Supporting information

Spherical Nucleic Acids with Tailored and Active Protein Coronae

Wuliang Zhang, Brian Meckes, Chad A. Mirkin
Department of Chemistry, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208, United States
International Institute for Nanotechnology, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208, United States

Experimental Methods

Safety statement. No unexpected or unusually high safety hazards were encountered.

General information. Human serum albumin crystal structure was accessed through PDB entry 1AO6; DNase I structured accessed through 4AWN, and IgG via 1IGT.

Materials. All materials were purchased from Sigma-Aldrich Co., MO, USA, and used without further purification or modification unless otherwise stated. All cell culture reagents were purchased from ThermoFisher Scientific, MA, USA. Human ErbB2 mAb was purchased from R&D Systems, MN, USA (accession number P04626).

Oligonucleotide synthesis and purification. The oligonucleotides for this study were synthesized on a solid support using a MerMade 12 automated DNA synthesizer (MM12, BioAutomation, TX, USA) using standard protocols. Reagents were purchased from Glen Research (VA, USA). After synthesis, the oligonucleotides were cleaved off from the solid support via addition of concentrated ammonium hydroxide (28-33% in water). Deprotection was carried out in ammonium hydroxide for 16 h at room temperature, after which ammonia was removed from the solution by purging with nitrogen for three hours at room temperature. The oligonucleotides were separated from the solid support by syringe filtration through a 0.22 μm filter. The resulting oligonucleotide solution was purified using reverse-phase high-performance liquid chromatography (RP-HPLC; Agilent, CA, USA) using a water/3% acetonitrile/3% triethylammonium acetate (A)-acetonitrile (B) solvent system. Label-free oligonucleotides were purified on a C18 column (250 mm × 10 mm, Microsorb 300 Å/10 μm) using a gradient from 0-75% solvent B in 40 minutes at 3 mL/min. Fluorophore-labeled sequences were purified on a C4 column, using the same gradient and flow rate. Peaks corresponding to the product were manually collected and lyophilized for 1-2 days to fully remove the solvent. The dried products were dissolved in a 20% acetic acid solution to remove the trityl protecting groups for 1 h at room temperature. The protecting group was then extracted with ethyl acetate three times prior to collecting and lyophilizing the aqueous layer. The final product was dissolved in water and characterized by mass-to-charge ratio using MALDI-ToF-MS (AutoFlex-III, Bruker, MA, USA) in linear negative mode in the presence of dihydroxyacetone phosphate (DHAP) matrix.

Gold nanoparticle synthesis and oligonucleotide functionalization. The protocol used for the synthesis of citrate-stabilized 13-nm gold nanoparticles (AuNPs) was adapted from the Frens method.[1] Following synthesis and purification, the concentration of AuNPs and oligonucleotides was determined using a Cary 5000 UV-vis (Agilent Technologies, CA, USA); the absorbance of AuNPs at 520 nm was recorded, and the concentration was calculated using Beer's Law with an

extinction coefficient of 2.76×10^8 M-1 cm-1.[2] For oligonucleotides, the absorption at 260 nm was recorded, and the extinction coefficient was obtained for each sequence using the IDT Oligo Analyzer (IL, USA). Oligonucleotides (300-fold excess) were then functionalized onto AuNPs through freezing directed synthesis.[3] For the SNAs used in cellular uptake studies, 10% of thiolated Cy5-T20 strands, along with the thiolated label-free control 1826 ODN, were initially functionalized onto AuNPs. To ensure equivalent structures, SNAs made in the same batch were used in all experiments. Unbound oligonucleotides were removed via spin filtration (50k Da MWCO; 4,000 rpm for 10 minutes). The eluent was discarded, and the particles were washed with Dulbecco's phosphate buffered saline (DPBS) containing 0.01% Tween 20, and then they were centrifuged, and the filtrate was discarded. After repeating the wash step three times, the particles were resuspended in PBS, and the SNA concentration was measured using UV-vis spectroscopy as described above. To determine the oligonucleotide loading density on the AuNPs, known concentrations of SNAs were treated with 0.1 M KCN to dissolve the AuNP core. Oligonucleotide concentration was then determined by measuring the ODN absorbance at 260 nm. The average number of oligonucleotides functionalized on a nanoparticle was calculated by dividing the concentration of oligonucleotides by that of the nanoparticles. The SNA solution was stored at 4 °C in the dark.

Protein labeling. To study the desorption kinetics of adsorbed proteins, human immunoglobulin G (IgG), human anti-HER2 antibody (anti-HER2), human serum albumin (HSA) (Sigma-Aldrich, MO, USA) were labeled with Texas Red-X succinimidyl ester, mixed isomers (ThermoFisher, MA, USA) following the manufacturer's protocol. The labeling was found to be 2.8, 2.9, and 1.7 of Texas Red-X fluorophores per IgG, anti-HER2, and HSA, respectively.

Adsorption of Proteins on SNAs. To adsorb functional proteins on the surface of SNAs, 1 mL of a 10 nM SNA solution was prepared in 2-(N-morpholino) ethanesulfonic acid (MES) buffer (pH 6.0 for HER2 and IgG, BioWorld, TX, USA; or pH 4.7 for HSA, ThermoFisher, MA, USA). The SNA solution was then slowly added to 1 mL of protein solution (20-fold excess of HER2 mAb and IgG at pH 6, 200-fold excess of HSA at pH 4.7 and IgG at pH 6) in MES buffer while stirring. The mixture was incubated for 4 h at room temperature with constant stirring. The product was pelleted by centrifugation for 1 h at 15,300 x g at 4 °C, and the supernatant was removed.[4] The pellet was then washed with PBS twice at the same centrifugation setting. The protein-adsorbed SNA pellet was then suspended in ~100 μL of PBS, and the AuNP content was determined by absorbance using UV-vis spectroscopy.

Protein-adsorbed SNA characterization. DLS measurements were taken on a Zetasizer NanoZS (Malvern Instruments, UK) using the refractive index of gold. Samples were prepared in water with an AuNP concentration of 1 nM. Measurements were conducted at 25 °C. The diameter was reported for the number average of five measurements of each sample, and for zeta potential, the average of five measurements was reported. The protein- immobilized SNAs were also analyzed by 1% agarose gel in 1x TBE (Tris/borate/EDTA) buffer. The samples were loaded in the wells with 6x gel loading dye (10 μL of 20 nM [AuNP]/well; 2 μL loading dye/well; New England BioLabs, MA, USA). The chamber was filled with 300 mL of 1x TBE. The gel was run at 120 V for 1 h at room temperature and was imaged with Amersham Typhoon 5 Biomolecular Imager (GE Healthcare Life Sciences, PA, USA) in the densitometry mode. To determine the amount of mAb or proteins adsorbed onto SNAs, the Texas Red-X-labeled mAb/proteins were first

dissociated from the SNA surface via sodium dodecyl sulfate (SDS) treatment (0.2 % v/v, 1 h, room temperature). The fluorescence of the dissociated proteins in solution was measured at room temperature on a plate reader (Cytation 5, BioTek Instruments, VT, USA) at 595/616 nm (10 nm slit width). Estimation of the mAb/protein concentration in solution was calculated by comparing the fluorescence intensities of the unknowns to those generated from a standard curve of Texas Red-X-labeled mAb/proteins mixed with the same concentration of SNA as the unknowns. The number of mAb/proteins per SNA was then calculated by dividing the protein concentration by the SNA concentration by AuNP. The oligonucleotide density on the protein-adsorbed SNAs was estimated in the same way (i.e. KCN dissolution followed by absorbance at 260 nm) as bare SNAs.

DNA degradation assay. To quantify the amount of oligonucleotides degraded by DNase I on SNAs with and without the functional protein corona, a degradation assay was performed. Texas Red X-labeled IgG, anti-HER2, and HSA were immobilized on SNAs functionalized with 100% Cy5-labeled oligonucleotides. In a black 96-well plate, IgG-SNA, anti-HER2 SNA, HSA-SNA, or SNA (100 pmol by ODN) was diluted in 1x DNase I reaction buffer and mixed with 1 μL of DNase I (New England BioLabs, MA, USA) to make a final volume of 100 μL. The fluorescence was monitored on a plate reader (Cytation 5, BioTek Instruments, VT, USA) at 650/672 nm (10 nm slit width) at 37 °C, over 12 h at 5 min intervals for the first two hours, and 20 min intervals thereafter.

Protein displacement assay in the presence of serum. The dissociation rate of pre-adsorbed functional proteins in the presence of serum is determined by a fluorescence-based assay. Texas Red X-labeled IgG, anti-HER2 and HSA were immobilized on SNAs functionalized with 100% Cy5-labeled oligonucleotides. As the protein coating leaves the Cy5-labeled oligonucleotide shell, the fluorescence of Texas Red X will be restored. In a black 96-well plate, IgG@SNA, anti-HER2@SNA, HSA@SNA, or SNA was diluted in PBS supplemented with 10% type AB male human serum (HS) to 5 nM by AuNP concentration. The fluorescence was monitored on a plate reader (Cytation 5, BioTek Instruments, VT, USA) at 595/616 nm (10 nm slit width) at 37 °C, over 12 h at 5 min intervals for the first two hours, and at 20 min intervals thereafter. A final reading was taken after the addition of 1 μL of 10% SDS (final SDS content was 0.1% v/v) to each well to establish the full protein dissociation point. In addition to SDS, ethanol up to 20% v/v, NaCl up to 1M, or $MgCl_2$ up to 1 M were also added to protein-adsorbed SNA solution, but none resulted in complete protein dissociation as SDS did. Hence, SDS was selected to fully dissociate the protein coronae.

On-particle DNA hybridization assay. The accessibility of the oligonucleotides on the SNA surface in the presence of protein corona was measured by a fluorescence-based DNA hybridization assay. In a black 96-well plate, SNA functionalized with label-free ODNs (500 nM final concentration by ODN) and its Cy3.5-labeled complementary strand (600 nM by final concentration) were combined in PBS. The mixture was incubated for 5 min at room temperature with constant shaking to allow for hybridization. The fluorescence intensity of Cy3.5 was measured on a Cytation 5 plate reader (excitation at 591 nm, emission at 611 nm, 9 nm slit width) at room temperature. The % DNA hybridized to protein-immobilized SNAs was calculated by assuming 100% hybridization for protein-free SNAs and 0% hybridization for SNAs functionalized with non-complementary (i.e., T20) strands.

Cell culture. Cells were cultured in a 5% CO_2 incubator following the suppliers' protocols. SK-BR-3 cells were cultured in McCoy 5A supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). MDA-MB-231 GFP cells were cultured in DMEM containing 10% FBS, 1% P/S, 1x non-essential amino acids (NEAA) and 1x GlutaMAX. THP-1 macrophages were cultured in Rosewell Park Memorial Institute (RPMI) containing 10% FBS, 1% P/S, and 50 μM of 2-mercaptoethanol.

Antibody-adsorbed SNA uptake in breast cancer cells. To examine if ErbB2 mAb adsorption on SNAs can increase the uptake by cells overexpressing HER2, SK-BR-3 (HER2-positive), and MDA-MB-231 GFP (HER2-negative) cell lines were used. In a typical experiment, SK-BR-3 cells were plated in a 96-well plate at 1×10^4 cells/well and MDA-MB-231 GFP were plated at 0.7×10^4 cells/well in the same wells as SK-BR-3 cells. Both cells were plated in their corresponding complete growth media. The cells grew for at least 20 hours before being treated with SNAs. The cells were incubated with mAb-SNA, IgG-SNA, or SNAs (2 nM by AuNP, with 10% Cy5-labeled oligonucleotides) for 1, 2, 4, 6, 8 h, and the cells without treatment were used as blank control. After each time point, the cells were washed with PBS once and detached by adding 30 μL of TrypLE per well followed by incubation at 37 °C for 10 minutes. The cells were then fixed in a 3.7% paraformaldehyde (PFA) solution for 15 minutes at room temperature. 100 μL of PBS was added per well before flow cytometry analysis. Flow cytometry (LSRFortessa, BD Biosciences, CA, USA) measurements were conducted using the green and red laser; FITC and APC channels were selected for GFP and Cy5 detection, respectively. The distribution of cell fluorescence of the gated cells was collected. The two cell populations were separated based on FITC signal and the median fluorescence intensity (MFI) in the APC channel was calculated using FlowJo v10 (FlowJo LLC, OR, USA).

Opsonin-adsorbed SNA uptake in macrophages. To investigate if adsorption of opsonin (IgG) and a control protein (i.e. HSA) on SNAs can affect uptake by macrophages, THP-1 monocytes were first differentiated into macrophages. One million THP-1 cells were seeded in a 1.2 mL polystyrene test tube. Cells were then differentiated with 100 nM PMA in complete growth media for 72 h. On the fourth day, cells were pelleted (1,000 rpm, 5 min) and allowed to rest in PMA-free media for 24 h before SNA treatment. Differentiated THP-1 macrophages were then treated with 2 nM of IgG-SNA, HSA-SNA, or SNAs (labeled with 10% Cy5-oligonucleotides) for 1, 2, 4, 6, and 8 h; cells without treatment were used a blank control. After each time point, cells were pelleted (1,000 rpm, 5 min) and washed once with PBS. After that, cells were stained with LIVE/DEAD fixable violet dead cell stain kit (ThermoFisher) following the manufacturer's instructions. After washing with PBS twice, the cells were then fixed in 1 mL of 3.7% PFA solution for 15 minutes at room temperature, washed once with 1 mL of PBS with 1% bovine serum albumin (BSA). Cells were suspended in 0.15 mL of PBS with 1% BSA and analyzed by flow cytometry (LSRFortessa, BD Biosciences, CA, USA) in the APC channel for Cy5 signal quantification. The distribution of Cy5 fluorescence of the gated live cells (Pacific Blue channel) were collected and the corresponding MFI values were calculated using FlowJo v10.

Receptor inhibition studies. In 1.2 mL polystyrene test tubes, THP-1 macrophages were rested in full growth media for 5 days, before being suspended in 100 μL of media. Cells were pretreated with 50 μg/mL of fucoidan, 10 μM of cytochalasin D, or 5 μL of the FcX Fc receptor blocking solution (BioLegend, CA, USA) for 30 minutes at 37 °C before treatment with 5 nM (by AuNP)

of 10% Cy5-labeled SNAs, and the cells were incubated for another 1 h at 37 °C. To measure the extent of blocking, the cells were pelleted, fixed in 3.7% PFA (15 min, room temperature), and resuspended in 0.15 mL of PBS with 1% BSA for flow cytometry analysis in the APC channel for Cy5 signal quantification.

THP-1 macrophage surface marker labeling. To confirm that PMA treatment caused differentiation of THP-1 cells, a surface marker of macrophages, PE anti-human CD14 antibodies (mouse IgG1, κ; BioLegend, CA, USA), was used as a label. The labeling procedure follows the manufacturer's protocol. After labeling, the cell suspension was rinsed with PBS and then fixed for flow cytometry analysis in the PE channel. The distribution of PE fluorescence of the gated cells was collected, and the corresponding MFI values were calculated by FlowJo v10.

Confocal microscopy. To visualize the effect of cytochalasin D on actin disruption, fixed cells were stained with AlexaFluor 488 (AF488)-phalloidin (ThermoFisher, MA, USA) overnight at 4°C, rinsed with PBS for three times, and then mounted in an antifade mounting medium (ThermoFisher, MA, USA) for confocal imaging. To analyze the effects of actin de-polymerization on the location of SNAs, membrane of cells treated with and without cytochalasin D was stained with AF488-wheat germ agglutinin conjugates (WGA; ThermoFisher, MA, USA) following the manufacturer's protocol. All cells were imaged using a Zeiss LSM 800 (Carl Zeiss, Germany) with a 63x oil immersion objective.

Isolation of serum proteins on SNAs. IgG@SNA, anti-HER2@SNA, HSA@SNA, and bare SNAs (10 nM) were incubated in 10% HS for 90 min at 37°C with constant shaking. Unbound protein was then removed by centrifugation for 50 min at 153,000 rcf at 4°C. After removal of the supernatant, the pellet was washed with PBS twice. The SNA pellet was then re-dissolved in ~20 μL of 0.1% SDS. The mixture was heated for 5 min at 95°C to dissociate the bound protein. The released protein was then separated from SNAs by centrifugation for 50 min at 153,000 rcf at 4°C, and the supernatant was collected for gel electrophoresis analysis.

SDS-PAGE. The proteins collected from 3 pmol of SNAs were analyzed by SDS-PAGE using a precast Mini ProTEAN TGX 4%-15% polyacrylamide gel (10 well, 50 μL, BioRad, CA, USA). Each sample was mixed with 2x Laemmli sample buffer (BioRad) at 1:1 ratio and loaded into the precast gel. Precision Plus Protein Dual Xtra Standards (BioRad) were used as a molecular weight marker. The gel was run for 1 h at 100 V in Tris-Glycine-SDS buffer. After rinsing the gel with 20% w/v NaCl solution (5 min) three times, the gel was then stained in 50 mL of Bio-Safe Coomassie Stain (BioRad, CA, USA) for 1 h at room temperature with gentle shaking. After that, the gel was de-stained in 100 mL of water for 1 h, and 20 mL of 20% w/v NaCl solution was added to the gel and left incubate for 2 h at room temperature. The protein bands in the gel were visualized using ChemiDoc™ MP Imaging System (BioRad, CA, USA) in the red (Cy5) fluorescence channel with automatic exposure.

Table S1. Oligonucleotide sequences used in this study

Name of strand	Application	Sequence
1826 control- SH	AuNP functionalization; for DLS, zeta potential, agarose	5'-TCC ATG AGC TTC CTG AGC TT-$(Sp18)_2$-SH-3'

	gel, DNA degradation and DNA accessibility studies	
T20-SH	AuNP functionalization; control sequence for DNA accessibility study	5'-TTT TTT TTT TTT TTT TTT TT-$(Sp18)_2$-SH-3'
Complementary scrambled	Hybridize to scrambled 1826 for DNA accessibility quantification	5'- Cy3.5-AAG CTC AGG AAG CTC ATG GA -3'
Cy5-T20-SH	AuNP functionalization and labeling; for protein displacement (100% Cy5), cellular uptake studies (10% Cy5)	5'-Cy5-TTT TTT TTT TTT TTT TTT TT-$(Sp18)_2$-SH-3'

Table S2. Average number of DNA on the surface of AuNPs for bare and protein-adsorbed SNAs.

Sample	**SNA**	**IgG@SNA**	**HSA@SNA**
ODN strands/NP	**253**	**315**	**244**

Table S3. Amount of adsorbed antibodies/proteins on the surface of SNAs and their corresponding isoelectric points.

Sample	**IgG@SNA**	**HER2@SNA**	**HSA@SNA**
Adsorbed proteins/NP	40 ± 2	26 ± 2	23 ± 2, 19 ± 1, 25 ± 1, and 20 ± 1 (four batches)
Isoelectric point of protein	6.6-7.2	8.6 ± 0.4	4.7

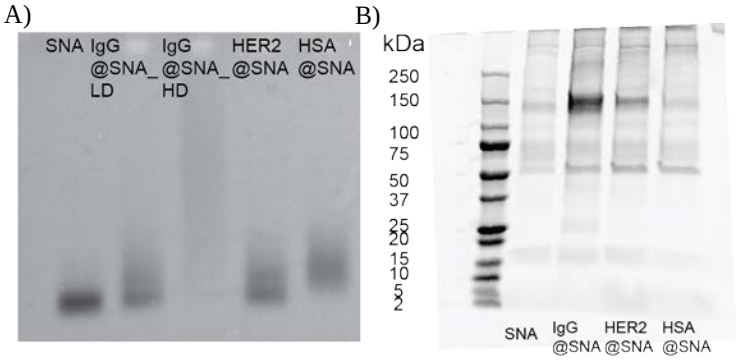

Figure S1. Gel electrophoretic analysis of protein-adsorbed SNAs (A) and the extracted hard protein corona (B). (A) 1% agarose gel of SNAs (lane 1), low-density IgG-adsorbed SNAs (lane 2), high-density IgG-adsorbed SNAs (lane 3), anti-HER2-adsorbed SNAs (lane 4), and HSA-

adsorbed SNAs (lane 5). (B) SDS-PAGE of proteins isolated from SNAs with and without tailored protein pre-adsorption after incubation in 10% HS.

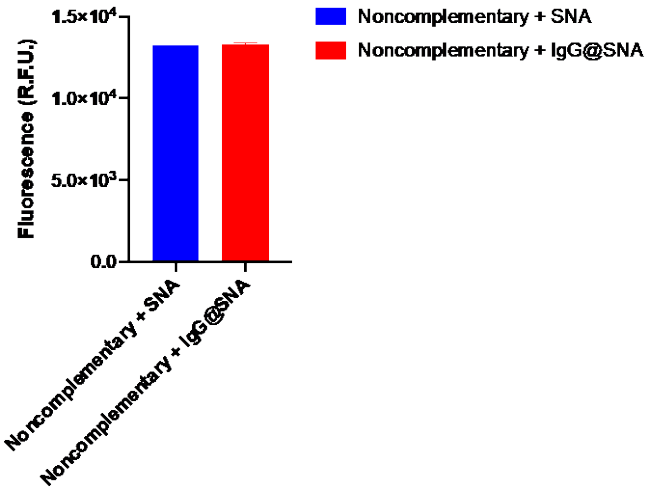

Figure S2. Fluorescence intensity of noncomplementary ssDNA strands mixed with SNAs (blue) and IgG@SNAs (red).

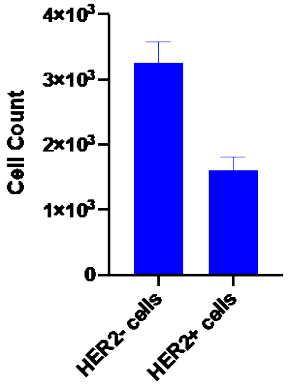

Figure S3. Cell count of co-cultured GFP-expressing HER2- (MDA-MB-231) and nonfluorescent HER2+ cells (SK-BR-3) from flow cytometry.

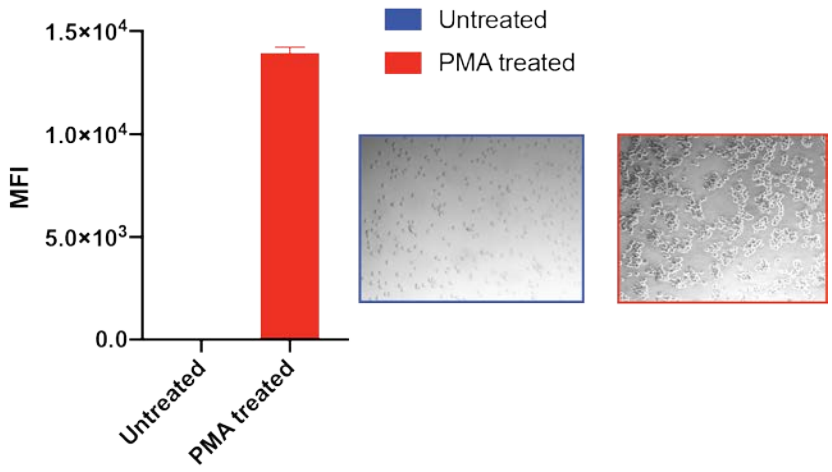

Figure S4. Expression level of the surface marker, CD14, by THP-1 cells before (blue) and after (red) PMA treatment. Insert: Micrographs of the cell morphology before and after PMA induced differentiation.

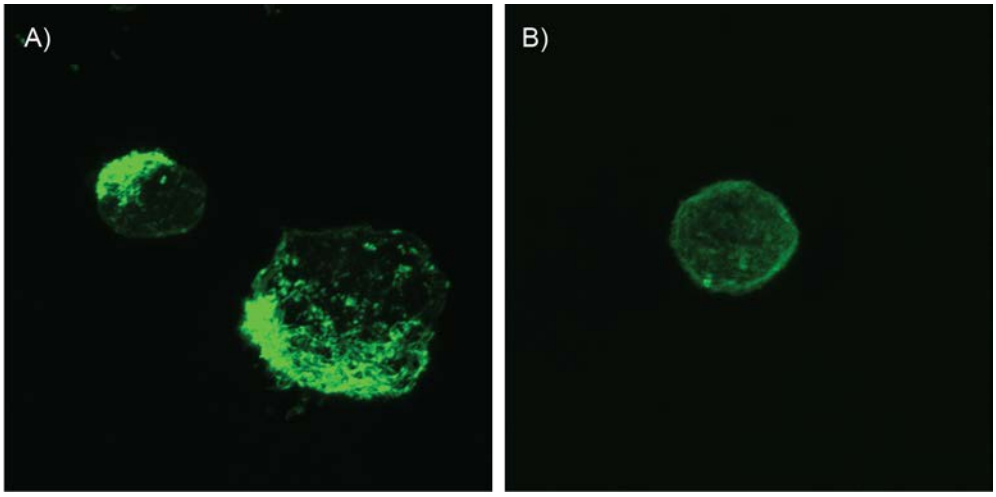

Figure S5. Fluorescence micrographs of the actin cytoskeleton with (A) and without (B) cytochalasin D treatment of the THP-1 macrophages. Actin was stained with AF488 phalloidin.

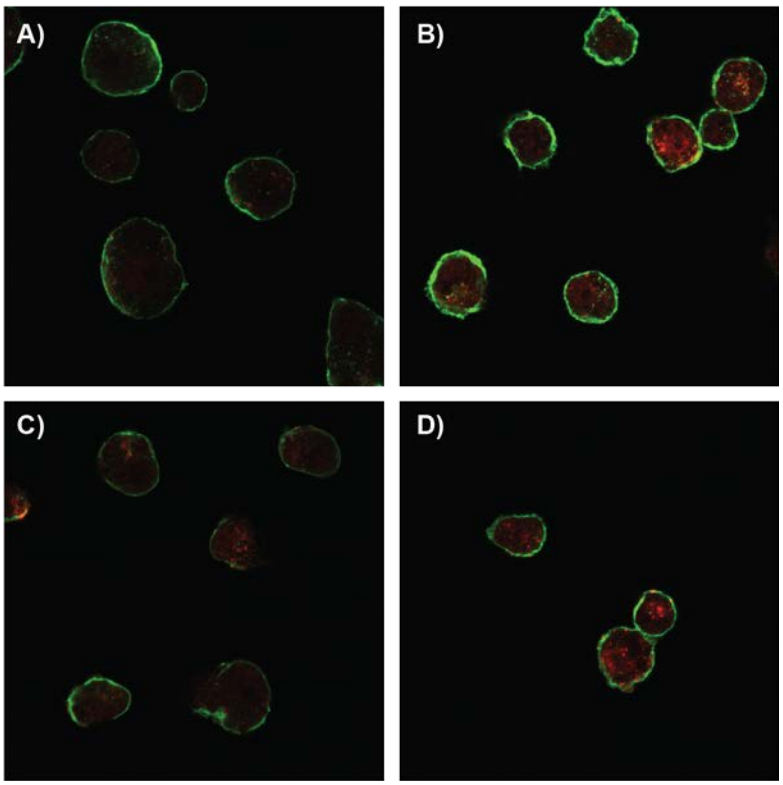

Figure S6. Overlay of fluorescence micrographs of the SNA oligonucleotide shell (red) and cellular membrane (green) of the THP-1 macrophages with (A, C) and without cytochalasin D (B, D) treatment. (A, B) Images of cell uptake of SNAs not precoated with proteins prior to incubation. (C, D) Images of cell uptake of IgG-adsorbed SNAs. Oligonucleotide shell was filled with 10% of Cy5-labeled DNA and cellular membrane was stained with Alexa 488-wheat germ agglutinin conjugates.

References

1. Frens, G., Controlled Nucleation for the Regulation of the Particle Size in Monodisperse Gold Suspensions. *Nature Physical Science* **1973,** *241*, 20.
2. Giljohann, D. A.; Seferos, D. S.; Patel, P. C.; Millstone, J. E.; Rosi, N. L.; Mirkin, C. A., Oligonucleotide Loading Determines Cellular Uptake of DNA-Modified Gold Nanoparticles. *Nano Lett.* **2007,** *7* (12), 3818-3821.
3. Liu, B.; Liu, J., Freezing Directed Construction of Bio/Nano Interfaces: Reagentless Conjugation, Denser Spherical Nucleic Acids, and Better Nanoflares. *J. Am. Chem. Soc* **2017,** *139* (28), 9471-9474.
4. Docter, D.; Distler, U.; Storck, W.; Kuharev, J.; Wünsch, D.; Hahlbrock, A.; Knauer, S. K.; Tenzer, S.; Stauber, R. H., Quantitative profiling of the protein coronas that form around nanoparticles. *Nat. Protoc* **2014,** *9*, 2030.