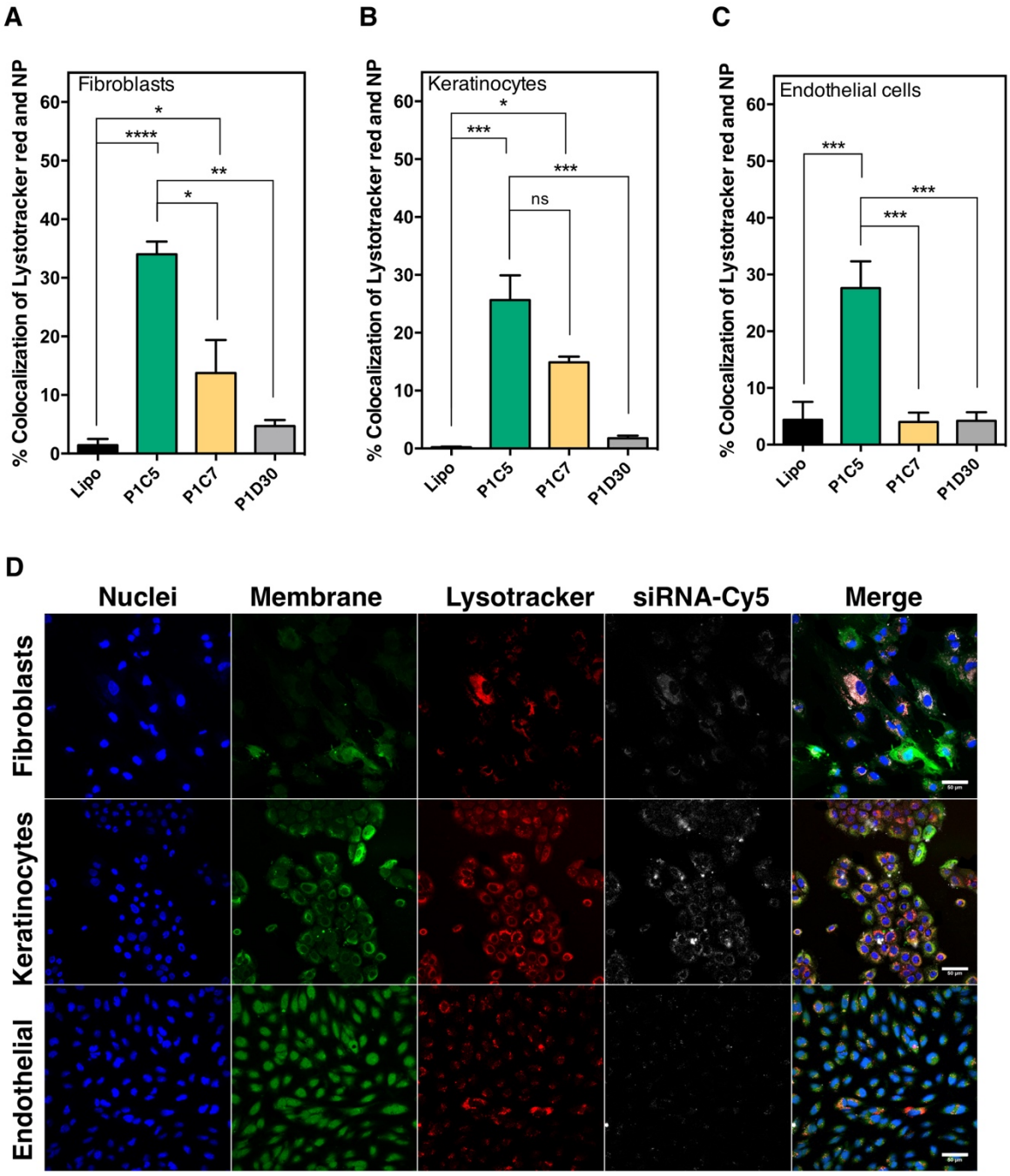

Figure S9. NP internalisation in human skin cells. Fibroblasts (A), keratinocytes (B) and endothelial cells (C) were transfected with NP@siRNA-Cy5 formulations (20 µg/mL) for 1 h, washed to remove the non-internalised NPs and finally stained (CFSE for cell membrane; Lysotracker red for endolysosome; H33342 for cell nuclei) for confocal microscopy examination. Formulations with high (P1C5 and P1C7) and low (P1D30) gene knockdown efficiency as well as lipofectamine (Lipo) were evaluated for internalisation studies. Cell internalisation was monitored by the co-localization of siRNA-Cy5 with Lysotracker red. Results are presented as Mean ± SEM (n=2 independent samples, 3-9 microscope fields). Statistical analyses were performed by one way ANOVA followed by a Bonferroni multi-comparison test (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001). (D) Representative confocal microscopy images showing the co-localization of P1C7@siRNA-Cy5 formulation with endolysosomal compartment (Lysotracker red) for 3 cell types. Scale bar is 50 µm.

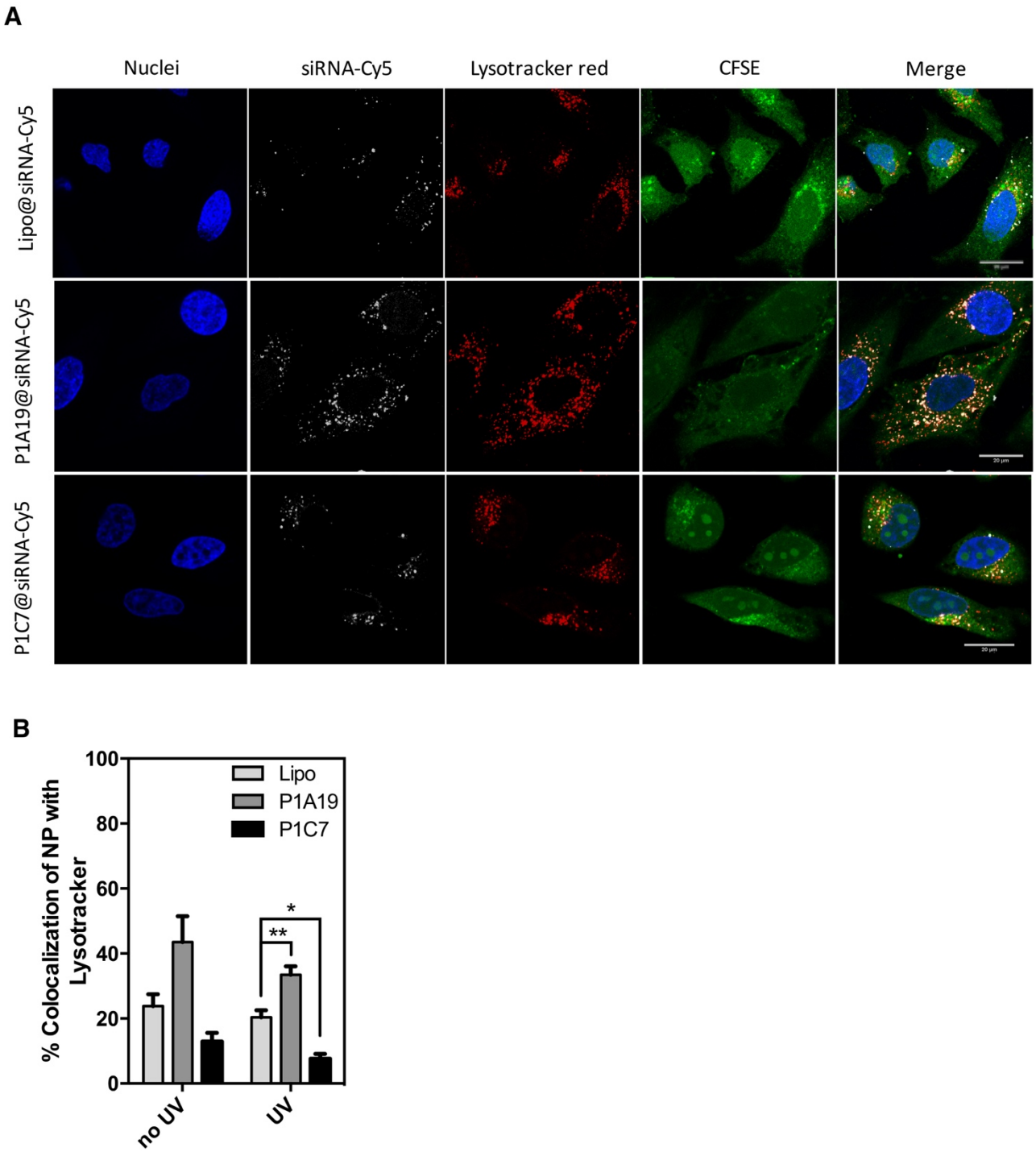

Figure S10. Intracellular release of siRNA. HeLa cells were transfected with P1C7@siRNA-Cy5, P1A19@siRNA-Cy5 (20 µg/mL) or Lipo@siRNA-Cy5 for 10 min, washed to remove the non-internalised NPs, irradiated or not with UV light for 10 min and stained (CFSE for cell membrane; Lysotracker red for endolysosome; H33342 for cell nuclei) for confocal microscopy examination 1 h after the incubation. (A) Representative confocal images showing the co-localization of siRNA-Cy5 formulation with endolysosomal compartment. Scale bar is 20 µm. (B) Co-localization between NPs@siRNA-Cy5 and endolysosomal compartment expressed as the Manders' overlap coefficient quantified by ImageJ analyses. Results are presented as Mean ± SEM (3-9 microscope fields). Statistical analyses were performed by One-Way ANOVA followed by followed by Tukey's post-hoc test. * $P<0.05$; ** $P<0.01$.

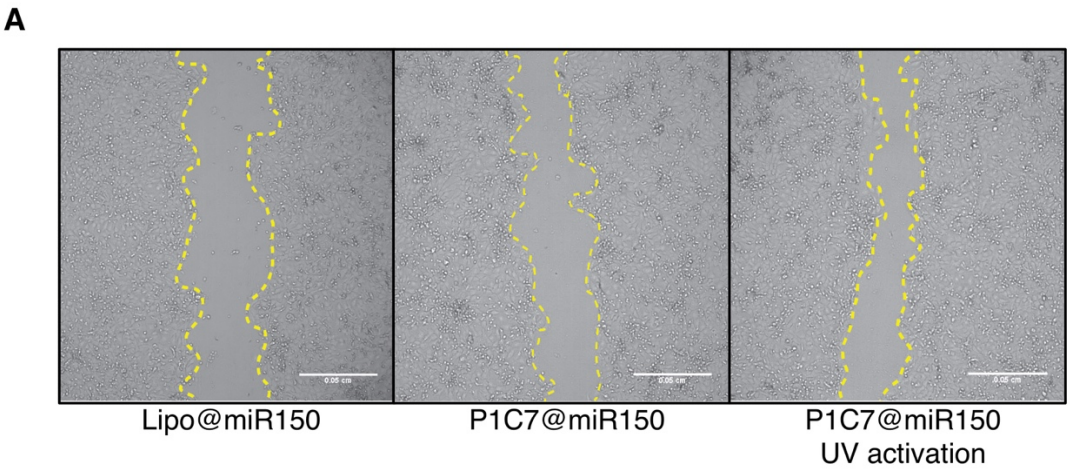

Figure S11. ***In vitro*** **wound healing activity of P1C7@miR150 NPs.** Confluent human keratinocytes were treated for 4 h with P1C7@miR150 without light activation or P1C7@miR150 with light activation. Lipofectamine complexed with miR150 (Lipo@miR150) was used as control. The wound was created by scratching the monolayer of cells. Cell migration was monitored by high-content microscopy. (A) Representative images of the wound healing process at 48 h post wounding. Scale is 0.05 cm.

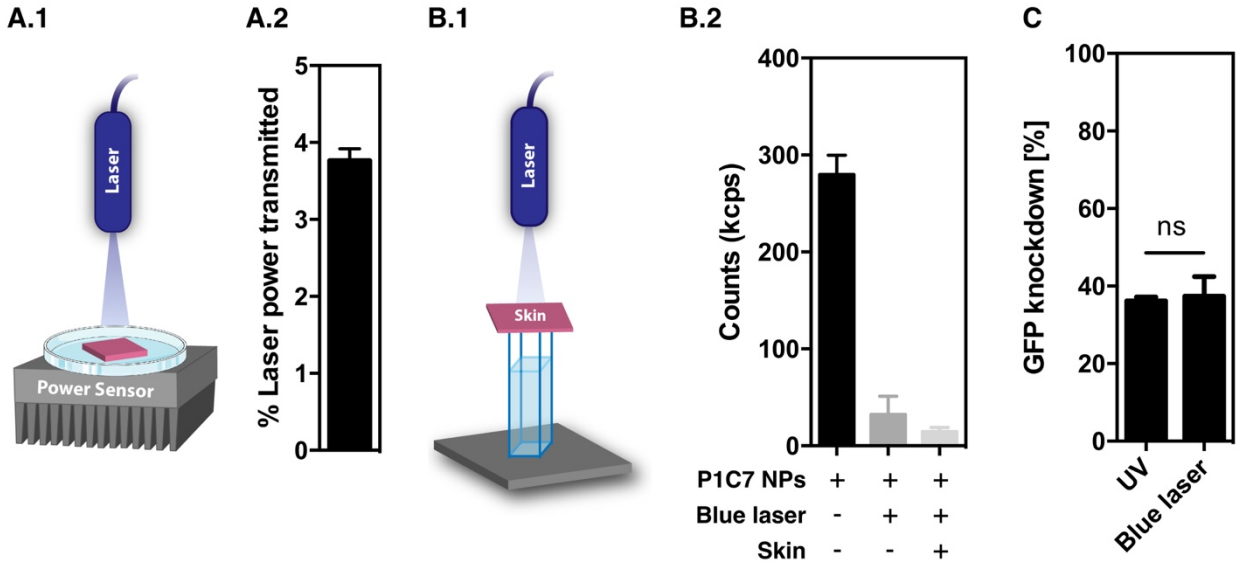

Figure S12. Photo-disassembly of NPs through a skin barrier. (A.1) Schematic representation of the experiment. A 2 cm × 2 cm skin (thickness of 260-290 µm as measured by a caliper) was placed in a plastic petri dish on top of a thermal power sensor (Thorlabs s310c). The tissue was then irradiated with a 405 nm laser at 80 mW/cm^2 during 1 min. Laser attenuation values were calculated by normalising against laser power values obtained with the empty petri dish. (A.2) Blue laser attenuation. Results are expressed as Mean ± SEM (n = 3). (B.1) Schematic representation of the methodology used. A sample of skin (thickness: 260-290 µm) was placed on top of a plastic cuvette containing an aqueous suspension of P1C7@miRNA150 complexes followed by its irradiation with a blue laser (405 nm, 80 mW/cm^2) for 10 min. As a control, a cuvette with an aqueous suspension of P1C7@miRNA150, without the skin sample on top, was irradiated by a blue laser for the same time. At the end, both NP suspensions were characterized by DLS analyses. (B.2) Blue laser disassembly of P1C7 NPs placed below skin. Results are presented as Mean ± SEM (n = 3). (C) Cells transfected with P1C7@siRNA complexes (20 µg/mL) and activated by a blue laser (405 nm at 80 mW/cm^2) yielded similar GFP knockdown efficiency as cells activated by a UV laser (365 nm at 1 mW/cm^2). Cell transfection was performed for 10 min followed by 10 min of irradiation. GFP knockdown was analysed at 48 h. Results are Mean ± SEM (n = 4). Statistical analysis was performed using an unpaired student t-test.

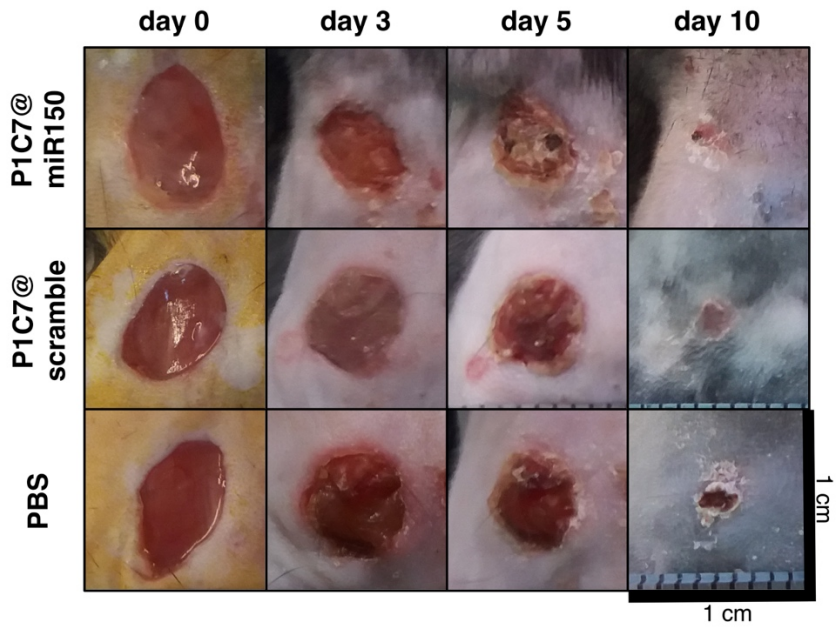

Figure S13. Wound healing activity of P1C7@miR150 formulations. Representative images of the wound healing process immediately after the surgery and at days 0, 3, 5 and 10.

Table S1. Information about the chemical name, CAS and vendor of the monomers used to generate the NP library.

Monomer	Chemical Name IUPAC	CAS	Vendor
A	Methylenebisacrylamide	110-26-9	Aldrich
B	Hexamethylenebisacrylamide	7150-41-6	Polyscience
C	Cystaminebisacrylamide	6098-457-8	Polyscience
D	Dihydroxyethylenebisacrylamide	868-63-3	Aldrich
E	Bisacryloylpiperazin	6342-17-2	Sigma
P1	(2-Nitro-1,3-phenylene)bis(methylene)diacrylate	1599460-50-0	Synthesized
1	Ethylenediamine	107-15-3	Merck
2	1,4-Diaminobutan	110-60-1	Aldrich
3	1,6-Diaminohexan	124-09-4	Alfa Aesar
4	Diethylenetriamine	111-40-0	Alfa Aesar
5	Triethylenetetraamine	112-24-3	Acros Organics
6	Pentaethylenehexamine	4067-16-7	Aldrich
7	3,3'-Diamino-N-methyldipropylamine	105-83-9	Aldrich
8	1,2-Diaminocyclohexane	694-83-7	Aldrich
9	1,8-Diamino-3,6-dioxaoctane	929-59-9	Acros Organics
10	1,13-Diamino-4,7,10-trioxatridecane	4246-51-9	Aldrich
11	1,4-Bis(aminopropyl)piperazine	7209-38-3	Aldrich
12	1,4-Phenylenedimethanamine	539-48-0	Merck
13	1,5-Diaminonaphthalene	2243-62-1	Aldrich
14	4,4'-methylenedianiline	101-77-9	Aldrich
15	1,3-Phenylenediamine	108-45-2	TCI Chemicals
16	1,3-Diaminopropane	109-76-2	TCI Chemicals
17	2,2-Dimethyl-1,3-propanediamine	7328-91-8	TCI Chemicals
18	1,3-Diaminopentane	589-37-7	TCI Chemicals
19	2,2'-Diamino-N-methyldiethylamine	4097-88-5	TCI Chemicals
20	Agmatine sulfate	2482-00-0	TCI Chemicals
21	1,4-Bis(aminomethyl)cyclohexane	2579-20-6	TCI Chemicals
22	4,4'-Methylenebis(cyclohexylamine)	1761-71-3	Aldrich
23	4,4'-Diaminobenzanilide	785-30-8	Aldrich
24	DL-Lysine	70-53-1	Aldrich
25	3-Amino-1-propanol	156-87-6	Aldrich
26	4-Amino-1-butanol	13325-10-5	Aldrich
27	5-Amino-1-pentanol	2508-29-4	Sigma Aldrich
28	6-Amino-1-hexanol	4048-33-3	Alfa Aesar
29	1-(3-Aminopropyl)pyrrolidine	23159-07-1	Alfa Aesar
30	1-(3-Aminopropyl)imidazole	5036-48-6	Aldrich
31	1-(3-Aminopropyl)-4-methylpiperazine	224-954-4	Alfa Aesar
32	Histamine	51-45-6	Sigma Aldrich

Table S2. Polymer molecular weight change of purified P1C5 and P1C7 in DMSO (0.8 M) upon irradiation with a UV lamp at 365 nm for 10 min and summary of the NPs properties.

	Polymer				Nanoparticle					
Sample	UV light	M_n (kDa)	M_w (kDa)	PDI	Size (nm)	Zeta (mV)	Count decrease (%)	siRNA complexation (%)	Internalisation (%)	Fold increase rela. to Lipo with UV
P1C5	No	3.3	9.5	2.88	353.1 ± 34.6	20.6 ± 2.3	95.1 ± 3.4	93.2 ± 3.9	6.4 ± 4.7	2.8 ± 0.6
P1C5	Yes	2.4	6.4	2.67						
P1C7	No	6.8	33.0	4.85	73.5 ± 10.5	18.9 ± 3.5	88.2 ± 5.4	84.2 ± 7.8	30.0 ± 1.9	5.0 ± 0.8
P1C7	Yes	5.0	26.7	5.34						

Table S3: Sequence of primers used in qRT-PCR experiments.

Gene		Sequence	species
h-MYB	FW	CCGAATATTCTTACAAGCTCC	human
	RW	GGACCTGTTTTTAGGTACTG	
h-GAPDH	FW	AGCCACATCGCTCAGACACC	human
	RW	GTACTCAGCGCCAGCATCG	
m-MYB	FW	CACAAAACATCTCCAGTCAC	mouse
	RW	TCTTCGTCGTTATAGTGTCTC	
m-GAPDH	FW	AAGGTCATCCCAGAGCTGAA	mouse
	RW	CTGCTTCACCACCTTCTTGA	
has-miR-150-5p	FW	TCTCCCAACCCTTGTACC	human
RNU6	FW	ACACGCAAATTCGTGAAG	human