Conjugated polymer nanoparticles for small interfering RNA delivery

Joong Ho Moon,[*,†] Eladio Mendez,[†] Yong Kim[‡], and Aman Kaur[†]

[†]*Department of Chemistry and Biochemistry, Florida International University, 11200 SW 8th St., Miami, FL 33199*

[‡]*Division of Oral Biology and Medicine, UCLA School of Dentistry, Dental Research Institute, UCLA's Jonsson Comprehensive Cancer Center, 10833 Le Conte Ave. 73-052 CHS, Los Angeles, CA 90095*

General

Nuclease free water was purchased from Acros organics. siRNA for actin B (siACT), siRNA for lamin (siLamin), siGLO Red and trypsin (0.25% with ethylenediamine tetraacetic acid , EDTA) were purchased from Thermo Fischer Scientific. siGLO is a synthetic siRNA indicator labeled with a DY-547 fluorophore. A549 (a carcinomic human alveolar basal epithelial cell line, CCL-185), HeLa (human epithelial cervical cancer), and F-12K Cell growth medium were purchased from American Type Culture Collection (ATCC, Manassas, VA). Fetal bovine serum (FBS) and penicillin-streptomycin were purchased from MP Biomedical. 75cm^2 cell culture flask and glass coverslips were purchased from Thermo Fischer Scientific. 12-well cell culture plates were purchased from Corning Inc. 0.45μm and 0.20 μm syringe filters were purchased from VWR. Quartz cuvettes (Catalog# 16.100-Q-10/Z15 and 16.100-Q-10/Z8.5) were purchased from Starna cells. Disposable microcuvettes (Catalog # 13-878-121) were purchased from Fisher Scientific. Dulbecco's phosphate buffered saline (DPBS) was purchased from Cellgro (Mediatech Inc., Manassa, VA). ECL Western blot detection kit was purchased from GE healthcare. Dynamic light scattering and zeta potential experiments were performed by Zetasizer nano–ZS (Zen 3600, Malvern Instruments Ltd.) using a microcuvette and a folded capillary cell (Catalog # DTS1060), respectively, at room temperature. At least three independent samples were prepared and each sample was measured three times. Fluorescence spectra were recorded

using Fluoromax-3 spectrofluorometer (Horiba Jobin Yvon) at room temperature with excitation at 400nm using a disposable cuvette. To minimize scattering and interference variation among the samples, the fluorescence intensity at the emission maximum was divided by that at 610 nm (for complexation with siLaminin). Similar normalization used for FRET analysis: the FRET intensity was divided by the CPN emission maximum. ViCell Counter from Beckman Coulter was employed to count viable cells (cell toxicity).

CPN/siRNA complex formation

25 μM of CPNs in water (100 μl) was used for the complexation experiments (i.e., fluorescence and dynamic light scattering). To make a 25 nM siRNA in the complex (i.e., 25CPN/25siRNA), 0.5 μl of 5 μM siRNA solution was added into the CPN solution. After measurement, additional 0.5 μl of 5 μM siRNA solution was added to make the complex with 25CPN/50siRNA. Similarly, 25CPN/100siRNA and 25CPN/200siRNA were prepared by adding additional 1 and 2 μl of siRNA solution, respectively. Total volume of siRNA added in the CPNs was 4 μl. The dilution effect in fluorescence intensity was negligible.

Cell culture

A549 cell line (ATCC) were revived using F-12K cell growth media (ATCC) containing 10% FBS and 1% streptomycin-penicillin and grown in 5% CO_2 incubator at 37°C. 24hrs before experiment, A549 cells were seeded ($3x10^4$ cells per well) on a 12-well culture plate containing glass coverslips.

CPN/siGLO Red complex preparation

CPN/siGLO complexes prepared (300µl) in water were mixed with 300µl of F-12K Growth medium (ATCC) without FBS and incubated for 10 min. Before incubating with cells, the complexes were further mixed with a culture medium containing 10% FBS to produce final volume of 1ml of CPN/siGLO solution in a culture medium per well.

Gel Electrophoresis for complexation efficiency

400nM of siLamin was mixed with CPN of varying N/P ratios (0, 2.2, 4.3, 8.7, 17.4, and 34.8) and left to incubate at room temperature for 15minutes. A 25 µL of the samples mixed with 2 µL of loading buffer (5X RNA loading buffer) was loaded on a 20% poly (acryl amide) gel (% cross-linking of 2.67) prepared in 1X Tris-boric acid-EDTA (TBE) Buffer. Gel electrophoresis was run in 1X TBE buffer at 90V for 80min. Free siRNA bands were visualized using 0.5µg/mL ethidium bromide solution.

Cell Imaging

Cells attached on coverslips were treated with the CPN/siGLO Red complexes for 12 hrs in 5% CO_2 incubator at 37°C. After 12 hrs, the treated cells were washed with DPBS (37°C) followed by fixation with 4% paraformaldehyde. After rinsing thrice with DPBS, the slides were mounted onto glass slides with a drop of DPBS and sealed. Fixed, mounted cells were imaged with an inverted fluorescence microscope (Olympus IX51) equipped with a 10X objective (Olympus).

Western Blotting (WB)

HeLa cells were seeded on 6 well plates (50,000 cells per well) one day before transfection. The transfection solution was prepared by combining CPNs, siActin B (siACT) and siGLO in 100 µl

of serum free medium. After 30 m incubation, the solution was mixed with a 2 ml of serum containing medium. Final CPN, siACT, and siGLO concentration in the transfection solution was 20 μM, 100 nM, and 100 nM, respectively. The medium was removed from the plate and replaced with the transfection solution. The transfection solution was removed after 24 h incubation at 37 ^{o}C. The cells were cultured in a fresh medium for another 24 h. The cells were lysed in a cell lysis buffer (New England Biolabs) containing protease inhibitors on ice for 30 m. After sonication, the lysate was centrifuged for 15 m at 14K rpm at 4^{o}C. The supernatant was recovered and equal amount of protein was used for western analysis. Samples (30 μg) were loaded on 9 % SDS-PAGE gel for electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked in 5 % milk in Tris-buffered saline tween-20 (TBST) for 1 h at room temp followed by incubation with primary antibody (1:5000 for anti-Actin; 1:10,000 for anti-tubulin in 5% milk/TBST) for overnight at 4 ^{o}C. After washing three times for 5 m at room temp in TBST, the membrane was incubated with secondary antibody (1:5000 in 5% milk/TBST) for 1 h at room temp. After washing three times for 10 m at room temp in TBST, the signal was detected by using ECL Western blot detection kit (GE Healthcare). For quantifying image intensity, Un-Scan-It software was used (Silk Scientific, Inc. Salk Lake City, UT).

Cell cytotoxicity assay

HeLa cells were seeded on 12 well plate ($10x10^4$ cells per well) and grown for 18 hrs. Cells were treated with CPN control (0, 10 and 20μM) in triplicates. Viable cell number was determined by using ViCell Counter (Beckman Coulter) after harvesting cells (day1, 2, 3 and 4).

Fig. S1. Microscopic images of A549 cells incubated with the CPN/siGLO complexes. Red fluorescent signals increased proportionally to the amount of siGLO in the complexes.

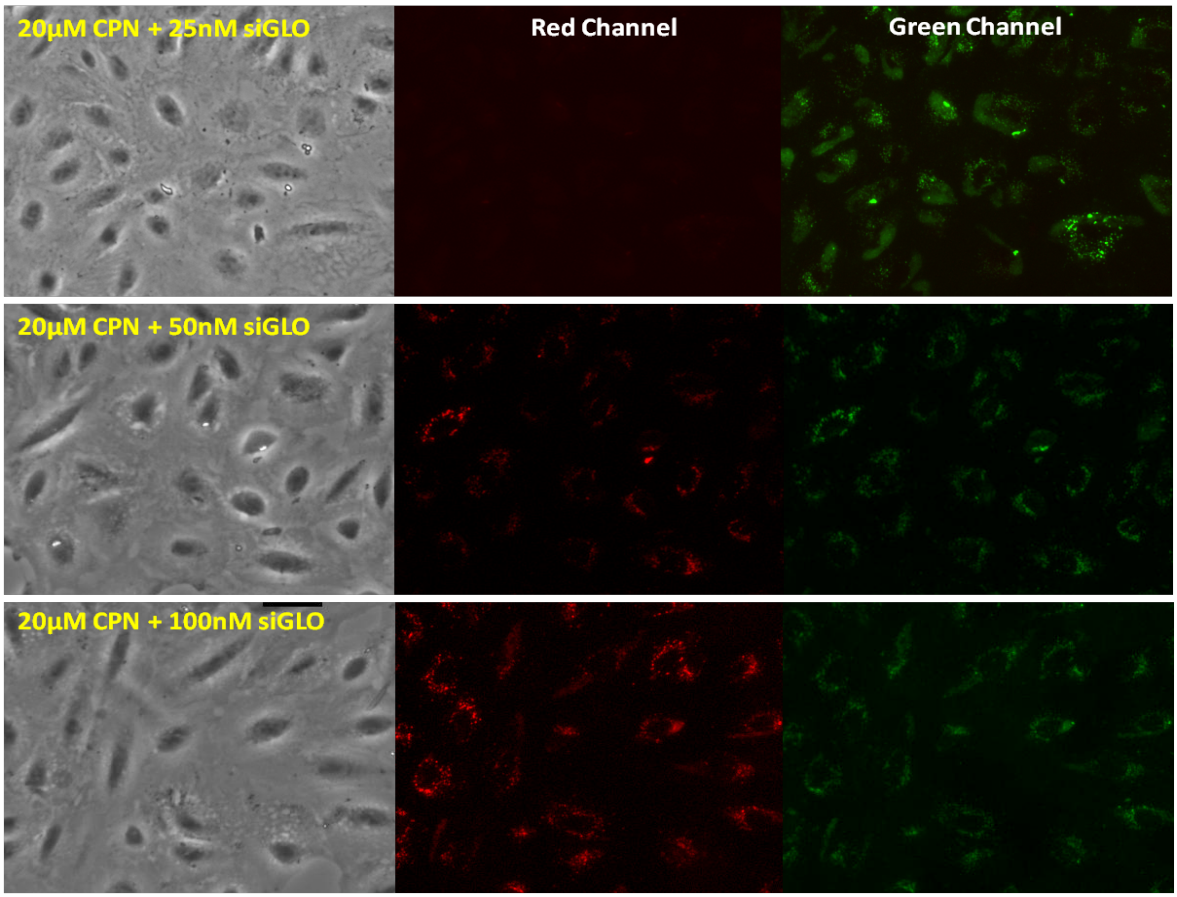

Figure S2. Microscopic images of A549 cells incubated with CPN only (top), siGLO only (middle), and the CPN/siGLO complexes (bottom). siGLO was delivered by CPNs.

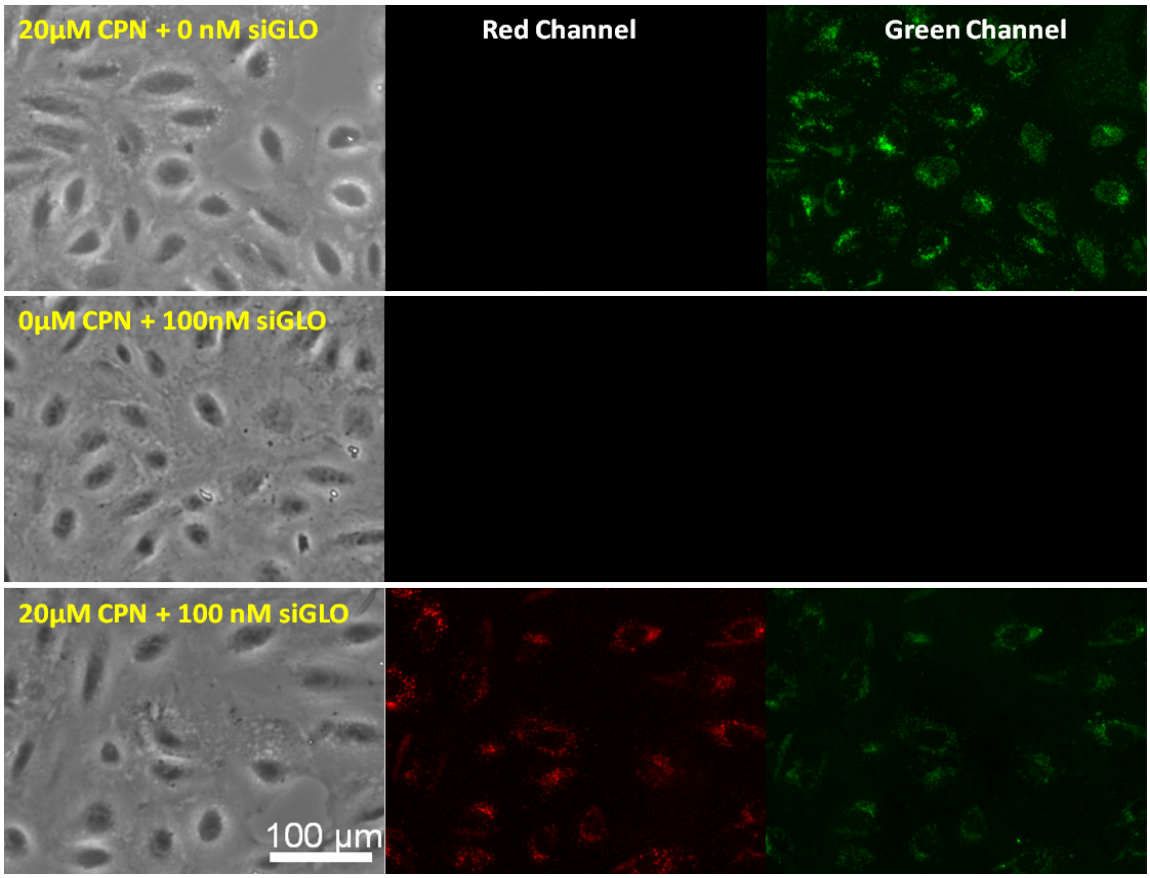

Fig. S3. Microscopic images of HeLa cells incubated with CPN/siACT/siGLO transfection solution containing no siGLO/siACT (top), 50 nM of siGLO and 50 nM of siACT (middle), and 100 nM of siACT and 100 nM of siGLO (bottom).

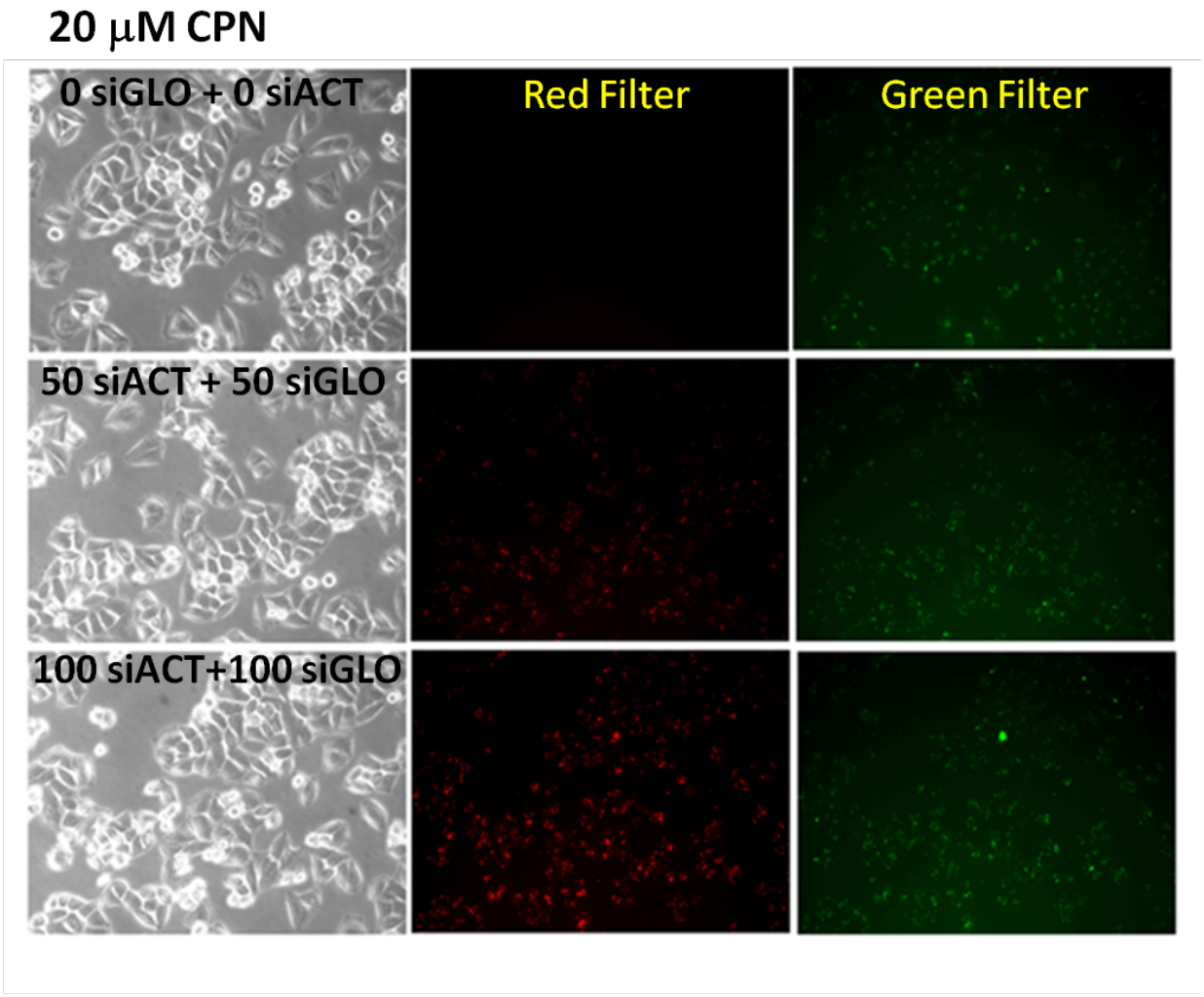

Fig. S4. Cell (HeLa) viability measurement in the presence of CPNs. No measurable viability inhibition was observed. Error bars are smaller than the size of symbols.

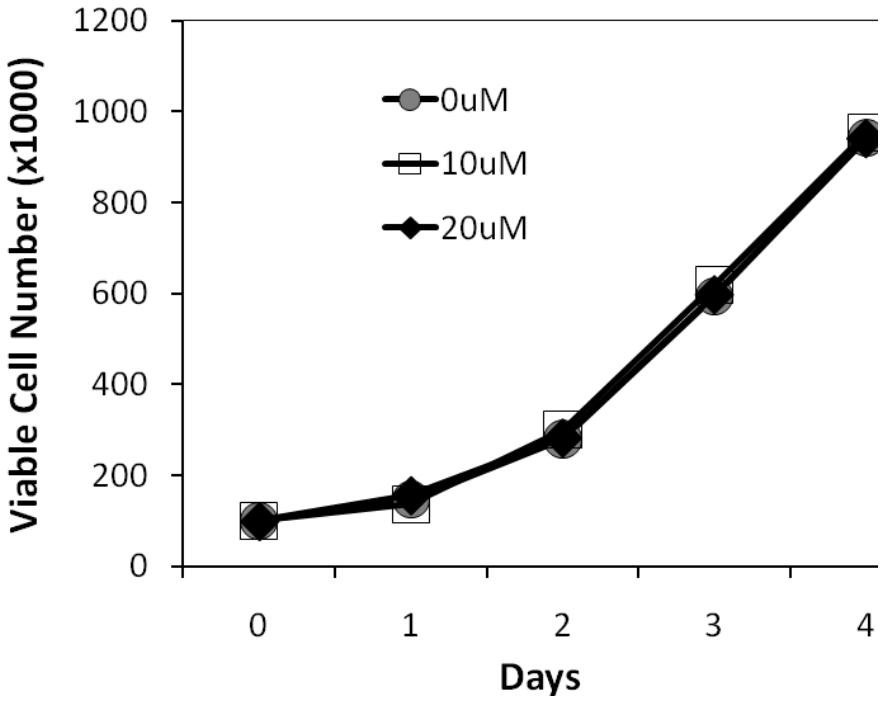

Fig. S5. Gel electrophoresis of CPN/siRNA complexes. Free siRNA were stained by ethidium bromide. Lane 1: 400 nM siRNA, 2: siRNA ladder, 3: 160 μM CPN, 4: 10 μM CPN/400 nM siRNA (N/P: ~2.2), 5: 20 μM CPN/400 nM siRNA (N/P: ~4.3), 6: 40 μM CPN/400 nM siRNA (N/P: ~8.7), 7: 80 μM CPN/400 nM siRNA (N/P ~17.4), and 8: 160 μM CPN/400 nM siRNA (N/P ~34.8)