Intracellular delivery of more than one protein with spatio-temporal control

Miguel M. Lino [a], Susana Simões [a], Sónia Pinho [a] and Lino Ferreira [a,b].*

[a] CNC-Center for Neuroscience and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal.

[b] Faculty of Medicine, University of Coimbra, 3000-548 Coimbra, Portugal.

Supporting Information

Experimental Section

Materials. β-Galactosidase (β-Gal) from *Escherichia coli*, thiolated oligonucleotide strands, hexadecyltrimethylammonium bromide (CTAB), sodium borohydride ($NaBH_4$), silver nitrate ($AgNO_3$), hexanethiol and mercaptohexanoic acid, and other chemicals were all purchased from Sigma-Aldrich and used as received without further purification. Sodium dodecyl sulfate (SDS) was purchased from VWR. Bovine Serum Albumin (BSA), DyLight-NHS ester, N-[γ-maleimidobutyryloxy] sulfosuccinimide ester (sulfo-GMBS), LysoTracker® Green DND-26, LysoTracker® Red DND-99, fetal bovine serum (FBS) and trypsin-EDTA solution were purchased from Thermo Fisher Scientific. Dulbecco's Modified Essential Medium (DMEM) is a commercial product of Merck Millipore. Purified water with resistivity above 18.2 $M\Omega.cm^{-1}$ was obtained by reverse osmosis (MilliQ, Millipore). Other reagents were analytical grade.

Characterization of protein conjugated with ssDNA by non-denaturing PAGE. The reaction mixture obtained after reacting the proteins with ssDNA and the fractions obtained after HPLC purification of the reaction mixture were analysed by gel electrophoresis. Reaction mixture (15 μL) and reaction mixture fractions obtained after HPLC purification (15 μL) were mixed with glycerol (5 μL; glycerol in 50% v/v of H_2O), loaded in a polyacrylamide gel (12%, w/v) and run for 45 min in 0.5 x TBE at 140 V. The gel was stained with SyBr Gold (1:5000 in 1 × TBE) for 10 min and imaged in a UV transilluminator (Molecular Imager Gel DOC, Biorad).

Heating profile of AuNR suspensions. To study the temperature variation induced by NIR-irradiation, AuNR suspensions (20 μg mL^{-1}, in PBS) were irradiated with a continuous wave NIR laser (780 nm). PBS solution was used as control. A 24 well plate was used and the volume for each well was fixed at 0.4 mL. The laser beam was collimated and expanded to a circular Gaussian beam with a diameter of about 9 mm. The laser was tuned to work at two power densities, 1.25 and 2 Wcm^{-2}. A FLIR SC5650 infrared camera was used to record and analyze the spatiotemporal temperature of the wells.

Thermal-induced release of BSA from AuNR. Suspensions of DL_{488}-BSA-dsDNA-AuNR (20 μgmL^{-1}), in 10 mM phosphate buffer pH 7.4 containing 30 mM NaCl, were heated at 1 ºC per minute in a thermocycler (Eppendorf). Immediately after reaching specific temperatures (25, 35, 45, 50, 55 and 60 ºC) the suspensions were centrifuged (30 min at 10000 g), the supernatant was collected and the amount of protein in the supernatant was determined by fluorescence using a calibration curve.

Uptake kinetics of βGal-dsDNA-AuNR. SC-1 cells were plated in a 24 well plate at a density of 5×10^4 cells/well and left to adhere overnight. The cells were incubated with βGal-dsDNA-AuNR (50

μg mL^{-1}) for 1, 2, 4, 6 and 24h. After incubation, in order to remove non-internalized nanorods, the cells were washed three times with PBS, dissociated with trypsin and counted. Finally, the samples were freeze-dried and the amount of gold was determined by inductive coupled plasma mass spectrometry (ICP-MS). In order to evaluate the levels of internalized AuNRs along the time, cells were incubated with βGal-dsDNA-AuNR for 4 h and after washing three times with PBS, cells were left in the incubator for 3, 6, 12 or 24 h. In a subset of samples, after incubation with βGal-dsDNA-AuNR, cells were treated with Mitomycin C (8 μg mL^{-1}, Sigma) for 2h30, to inhibit cell proliferation.

Uptake of BSA-dsDNA-AuNR in the presence of chemical endocytosis inhibitors. SC-1 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 4 h co-incubation with BSA-dsDNA-AuNR-TRITC. The following inhibitors were tested: EIPA (80 μM), nocodazole (1 μM), cytochalasin D (25 μM), polyinosinic acid (80 μM), filipin III (1 μM), dynasore (100 μM). In order to block the albumin receptor, pre-incubation and co-incubation with BSA (10 mg mL^{-1}) was also performed. In order to test the inhibitory effect of dynasore, FITC-labelled transferrin was used as control of clathrin-mediated endocytosis. Cells were exposed to culture medium with and without each inhibitor for 30 min and then incubated with FITC-labelled transferrin (15 μgmL^{-1}) for 3 min at 4 ºC and finally analysed by flow cytometry.

Cytotoxicity of BSA-dsDNA-AuNR. To assess the cytotoxicity of AuNRs, SC-1 fibroblasts were seeded on a 96 well plate (4×10^3 cells/well), left to adhere for 24 h and then incubated with AuNR-MHA or BSA-dsDNA51.7-AuNR (concentrations between 10 and 200 μg mL^{-1}) for 4 h. After incubation, cells were washed with medium to remove non-internalized AuNRs. In some conditions, after incubation with BSA-dsDNA-AuNR, cells were washed and irradiated with a fibercoupled Roithner laser (780 nm). Each well was placed below the end of the fibre and irradiated with a power

density of 1.25 or 2 W cm^{-2} for 2 min. Then cells were left in the incubator for 24 h and the ATP production was measured by a Celltiter-Glo Luminescent Cell Viability Assay (Promega).

In a separate experiment, the cytotoxicity of AuNRs was evaluated by an Annexin/PI assay. Cells were seeded in a 48 well plate (20000 cells/well), left to adhere for 24 h and incubated with BSA-dsDNA51.7-AuNR at 20, 50 and 100 μg mL^{-1}, for 4 h. After washing to remove non-internalized AuNRs, cells were irradiated at 1.25 or 2 W cm^{-2} for 2 min. After 24 h incubation at 37 ºC, the medium containing detached cells was collected and the adherent cells were rinsed with PBS and trypsinized. After centrifuging, the pellet was resuspended in 100 μL of binding buffer containing 2.5 μL of Annexin V-FITC conjugate (Invitrogen). After 15 min incubation, 100 μL of propidium iodide (2 μg mL^{-1}) were added to each tube and then the cells were kept on ice until analysis by flow cytometry.

Immunocytochemistry. Cells were seeded in gelatin coated coverslips and left to adhere for 24 h. After 4h incubation with βGal-dsDNA-AuNR (50 μg mL^{-1}), cells were irradiated with 780 nm laser at 1.25 W cm^{-2}. The samples were immediately fixed after irradiation with paraformaldehyde 4% (v/v) for 15 min at room temperature followed by the washing (3 times) with PBS. After blocking (PBS solution with 1% BSA), cells were incubated with a rabbit anti-β-galactosidase antibody (Invitrogen) for 60 min, washed three times with blocking buffer and incubated with alexa-fluor488 conjugated goat anti-rabbit IgG (dilution 1:1000) for 60 min. The excess of antibody was removed by washing with PBS before staining with DAPI (1 μg mL^{-1}) for 5 min. Coverslips were analyzed in a confocal microscope (LSM 710, Carl Zeiss). The corrected total cell fluorescence was quantified with ImageJ and corrected for background fluorescence. Manders' colocalization coefficient was calculated using Image J and JACoP plugin.

Intracellular localization of AuNR-TRITC. Cells were seeded in an IBIDI 15 well slide (5000 cells/well), left to adhere for 24 h and then incubated with BSA-dsDNA-AuNR-TRITC (50 μg mL^{-1}) for 4 h. After incubation, cells were washed with medium to remove non-internalized AuNRs. Then, the cells were incubated with LysoTracker® Green (100 nM) for 30 min to stain the endosomes and with Hoechst 33342 (1μg mL^{-1}) to stain the nuclei. Cells were observed under confocal microscope, immediately after laser irradiation at 780 nm (1.25 and 2 Wcm^{-2}). The images were analyzed in ImageJ and the colocalization was determined by calculating the Manders' colocalization coefficient between AuNR-TRITC and Lysotracker green.

Light-induced endosomal escape. To study endosomal escape, cells were incubated with BSA-dsDNA-AuNR in the presence of a membrane impermeable molecule, calcein. Briefly, 5000 cells/well in a IBIDI slide were incubated with 50 μg mL^{-1} of BSA-dsDNA-AuNR and 0.25 mM calcein for 4h. After removing AuNRs, cells were incubated with lysotracker red (100 nM) for 30 min. Then, medium was replaced and cells were irradiated with 780 nm laser for 2 min (1.25 and 2 Wcm^{-2}) and analyzed under confocal microscope.

Supplementary Data

Supplementary table 1. Oligonucleotide strands used for the conjugation to AuNRs and for protein modification. The melting temperature refers to a theoretical melting temperature relative to the portion of the strand that is able to hybridize with the complementary strand.

	Tm / ºC	sequence
AuNR conjugation	51.7	5'-HS-C6-TTTTTTTTTTTTTTTATAACTTCGTATA-3'
	68.9	5'-HS-C6-TTTTTTTTTTTTGTCCGGGTCCAGGGC-3'
DNA-protein conjugates	51.7	5'-HS-C6-TATACGAAGTTATAAAAAAAAAA-3'
	68.9	5'-HS-C6-TGCCCTGGACCCGGAC-3'

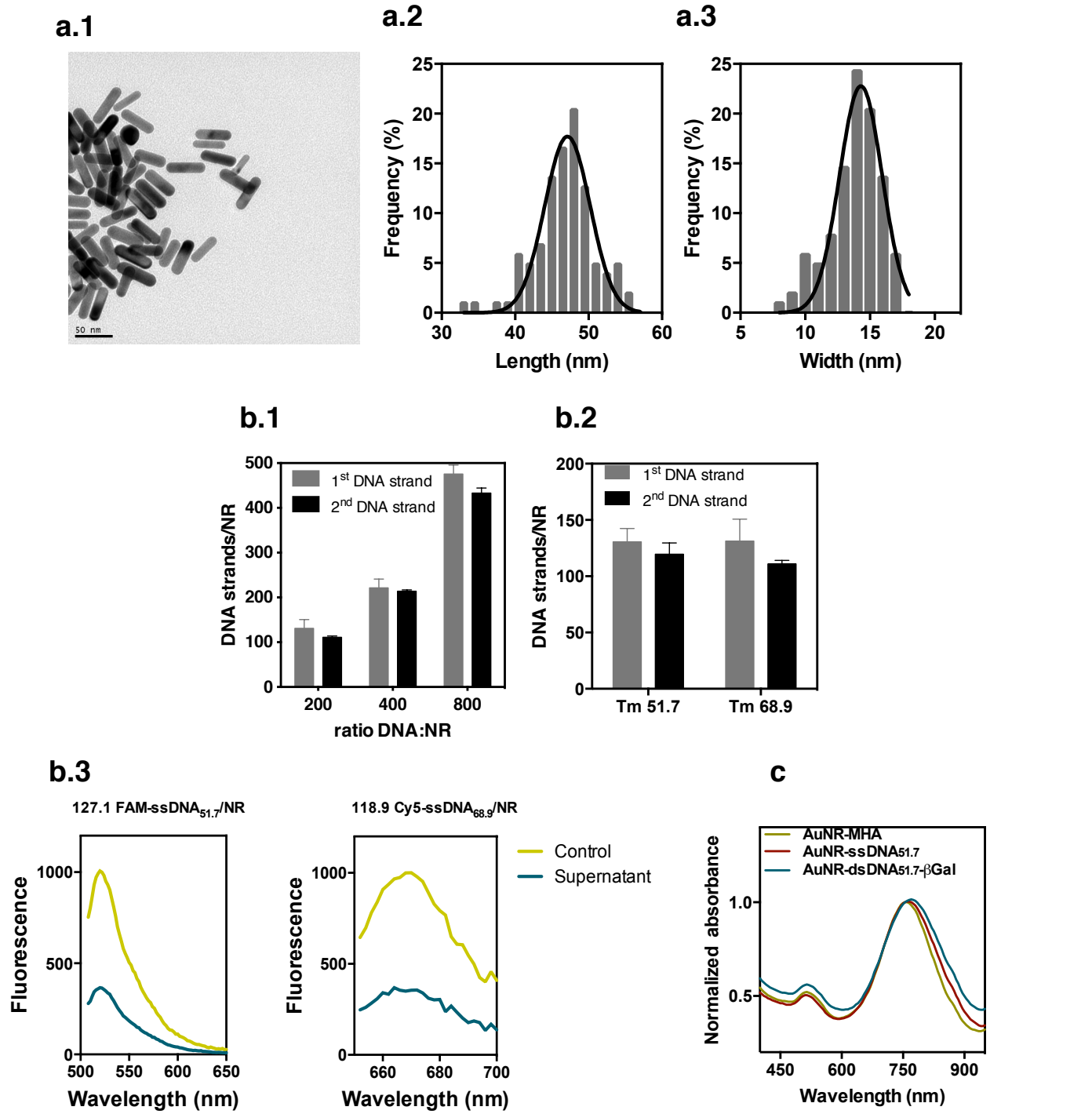

Figure S1. Characterization of AuNRs. (a) TEM analysis. (a.1) Representative TEM image of AuNRs. (a. 2-a.3) NR length (a.2) and width (a.3) distribution obtained from TEM images. The AuNRs showed an average length of 46.7 ± 4.1 nm, n=100 AuNRs and an average width of 13.8 ± 1.9 nm, n=100 AuNRs. (b.1) Quantification of oligonucleotides immobilized on NR surface via thiol gold chemistry (1st DNA strand) or hybridized to the ssDNA conjugated to the NR (2nd DNA strand). The amount of strands per AuNR was determined indirectly in the supernatant by measuring absorbance at 260 nm in Nanodrop. (b. 2) Comparison of the binding efficiency of the two sequences with different melting temperatures (91.7% efficiency for DNA strand with Tm 51.7 ºC and 84.5% efficiency for DNA strand with Tm 68.9 ºC). Results in b.1 and b.2 are Average ± SD, n=3. (b.3) Quantification of 2 oligonucleotides immobilized on AuNRs. AuNRs were reacted simultaneously with equivalent amounts of ssDNA51.7 labelled with fluoresceineamine and ssDNA68.9 labelled with Cy5 (the ratio between AuNR and each ssDNA was 1:200). Fluorescence in the supernatant was measured and compared with a control solution containing the same amount of ssDNAs used for immobilization. (c) Absorbance spectra of AuNRs after conjugation with mercaptohexanoic acid (AuNR-MHA); after conjugation with ssDNA51.7 (AuNR-ssDNA51.7) and after hybridization with β-Gal (AuNR-dsDNA51.7-βGal). Surface plasmon resonance band does not change significantly during surface modification.

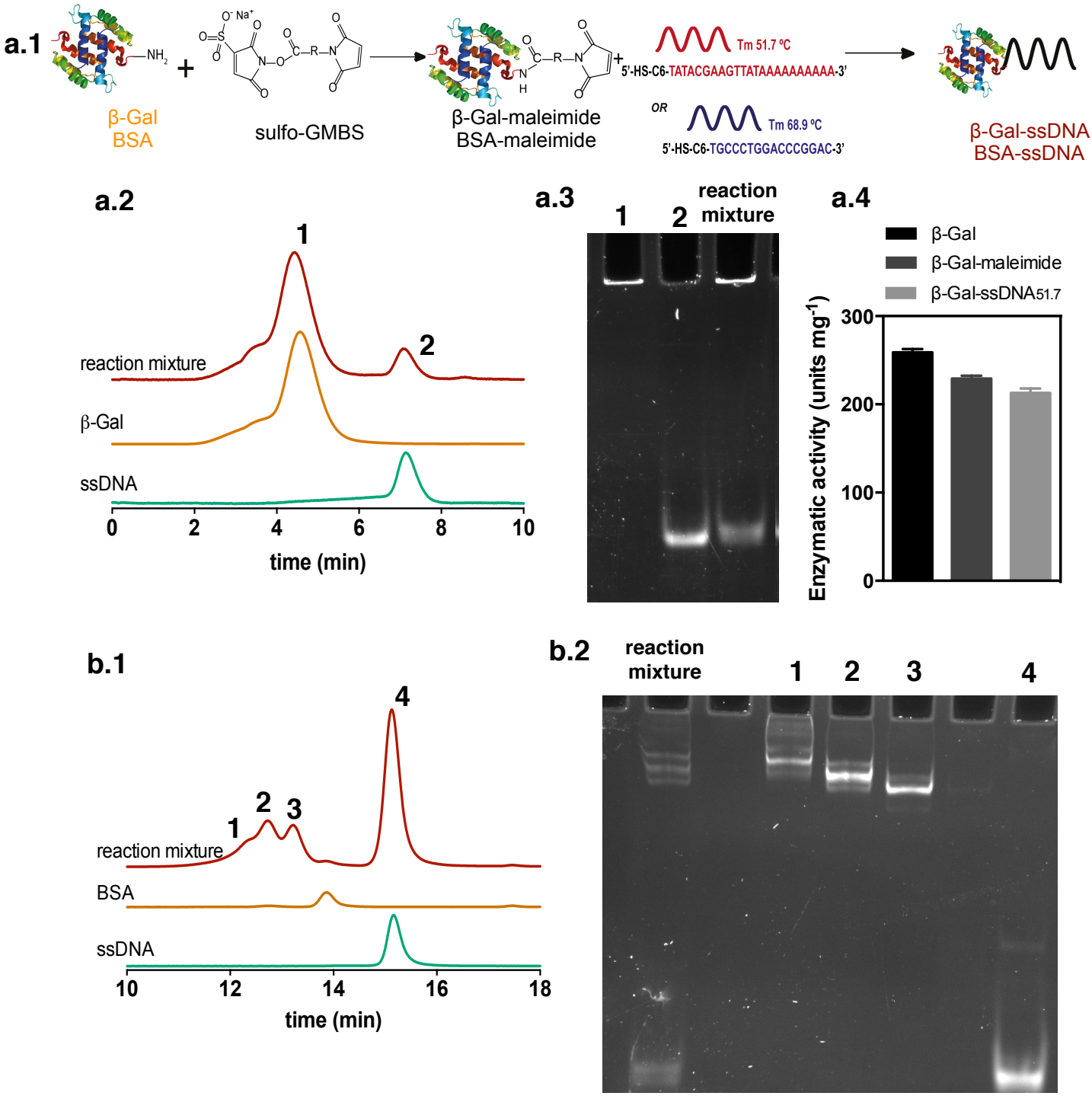

Figure S2. Characterization of oligonucleotide-protein conjugates. (a.1) Scheme showing the coupling reaction of ssDNA to the proteins. (a.2) HPLC characterization of the reaction mixture between β-Gal and ssDNA. Representative chromatograms of β-Gal, ssDNA and the final reaction mixture of β-Gal with ssDNA. Integration of DNA peaks indicate a conjugation value of 37% of the total DNA. This means that each protein molecule was conjugated with at least one oligonucleotide. (a.3) Electrophoresis characterization of the HPLC fractions. Fractions 1 and 2 were analysed. Samples were run in polyacrylamide gel and DNA (and not protein) was stained with Sybr Gold. Quantification of free DNA indicates a conjugation value of 35% of the total DNA. Fraction 1, which corresponds to the β-Gal-ssDNA conjugate was used for subsequent experiments. (a.4) Enzymatic activity β-Gal, β-Gal conjugated with sulfo-GMBS (β-Gal-maleimide) and β-Gal conjugated with ssDNA51.7 (β-Gal-ssDNA51.7). (b.1) HPLC characterization of the reaction mixture between BSA and ssDNA51.7. Representative chromatograms of BSA, ssDNA and the final reaction mixture of BSA with ssDNA. (b. 2) Electrophoresis characterization of the HPLC fractions. Fractions 1-4 were analyzed. Fraction 3 was used for conjugation with AuNR-ssDNA. We selected this fraction because each protein is conjugated with one ssDNA.

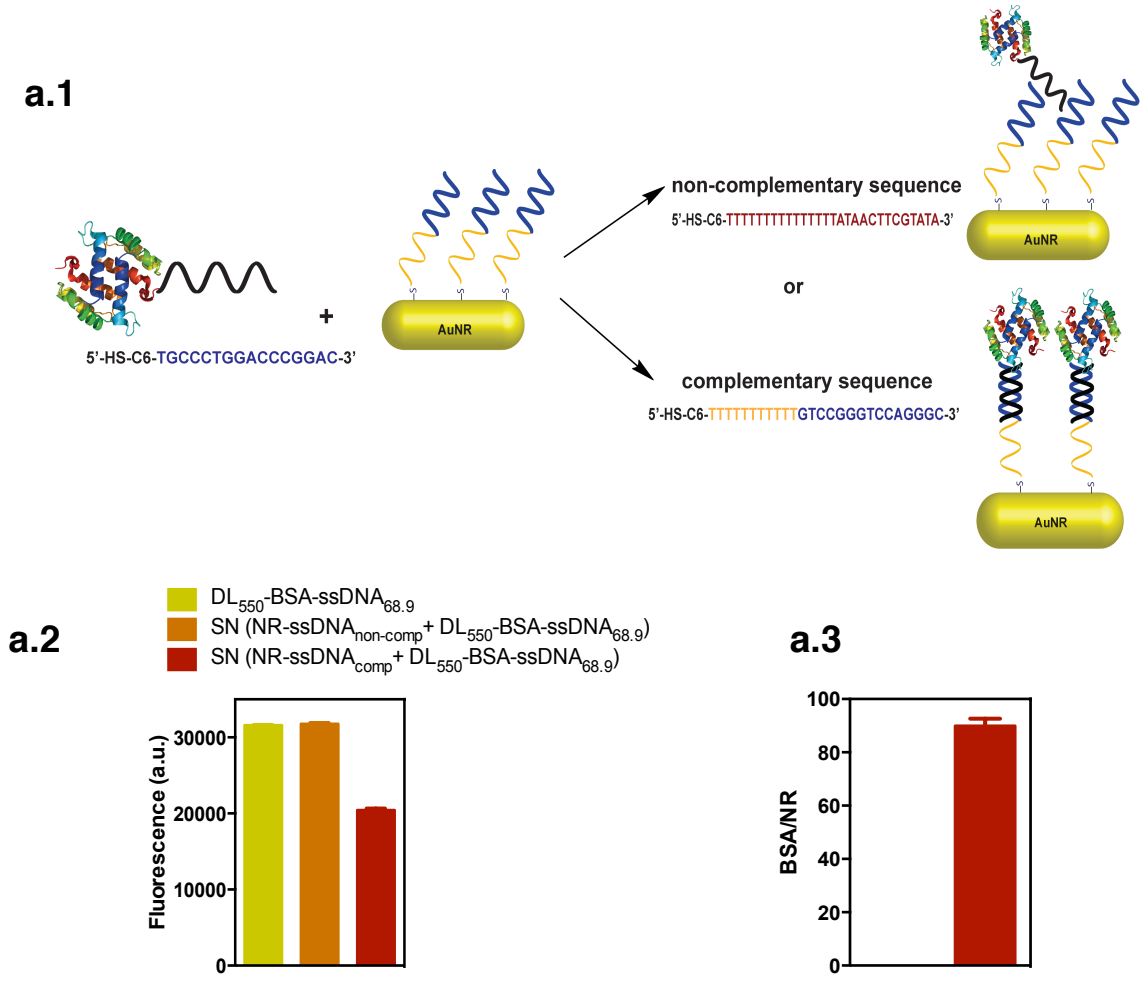

Figure S3. Specificity for the hybridization between AuNR-ssDNA and BSA-ssDNA. (a.1) Scheme showing the experiment set-up to demonstrate that the hybridization between AuNR-ssDNA$_{68.9}$ and BSA-ssDNA$_{68.9}$ was specific. (a.2) Fluorescence intensity of the supernatants collected after the hybridization of DL$_{550}$-BSA-ssDNA$_{68.9}$ to AuNRs conjugated with complementary (ssDNAcomp) or non-complementary (ssDNAnon-comp) oligonucleotide sequences. The ratio BSA to AuNRs was 1:300. DL$_{550}$-BSA-ssDNA$_{68.9}$ solution for hybridization was used as a control. (a.3) Number of proteins immobilised per AuNR, calculated indirectly from the fluorescence in the supernatant through a calibration curve (concentration between 2.5 and 50 nM, excitation at 480 nm and emission at 515 nm). In a.2 and a.3, results are Average ± SD, n=3.

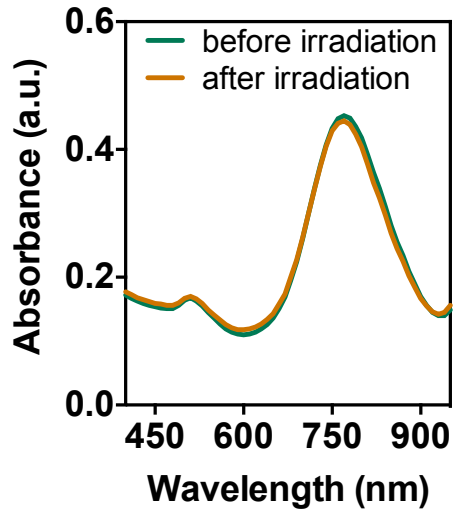

Figure S4. Absorbance spectra of BSA-dsDNA-AuNR (20 μg/mL) before and after laser irradiation (2 Wcm^{-2}, 2 min).

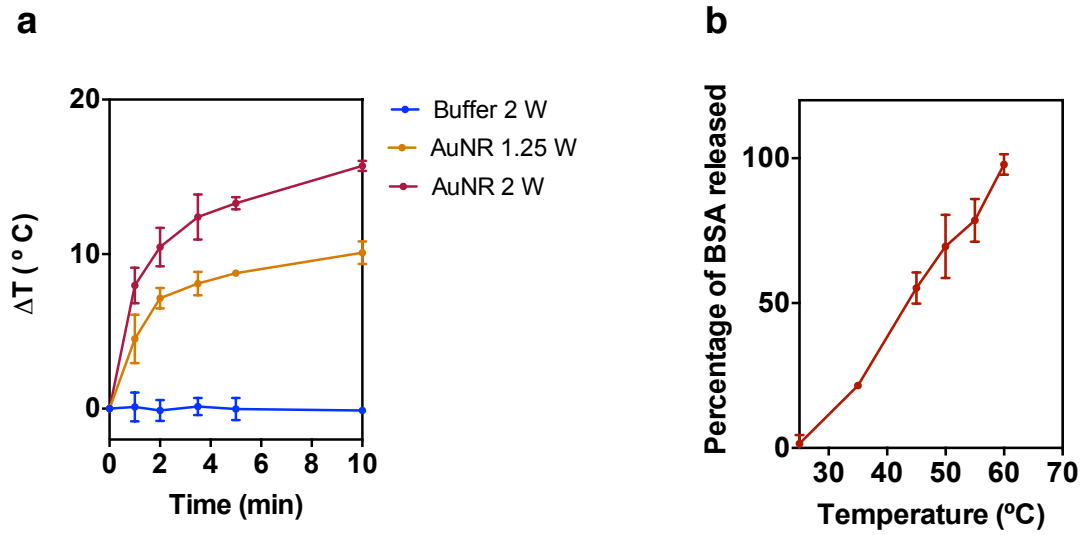

Figure S5. Heating profile of AuNRs. (a) Heating profile of AuNR suspensions irradiated with 780 nm laser with variable laser power. AuNR suspensions (20 μgmL^{-1}), in 10 mM phosphate buffer containing 30 mM NaCl, were irradiated with 780 nm laser and the temperature was measured using a FLIR SC5650 infrared camera. (b) Thermal release of BSA from AuNRs. Suspensions of DL488-BSA-dsDNA51.7-AuNR (20 μgmL^{-1}) were heated at 1 ºC per minute. The amount of BSA released is plotted as a function of the temperature of the suspension. After reaching different temperatures, the suspensions were immediately centrifuged, the supernatant was collected and the amount of DL488-BSA in the supernatant was determined by fluorescence using a calibration curve.

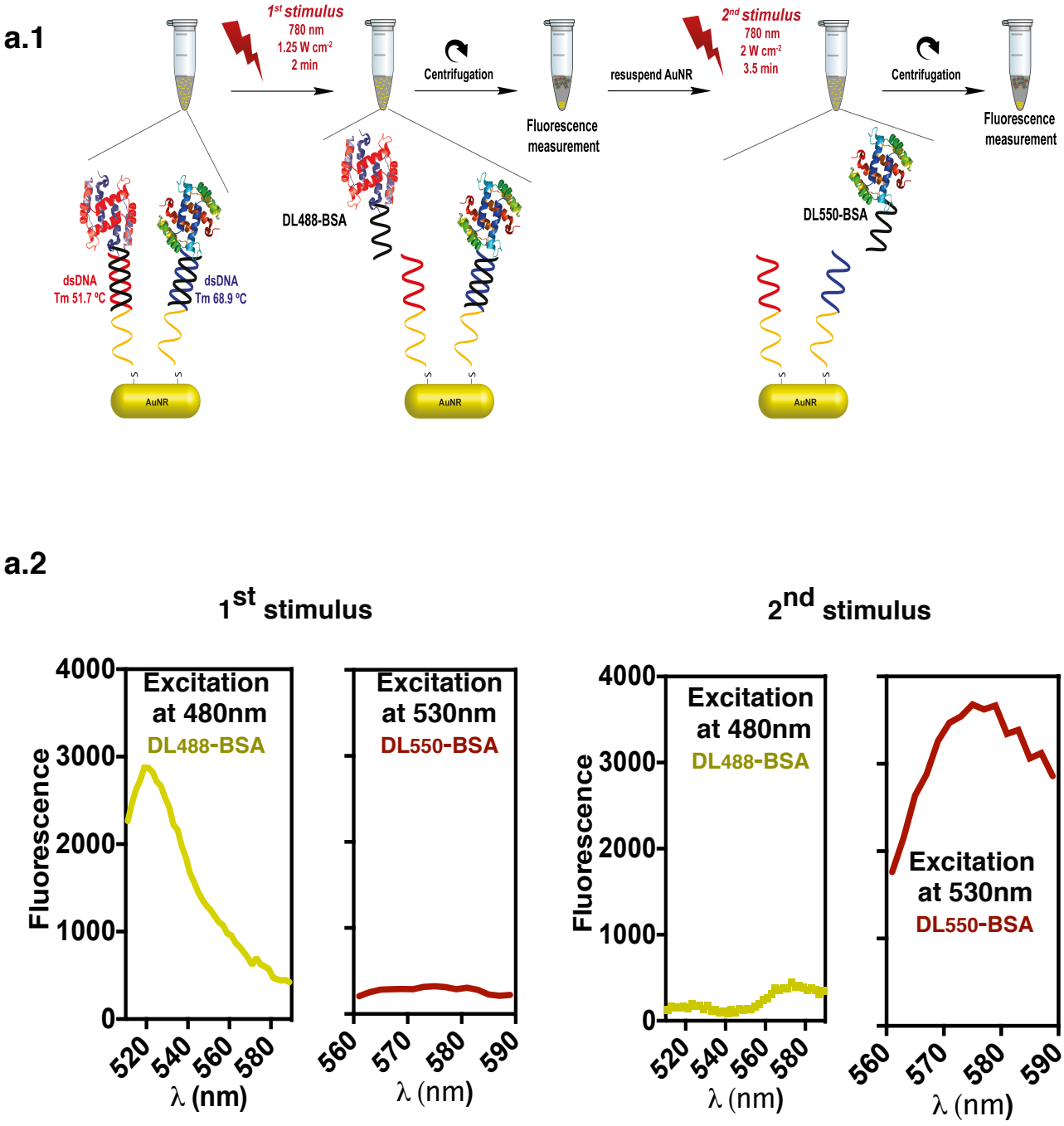

Figure S6. Sequential release of DL488-BSA-ssDNA51.7 and DL550-BSA-ssDNA68.9 from AuNRs after light activation. (a.1) Scheme illustrating dual release experiment. A suspension of DL488-BSA-dsDNA51.7-AuNR-DL550-BSA-dsDNA68.9 was first irradiated for 2 min at 1.25 W cm^{-2} and then centrifuged in order to collect the supernatant. The NR were resuspended and irradiated again at 2 W cm^{-2} for 3.5 min and then centrifuged. AuNRs used in this experiment were conjugated with 32 molecules of DL488-BSA-ssDNA51.7 and 34 molecules of DL550-BSA-ssDNA68.9. (a.2) The fluorescence of both supernatants was measured in a fluorimeter using an excitation wavelength at 480 nm for DL488-BSA or 530 nm for DL550-BSA. The first stimulus caused the release of 86% of DL488-BSA and 7% of DL550-BSA. The second stimulus released 14% of DL488-BSA and 93% of DL550-BSA.

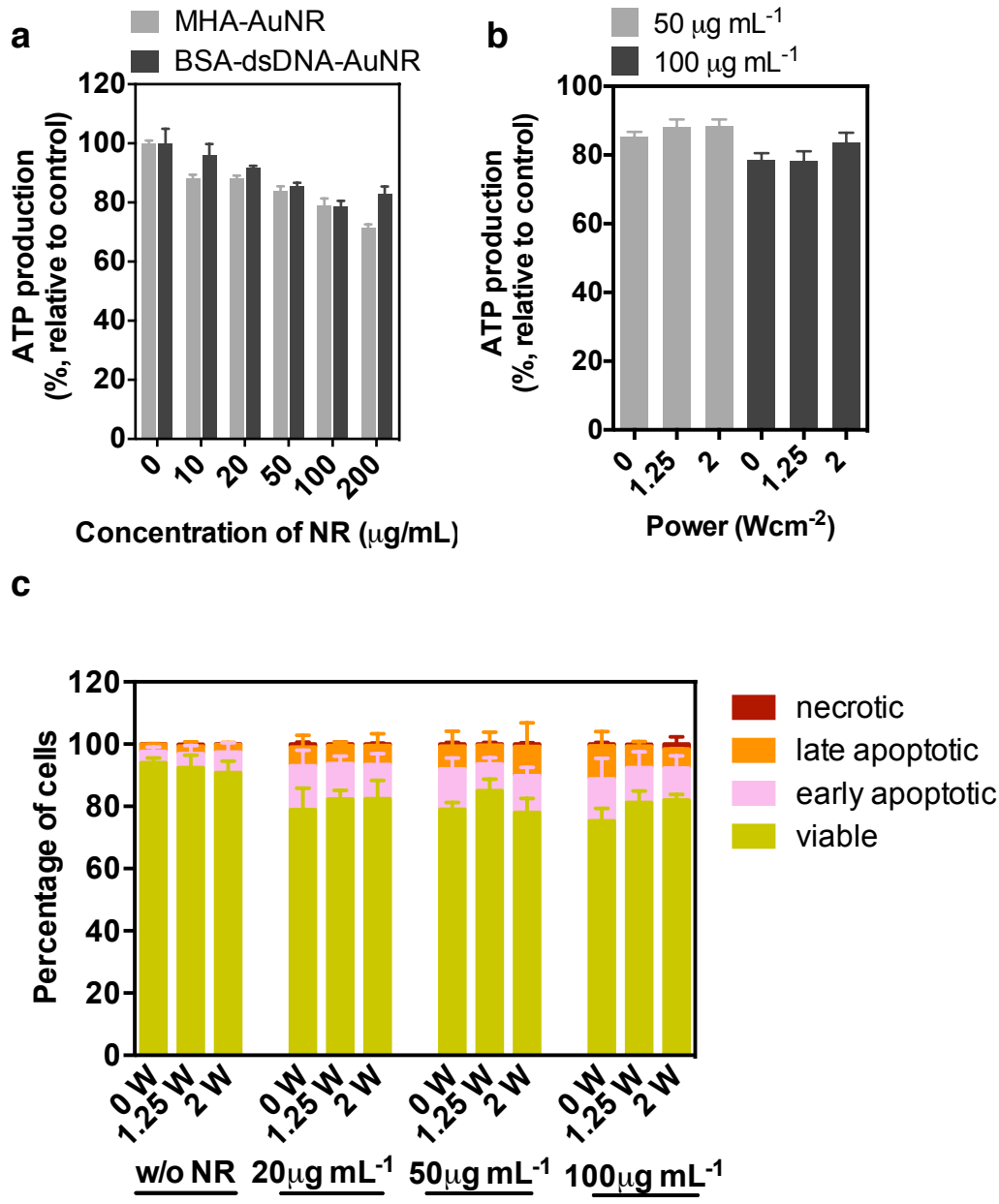

Figure S7. Cytotoxicity of BSA-dsDNA51.7-AuNR. Fibroblasts were incubated with different concentrations of MHA-AuNR or BSA-dsDNA51.7-AuNR for 4 h, washed, incubated in cell culture media for 20 h after which cell metabolism (a and b) or cell viability (c) was evaluated by an ATP (a and b) or annexin V/PI (c) assays. Control are cells cultured without AuNRs. (a and b) Cytotoxicity of non-irradiated BSA-dsDNA51.7-AuNR (a) and irradiated BSA-dsDNA51.7-AuNR (780 nm laser for 2 min; laser powers: 1.25 and 2 W cm^{-2}) (b) as evaluated by an ATP assay. (c) Cytotoxicity of non-irradiated and irradiated BSA-dsDNA51.7-AuNR as measured by an annexin V/PI assay. In a , b and c, results are expressed as Average ± SD (n=3).

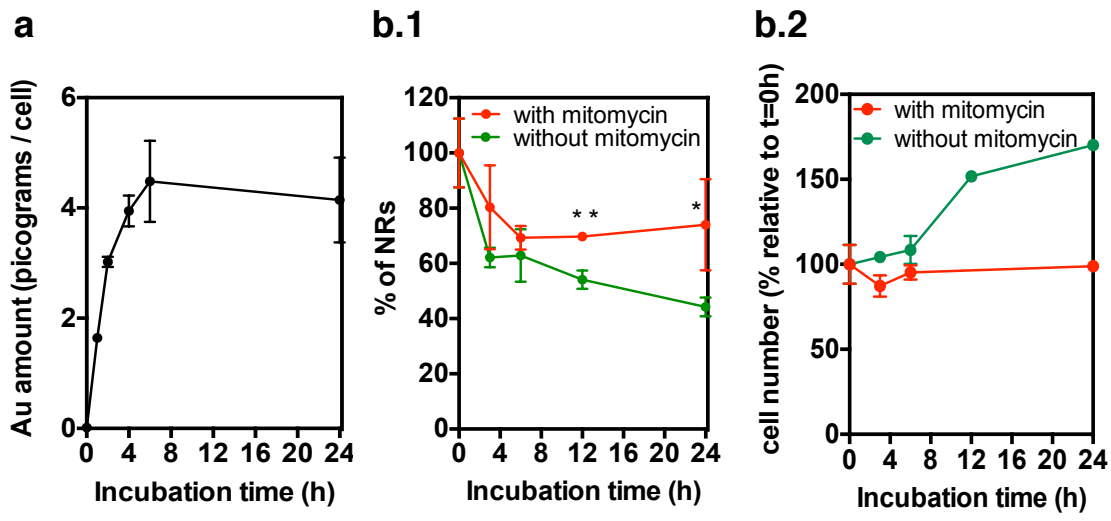

Figure S8. Uptake of β-Gal-dsDNA51.7-AuNR by fibroblasts. (a) Amount of Au in cells incubated with (50 μg mL^{-1}) for different times. At each incubation time, cells were washed to remove the non-internalized AuNR after which they were trypsinized, counted and freeze-dried for ICP-MS analysis. (b) Amount of internalized Au during fibroblast proliferation. Cells were incubated with β-Gal-dsDNA51.7-AuNR (50 μg mL^{-1}) for 4 h, washed to remove the non-internalized AuNR, and cultured for additional 0, 3, 6, 12 and 24 h in cell culture media. In a set of experiments, cells were treated with mitomycin for 150 min after the 4 h uptake period. To determine the Au amount in the cells treated or not with mitomycin, cells were trypsinized, counted and freeze-dried for ICP-MS analysis. (b.1) Intracellular content of Au as quantified by ICP-MS. Unpaired t-test was used to compare both groups. * and ** mean $p<0.05$ and $p<0.01$. (b.2) Cell number relative to time 0 h (end of the incubation with NR). In a, b.1 and b.2, results are Average ± SD, n=3.

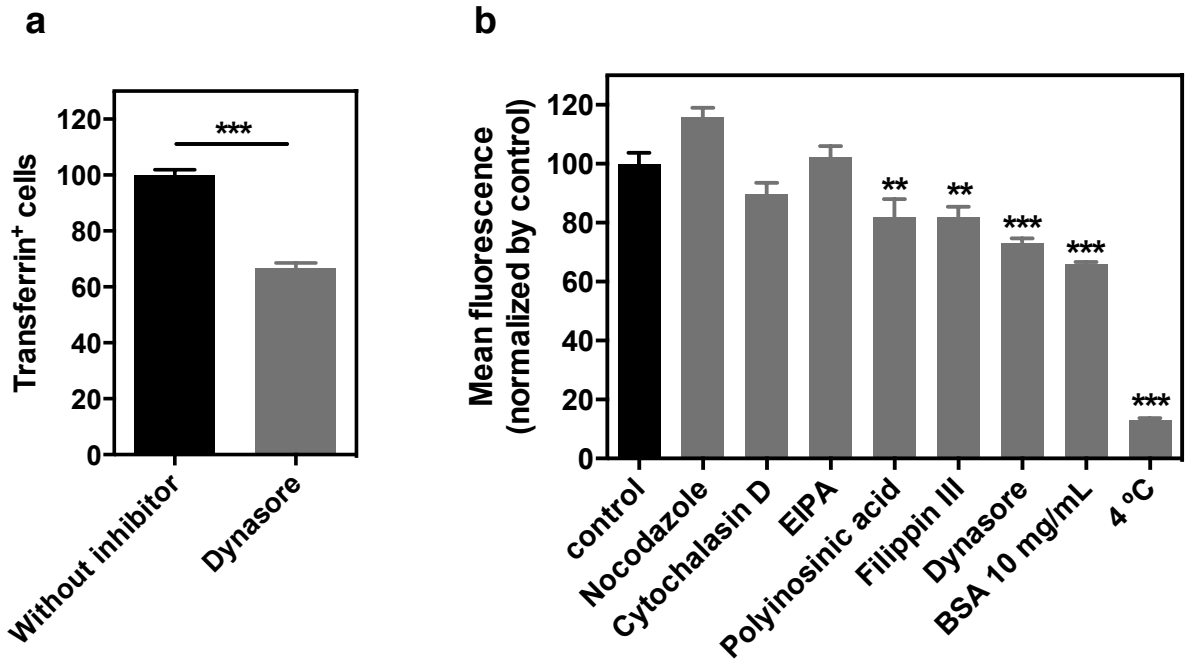

Figure S9. Internalization mechanism of BSA-dsDNA-AuNR. a) Uptake of BSA-dsDNA-AuNR-TRITC in the presence of different endocytosis inhibitors. Mouse fibroblasts were pre-incubated for 30 min with different endocytosis inhibitors. Afterwards, cells were incubated for 4 h with BSA-dsDNA-AuNR-TRITC (50 μg mL^{-1}) in the presence of each inhibitor, followed by two washings steps with PBS. In a separate condition, cells were incubated with BSA-dsDNA-AuNR-TRITC at 4 ºC. Finally, cells were trypinized and analyzed by flow cytometry. Control refers to cells incubated with BSA-dsDNA-AuNR-TRITC without being exposed to any inhibitor. b) Inhibition of transferrin endocytosis with dynasore. Dynasore (100 μM), inhibitor of clathrin mediated endocytosis, is able to inhibit the uptake of FITC-labeled transferrin in SC-1 cells. Cells were exposed to culture medium with and without each inhibitor for 30 min and then incubated with FITC-labeled transferrin (15 μg mL^{-1}) for 3 min at 4 ºC and finally analysed by flow cytometry. **, *** denote statistical significance ($p<0.01$; $p<0.001$) against the control group, assessed by one-way ANOVA followed by Tukey's *post-hoc* test.

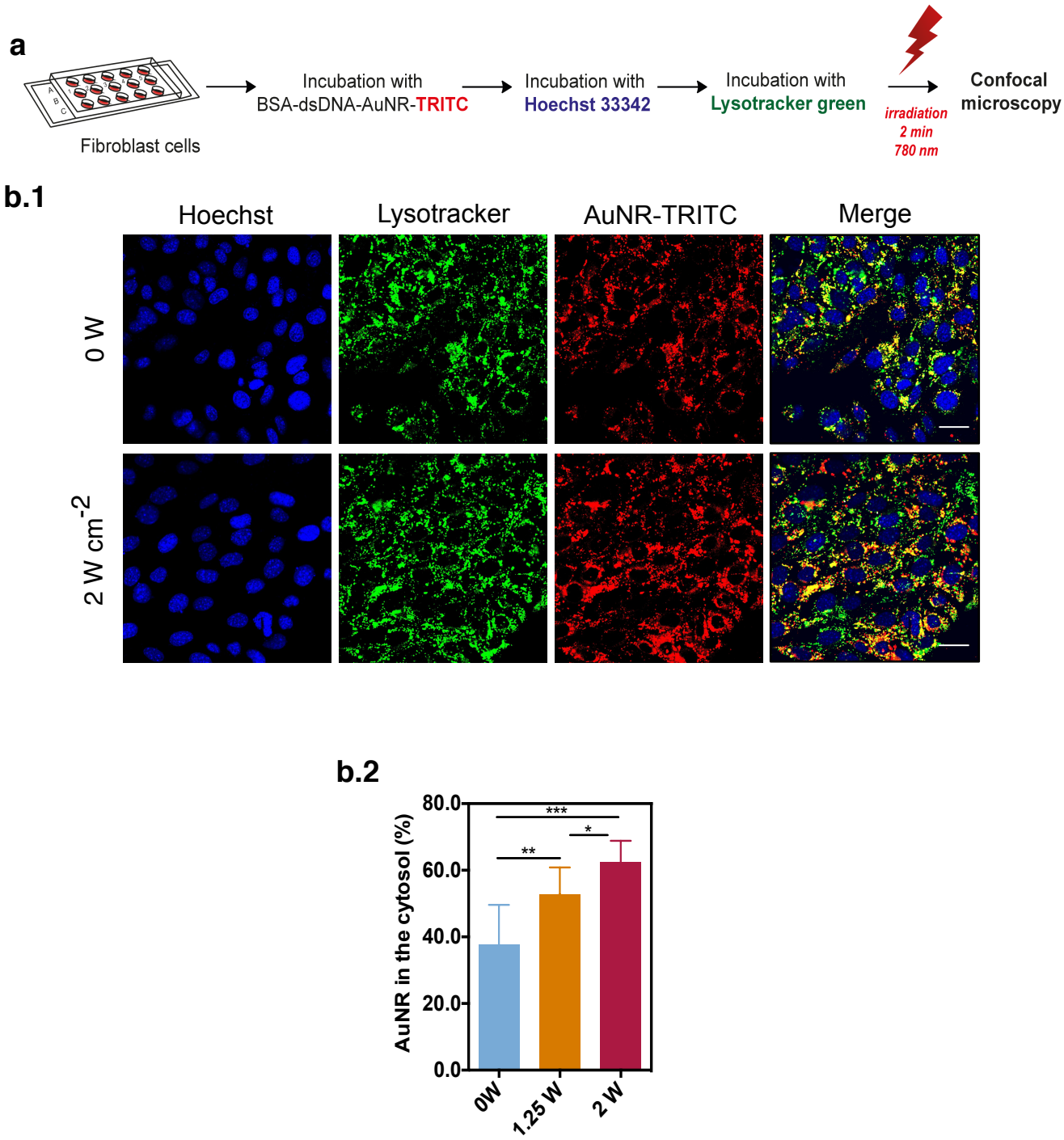

Figure S10. Intracellular trafficking of BSA-dsDNA51.7-AuNR-TRITC. (a) Schematic representation of the protocol used. (b.1) Confocal images of cells stained with lysotracker green after 4 h of incubation with BSA-dsDNA51.7-AuNR-TRITC (50 μg mL^{-1}) and laser irradiation (2 W cm^{-2}, 2 min). Scale bar is 30 µm. (b.2) Percentage of AuNR-TRITC outside the endolysosomal compartment, determined by calculating the overlap coefficient between TRITC and lysotracker green in ImageJ. Results are Average ± SD, n=9 (3 samples, 3 microscope fields per sample). *, **, *** denote statistical significance ($p<0.05$; $p<0.01$; $p<0.001$) assessed by one-way ANOVA followed by Tukey's *post-hoc* test.

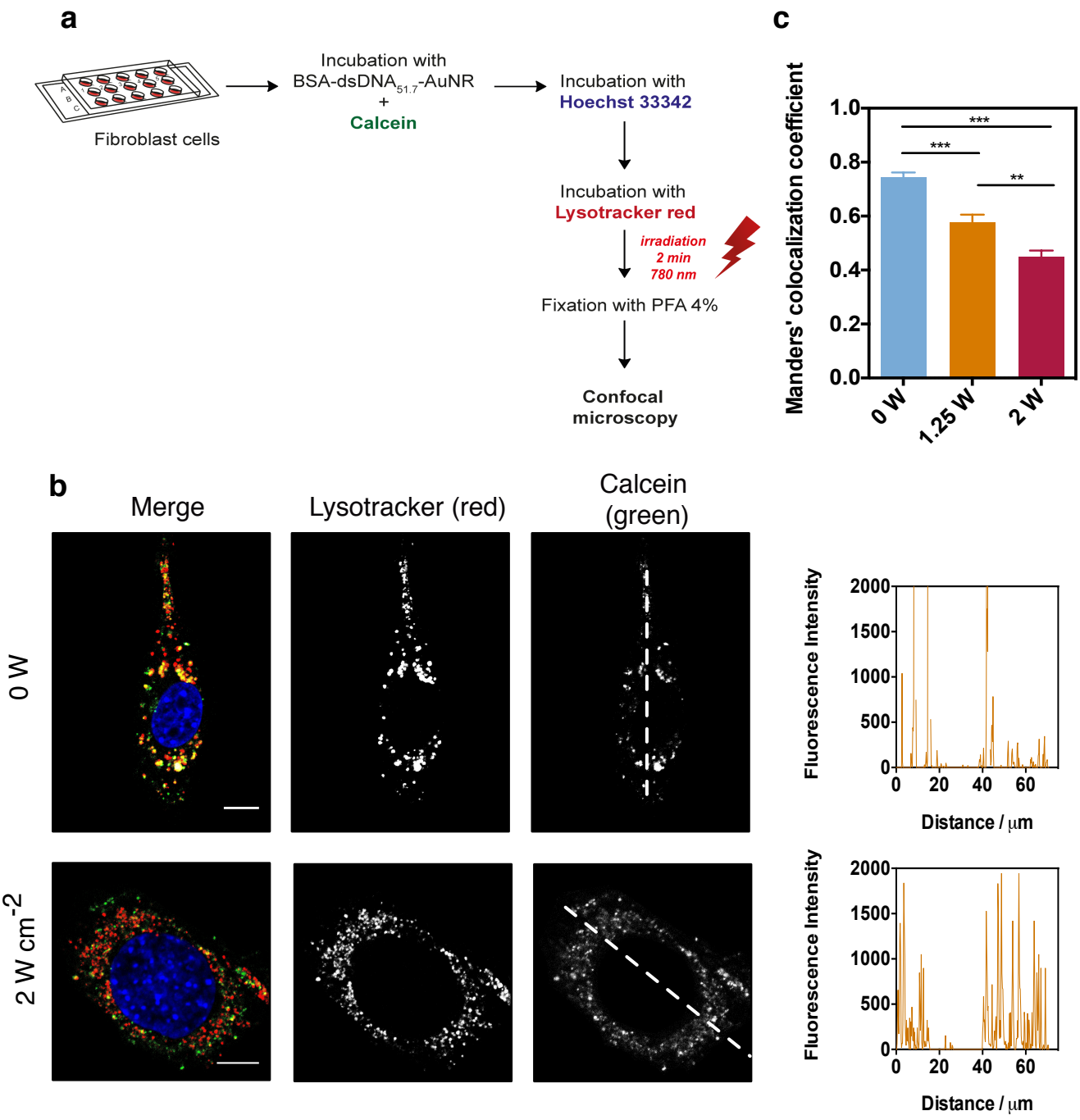

Figure S11. Effect of the NIR irradiation in the endolysomal escape of AuNRs. (a) Schematic representation of the protocol used. Cells were incubated with BSA-dsDNA51.7-AuNR (50 μg mL^{-1}) and calcein (25 mM) for 4 h. After replacing the medium, cells were incubated with lysotracker red (100 nM) for 15 min and then irradiated for 2 min with a laser at 780 nm (power: 1.25 or 2 W cm^{-2}). Cells were then observed in a confocal microscope. (b) Co-localization between calcein and lysotracker red in cells incubated with BSA-dsDNA51.7-AuNR. Scale bar 10 μm. Fluorescence intensity plots were obtained from cell regions labeled with a dash. (c) Co-localization between calcein and lysotracker red expressed as Manders' overlap coefficient (calculated using ImageJ). The results are expressed as Average ± SD, n=3 (3 samples, 4 microscope fields per sample). **, *** denote statistical significance ($p<0.01$; $p<0.001$) assessed by one-way ANOVA followed by Tukey's *post-hoc* test.

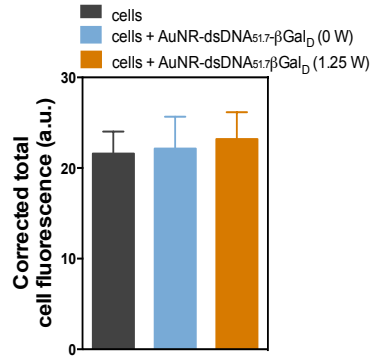

Figure S12. Enzymatic activity quantified after 4h of incubation with AuNRs conjugated with denatured β-Gal (AuNR-dsDNA51.7-βGalD) (50 μg mL^{-1}) followed by irradiation for 2 min at 1.25 W cm^{-2}. No significant change in the fluorescence was observed. Therefore, increase in the fluorescence requires an active enzyme (β-Gal). In a.2 and a.3, results are expressed as Average ± SD, n=3 (3 samples, 5 microscope fields per sample). **, *** denote statistical significance ($p<0.01$; $p<0.001$) assessed by one-way ANOVA followed by Tukey's *post-hoc* test.

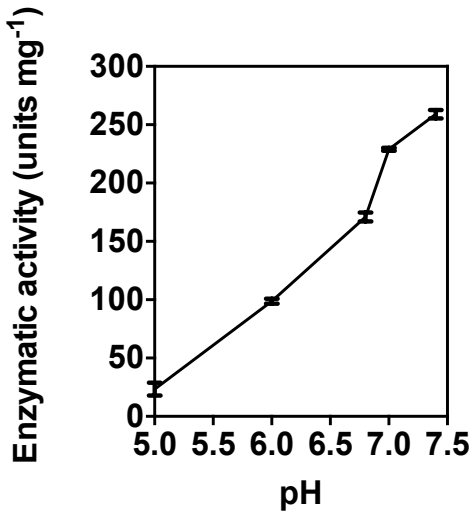

Figure S13. Enzymatic activity of β-Gal at different pHs. β-Gal (50 μL, 0.4 μg mL^{-1}) prepared in 0.1 M phosphate buffer (pH 5.0, 6.0, 6.8, 7.0 and 7.4) was added to ONPG (100 μL, 13 mg mL^{-1}) also prepared in 0.1 M phosphate buffer (pH 5.0, 6.0, 6.8, 7.0 and 7.4). The absorbance at 420 nm was measured for 30 min at 37 ºC in a 96 well plate using a Synergy HT microplate reader. One unit corresponds to the hydrolysis of 1 μmol of substrate (ONPG) per minute per mg of enzyme powder. The results are expressed as Average ± SD, n=3.

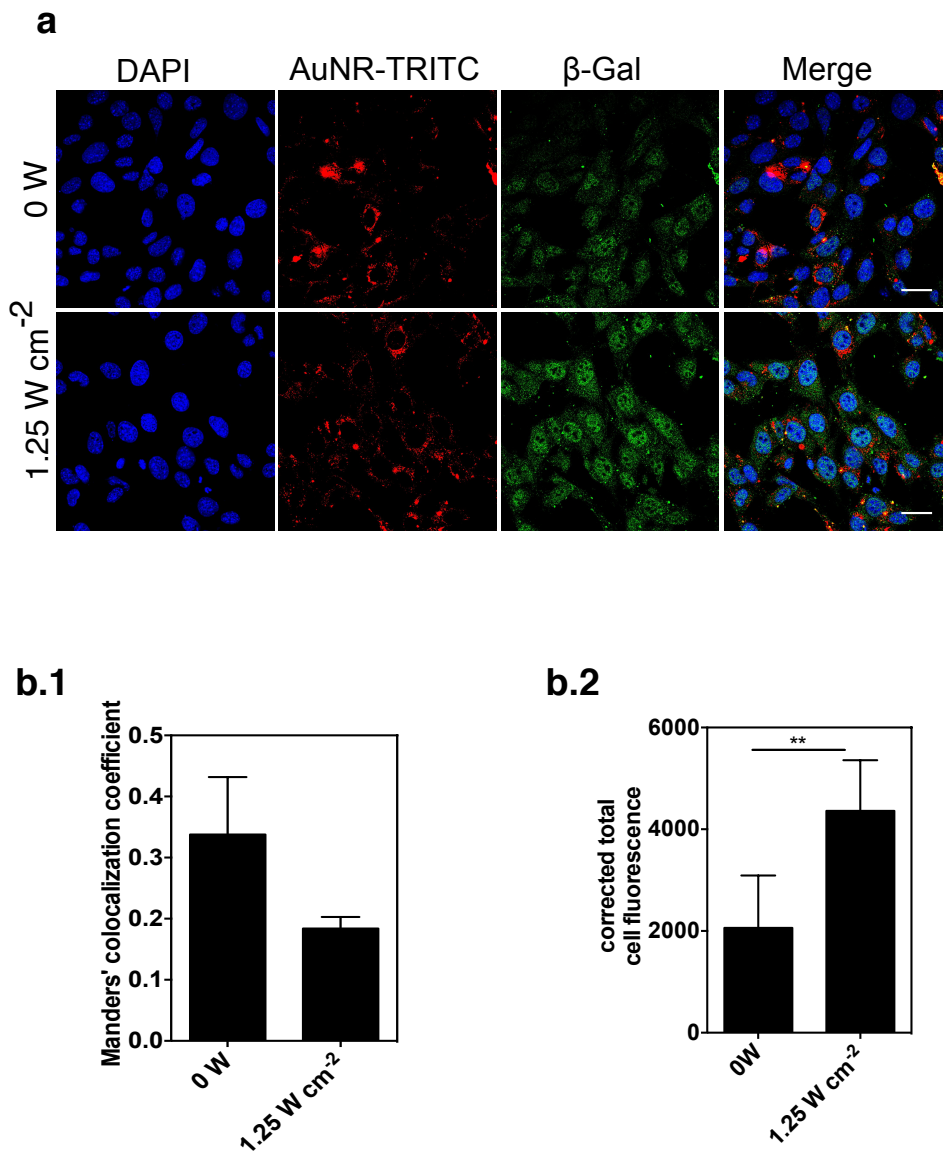

Figure S14. Intracellular levels of β-Gal assessed by immunocytochemistry. (a) Confocal images of fibroblasts incubated with βGal-dsDNA51.7-AuNR-TRITC. Cells were incubated for 4 h with βGal-dsDNA51.7-AuNR-TRITC (50 μg mL^{-1}), washed to remove the non-internalized AuNRs, feeded with new cell culture media and irradiated for 2 min with a 780 nm laser (power: 1.25 W cm^{-2}). Cells were fixed immediately after laser treatment (0 min) using a β-Gal antibody. Scale bar is 30 μm. (b.1) The co-localization between AuNR-TRITC and β-Gal was determined using ImageJ and is expressed as Manders' co-localization coefficient. After irradiation, and immediate (time 0 min) evaluation of the co-localization between β-Gal and AuNR-TRITC, our results show a decrease in the co-localization of both entities, which indicates the release of the protein from the AuNR. (b.2) Cell fluorescence in each experimental condition was corrected to the corresponding background fluorescence and thus designed as corrected total cell fluorescence. Results are Average ± SD, n=9 (3 samples, 3 microscope fields per sample). ** denotes statistical significance ($p<0.01$) assessed by an unpaired t-test.