Supplementary information

Intracellular delivery of functional proteins with DNA-protein nanogels-lipids complex

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1. Experimental section

Materials

Streptavidin, biotin, biotinylated primers, fetal bovine serum (FBS), 4',6-diamidino- 2-phenylindole (DAPI) and dimethyl sulfoxide (DMSO) were purchased from Merck Sigma-Aldrich. Green fluorescent protein conjugated to biotin (bt-GFP) was from ProteinMods. Potassium aluminosilicate (Muscovite Mica) was from Goodfellow. Biotin-16-dCTP and dNTP nucleotides were from Jena Bioscience. Streptavidin labeled with AlexaFluor555 (strep-555) and with AlexaFluor633 (strep-633), streptavidin alkaline-phosphatase conjugate (strep-ALP), DDAO phosphate (DDAO-P), Gibco DMEM Glutamax, Gibco DMEM/F-12, PenStrep penicilline-streptomycine (10,000 U/ml), AlexaFluor-Plus405, phalloidin and phosphate buffer saline (PBS) pH 7.4 and antibodies (EEA1 rabbit monoclonal antibody, RAB5 rabbit polyclonal antibody and CD107a (LAMP-1) recombinant rabbit monoclonal antibody) were from Invitrogen. Q5 High-Fidelity DNA Polymerase (2,000 units/mL) and gel loading dye were from NEB. GFP-Twin-Strep-tag (st-GFP) and streptactin conjugated to DY-649 were from IBA Lifesciences. Mouse melanoma (B16-F0, ATCC CRL-6322) cells were from LGC Promochem. 32% solution of paraformaldehyde (PFA) was from Electron Microscopy Sciences. Phalloidin-X5-FluoProbes 505 was from Interchim. Vectashield antifade mounting medium was from Vector Laboratories. Midori Green Advance DNA stain was from NipponGenetics. Deionized MQ water (18 MΩ·cm) was from Millipore.

Methods

Wide field fluorescence microscopy. Epifluorescence microscope (Observer D1, Zeiss) equipped with a Zyla 4.2+ sCMOS camera (Andor) and multi-wavelength LED source (SpectraX, Lumencor) was used. The acquisition was made with a 40 X LD Plan Neofluar (Zeiss) or 10 X Achroplan objective, and the exposure time was set to 70-100 ms with 1x1 bining. Spectral characteristics of used filters: Semrock LED-YFP-A (λ_{Ex} = 509 ± 11 nm / λ_{Em} = 544 ± 12 nm); Semrock LED-mCherry-A (λ_{Ex} = 578 ± 11 nm / λ_{Em} = 641 ± 38 nm) and Semrock Cy5-4040A (λ_{Ex} = 628 ± 20 nm / λ_{Em} = 692 ± 20 nm).

Confocal fluorescence microscopy. The glass slides on which the cells were grown were removed from the well plates, mounted with Vectashield mounting medium on additional glass slides and sealed with nail polish. The slides were examined by SP8 Leica Microsystems confocal fluorescence microscope at 400X magnification. Images were acquired using the software Leica Acquisition System X and treated using open source FiJi software.

Atomic Force Microscopy (AFM). Freshly cleaved mica slides were treated with 30 µL of 1 mM spermine for three minutes. Mica slides were abundantly washed with MQ water and nanogel samples were allowed to absorbed on the cationic surface for 1 hour before been washed and dried under the jet of compressed air. AFM images were taken with a Cypher ES Atomic Force Microscope (Oxford Instruments) using silicon probes (Applied NanoStructures-FORT) in the tapping mode in air. The images were analyzed with Gwyddion software.

Flow cytometry. Flow cytometry analysis was performed on Guava easyCyte 5 HPL Benchtop Flow Cytometer (Merk Millipore). The maximum acquisition time was set at 3.30 minutes and the maximum number of counts at 5000.

Protocols

Preparation of DNA-streptavidin nanogels (NG, NG-555 and NG-633). 3480 bp multibiotinylated DNA (bt-DNA) was obtained by PCR in presence of 50% of biotinylated dCTP (biotin-16-dCTP) in the dNTP mixture as described in [1]. For preparation of the nanogels, typically 2 µM (in phosphate groups) of bt-DNA was mixed on ice with 100 nM of either native streptavidin or streptavidin labeled with AlexaFluor633 (strep-633) in 10 mM phosphate buffer (pH 7.4) containing 75 mM NaCl and incubated on ice for 1h to give bare nanogels (NG) or fluorescent nanogels labeled with AlexaFluor555 (NG-555) or AlexaFluor633 (NG-633) respectively. When stated, proportional concentrations of bt-DNA and streptavidin have been used.

Preparation of cationic liposomes. Unlabeled liposomes (DMAPAP/DOPE 1:1 M/M) or FITC-labeled liposomes (containing 5% molar DOPE-FITC) at 20 mM were prepared as described in [2]. The liposomes were then diluted to the working concentration with NaCl 150 mM for further experiments. The diameter of the resulting liposomes was about 120 nm.

Formation of lipoproplexes (LPX). In a regular experiment, 1 volume of NGs in 10 mM phosphate buffer and 1 volume of the desired concentration of liposomes in 150 mM NaCl were mixed together and vortexed for one minute before being incubated at room temperature for at least 30 minutes prior to further experiments. Charge ratios (R+/- = [lipid charge]/[nanogel charge]) were calculated by taking the charge of DMAPAP to be +3 at physiological pH, and by considering that the NG charge originates solely from its DNA component (concentration of DNA's phosphate groups).

Flow cytometry analysis of LPX. NG and NG-633 have been obtained as described above with streptavidin (or strep-633) centrifugated at 10.000 rpm for 10 minutes to remove eventual aggregates, and the solutions of nuclease free water, 150 mM NaCl and 10 mM PB filtered with a 0.2 µm PVDF filter. 1 volume of NGs or controls (100 nM streptavidin only, 100 nM strep-633 only, 10 mM Phosphate Buffer only) was mixed with 1 volume of increasing amounts of either unlabeled DMAPAP/DOPE liposomes, liposomes labeled with 5% DOPE-FITC, or controls (NaCl 150 mM), vortexed and incubated at RT for at least 30 minutes. The charge ratios R+/- that have been analyzed are 0, 0.75, 1.5, 3, 4.5 and 9 and the conditions tested are FITC-LPX-633, LPX-633, FITC-LPX and liposomes-FITC. Aliquots of each sample were then analyzed by flow cytometry. FITC from the liposomes was detected with laser excitation at 488 nm and emission at 525±30 nm, whereas Alexafluor633 in the nanogels was detected with laser excitation at 640 nm and emission at 661±19 nm. Data analysis was made with InCyte 2.7 software.

Fluorescence microscopy analysis of LPX aggregation. 1 volume of NGs-633 prepared as previously described was mixed with 1 volume of liposome-FITC at 0, 0.5, 1, 2, 3 or 6 µM in 150 mM of NaCl to give charge ratios R+/- of 0, 0.75, 1.5, 3, 4.5 and 9 respectively, and incubated at room temperature for 30 minutes. Resulting samples were imaged at 37°C by epifluorescence microscope with Semrock LED-mCherry-A and Semrock LED-YFP-A filter for AlexaFluor633 and FITC fluorescence respectively. The average areas of the fluorescent signal with a cutoff area of 0.25-250 and 0.10 circularity were measured with particle analysis tools in opensource Fiji software.

B16 cells culture. Mouse melanoma cells (B16 cells) were grown in DMEM with Glutamax in 10% FBS (Fetal Bovine Serum), streptomycin (100 µg/ml) and penicillin (100 U/ml) at 37°C in presence of 5% CO_2.

Lipoproplexes intracellular delivery. On B16 cells, seeded at 60,000-70,000 cells/mL one day before the in 24 well plates, were added 400 µl of lipoproplex solution (or controls) and 400 µl of DMEM medium without serum. After 2h incubated at 37°C in the presence of 5% CO_2, the cells were washed with PBS 1X, fixed with 2% paraformaldehyde for 10 minutes and imaged by epifluorescence microscope equipped with Semrock LED-mCherry-A and Semrock LED-YFP-A filters. For confocal microscopy observation, B16 cells were seeded in on glass slides and deposited on 24-well plates before being transfected and fixed. First, cells were washed with PBS 1X and permeabilized with 0.1% Triton X-100 for 10 minutes, washed again three times with PBS 1X and stained with 250 µL of 0.1X Phalloidin 505 and 1 µg/mL DAPI for 30 minutes at room temperature and subsequently washed again with PBS 1X.

Kinetics of nanogels internalization. 1 volume of NGs and 1 volume of 1.5 µM liposomes or FITC-liposomes were mixed together with charge ratio R+/- = 1.5 and incubated at room temperature for 30 minutes to form lipoproplexes. Then, 400 µl of lipoproplexes (or control samples) and 400 µL of DMEM medium without serum were loaded on sub-confluent cells plated one day before the experiment and incubated for 30, 60 or 120 min at 37°C before been washed and fixed with 2% PFA. Cells were then imaged with epifluorescence microscope equipped with Semrock LED-mCherry-A filter to detect AlexaFluor633 fluorescence. The average areas of fluorescence intensity of AlexaFluor633 fluorophore (corresponding to nanogels) of three individual images were analyzed with ImageJ opensource software. The background subtraction, rolling ball radius=50 and sliding paraboloid were applied and the thresholder was set between 30 and 65535. Area of particles were analyzed with 50-Infinity μm^2 cut-off.

Intracellular delivery of GFP

Preparation of GFP-functionalized nanogels (btGFP-NG) and lipoproplexes (btGFP-LPX). Strep-633 at 2 mg/mL and biotinylated GFP (bt-GFP) at 1 mg/mL were centrifuged for 10 min at 6°C at 12700 rpm in order to remove any eventual aggregates. Nuclease-Free Water, phosphate buffer and NaCl were filtered with 0.2 µm PES filter prior to further analysis. Nanogels were prepared by mixing 4 µM in phosphate groups of bt-DNA with 200 nM of either strep-633 or native streptavidin. Then, 1 volume of 200 nM bt-GFP in 10 mM phosphate buffer was added to the NGs-633, NGs or the control, and the sample was vortexed, and incubated for 1 hour on ice protected from the light. As control, a solution containing only 200 nM bt-GFP and a solution containing NGs-633 not functionalized with GFP were also prepared. 1 volume of GFP-NGs, NGs-633 and GFP-NGs-633 was then mixed with 1 volume of 1 µM of liposomes in 150 mM NaCl, vortexed extensively for 1 minute and incubation for 30 minutes at room temperature. As control, GFP-NGs, NGs-633, GFP-NGs-633, bt-GFP alone and streptavidin AlexaFluor633 alone were mixed with 1 volume of 150 mM NaCl and incubated in the same conditions.

Preparation of DNA-streptactin nanogels (NG-649) and GFP-functionalized DNA-streptactin nanogels (stGFP-NG-649). Similarly to the formation of NG-633, the NG-649 were formed by mixing on ice of 2 µM bt-DNA with increasing amounts of streptactin labeled with DY649 in 10 mM phosphate buffer (pH 7.4). To form the stGFP-NG-649, first 7.32µM of streptactin was incubated with equimolar amount of twin-streptag-bearing GFP (st-GFP) on ice. Different amounts of the resulting stGFP-streptactin complex were then added to 2 µM bt-DNA and incubated on ice for 2 hours. To obtain the corresponding lipoproplexes (stGFP-LPX-649), 1 volume of stGFP-

NGs-649 in 10mM phosphate buffer, was mixed with 1 volume of 1 µM of liposomes in 150 mM NaCl, vortexed for 1 minute and incubation for 30 minutes at room temperature.

Intracellular delivery of GFP-functionalized lipoproplexes. B16 cells were seeded at 60,000-70,000 cells/mL one day before the delivery experiment in 24 well plates. Then, the cell medium was removed and 400 µL of each sample were loaded to the plates. Then 400 µL of DMEM without serum was added to each well. B16 cells were incubated with GFP-LPX for 2h at 37°C in the presence of 5% CO_2. After 2 hours, the medium was replaced with 1 mL of fresh DMEM/FBS. After 1h, the cells were washed two times with PBS 1X and fixed with 500 µL of 4% PFA for 10 minutes. Then, cells were then washed again three times with PBS 1X, and nuclei stained with 250 µL of 1 µg/mL DAPI for 5 minutes at room temperature and subsequently washed again with PBS 1X. The samples were then imaged by confocal microscopy.

Transfection of alkaline phosphatase (ALP)

Preparation of ALP-functionalized nanogels and lipoproplexes. bt-DNA was mixed with strep-ALP conjugate to give final concentrations 1 µM in DNA phosphate groups and 15 nM in strep-ALP. 1 volume of NGs-ALP was then mixed with 1 volume of 500 nM liposomes and incubated at room temperature for 30 minutes to form ALP-LPX.

Transfection of ALP-LPX in B16 cells. In 24-well plates, 400 µl of ALP-LPX or controls and 400 µL of DMEM medium without serum were loaded on sub-confluent B16 cells and incubated for 2 hours at 37°C in the presence of 5% CO_2. Then, cells were washed three times with pre-warmed DMEM/F-12 medium. 500µL of 20 µM DDAO-P substrate in DMEM/F-12 were added followed by additional 40 min of incubation at 37°C in the presence of 5% CO_2. Substrate was then removed and cells were washed twice before been fixed with 4% PFA. Cells were then imaged by epifluorescence with Semrock Cy5-4040A filter to visualize the fluorescence of hydrolyzed substrate (DDAO). Images were treated using open source FiJi software. Mask for particle analysis of individual cells was obtained by combining 'find maxima segmentation' and fluorescence thresholding, as described in [3]. Mean fluorescence intensity for each cell was extracted and analyzed after subtraction of the mean fluorescence intensity of the background.

Flow cytometry analysis for determination of ALP functionalized nanogels internalization in B16 cells. After removing the DDAO-P substrate, the cells were gently washed with PBS 1X, detached using Trypsin-EDTA and centrifuged for 10 minutes at 600 g. The supernatants were removed and the cells pellets were resuspended in 4% PFA for 10 minutes and diluted in PBS 1X prior to further analysis. Each sample was analyzed by flow cytometry. The fluorescence of hydrolyzed DDAO was detected inside the cells with laser excitation at 640 nm and emission at 661/19 nm. Data analysis was performed with online https://floreada.io/ Flow cytometry analysis platform.

In vitro ALP enzymatic activity assay. 1 volume of ALP-LPX or controls was mixed with 1 volume of DMEM without phenol red. Then, 1 volume of DDAO-P substrate (20 µM) in DMEM/F-12 was added to 1 volume of the sample and each control. The substrate hydrolysis was followed by measuring the fluorescence of DDAO product in 96 well plates at 590/645 nm for 1h at 37°C using Synergy HT microplate reader (from BioTek) with Gene5

interface. The measured fluorescence intensity for ALP alone is taken as 100% enzymatic activity. Each sample was prepared in triplicate. Final [NaCl]=75 mM.

Immunolabeling assay. For immunolabeling assay, cells were grown at 30000 cells per well 24h before experiment on 96-well plates with coverslip bottom for high throughput microscopy (Ibidi). Immunolabelling was directly performed on well plate after cell fixation. Antibodies against EEA1 (1/200; rabbit monoclonal antibody, MA5-14794; Invitrogen), LAMP1 (1/200; rabbit monoclonal antibody, MA5-32491; Invitrogen) and tubulin (1/100; rabbit polyclonal antibody, LiCOR) were used for immunolabeling of B16 cells after incubation with fluorescent LPX. Depending on the king of fluorescent nanogel used for experiments, fluorescent secondary antibody was chosen to allow the observation without crossover of dye fluorescence spectra. Then alexaFluor647 conjugated secondary anti-rabbit antibody (Fisher Scientific, A21244; 1/500) or AlexaFluor55 conjugated secondary anti-rabbit antibody (Fisher Scientific, A21428; 1/500) was then incubated for 1h at RT and nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI; 0.3 µg/mL, Sigma-Aldrich, D1388). The cells were then examined under confocal fluorescence microscopy, (SP8 Leica Microsystems) at 400X magnification. Images were acquired using the software Leica Acquisition System X and treated using open source FiJi software.

MTT assay. The metabolism of viable cells is able to reduce yellow MTT into purple formazan crystals. Different concentrations of LPX were formed my mixing NGs and liposomes at fixed ratio R +/- = 1.5 ([NG] = 0.25, 0.5; [liposomes] = 0.125, 0.25 µM). After 2 hours of incubation of samples on B16 cells, the medium was removed and replaced with fresh DMEM medium in presence of serum and incubated. The supernatant was then replaced with 1 mM of MTT solution in DMEM medium. After 2h of incubation, the supernatant was removed, and the formed formazan crystals were suspended in 50 µL of isopropanol containing 0.06 M HCl and 0.5% SDS. The results are given as absorbance of the resulting samples measured at 570 nm on a BioKinetics microplate reader EL340, and are compared with the control sample where cells were incubated in the buffer alone.

Statistical analysis. Prism 10.1.1 (GraphPad Software Inc.©, USA) was used for statistical analysis. Data were analyzed by Kruskal-Wallis test after being assessed for non-normal distribution. The number of repetitions and statistical tests used for each data set is indicated in the figure legends.

2. Supplementary figures

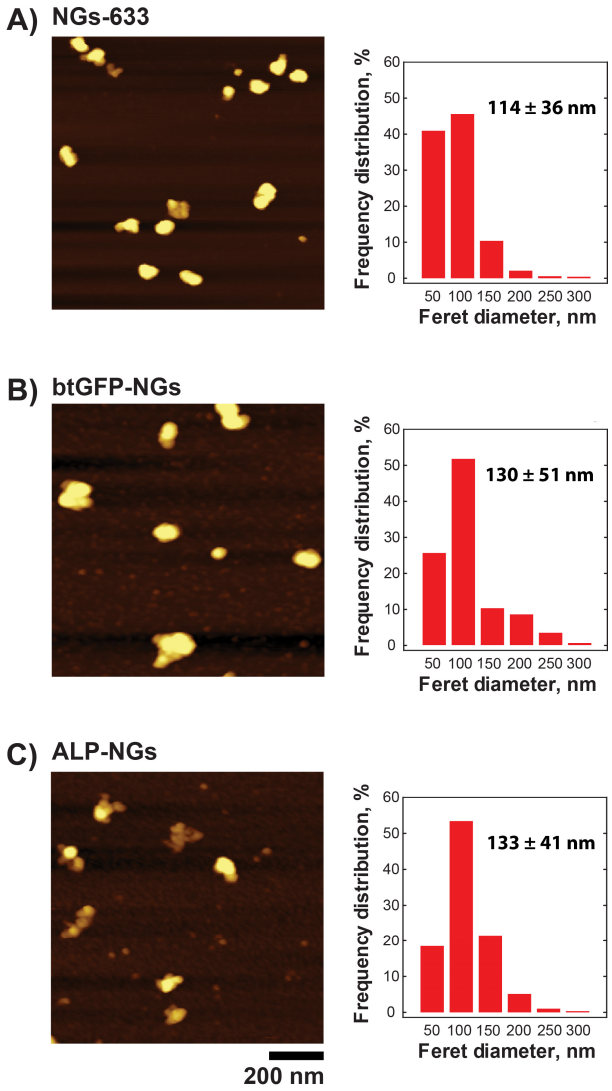

Figure S1. AFM images and frequency size distribution for different nanogels. A) AlexaFluor633-labeled nanogels (NGs-633), B) Nanogels functionalized with biotinylated GFP (btGFP-NGs), and C) Alkaline phosphatase-functionalized nanogels (ALP-NGs). Normalized frequency shows distributions of the long axis length (Ferret diameter) of individual objects observed on two 5x5 μm^2 AFM images for each condition. NGs-633: count = 714; btGFP-NGs: count = 176; ALP-NGs: count = 292.

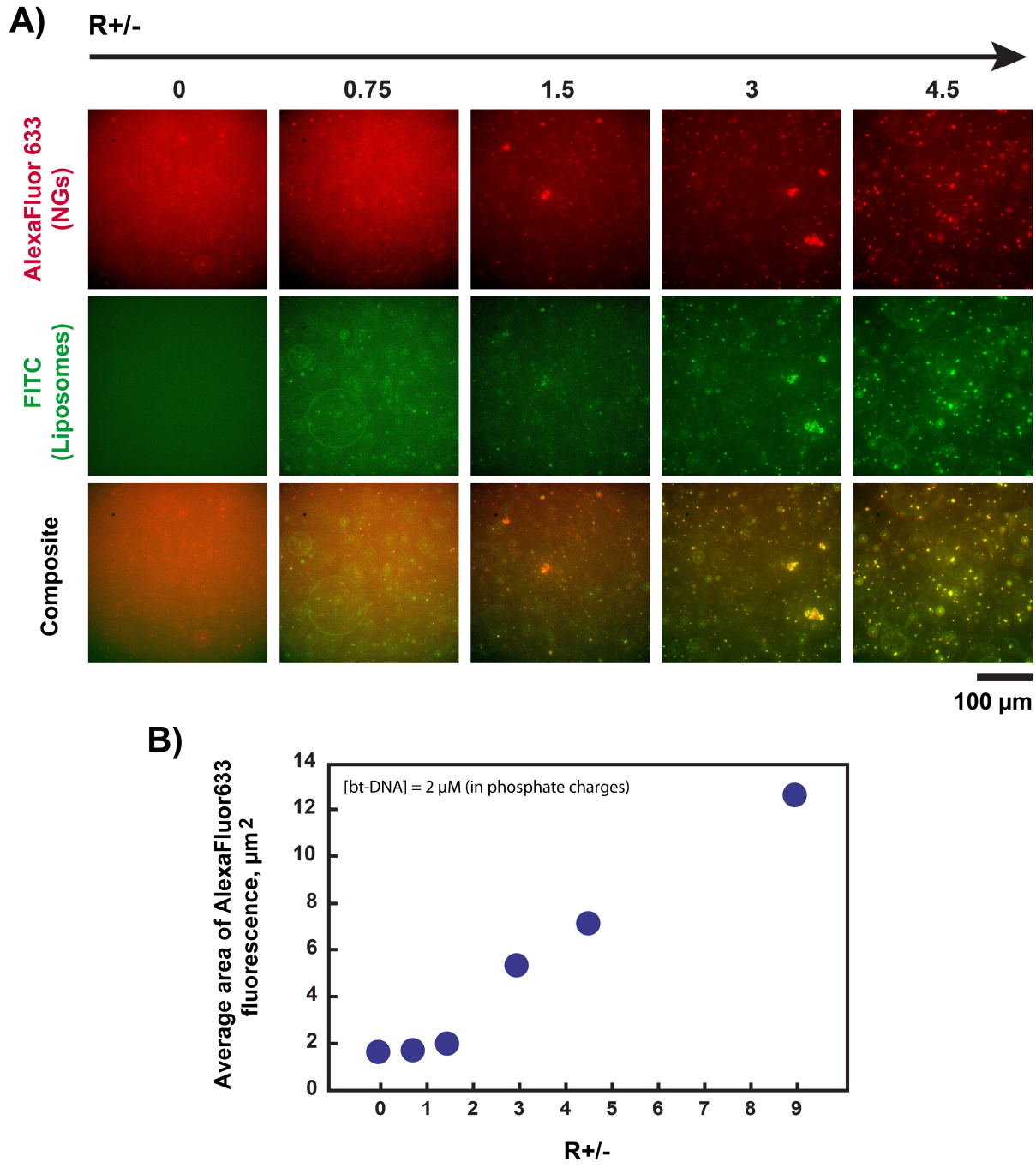

Figure S2. Fluorescent microscopy analysis of lipoproplex aggregation. A) Fluorescent microscopy observation of NG-633/liposome-FITC complexes at increasing R+/- charge ratios. Concentration of fluorescent AlexaFluor633-labeled nanogels (NG-633) is fixed at 2 µM (in term of DNA's phosphate groups). Composite images are obtained by overlaying of both fluorescence emissions. B) The average area of AlexaFluor633 emission signal as function of R+/-. The count for analyzed objects varied from 35 to 175. Halos are observed around the positions of out-of-focus bright aggregates.

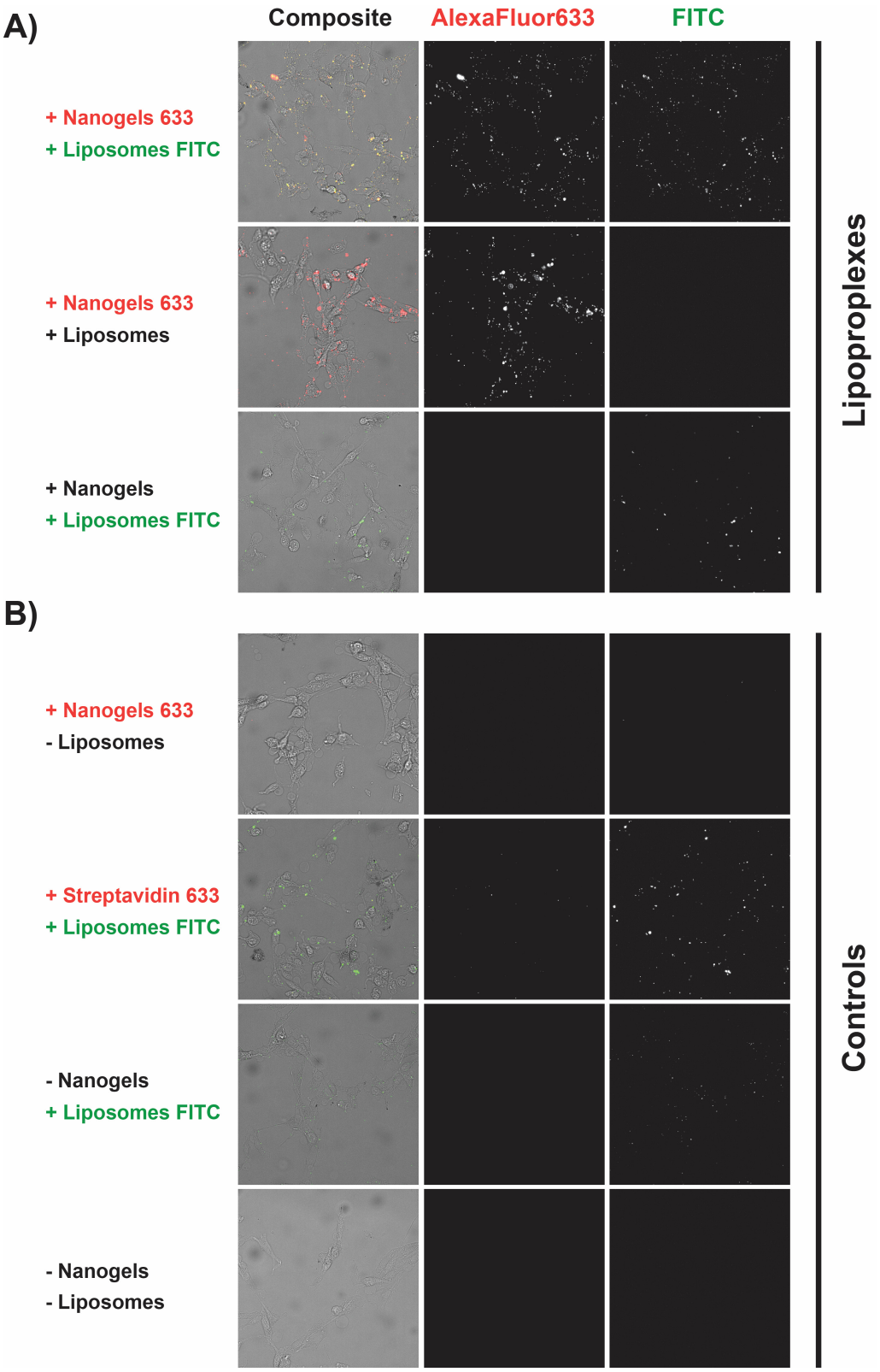

Figure S3. Wide-field microscopy observation of B16 cells treated with different lipoproplexes and controls. FITC fluorescence (liposomes), AlexaFluor633 fluorescence (NGs) and composite of fluorescence images with

transmission microscopy for: **A) Different lipoproplexes** (formed with fluorescent AlexaFluor633 nanogels and fluorescent FITC-labeled liposomes (+Nanogels 633, +Liposomes FITC); fluorescent nanogels and non-labeled liposomes (+Nanogels 633, +Liposomes); or non-labeled nanogels and FITC-labeled liposomes (+Nanogels, +Liposomes FITC)). R+/- = 1.5 for all samples. **B) The controls** (fluorescent NGs in absence of liposomes (+Nanogels 633, -Liposomes), fluorescent AlexaFluor633-streptavidin with FITC-labeled liposomes (+Streptavidin 633, +Liposomes FITC), labeled liposomes only (-Nanogels, +Liposomes FITC) and in absence of both NGs and liposomes (-Nanogels 633, -Liposomes).

For all conditions, B16 cells were incubated for 2 hours in the presence of sample or control solutions. The cells were imaged after 2h incubation washing and fixing. AlexaFluor633 fluorescence was observed with Semrock LED-mCherry-A filter (λ_{Ex} = 578 ± 11 nm / λ_{Em} = 641 ± 38 nm), and FITC was detected with Semrock LED-YFP-A filter (λ_{Ex} = 509 ± 11 nm / λ_{Em} = 544 ± 12 nm).

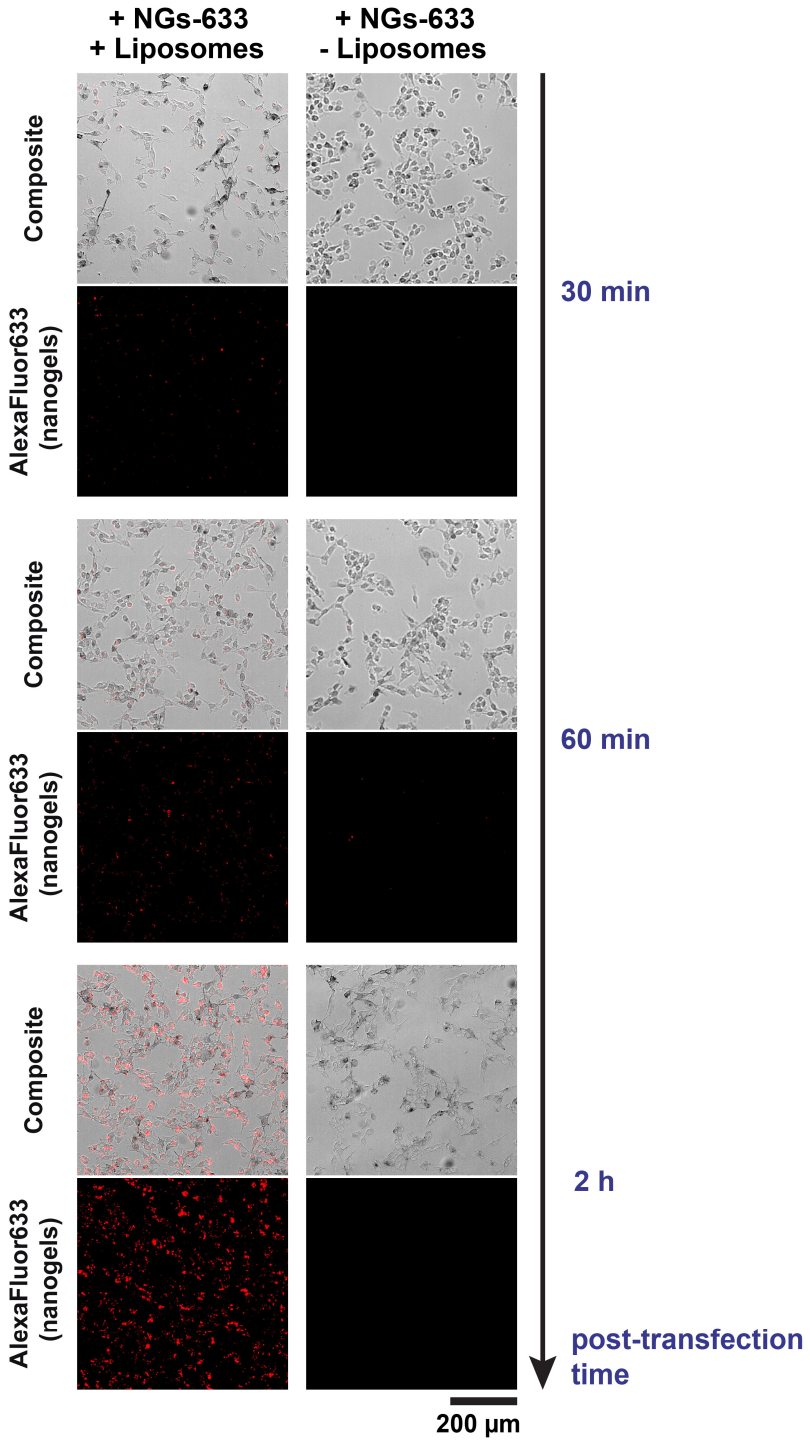

Figure S4. Kinetics of lipoproplexes internalization. Epifluorescence images for B16 cells incubated for 30, 60, or 120 min in the presence of either fluorescent nanogels (+NGs-633, - Liposomes) or lipoproplexes composed of fluorescent nanogels and liposomes (+NGs, +Liposomes). AlexaFluor633 signal from fluorescent nanogels is detected with Semrock LED-mCherry-A filter (λ_{Ex}= 578 ± 11 nm and λ_{Em} = 641 ± 38 nm) and B16 cells are visualized with a bright field filter.

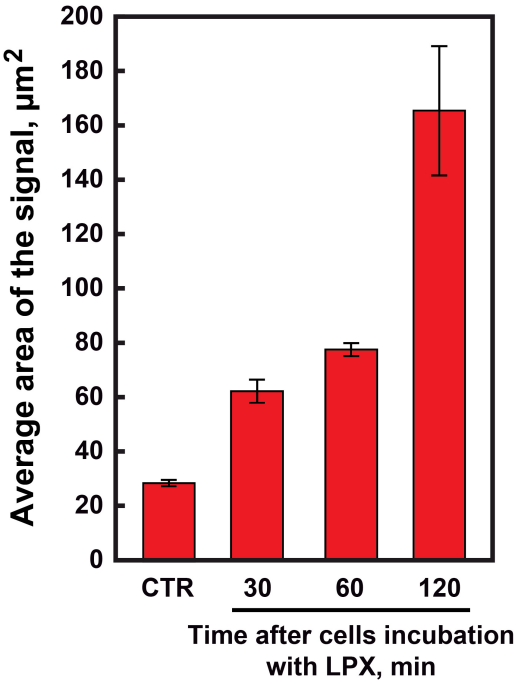

Figure S5. Analysis of the kinetic of lipoproplexes internalization. Fluorescence of AlexaFluor633 labeled NGs for conditions shown in the Figure S4. Each histogram and the standard deviation represent the analysis of the average areas of three individual images. The average counts were 336, 2446, 2696, 2210 for CTRL, 30 minutes, 60 minutes and 2 hours incubation respectively. The control (CTR) represents background fluorescence intensity of cells incubated with buffer solution.

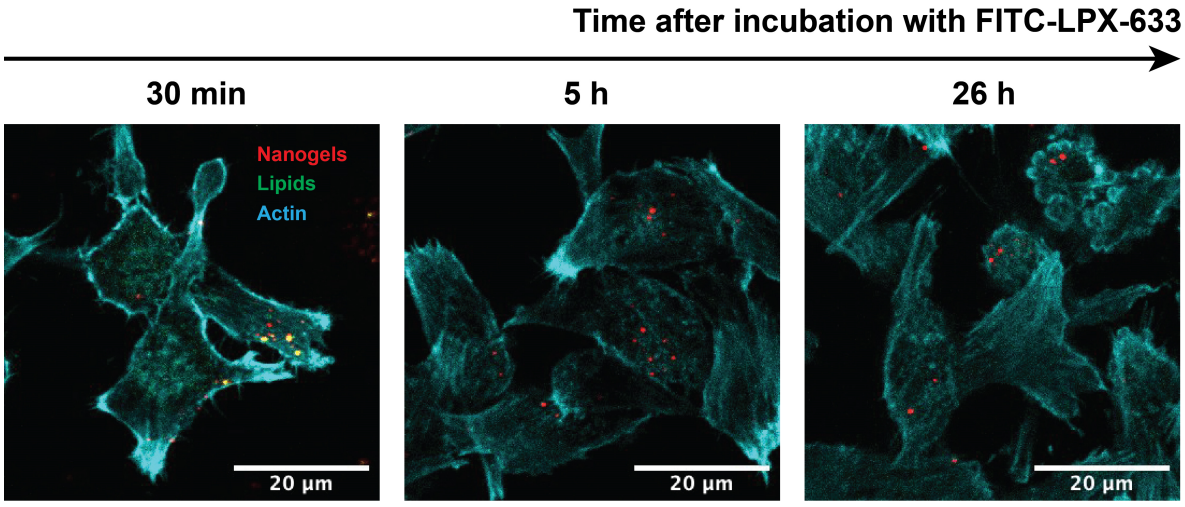

Figure S6. Confocal microscopy images of B16 cells at different times after incubation with FITC-LPX-633. Composite images for B16 cells observed 30 min, 5 hours and 24 hours after treating them with lipoproplexes formed from AlexaFluor633-labeled nanogels with FITC-labeled liposomes (FITC-LPX-633). Actin filaments labeled with phalloidin are shown in cyan, NG-633 are shown in red, and FITC-labeled lipids are shown in green. Yellow spots observed at 30 minutes after incubation thus correspond to the colocalization of nanogels with lipids.

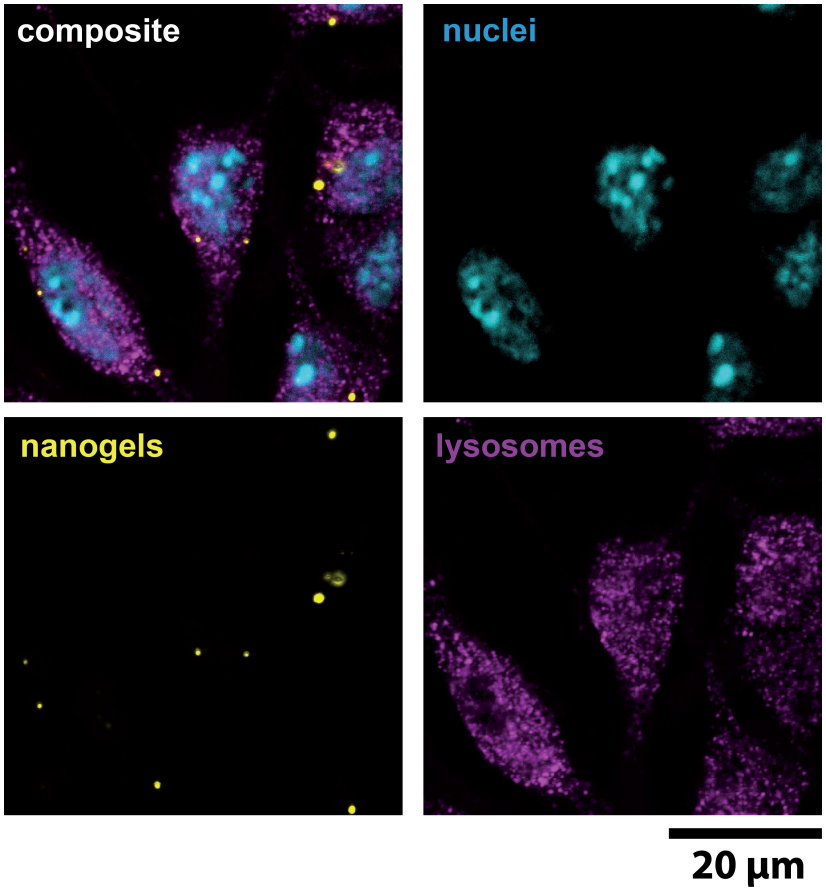

Figure S7. LAMP1 immunolabeling on B16 cells after LPX-633 incubation. Overlap of LAMP-1 (lysosome immunolabeling), NG-555 and DAPI (nuclei staining) signals obtained by confocal microscopy analysis (Top left panel), DAPI signal (cyan color, top right panel), NG-555 signal (yellow, bottom left panel) and LAMP-1 lysosomes immunolabeling signal (magenta, bottom right panel).

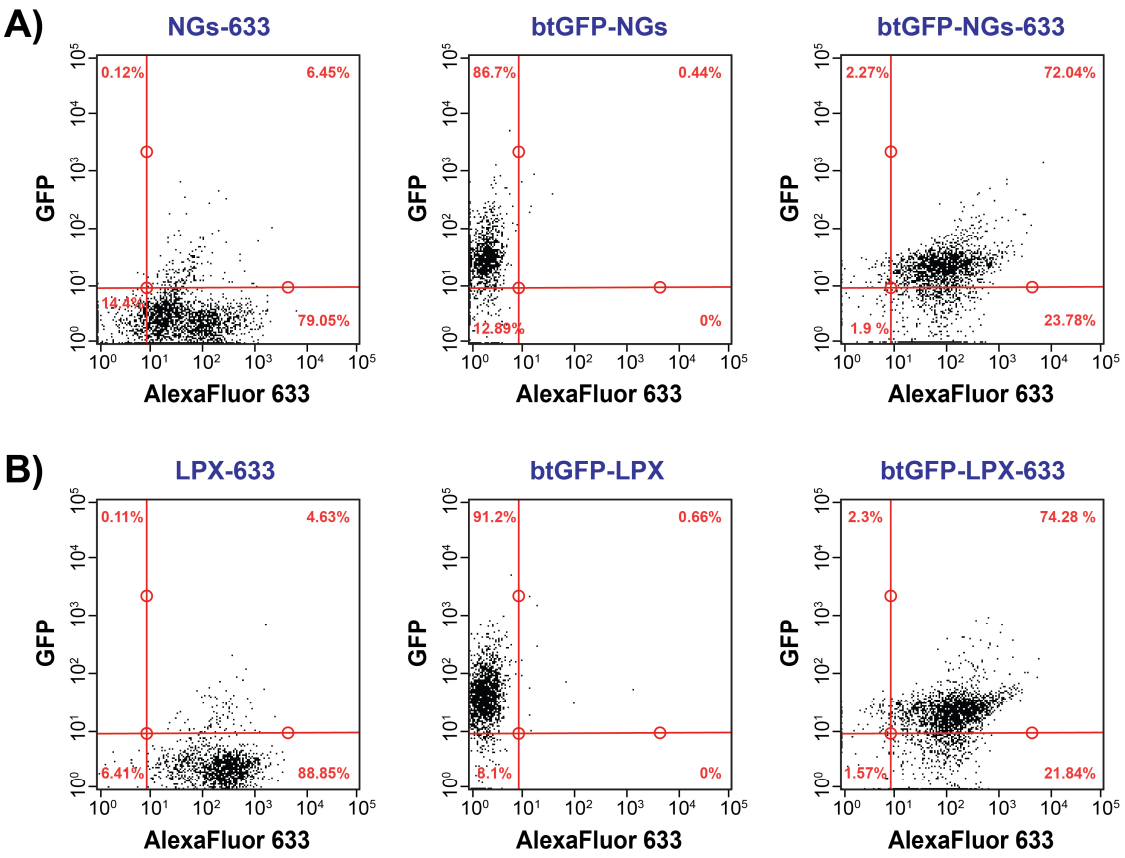

Figure S8. Flow cytometry analysis of GFP-functionalized nanogels and lipoproplexes. A) Dot plots of nanogels functionalized with AlexaFluor633 only (NG-633), GFP only (GFP-NG) and both AlexaFluor633 and GFP (GFP-NGs-633). B) Dot plots for the same NGs as in (A), but in the form of lipoproplexes (complexed with non-labeled liposomes). Red lines separate the dot plots into 4 quadrants. Red numbers give the percentage of the observed events in each quadrant.

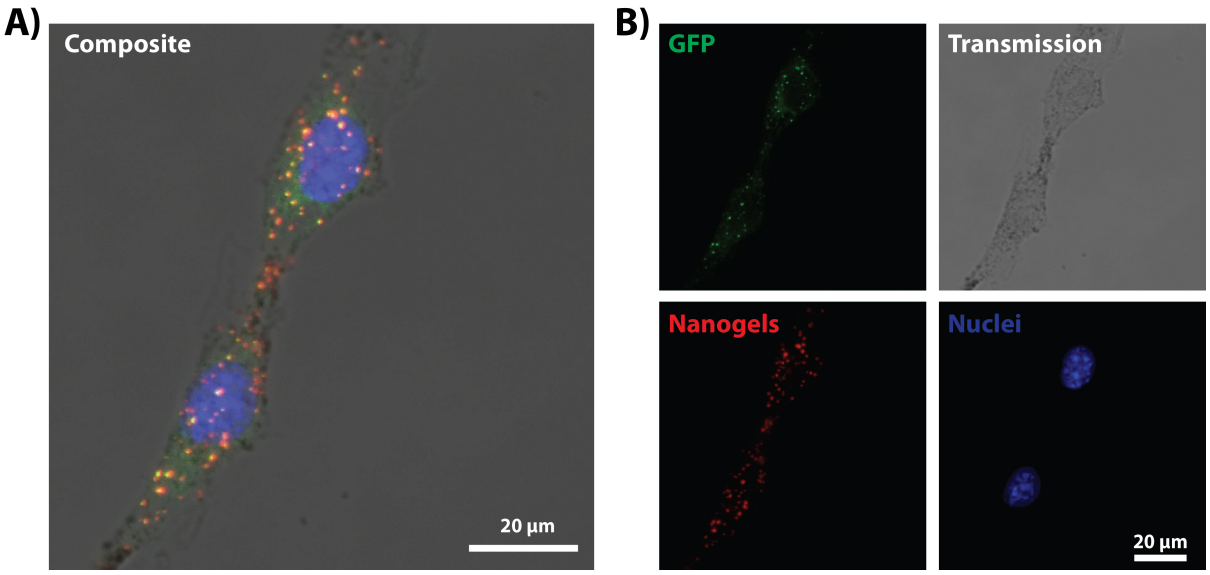

Figure S9. Z-stack projection of confocal planes for B16 cells treated with GFP-functionalized lipoproplexes (btGFP-LPX-633). Transmission and confocal microscopy images of B16 cells incubated with btGFP-LPX-633. Cells are imaged in transmission light and overlayed with fluorescence signal from confocal observations. Green color corresponds to the GFP (λ_{Ex}= 488 nm and λ_{Em} = 495-550 nm), blue to the nucleus stained with DAPI (λ_{Ex}= 405 nm and λ_{Em} = 415-450 nm), and red to nanogels containing AlexaFluor633-labeled streptavidin (λ_{Ex}= 638 nm and λ_{Em} = 645-700 nm). Composite image is shown in (A), and separate channels in (B). Z-depth = 5.79 µm.

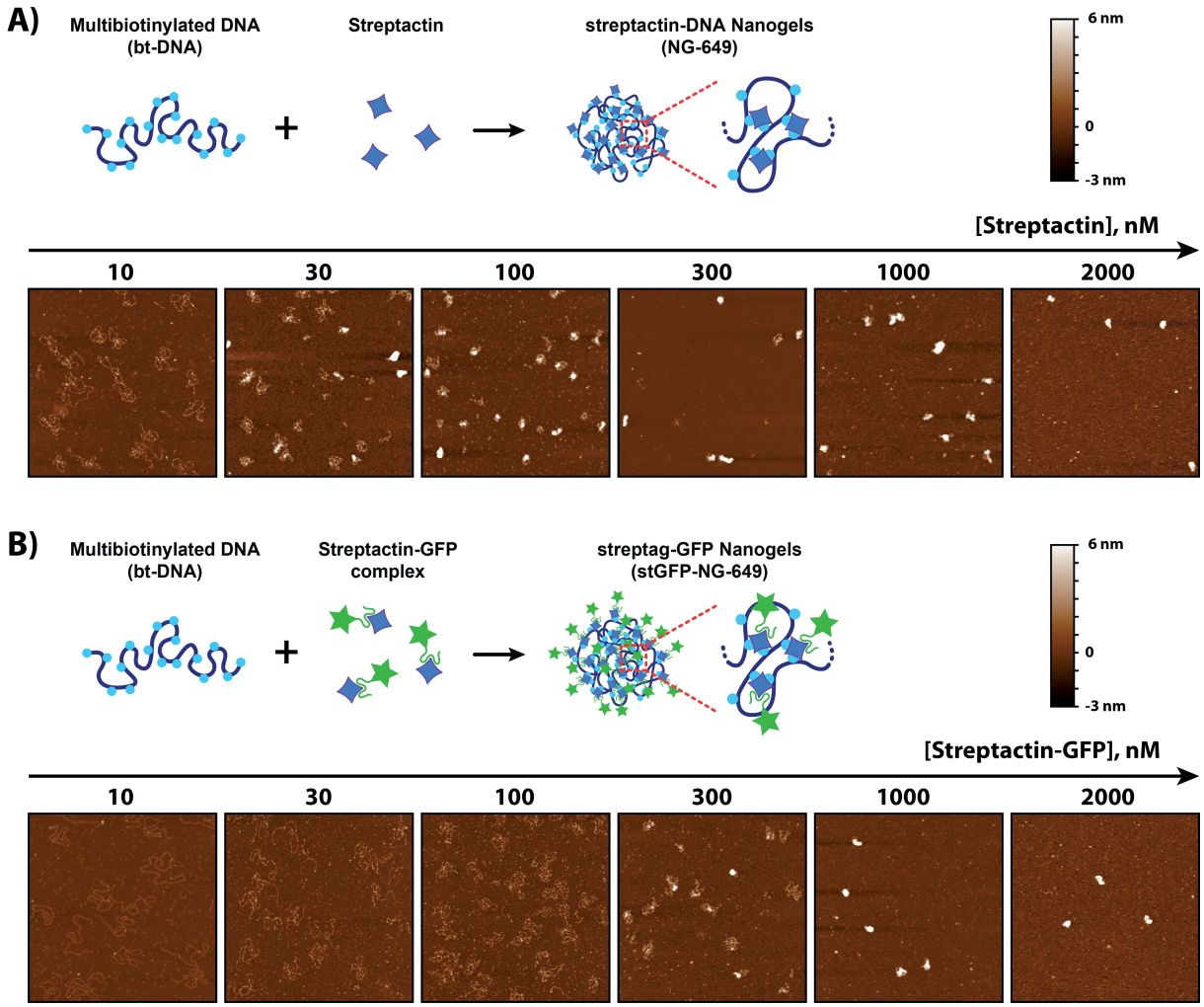

Figure S10. DNA-streptactin nanogels and their functionalization with st-GFP. Schemes (top) and AFM observations (bottom) of formation of nanogels with streptactin alone (A) and equimolar stGFP-sreptactin complex (B). Streptactin is labeled with DY649, and st-GFP bears the twin-strep-tag. All images are 1700 nm x 1700 nm.

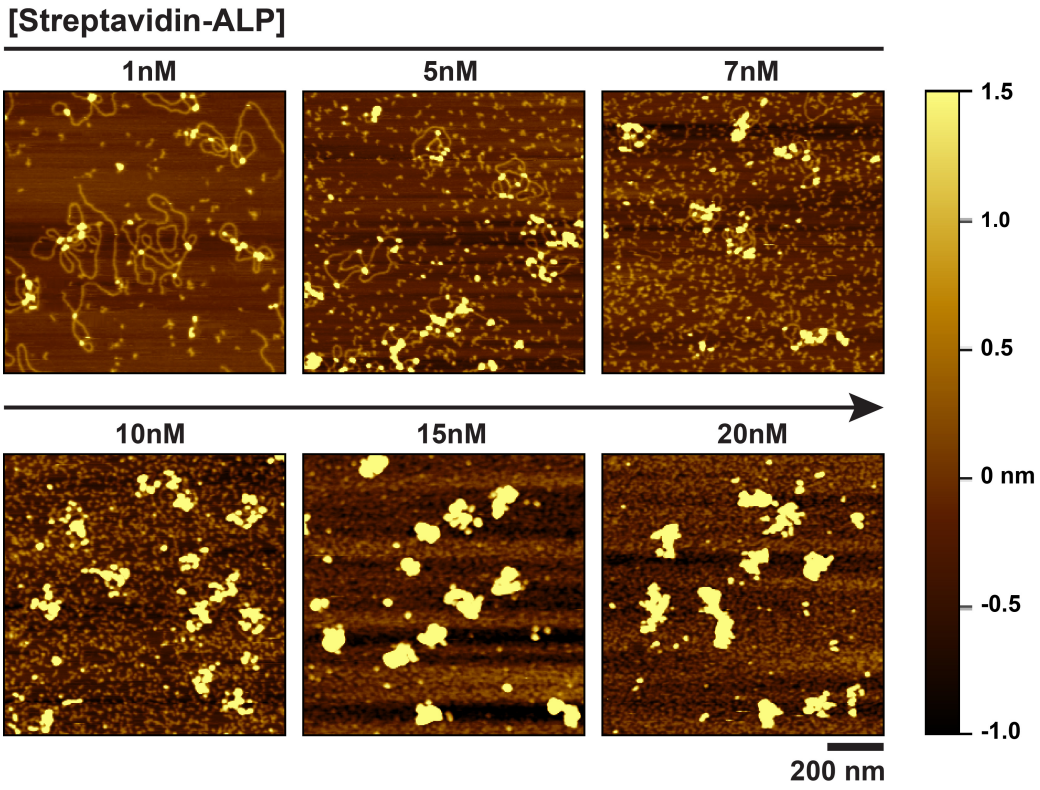

Figure S11. AFM observation of formation of nanogels functionalized with streptavidin-Alkaline phosphatase conjugate (NGs-ALP). AFM observation of bt-DNA (1 μM in phosphate groups) in presence of increasing concentrations of streptavidin-ALP conjugate. The height color scale bar is given at the right.

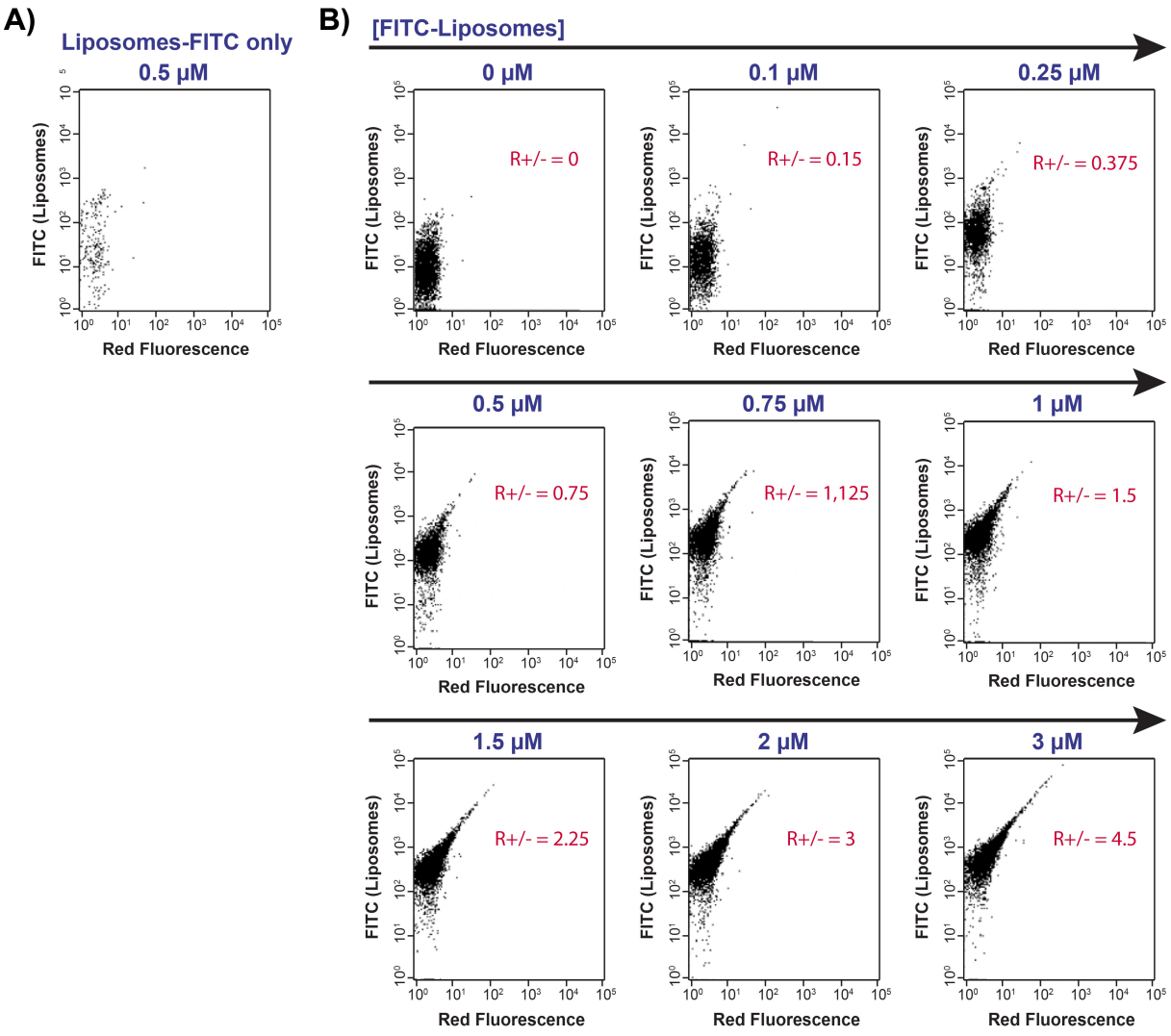

Figure S12. Flow cytometry analysis of formation of ALP-functionalized lipoproplexes. A) Dot-plot for FITC-labeled liposomes only (0.5 µM). B) Dot plots for ALP-NGs (2 µM of bt-DNA with 30 nM strep-ALP) in presence of increasing concentrations of FITC-labeled liposomes. Corresponding R+/- charge ratios are given in red.

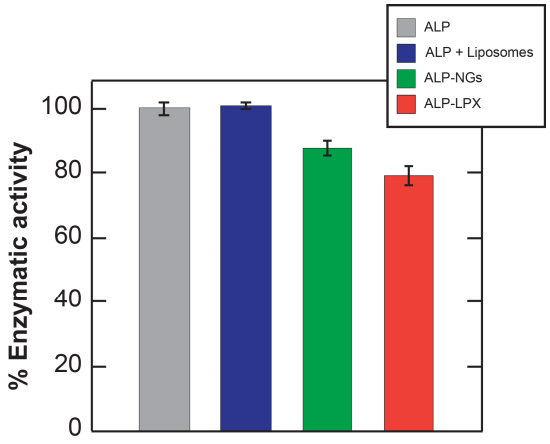

Figure S13. Effect of incorporation into nanogels and lipoproplexes on the acellular enzymatic activity of alkaline phosphatase. Comparison of enzymatic activity for ALP-streptavidin conjugate alone (ALP), ALP in presence of DMAPAP/DOPE liposomes (ALP+liposomes), ALP incorporated into DNA nanogels (ALP-NGs), and lipoproplexes formed by complexation between the liposomes and ALP-NGs (ALP-LPX) at R = 0.75. The measured fluorescence intensity of hydrolyzed substrate (DDAO) for ALP alone is taken as 100% enzymatic activity.

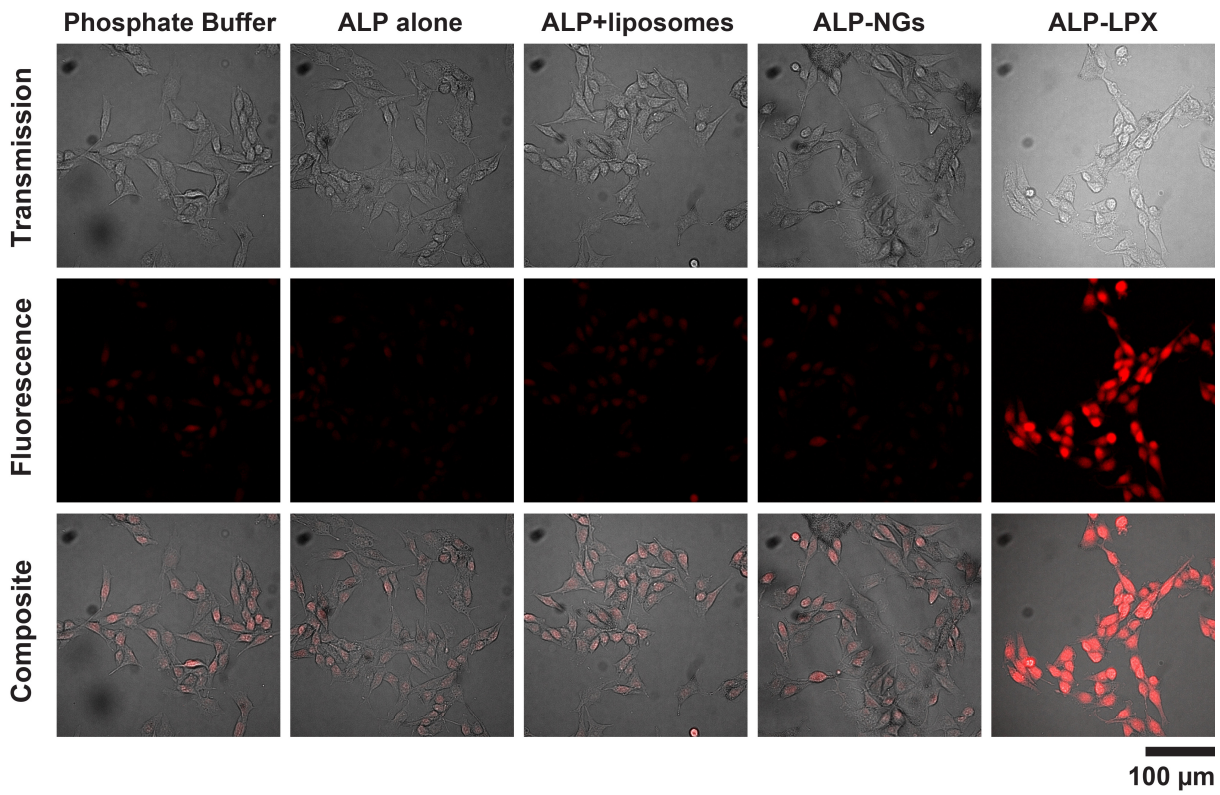

Figure S14. Fluorescence microscopy detection of intracellular catalytic activity of ALP after intracellular delivery of ALP-LPX. Transmission, fluorescence and composite images for B16 cells treated with ALP-LPX (R+/- = 0.75) and controls (ALP-NG without liposomes; ALP in presence of liposomes, ALP alone and buffer only). Observed fluorescence emission was due to hydrolysis of DDAO-P substrate to give fluorescent DDAO (detected with λ_{Ex} = 628 ± 20 nm and λ_{Em} = 692 ± 20 nm). Mean fluorescence distributions for this experiment are given in the Figure S15B.

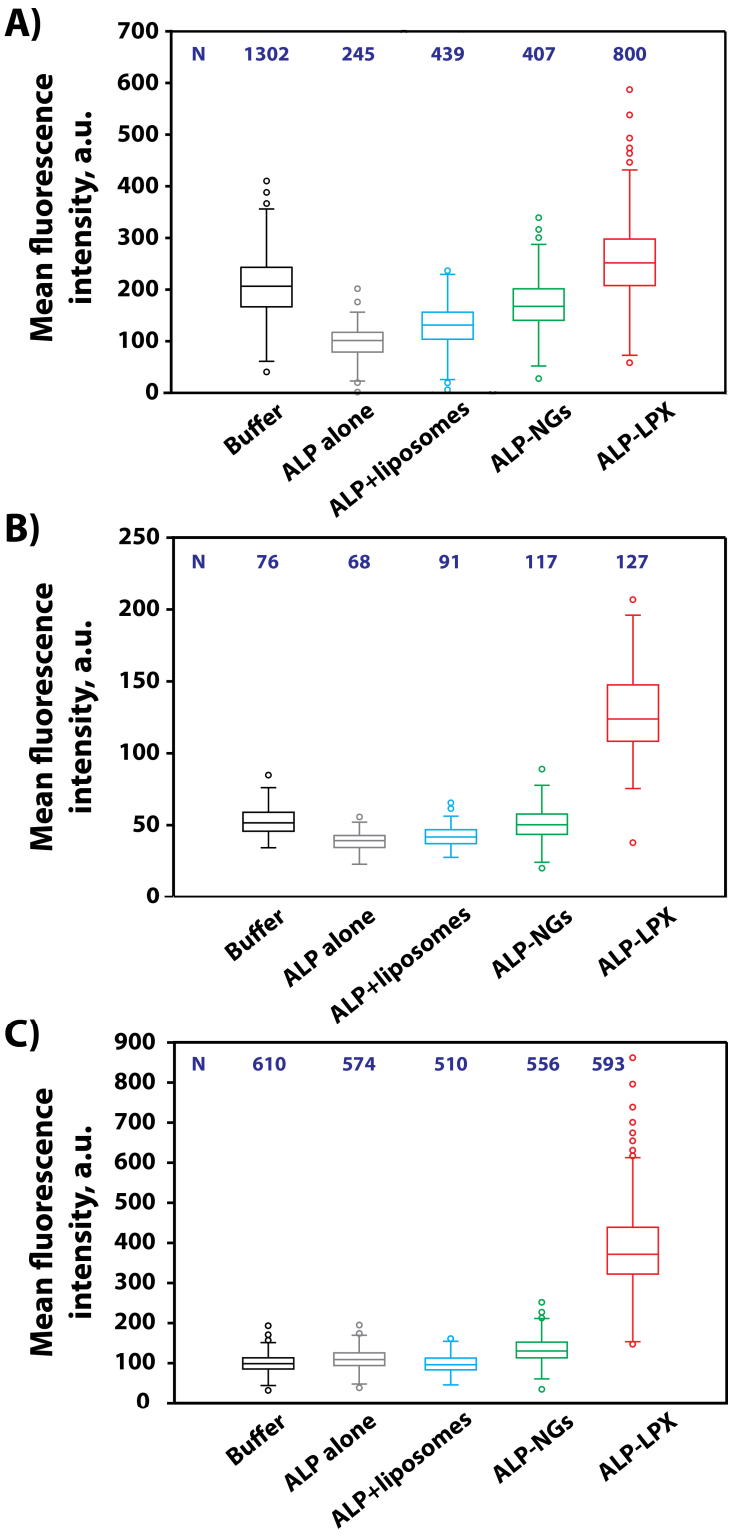

Figure S15. Box plot fluorescence distributions for 3 individual experiments of ALP-LPX delivery. Boxes delimitate 1st and 3rd quartiles of mean fluorescence intensity of hydrolyzed substrate (DDAO) measured by fluorescence microscopy. Solid horizontal lines indicate the median values, whiskers are 1.5 times the interquartile range, and circles show the outlier points. Numbers on the top (N) show the count of analyzed cells in each sample. Mean fluorescence intensity corresponds to the absolute values of measured mean fluorescence of DDAO per cell.

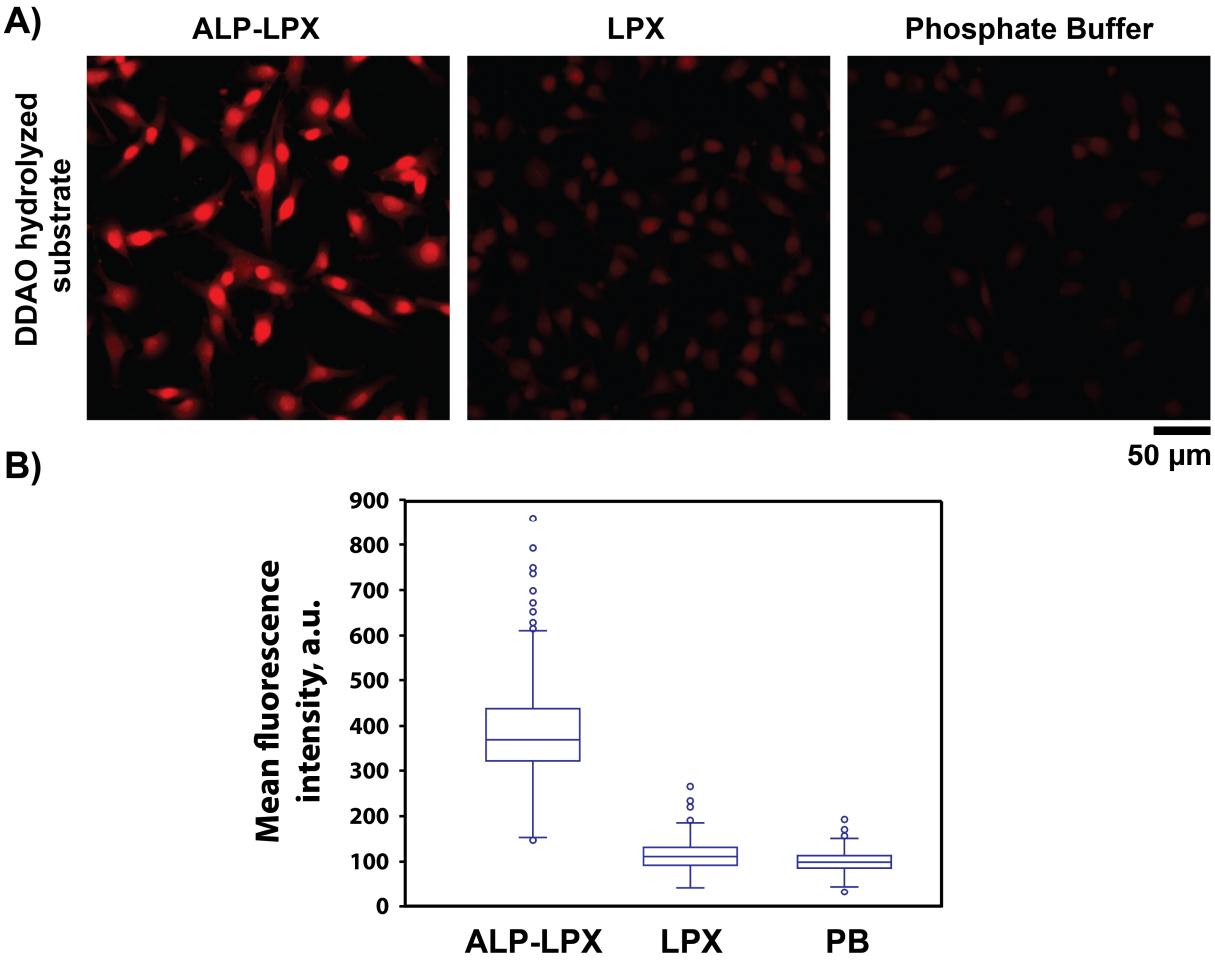

Figure S16. Comparison of the ALP enzymatic activity of B16 treated with ALP-functionalized and non-functionalized lipoproplexes. A) Fluorescence microscopy observation of hydrolyzed DDAO-P substrate (detected at λ_{Ex} = 628 ± 20 nm and λ_{Em} = 692 ± 20 nm). B) Box plot distribution of the mean fluorescence intensity hydrolyzed DDAO-P for each condition from A (measured by fluorescence microscopy). Boxes delimitate 1st and 3rd quartiles of mean fluorescence intensity. Solid horizontal lines indicate the median values, whiskers are 1.5 times the interquartile range, and circles show the outlier points.

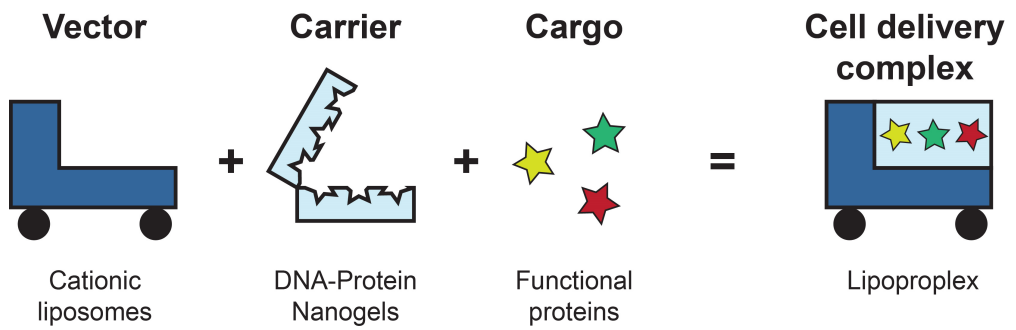

Figure S17. Schematic representation of lipoproplex-based proteins delivery platform. Lipoproplex platform is constructed from three elements: cargo molecules are loaded into the DNA-streptavidin nanogels carrier via streptavidin-biotin interactions. Successful cell delivery then requires a cationic vector (in our case – cationic liposomes) to enable the internalization by cells of the DNA-protein nanogels.

3. References

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