Polydiacetylenic Nanofibers as new siRNA vehicles for *in vitro* and *in vivo* delivery

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Chemistry Part:

Materials and methods:

10,12-pentacosediynoic acid, N-hydroxysuccinimide (NHS), 1-ethyl-(3-dimethylaminopropyl)carbodiimide hydrocloride (EDC), N,N-diisopropylethylamine, 4,7,10-trioxa-1,13-tridecanediamine were purchased from Sigma-Aldrich (Saint-Quentin Fallavier,

France).Column chromatography was performed on silica for chromatography: Silicagel 60 (40-63 µm) Geduran Si60 (Merck, Darmstadt, Germany).The progress of all reactions and column chromatography was monitored by thin-layer chromatography (TLC). TLC was performed with Merck silica gel 60 F254 pre-coated plates and visualized by UV absorption and/or stained with a solution of vanillin – H_2SO_4 stain, Dragendorff reagent, ninhydrin or iodine vapors. ^1H and ^{13}C NMR spectra were recorded on a Bruker Ultrashield plus 400 Spectrometer (operated at 400 or 100 MHz). The ^1H NMR chemical shifts are reported in parts per million, using the signal for residual solvent protons (7.27 for $CDCl_3$, 3.31 for MeOD) as reference. The ^{13}C NMR chemical shifts are reported in parts per million, using the signal for residual solvent protons as reference (77.23 for $CDCl_3$, 48.95 and for CD_3OD). Data were represented as follows, chemical shift, multiplicity (s = singlet, d = doublet, t = triplet and m = multiplet), integration and coupling (J in Hz). High Resolution Mass Spectra (HRMS) were obtained using an Agilent Q-TOF (time of flight) 6520. NMR analysis and Mass analysis weredone by the analytical platform (GDS 3670, CNRS & Université de Strasbourg) at the Faculty of Pharmacy.

Synthesis of surfactant (1): C25-diyne-trioxaamine,(N-(3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)pentacosa-10,12-diynamide:

Commercial 10,12-pentacosediynoic acid (1 equivalent, 10 g, 26.7 mmoles, Mw 374.6; Sigma-Aldrich,) and N-hydroxysuccinimide (1.63 equivalent, 5 g, 43.4 mmoles; Mw 115.09) were dissolved in dichloromethane (200 mL) and purged with argon. 1-ethyl-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.49 eq, 8.3 g, 43.3 mmol; Mw 191.7) was added as a solid, followed by slow drop-wise addition of dry N,N-diisopropylethylamine (1.49 eq, 7 mL, 5.18 g, 40 mmol). The mixture was stirred overnight at room temperature in brown glassware, which protects the reagents from light. Solvents and excess amine were evaporated in a rotary evaporator, then dissolved again in dichloromethane (200 mL) and extracted with distilled water (100 mL). Aqueous phase was re-extracted with dichloromethane (200 mL). The combined organic phases were dried over anhydrous sodium sulfate, filtered on a paper disc under vacuum, evaporated in a rotary evaporator and dried under vacuum The obtained white solid (12.54 g, 26.6 mmoles, Mw 471.67) is the expected NHS ester obtained in quantitative yield was confirmed by NMR analysis.

This activated hydroxysuccinimidyl ester was used in the next step with no further purification.

The NHS ester (4.716 g, 10 mmoles, Mw 471.67) was dissolved in 200 mL dichloromethane (stabilized with amylene). 1,13-diamino-4,7,10-trioxatridecane (4.4 g, 20 mmoles, Mw 220.3), dissolved in 200 mL dichloromethane was added at room temperature. The reaction was stirred for 24 hours. Methanol (20 mL) was added to dissolve the formed precipitate. The reaction mixture was purified directly, without evaporation, on a silica gel column, equilibrated with 5% methanol and 0.5% NH_4OH in dichloromethane (column with a diameter of 5 cm and 17 cm length). Column was first eluted with a mixture of methanol/dichloromethane/ammonia 5:94:0.5 (400 mL), then with methanol/dichloromethane/ammonia (10:89:1). Two Dragendorff positive products were isolated. A major side product resulting from bi-coupling reaction (1.2 g, 26%, Mw 933.48) eluted in the first head fractions. The second corresponded to the desired mono-coupling product (3.7 g, 64% isolated yield, Mw 576.91). TLC analysis of the fractions was done with methanol/dichloromethane/ammonia 9:90:1 elution and staining with Dragendorff reagent. Bi-coupling product was poorly soluble in methanol, and NMR analysis was done in deuterated chloroform. The desired mono-coupling product was analyzed by NMR analysis at a concentration of 10 mg in 0.75 mL deuterated methanol-4D, and by NMR analysis of a sample in a MeOD-4d/10% TFA (trifluoroacetic acid) mixture to better resolve overlapping signals.

Analysis of C25-diyne-trioxaamine:

^{1}H NMR (400 MHz, MeOD 10%TFA) δ 3.64-3.71 (m, 8H), 3.59-3.64 (m, 2H), 3.53 (t, *J*=5.8 Hz, 2H), 3.27 (t, *J*=6.6 Hz, 2H), 3.11 (t, *J*=6.4 Hz, 2H), 2.26 (t, *J*=6.6 Hz, 4H), 2.20 (t, *J*=7.4 Hz, 2H), 1.91-1.99 (m, 2H), 1.73-1.82 (m, 2H), 1.73-1.82 (m, 2H), 1.47-1.57 (m, 4H), 1.39-1.46 (m, 4H), 1.29-1.37 (m, 23H, alkyles), 0.90 (t, *J*=6.8 Hz, 3H) ppm.

See Figure S2**:** ^{1}H-NMR analysis of the surfactant **(1)**

^{13}C NMR (100 MHz, MeOD 10% TFA) δ 76.6, 76.5, 70.0, 69.7, 69.6, 69.0, 68.3, 65.0, 38.7, 36.3, 35.7, 31.7, 29.3-28.1, 26.7, 25.6, 22.3, 18.3, 13.0 ppm.

High resolution mass spectroscopy coupled to liquid chromatography: LC-HRMS

Observed mass: 576.48527 (theoretical 576.48661; score 96.91%); 100% purity by LC-MS (from +ESI total ion current)

See Figure S3: LC-HRMS analysis of the surfactant **(1)**

Formulation and physicochemical characterization

Formulation and photopolymerization of Polydiacetylenic nanofibers

A concentrated solution of C_{25}-diyne-trioxaamine was prepared by dissolving the amine in ethanol at 60°C under sonication (60 mg/6 mL). This warm solution was filtered through a cotton pad (in a glass pipette, to remove minor polymerized contaminants) and injected into ethanol-water (12 mL/42 mL) mixtures to obtain final 30% ethanolic solutions at a concentration of 1 mg/mL trioxaamine. This solution was placed at 5°C for 18 hours, where it transformed into an opaque gel-like suspension. UV-polymerization was performed on 5 mL batches in a UV-Crosslinker (Bio-Link BLX-E254, 5 x 8 W 254 nm tubes). The batches were UV irradiated in open 6-well culture plates for 10 minutes at 254 nm. During the UV-irradiations the suspensions turned dark blue. See Figure S4: Photo-polymerization of ethanolic solutions of surfactant (**1**).

The dark-blue suspensions were acidified by adding 50 µL diluted chlorhydric acid (1 N) to each of the 5 mL batches, which were transferred in 15 mL Falcon tubes. The suspensions were then heated under sonication at 55°C for 1 hour. This led to a drastic color change. The suspensions turned crimson red, and the polymerized aggregates dissociated into small nano-fibers. These are highly fluorescent. Their homogeneity can be assessed by direct observation by fluorescence microscopy (rhodamine filter set, 600 fold magnification). UV-Vis spectra of each batch were recorded on 30 µg/mL suspensions in water. The samples with highest optical density OD were selected for further biological evaluation.

See Figure S5: Preparation of the fluorescent PDA-Nfs used for siRNA delivery. (A) Formation of the fluorescent crimson red Nanofibers PDA-Nfs

Dialysis of Polydiacetylenic nanofibers (PDA-Nfs)

A small batch was extensively dialyzed for 72 hours in a dialysis cassette (Float-A-Lyzer G2 MWCO 3500) in 1 L 30% ethanol/water solution acidified with 1 mL concentrated

hydrochloric acid. This procedure aimed to remove all traces of remaining monomer. Dialyzed and non-dialyzed samples showed unchanged siRNA complexation ability (see Figure S8: Gel retardation assays of siRNA complexation by the PDA-Nfs by electrolysis at varying N/P values).

Electron microscopy of the PDA-Nfs(LVEM)

The samples of PDA-nanofibers were analyzed by low voltage transmission electron microscopy at 5 kV with the benchtop Low-voltage Electron Microscope LVEM5 (Delong instruments). Observation at low voltage 5000 V permits the direct observation with high contrast of objects composed only of light elements without using heavy metal staining or shadowing. This way native structures of organic materials that exist in solution can be observed directly. For optimum contrast, we used Ultrathin carbon grids with 3 – 4 nm thickness (Ultrathin C Type-A grids from TED Pella INC). The protective formvar layer was removed by dipping the grids in chloroform following manufacturer's instructions. The carbon grids were then ionized in a Glow discharge system ELMO for EM grids (Cordouan technologies). A glow discharge treatment with air renders the hydrophobic carbon film surface negatively charged and hydrophilic, which allows for optimal wetting with the sample solutions and attachment of analyzed nanomaterial (that is positively charged in our case, with positive zeta potential). The parameters used are as follows: 25 seconds flow of plasma discharge at 0.1 – 0.15 mbar atmosphere and 1.2 – 1.4 mA. Ionized grids were treated for 1 minute with 5 µL samples at 1 mg/mL surfactant concentration, dried over filter paper and analyzed directly without further preparation with the LVEM5 in TEM mode at 150 000 – 200 000 fold magnification. See figure 1B in main text.

UV-VIS spectra of Nanofibers

UV-Vis spectra were recorded with the double beam spectrometer Cary 100 Bio UV-Vis (ELO50-73007; Varian). Nanofiber suspensions were diluted in pure water (30 µg polymerized C_{25}diyne-trioxaamine in 1 mL water). The absorption maxima were determined (lambda max.) from these spectra.

The red biologically active form has the following spectral characteristics:

$\lambda_{max(1)}$ = 506 nm (OD = 0.660) ; $\varepsilon_{506\ nm}$ = 220 (100 mL x g^{-1}x cm^{-1})

$\lambda_{max(2)}$ = 547 nm (OD = 0.836) ; $\varepsilon_{547\ nm}$ = 279 (100 mLx g^{-1}x cm^{-1})

See Figure S5**:** Preparation of the fluorescent PDA-Nfs used for siRNA delivery: (C) UV-Vis absorption spectrum of the diluted PDA-Nfs solution.

Fluorescence spectroscopy of PDA Nanofibers

Fluorescence emission and excitation spectra were recorded on Shimadzu Spectrofluorophotometer RF-5301. The maximum excitation and excitation wavelengths were first determined in a step-wise approach. The corresponding excitation and emission spectra were then recorded using the most efficient excitation and emission wavelengths using the following parameters: the excitation spectrum was recorded from 400 nm to 630 nm, at the maximum emission wavelength of 646 nm. A split width of 10 nm for both emission and excitation, low sensitivity and slow scan speed were used as standard settings. The emission spectrum was recorded from 565 nm to 800 nm at the maximum excitation of wavelength of 550 nm. A split width of 10 nm for both emission and excitation, low sensitivity and slow scan speed were used as standard settings.

$\lambda_{excit(max)}$ = 550 nm

$\lambda_{emission(max)}$ = 646 nm

Notably,a large energy gap (> 100 nm) separates fluorescence emission maximum from excitation maximum (Stokes shift). See Figure 2 (main text).

Size analysis by dynamic light scattering (DLS)

The mean size of the PDA-nanofibers was determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments). The samples were diluted in water (10 μg in 1 mL). We used following settings for size determination and for zeta-potential measurement: refractive index of the particles 1.43; viscosity of the solvent 0.8872 cP; dielectric constant 78.5 at 25°C. The mean size of the biologically active PDA-nanofibers was 778 nm (center weighed by number, mean of 5 measurements) and 744 nm center weighed by volume, mean of 5 measurements) showing a low polydispersity index PDI of 0.66. Setting the following specifications: automatic sampling; refractive index of water; temperature = 25°C. The values correspond to average size ± standard deviation of three runs. The zeta potential of the same sample showed to be as high as 52.5 mV (mean of 3 runs). See Figure S6 (A): Dynamic light scattering (DLS) and zeta potential measurement of the free PDA-Nfs.

The complexes formed with siLim and PDA-Nfs at N/P = 6 prepared under the same conditions as used for the *in vivo* studies were also extensively characterized by DLS and zeta potential measurements as described for the PDA-Nfs. The flocculation limit at N/P = 4 was explained by a drastic drop in the zeta potential to become negative for N/P = 2, as expected for complexes where the negatively charged groups resulting from excess siRNA predominate.

For the biophysical characterization of PDA-Nfs/siLim complexes prepared at the same conditions and buffer conditions (Hespes buffered glucose) as used for the *in vivo* see Figure S11: Biophysical characterization of siLim1/PDA-Nfs complexes.

Gel retardation assay

Fluorescently labelled siRNA (Cy5-siLuc, 23.8 pmoles siRNA, equivalent to 1000 pmoles phosphate groups 'P') were incubated within creasing amounts of PDA-nanofibers in Hepes Buffered Glucose (HBG, pH 7.5, completed to 20 µL). The resulting siRNA-nanofiber complexes have increasing N/P ratio ranging from 1/1 to 7/1. N/P ratios are defined by the number 'N' of amine groups from the trioxaamine Nanofibers (being positively charged), versus the number 'P' of negatively charged phosphate groups present in the siRNA (P). Using a constant concentration of 23.8 pmoles siRNA an N/P = 1 ratio was obtained by adding 0.6 µL of the PDA-Nf solution (1 mg/mL, 1.6 mM) containing 1000 pmoles monomer. For the different N/P values, simple multiples of the nanofiber solution were used accordingly. After 1 hour incubation time at 25°C we added 3 µL 50% Glycerol in HBG solution to each sample, then loaded them onto a 1.2% agarose gel in Tris-Acetate buffer (pH 7.5, 40 mM). The electrophoresis of the siRNA-nanofiber complexes was performed at 80 V during a run time of 30 minutes. The electrophoresis gel was analyzed with the ImageQuant LAS 4000 system (GE Healthcare Lifescience Inc) using Red Epi light (630 nm) and R670 Cy5 filter. For N/P values > 4no free siRNA could be detected. Free uncomplexed siRNAs such as shown in the left lane (see figure) migrate as a discrete band. Moreover the dialyzed nanofibers showed the same complexation potential as the non-dialyzed PDA-Nf solution. See Figure S7 (A) and (B): Complexation of siRNA by the PDA-Nfs, analyzed by gel retardation assays.

Biology part:

Materials and methods

siRNA used *in vitro* and *in vivo* experiments

siRNA were synthetized by Eurogentec, provided in annealed form and HPLC purified.

Sequences are as follows with: ***TT*** (DNA bases)

siLim1 was purchased from Ambion : LHX1 Silencer Select Pre-designed siRNA 40 nmoles in annealed form.

siLuc:

Gl3-ssSIRNA: 5'-CUU ACG CUG AGU ACU UCG A ***TT***-3' (sense strand)

Gl3-asSIRNA: 5'-U CGA AGU ACU CAG CGU AAG ***TT***-3' (antisense strand)

siLuc4.5:

Gl4.5-TT –ssRNA: 5'- GGA CGA GGA CGA GCA CUU C ***TT***-3'(sense strand)

Gl4.5-TT –asRNA: 5'-GAA GUG CUC GUC CUC GUC C ***TT***-3' (antisense strand)

siLim1:

Lim-ssRNA: 5'-CCGGUGUUUCGGUACCAAA***TT***-3' (sense strand)

Lim-asRNA: 5'-UUUGGUACCGAAACACCGGAA-3'(antisense strand)

siCtl:

Ctl-ssRNA: 5' -CGU ACG CGG AAU ACU UCG A***TT****-3'*(sense strand)

Ctl-asRNA: 5'-U CGA AGU AUU CCG CGU ACG ***TT***-3' (antisense strand)

siLuc-Cy5 :

ssRNA-Cy5: 5'- Cy5-CUU ACG CUG AGU ACU UCG A***TT****-3'*(sense strand)

asRNA : 5'-U CGA AGU ACU CAG CGU AAG ***TT***-3' (antisense strand)

Cell culture

A549-Luc cell line

A549-luc cells that stably express the reporter gene luciferase were established by Dr. J.-L. Coll's group (Institut Albert Bonniot, Université de Grenoble), by transfecting the human lung carcinoma cells A549 (CCL-185; American Type Culture Collection, Manassas, VA, USA)

with pGL3-Luc plasmid (Clontech, Mountain View, CA, USA). Cells are grown in RPMI 1640 medium (Eurobio, Les Ulis, France) supplemented with 10% fetal bovine serum (FBS, Eurobio), 1% antibiotic solution (penicillin-streptomycin, Gibco, Fisher Scientific, Illkirch, France) and maintained under 800 µg/mL of G418 selection antibiotic (Promega, Madison, WI, USA) at 37°C in a 5% CO_2 humidified atmosphere.

786-O cell line

The human renal cancer cell line 786-O, of the CCC type, was obtained from American Type Culture Collection (Manassas). Cells were maintained in DMEM (Gibco) supplemented with 10% FBS and 1% antibiotics (penicillin/streptomycin, Gibco) in a 10% CO_2 atmosphere. 786-O cells stably transfected either with pGL4.50 [luc2/CMV/hygromycin] (Promega), or pVITRO2-LGFP-Lim1 (InvivoGen, Toulouse, France) were also used and maintained in the same medium supplemented with the antibiotic hygromycin for selection of transfectants (200 µg/mL, Gibco).

RNA interference

A549-Luc cell line

Twenty-four hours prior to transfection, 2.5×10^4 A549 Luc cells were seeded per well in 24-well tissue culture plates (Corning, NY, USA). The required amounts of siRNA duplexes were diluted up to 100 µL in HBG (Hepes Buffered Glucose: 20 mM Hepes pH 7.5, 5% glucose, sterile filtered). Nanofiber solutions were added and the samples were mixed and incubated 60 min at room temperature to favor association between siRNA and PDA-nanofibers. Targeting luciferase (siLuc) or control (siCtl) siRNA-nanofiber solutions were then added onto the cells in serum free medium. After 4 h of incubation, the medium was replaced by fresh RPMI medium containing 10% FBS. For initial screening, experiments were done in duplicate, and then confirmed in quadruplicate assays. See Figure 3 in main text: Efficacy of Nanofibers (PDA-Nfs) to silence luciferase reporter gene in A549-Luc cell line at 10 nM of siRNA concentration.

See Figure S8: Transfection efficiency of PDA-Nfs/siRNA in the presence of 10% serum

786-O cell line[1,2]

As with the previous cell line 786-O wt or expressing luciferase or overexpressing Lim-1, cells were seeded in 24-well plates (2.5×10^4 cells/mL) and grown for 24 h. Complexes of PDA-Nfs/Lim-1-targeting siRNA (siLim1, Life technologies, Courtaboeuf, France), or control siRNA (siCtl, Life technologies) or luciferase-targeting siRNA (siLuc4.5, Eurogentec, Angers, France) were also prepared in HBG buffer and matured for 60 min. Naked siRNA diluted in the HBG buffer were also used as negative control. Cells were transfected in the absence of serum and after 4 h, serum was added (10% FBS). Luciferase or Lim-1 expression was analyzed after 48 hours.

Commercial transfection agent jetPRIME™ (POLYPLUS-transfection, Illkirch, France) was chosen as a positive control at 10 nM siRNA concentration using standard protocols from POLYPLUS-transfection, siRNA complexes being formed in provided jetPRIME buffer.

See Figure S9: Efficacy of PDA-Nanofibers (PDA-Nfs) to carry gene silencing in 786-O-Luc cell line.

See Figure S10: Efficiency of PDA-Nfs/siLim complexes to silence Lim-1 oncogene in 786-O cell line.

Luciferase quantification

Quantification with automated luminometer

Luciferase gene expression was determined on cell lysates (100 µL 1/5 diluted lysis buffer: Promega Cell Culture Lysis 5x Reagent). The luciferase activity was quantified on 2 µL of cell lysate (after a 5 minutes centrifugation at 14000 g) in white 96-well plates with the Centro LB luminometer (Berthold, Thoiry, France). Following parameters were used: 50 µL of the luciferase substrate (Luciferase Assay Substrate, Promega, France) were automatically distributed ahead of each measurement, plate shaken for 3 seconds then bioluminescence was quantified over 1 second. Luciferase activity was compared to non-transfected cells and with cells being transfected with control siRNA (siCtl, non-matching sequence).

Protein concentration in cell lysates was measured by using BCA assay kit (Interchim, Montluçon, France), according to manufacturer's instructions on 15 µL cell lysates. After expressing the luciferase activity as relative light units integrated over 10 s per mg of cell

protein (RLU/10 s/mg protein), the luciferase gene silencing was calculated relative to the luciferase activity of non-transfected A549-luc cells.

Quantification by bioluminescence imaging

After 48h of transfection, the culture medium of 786-O cells expressing luciferase was supplemented with luciferin at 150 µg/mL (Promega) and the cell plates were incubated for 10 minutes in the dark before imaging. This period of time allows the oxidation of the luciferin and the emission of quantifiable photons by the action of the luciferase. The intensity of the bioluminescence is then evaluated using the "Fusion FX7" imager (Vilber-Lourmat, Collegien, France) allowing the acquisition of the signal after 5 minutes of exposure and an Image capture with a cooled CCD camera.

Cell density

To evaluate the cytotoxicity of PDA-Nfs, we assessed cell density using the crystal violet staining method. Cells were fixed with 10% paraformaldehyde (Sigma-Aldrich) and then stained with 0.1% crystal violet dye (Sigma-Aldrich). After treatment with acetic acid at 33%, the coloration was measured with a UV-Vis spectrophotometer (Cary, Varian Instruments) at 590 nm. The intensity of coloration obtained is proportional to the cell density.

Real-time quantitative PCR analysis

786-O cells and tumors total RNAs were extracted using the Trizol method according to the manufacturer's protocol (Invitrogen, Fisher scientific). Then, 1 µg of total RNA were reverse transcribed using non-specific primer p(dT)15 according to the supplier's protocol (Iscript ™ cDNA synthesis script, Biorad, Marnes-la-Coquette, France). Complementary DNAs specific for each mRNA were amplified using the 'SsoAdvanced™ Universal SYBR® Green Supermix' kit (Biorad) in the CFX Connect™ Real-Time System (Biorad). Sense and antisense primers used were as follows: Lim-1 (primers designed by Biorad and provided as a mixture, PrimePCR™ SYBR® Green Assay, Bio-Rad), cyclophilin A (forward primer: CCACCGCCGAGGAAAACCG; reverse primer: TGGACAAGATGCCAGGACCCGT), 18S (forward primer: CTTCCACAGGAGGCCTACAC; reverse primer: CTTCGGCCCACACCCTTAAT, Eurogentec). Lim1 (forward primer: CTTCTTCCGGTGTTTCGGTA; reverse primer: TCATGCAGGTGAAGCAGTTC).

In vivo experiments[1,2]

Xenograft tumor Model

All animal studies were in compliance with the French animal use regulations. A total of 10 million 786-O cells were injected subcutaneously into the skin of the back of 4-weeks-old athymic male mice (SWISS nu-/nu-; Charles River Laboratories, l'Arbresle, France). After one month and when 786-O tumors had grown to an overall volume of 300 mm^3, mice were randomized in three groups. The protocol was intra-peritoneal (ip) injection of PDA-Nfs/siLim1 or PDA-Nf/siCtl complexes at 0.2 mg of siRNA per kg of mouse and a N/P = 6 ratio of PDA-Nfs. After 48 hours of treatment, animals were euthanized and the tumors were harvested and snap-frozen for PCR or Western blot analysis.

Histological analysis of the xenograft tumors and detection of PDA-Nfs

Harvested subcutaneous tumors from siRNA/PDA-Nfs injected mice were fixed in 10% neutral-buffered formalin and processed for histologic examination including embedding in paraffin, sectioning, and staining with hematoxylin and eosin. Four µm sections from selected paraffin were used for histopathology analysis to confirm the presence of PDA-Nfs. Histological sections were analyzed by fluorescence microscopy and polarizing filter. The presence of the PDA-Nfs was confirmed by their red fluorescence and bi-fringency.

Figure S13: Detection of PDA-Nfs in tumors from mouse sacrificed 48 hours after siRNA/PDA-Nfs i.p. injection.

Western blot analysis

Protein extraction and membrane preparation were performed as described by Massfelder *et al.*[1,2]. Membranes were incubated overnight at 4°C with the appropriate dilution of the primary antibody: anti-Lim1 (1:3000; Abcam, Cambridge, UK). For visualization of protein gel loading, an anti-β-actin (1:20000; Sigma-Aldrich) antibody was used. The appropriate horseradish peroxidase-conjugated secondary antibody was used. Immunoreactivity was visualized with the enhanced chemiluminescence immunoblotting detection kit (Luminata™, Millipore, Molsheim, France). See Figure 5B in main text: Efficacy of PDA-Nanofibers (PDA-Nfs) to carry small interfering RNA *in vivo* by Western blot analysis.

Biodistribution studies

The biodistribution of the fluorescent siRNA/PDA-Nf complexes was followed in Swiss CD-1 mice. They were treated by single i.p. injection and sacrificed 1 hour or 48 hours after administration of siLim1/PDA-Nfs by the TechMed'ill platform. No clinical signs of pain, discomfort or abnormal behavior were observed in the mice injected with the siRNA/PDA-Nfs.

As described in main article no sign of inflammation or redness/rashes at the site of injection and no visible local accumulation of the red PDA-Nfs, neither at 1 hour nor at 48 hours after injection were observed. Fresh blood was withdrawn at 1 hour and 48 hours and blood films were prepared, then analyzed by fluorescence microscopy. Major organs were fixed in PBS-paraformaldehyde solution and paraffin embedded on Leica Perl workstation.

20 µm thick sections were cut from organs at two levels, bleached with 0.05% potassium permanganate solution and permanently mounted on microscopic Superfrost™ glass slides with Eukitt™on Shanon VaristainXY workstation.

Organ sections and blood films were analyzed with Leica DM 4000B fluorescent microscope and micrographs were taken with Olympus DR72 camera module at 15 millisec exposure (red fluorescence; Ex : 515-560 et Em : 580-590 and Ex : 540-580) and 1.4 sec. exposure for unspecific tissue related green fluorescence.

Results: In mice sacrificed at 1 hour the fibers could be detected in blood samples (data not shown), a strong distribution is also observed in the spleen, liver, medium pharmacodistribution is observed in the lung and kidney. Mice sacrificed at 48 hours show no longer any detectable presence of the fluorescent PDA-Nfs in the analyzed organs, showing that the PDA-Nfs have been metabolized or cleared from the body. Notably at 48 hours some PDA-Nfs were detected in urine sample from sacrificed mouse (data not shown).

Figure S12: Fluorescence microscopy of sections of paraffin embedded organs from mice sacrificed 1 hour after siRNA/PDA-Nfs injection.

Statistical analysis

All values are expressed as mean ± s.e.m. Statistical analysis was performed using one-way ANOVA followed by the Student–Newman–Keul's test for multiple comparisons. A $P<0.05$ was considered significant (statistical analysis done with Prism 7™ scientific software by GraphPad™).

References:

1 V. Dormoy, D. Jacqmin, H. Lang and T. Massfelder, *Anticancer Res.*, 2012, **32**, 3609–3617.

2 V. Dormoy, C. Béraud, V. Lindner, L. Thomas, C. Coquard, M. Barthelmebs, D. Jacqmin, H. Lang and T. Massfelder, *Oncogene*, 2011, **30**, 1753–1763.

SI Figures

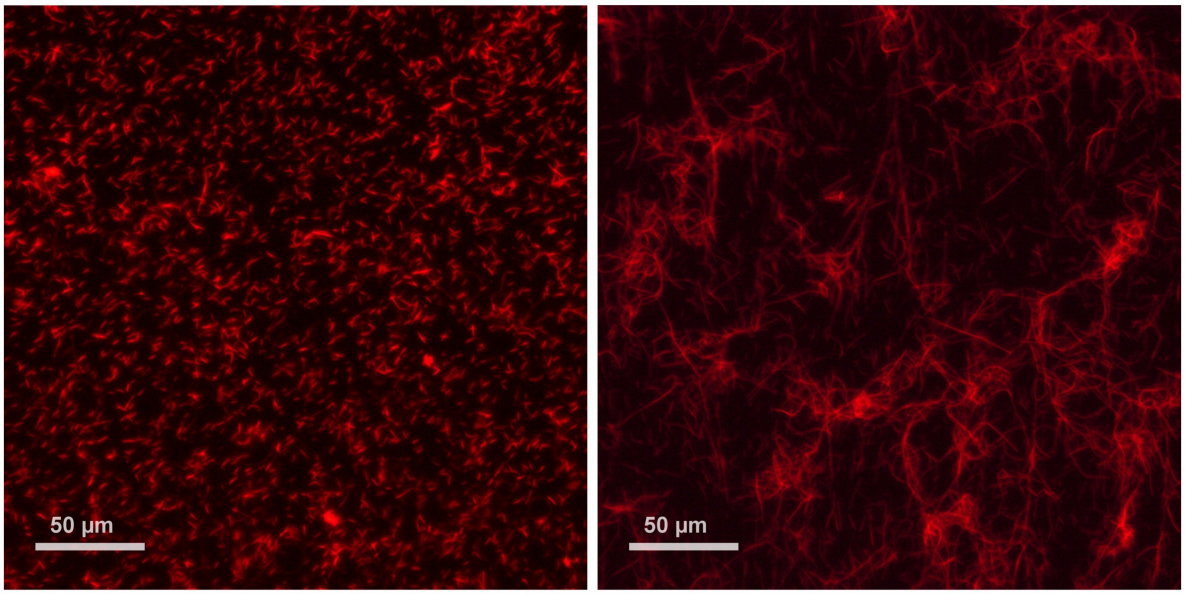

Figure S1: Fluorescence microscopy of **(A)** smallPDA nanofibers PDA-Nfs formed with surfactant **(1)**; **(B)** long microfibers formed withhistidine grafted analogue **(2).**

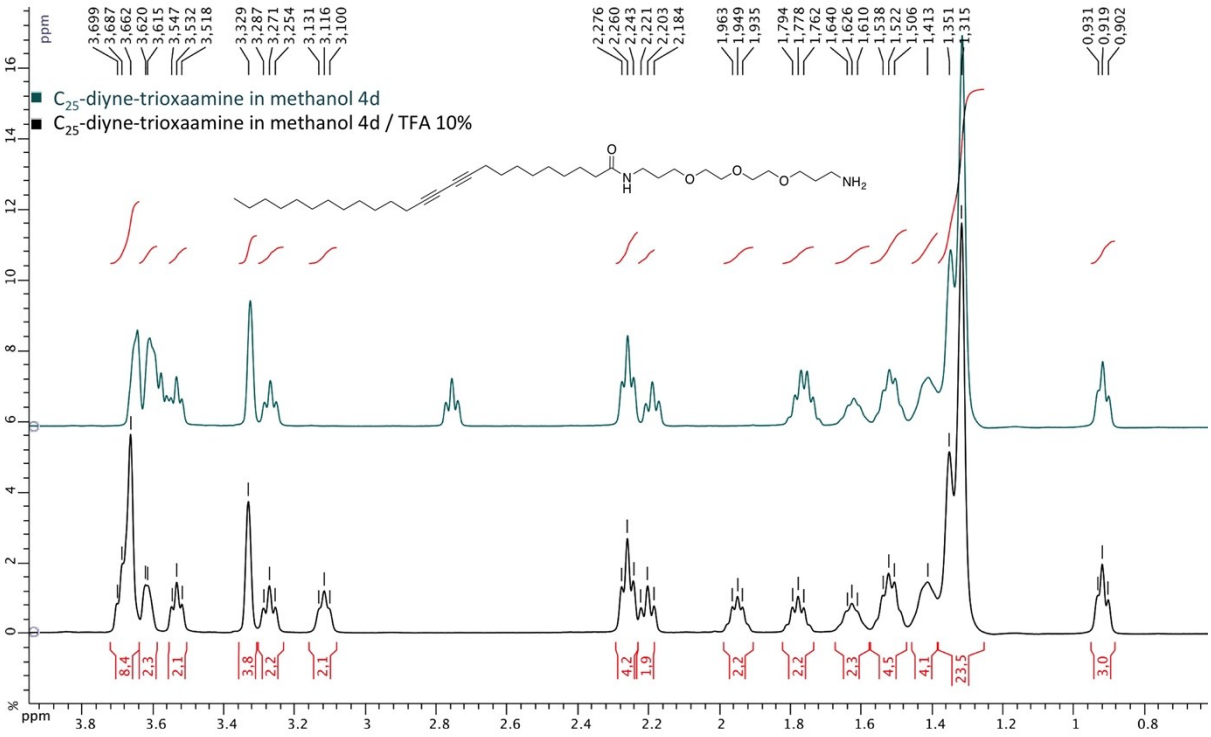

Figure S2: ^{1}H-NMR analysis of surfactant (1). In the upper part is given the spectrum of the neutral form, as isolated by chromatography; in the lower part is given the spectrum of the protonated surfactant **(1)** after addition of TFA in the deuterated methanol.

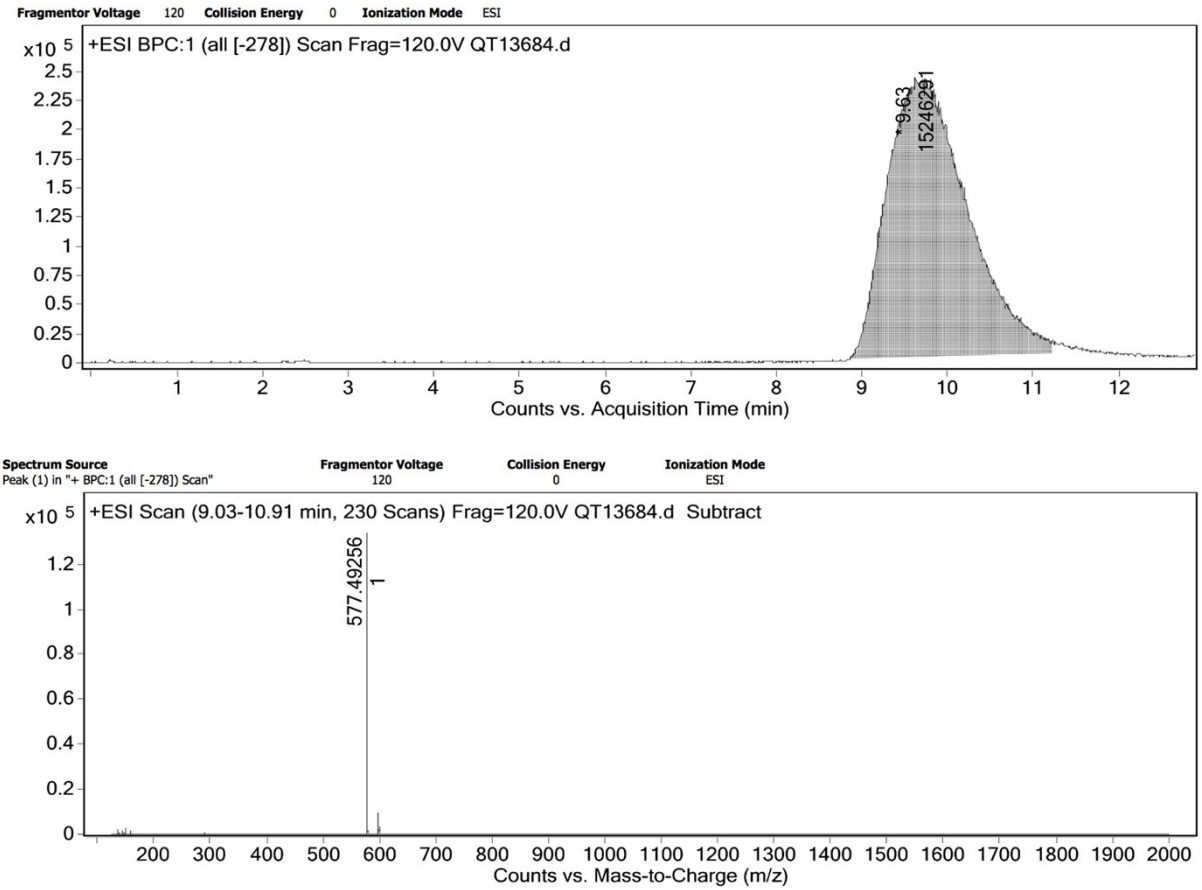

Figure S3: LC-HRMS analysis of surfactant (1). In the upper part is given the positive +ESI total ion from the chromatogram. In the lower part are shown the mass peaks in the peak. The exact mass $M+^{1}H^{+}$ 577.49256 ion confirms the molecular formula of the molecule (score 96.91%).

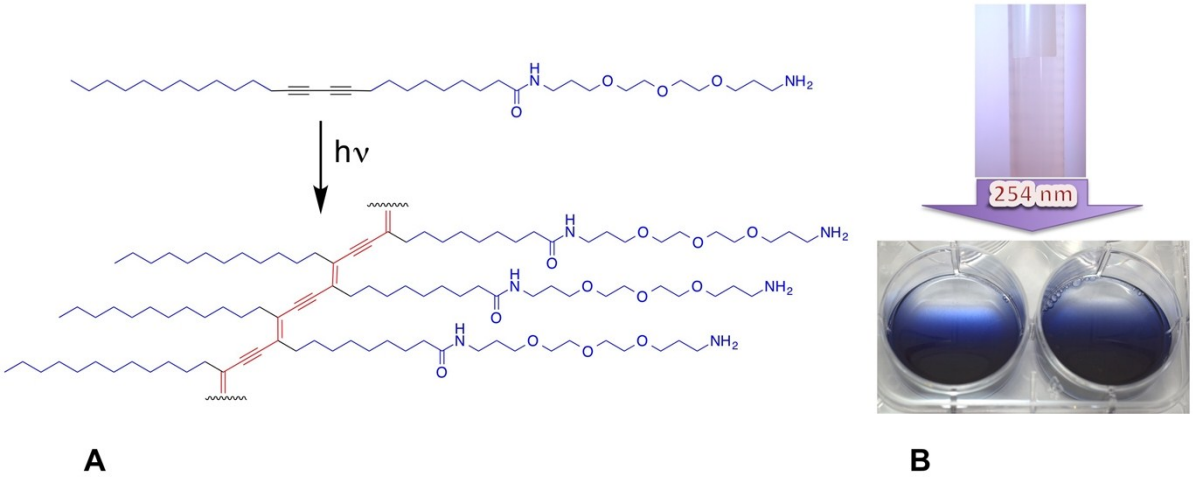

Figure S4: Photo-polymerization of ethanolic solutions of surfactant (1). The chemical structure of the conjugated polymer is given in (**A**); in (**B**) we show the initially formed suspension of the blue polymerized fibers (polymerized in sterile 6-well cell culture plates.

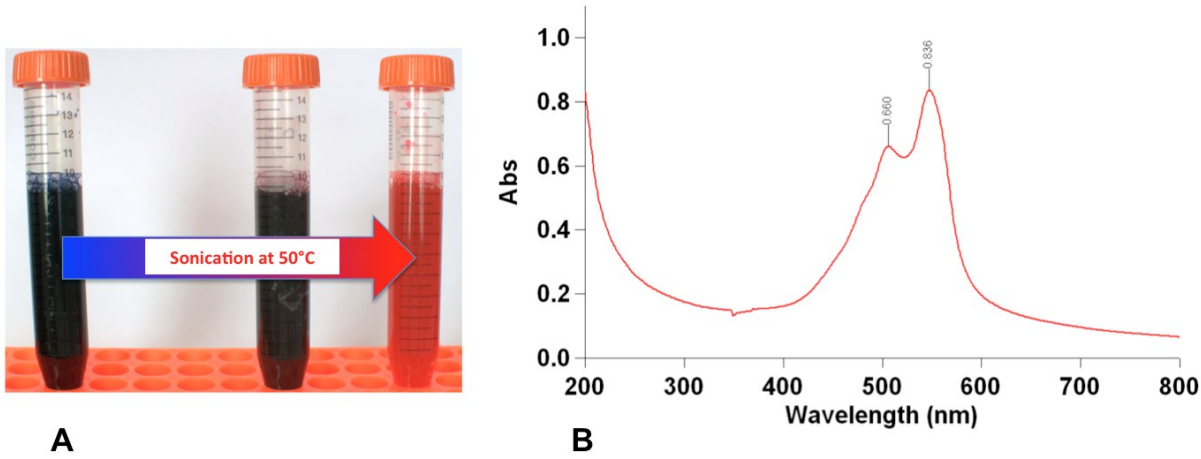

Figure S5: Preparation of the fluorescent PDA-Nfs used for siRNA delivery (**A**) Formation of the fluorescent crimson-red Nanofibers PDA-Nfs out of the initially blue solution, (**B**) UV-Vis absorption spectrum of the diluted PDA-Nfs solution (30 µg/mL).

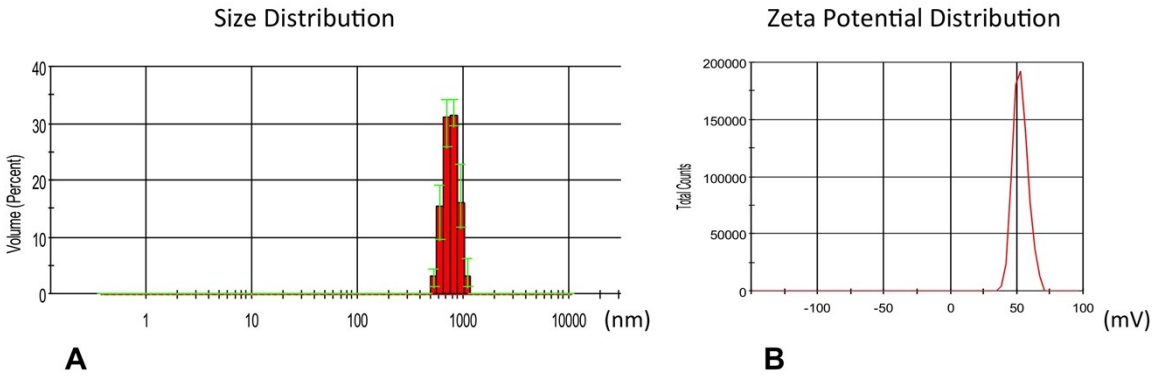

Figure S6: Dynamic light scattering (DLS) and zeta potential measurement of the free PDA-Nfs. (**A**) Dynamic light scattering (DLS) (Nanosizer, Malvern Instruments) showing a mean size of the PDA-Nfs around 780 nm; (**B**) Zeta-potential measurement of the PDA-Nfs in water (Nanosizer, Malvern Instruments) showing a high positive value of 52 mV.

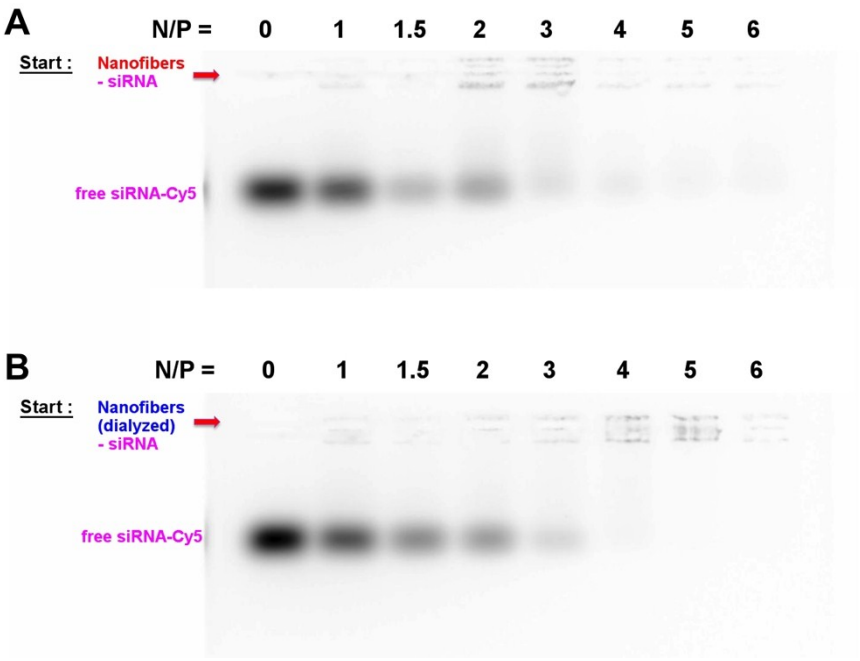

Figure S7: Gel retardation assays of siRNA complexation by the PDA-Nfs by electrolysis at varying N/P values. (A) Complexation of siRNA by the PDA-Nfs, analyzed by gel retardation assays, with fluorescently labeled Cy5-siRNA. The siRNAs are completely complexed at N/P > 4 (ratio between amino groups of PDA-Nfs and negatively charged phosphate groups of PDA-Nfs. (**B**) The siRNA complexation potential of dialyzed fibers is unchanged compared to non-dialyzed PDA-Nfs, showing indirectly absence of monomer in non-dialyzed PDA-Nf solution.

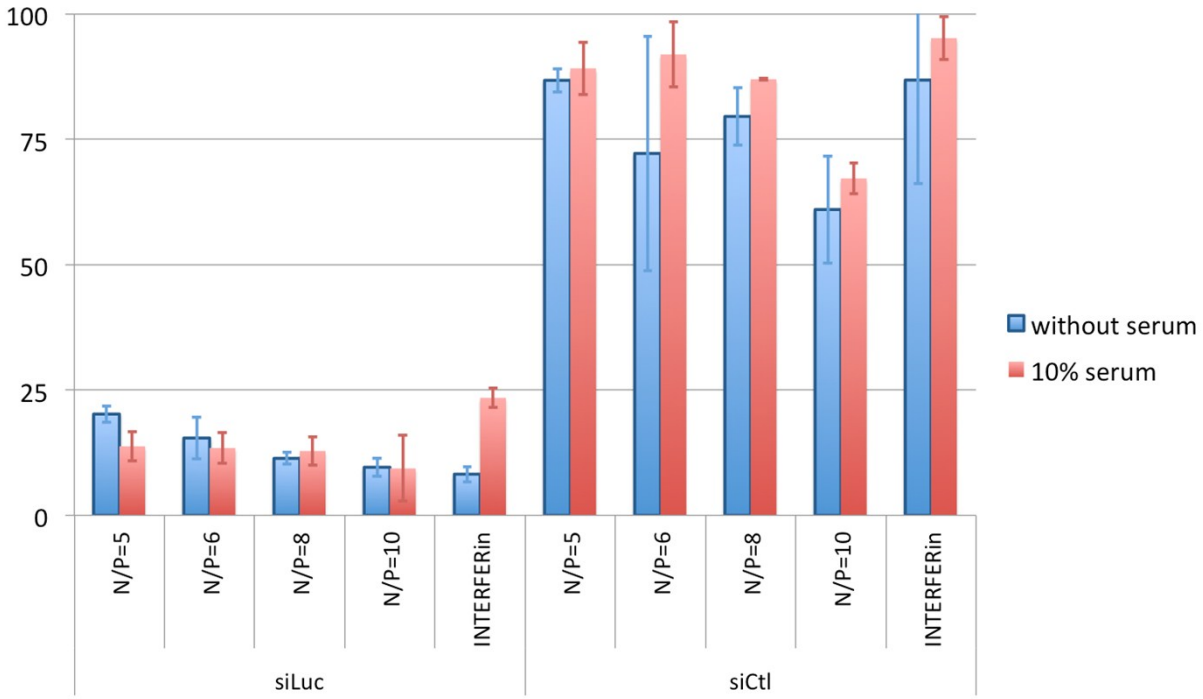

Figure S8: Transfection efficiency of PDA-Nfs/siRNA in presence of 10% serum. The experiment was performed using the A549-Luc cell line, at 20 nM siRNA concentration. The luciferase reporter gene is specifically silenced at a range of N/P values from N/P =5 to 10. The PDA-Nfs/siRNA compare favorably to commercial reagent INTERFERin from POLYPLUS-transfection (Illkirch, France).

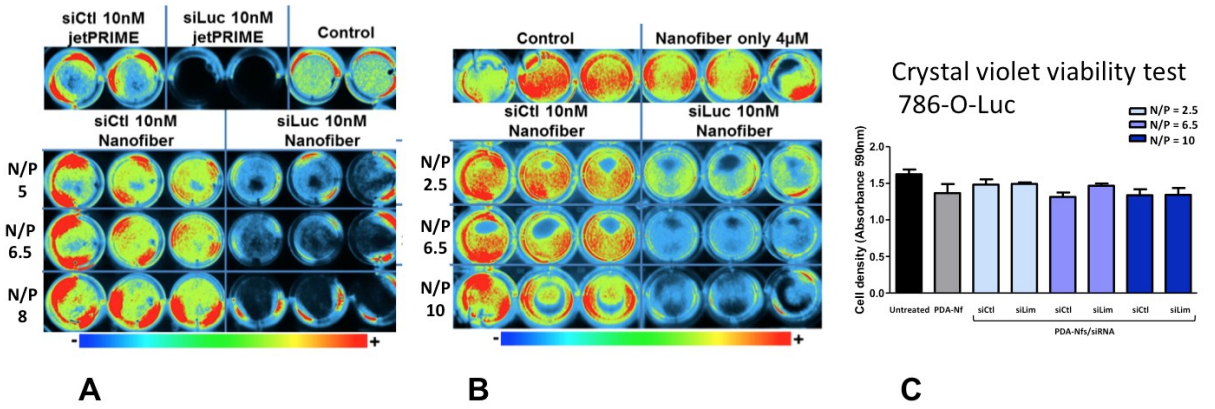

Figure S9: Efficacy of PDA-Nanofibers (PDA-Nfs) to induce gene silencing in 786-O cell line. (**A, B**) Bioluminescence imaging of the 786-O cells expressing the luciferase reporter gene that have been transfected for 48 hours with PDA-Nf/siRNA at various N/P ratios of PDA-Nfs. Visualization has been done on live cells after direct addition of luciferin substrate. So the luciferase expression is quantified by measuring the bioluminescent signal due to oxidation of luciferin substrate. In the two plates (**A**) and (**B**) each line has constant PDA-Nfs N/P ratio ranging from 2.5 to 10; on the left, triplicates of each plate are the cells transfected with control siRNA (siCtl), on the right side, triplicate are the conditions with the Luciferase targeting siRNA (siLuc) showing a high silencing of the luciferase expression. In the plate (**A**), the silencing efficiency of luciferase using the transfection agent jetPRIME routinely used is comparable to PDA-Nfs. (**C**) The cytotoxicity of PDA-Nfs was assessed by crystal violet staining after verification of the luciferase silencing by bioluminescence imaging (**B** plate). Results are expressed with the absorbance at 590 nm and shown as mean ± s.e.m., n=3. The commercial transfection reagent jetPRIME®(POLYPLUS-transfection, Illkirch, France) was used as a standard positive control in the siRNA transfection experiment (top lane)

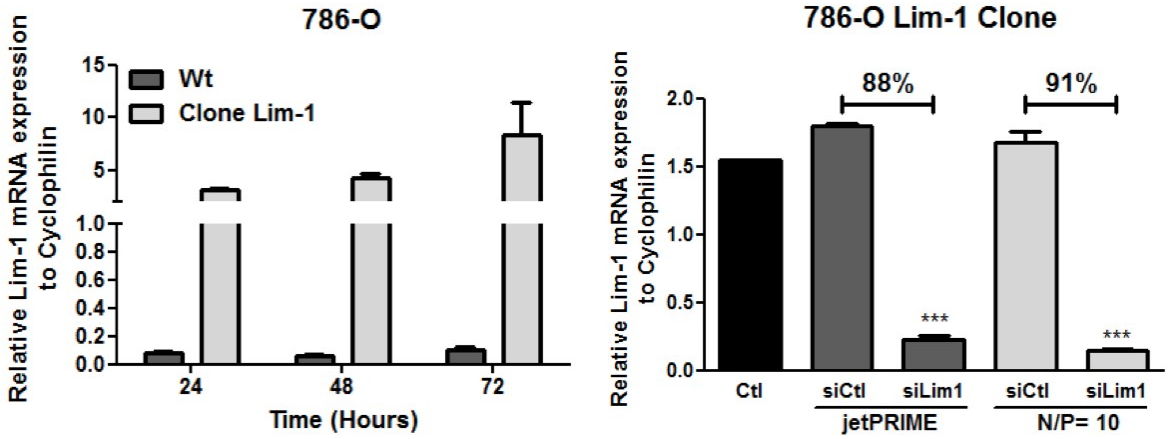

Figure S10: Efficiency of PDA-Nfs/siLim complexes to silence Lim-1 oncogene in 786-O cell line. (**A**) Lim-1 mRNA relative expression was measured by real-time quantitative RT-PCR on total RNAs from wildtype 786-O cells (Wt) or 786-O clone overexpressing Lim-1. Results are shown as mean ± s.e.m., n=4. (**B**) Lim-1 mRNA relative expression was measured by real-time quantitative RT-PCR on total RNAs from 786-O clone overexpressing Lim-1 transfected with PDA-Nfs/siLim1 versus PDA-Nfs/siCtl complexes at 10 nM siRNA concentrations and at optimum PDA-Nfs concentration (N/P=10). The transfection reagent jetPRIME®(POLYPLUS-transfection, Illkirch, France) was used as a positive control. 91% inhibition could be obtained with the PDA-Nfs/siLim1 system (100% - 100 x $\frac{Expression\ with\ siLim1}{Expression\ with\ siCtl}$). Cyclophilin expression was used as housekeeping gene and to calculate the relative expression of Lim-1. Results are shown as mean ± s.e.m., n=3; ***P<0.001 from siCtl.

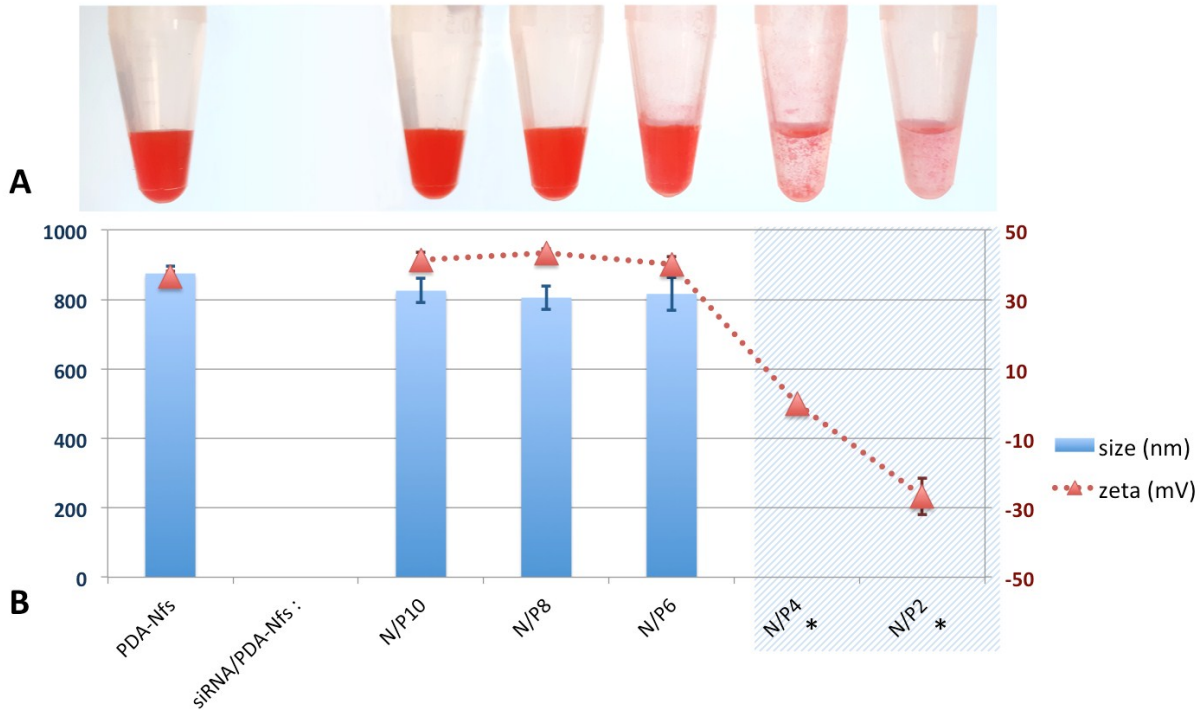

Figure S11: Biophysical characterization of siLim1/PDA-Nfs complexes. The complexes were prepared in the same conditions and buffer conditions (Hepes buffered glucose: 20 mM Hepes pH 7.5, 5% glucose, sterile filtered) as used for the *in vivo* studies, where complexes with N/P = 6 were used. (**A**) siLim1/PDA-Nfs complexes prepared at various N/P ratios (N/P = 10, 8, 6, 4, 2) (on the right side) compared to uncomplexed PDA-Nfs in the same HBG buffer (left side tube). (**B**) DLS size measurement of siLim1/PDA-Nfs complexes shown in (**A**), as well as the zeta potential values for the complexes. At the concentration N/P = 6 the complexes remain stable and aggregation occurs at N/P values <6 (flocculation visible in A, 5th tube). Between N/P = 6 and 10 the zeta potential values are higher than 30mV, which stands often for colloidal stability. The aggregates at N/P = 2 have a negative zeta potential due to excess negative charge resulting from siRNA sticking on the surface of the PDA-Nfs.

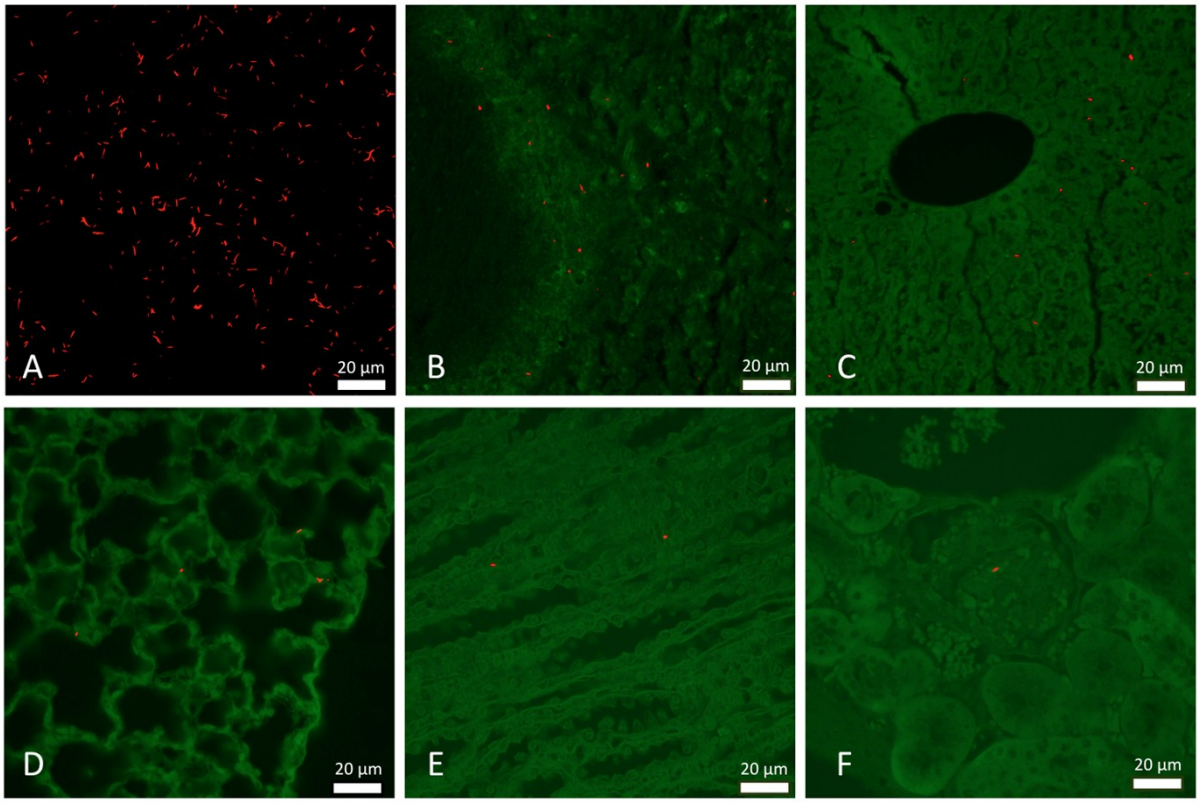

Figure S12: Fluorescence microscopy of sections of paraffin embedded organs from mice sacrificed 1 hour after siRNA/PDA-Nfs injection. The micrographs show an overlay of green fluorescence (autofluorescence of tissue) with specific red fluorescence of Nanofibers (Ex : 515-560 et Em : 580-590 and Ex : 540-580). (**A**) siLim1/PDA-Nfs solution such as prepared for *in vivo* studies (10 fold dilution in water, 40x oil immersion objective). (**B**) Presence of siLim1/PDA-Nfs in histological sections of the spleen (40x oil immersion objective). (**C**) Presence of siLim1/PDA-Nfs in histological sections of the liver (40x oil immersion objective). (**D**) Detection of siLim1/PDA-Nfs in histological sections of the lung (40x oil immersion objective). (**E, F**) Detection of siLim1/PDA-Nfs in histological sections of the kidney (40x oil immersion objective).

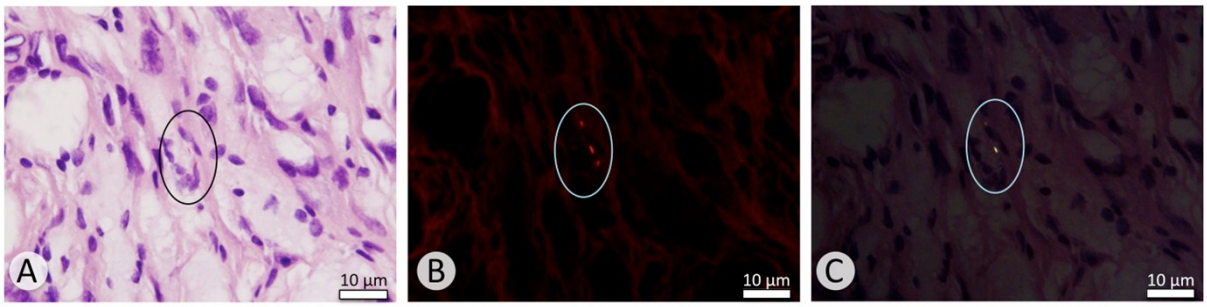

Figure S13: Detection of PDA-Nfs in tumors from mice euthanized 48 hours after siRNA/PDA-Nfs i.p. injection. Tumors from xenografted mice were harvested 48 hours after treatment with siLim1/PDA-Nfs and stored in liquid nitrogen prior to histological analysis. (**A**) Representative histological HE staining of paraffin embedded tumor sections. (**B**) Detection of PDA-Nfs by their red fluorescence Ex : 515-560 et Em : 580-590 and Ex : 540-580. (**C**) Detection of the PDA-Nfs by their birefringency undercross-polarized light illumination (100x objective).