Supporting Information

Endosomal Escape and Delivery of CRISPR/Cas9 Genome Editing Machinery Enabled by Nanoscale Zeolitic Imidazolate Framework

Shahad K. Alsaiari,[†] Sachin Patil,[†] Mram Alyami,[†] Kholod O. Alamoudi,[†] Fajr A. Aleisa,[§] Jasmeen S. Merzaban,[§] Mo Li,[‡] Niveen M. Khashab[*,†]

[†] Smart Hybrid Materials (SHMs) Laboratory, Advanced Membranes and Porous Materials Center, King Abdullah University of Science and Technology (KAUST), Thuwal 23955-6900, Saudi Arabia.

[§] Cell Migration and Signaling Laboratory, Division of Biological and Environmental Science and Engineering, King Abdullah University of Science and Technology (KAUST), Thuwal 23955-6900, Saudi Arabia.

[‡] Stem Cell and Regeneration Laboratory, Division of Biological and Environmental Science and Engineering, King Abdullah University of Science and Technology (KAUST), Thuwal 23955-6900, Saudi Arabia.

Materials and Methods

Materials. All chemicals were purchased from Sigma-Aldrich. Cas 9 incorporated with nuclear localization signal (NLS), sgRNA targeting EGFP with T7 promotor sequence, molecular probes including (Hoechst 33342, lysotracker and alexa fluor 647 (C2 maleimide), live-dead viability kit), primers (qRT-PCT and Surveyor assay) and GeneArt genomic cleavage detection kit were obtained from Thermo Fisher Scientific. RNA extraction kit (RNeasy mini kit) and SYBR Green Master mix were provided by Qiagen.

The sgRNA sequence with the T7 promoter, target sequence and crRNA is as follow GTTTTTTTTAATACGACTCACTATAGGGCACGGGCAGCTTGCCGGGTTTTAGAGCTA GAAATAGC.

Assembly of Cas 9/sgRNA@ZIF-8. Cas 9 and sgRNA were mixed at a molar ratio of 1:1 in phosphate buffer saline (PBS) and incubated at room temperature for 5 minutes. Cas 9/sgRNA complex was then dissolved in 2-methylimidazole (2.5 M). The mixture was stirred for 30 minutes followed by the slow addition of zinc nitrate solution (0.5 M) under mechanical agitation for 20 minutes. The resulting product was collected by centrifugation and washed with DI water three times to remove any residues. The supernatant was collected to calculate the loading capacity and encapsulation efficiency. CC-ZIFs were diluted in water for characterization or Opti-MEM medium for cell study to a concentration of 100 µg mL^{-1}. The size and Zeta potential of Cas 9/sgRNA@ZIF-8 were performed using a Malvern Zetasizer NanoZS at 25 °C at pH 7 in aqueous solutions. X-ray powder diffraction (PXRD) measurements were

performed using a Panalytical X'Pert Pro X-ray powder diffractometer using the Cu Kα radiation (40 V, 40 mA, λ = 1.54056 Å) in a θ – θ mode from 20 ° to 90 ° (2 θ). Standard deviations were calculated with 3 runs. Transmission electron microscopic (TEM) images were obtained using FEI Tecnai 12 microscope operating at 120 kV. For visualization by TEM, samples were prepared by dropping a solution of production on a copper grid 300 mesh (Electron Microscopy Sciences, LC 300-Cu). Cas 9 was labeled with Alexa fluor 647 C2 maleimide (AF 647) according to the manufacturer's protocol to monitor Cas 9/sgRNA loading and release. Fluorescence measurements were performed on a Cary Eclipse fluorescence spectrophotometer (Varian). The slits for excitation and emission were set at 10 nm.

Cas 9/sgRNA loading. Supernatants containing non-loaded Cas 9/sgRNA were collected to determine the loading capacities (LC) and the entrapment efficiency % via UV-Visible spectroscopy measured at a given wavelength (Cas 9/sgRNA: 260 nm). Absorbance spectra were measured using a NanoDrop ONE (Thermal Scientific). The Beer-Lambert law was used to quantify the concentration of loaded Cas 9/sgRNA. Equations used to calculate Cas 9/sgRNA concentration, loading capacity and entrapment efficiency are as follow:

Cas 9/sgRNA concentration = (OD_{260}/pathlength) × standard coefficient × dilution factor

LC = [m drug loaded /m NPs] ×100

EE= [m drug loaded/ m initial drug] ×100

Lipofectamine CRISPRMAX Cas9. The lipofectamine CRISPRMAX Cas9 was prepared according to the manufacturer protocol. Briefly, 1 µL Cas9 Plus reagent was added to the solution containing Cas9 protein and gRNA. After brief vortexing, the mixture was incubated at 25 °C for 5 minutes to allow the formation of Cas9/sgRNA. The Cas9/sgRNA remained active at 25 °C for up to 2 h. Then, 25 µl Opti-MEM medium was added to a separate sterile Eppendorf

tube, followed by addition of 1.5 μL of lipofectamine CRISPRMAX. After brief vortexing, the Lipofectamine CRISPRMAX solution was incubated at 25 °C for approximately 5 minutes. After incubation, the Cas9/sgRNA was then added to the Lipofectamine CRISPRMAX solution.

Release of AF-Cas 9/sgRNA via pH trigger. To evaluate the release of Cas 9/sgRNA from ZIF-8, AF 647 labeled Cas 9 was used. Aliquots of hydrochloric acid were added to 600 μg mL^{-1} Cas 9/sgRNA@ZIF-8 in PBS to reach pH of 5 and 6. PBS only was added to the sample of pH 7. The fluorescence of released AF-Cas 9/sgRNA was monitored by fluorescent spectroscopy (excitation/emission wavelength: 650 nm/668 nm).

Preparation of stable EGFP-CHO cells. pENTR11 containing the cDNA of the EGFP gene was subjected to Gateway® reaction (Invitrogen) in the presence of a pDEST26 vector. The EGFP-construct was then transfected into Chinese Hamster Ovary (CHO)-K1 cells (ATCC) using a calcium phosphate method as described previously.[1] Briefly, cells were cultured one day prior to transfection in 12-well plates (Corning) at a density of $1.5x10^5$ cells/mL in DMEM media (Gibco) supplemented with 10% FBS, 1X of Gibco MEM Non-Essential Amino Acids and 1X of Gibco GlutaMAX at 37°C in 5% CO_2. The following day, media was replaced with 1 mL of new media one hour prior to transfection. Next, pDEST26-EGFP was mixed with 50 μL of 250 mM $CaCl_2$ as 2.5 μg/mL then 50 μL 2X HEPES buffer was added and incubated for one minute to allow crystal to form and mixed gently for the first ~30s of the incubation. The mixture of DNA containing crystals was added to the cells drop wise and incubated with the cells for six hours at 37^oC. The transfection mixture was then removed and cells were washed twice with PBS (Gibco) prior to performing a glycerol shock by adding 1 mL of 10% glycerol and leaving it for one minute at room temperature. Cells were then washed twice with PBS and incubated in

fresh media for two days. To select stable transfectants, cells were amplified for 10-14 days and then were sorted for high GFP expression using BD Influx™ cell sorter in the KAUST Bioscience Core Lab. Stably transfected cells were expanded and used for the experiments outlined here.

Cell culture. CHO cells were seeded in 6 wells plate at a density of 5×10^5 cells. Cells were cultured in RPMI medium containing 10 % FBS and 0.1 % penicillin-streptomycin at 37 ºC in a humidified 5 % CO_2 atmosphere. After cell attachment, they were treated with free Cas 9/sgRNA (240 nM) and CC-ZIFs (100 μg mL^{-1}) dispersed in opti-MEM.

Cell transfection. AF 647 labeled Cas 9 (AF-Cas9) was used to track the uptake of the Cas 9/sgRNA. Transfected cells incubated for 1, 3 and 6 hours. Afterward, cells were washed and imaged by CLSM (Zeiss LSM 880 AIRYSCAN FAST System) or trypsinized, washed and analyzed by flow cytometry (BD LSRFortessa).

Cell viability. To study cytotoxicity, live-dead viability assay and cell counting kit-8 (CCK-8) assay were performed according to the manufacturer's protocol. Unlabeled Cas 9 was used for the treatment. Briefly, CHO cells (5×10^3 cells per well) were seeded onto a 96-well plate. After 12 hours, the culture medium was changed, and cells were incubated with different concentrations (250, 200, 150, 100 and 50 μg mL^{-1}) of Cas 9/sgRNA and Cas 9/sgRNA@ZIF-8 in 200 μL of opti-MEM medium at 37 °C for 3 hours. Only 250 and 100 μg mL^{-1} were selected for the live-dead assay. The medium was then discarded and replaced with fresh RPMI incubated for 9 hours.

Live-dead viability assay. After treatment, cells were washed and stained with the live-dead reagent for 15 minutes. Cells were washed again and collected for the flow cytometry analysis. **CCK-8 Assay.** Media was then discarded and prepared culture medium containing 10 % CCK-8 solution was added into each well, including a negative control of culture media alone. After 3 hours of incubation, the absorbance was measured at 450 nm using a microplate spectrophotometer (xMark™ Microplate Absorbance Spectrophotometer).

Endosomal escape. AF-Cas9 was used to assess the endosomal escape by CLSM. Transfected cells incubated for 1, 3 and 6 hours were washed three times and stained with lysotracker green according to the manufacturer's protocols for 30 minutes. Then, they were washed and imaged by CLSM.

EGFP expression assessment by flow cytometry, CLSM, quantitative real-time polymerase chain reaction (qRT-PCR) and Surveyor assay. EGFP transfected CHO cells were seeded in 6 wells plate at a density of 5×10^5 cells. Cells were cultured in RPMI medium containing 10 % FBS and 0.1 % penicillin-streptomycin at 37 ºC in a humidified 5 % CO_2 atmosphere. After cell attachment, they were treated with free Cas 9/sgRNA (240 nM) and CC-ZIFs (100 μg mL^{-1}) dispersed in opti-MEM for 3 hours. Media containing nanoparticles was replaced by fresh RPMI media. Cells were incubated for 2 and 4 days. Finally, they were washed and collected for flow cytometry analysis, CLSM or qRT-PCR.

qRT-PCR. After washing, transfected cells were collected for RNA extraction. Total RNA was extracted using RNeasy mini kit (Qiagen) according to the instructions of the manufacturer and total RNA concentration was calculated using Qubit. The first-strand cDNA was synthesized after the reverse transcription of the total RNA (2 μg) by using high-capacity cDNA reverse transcription kit in a 20 μl reaction volume. For RT-PCR analysis, EGFP pair of primers used as follow: EGFP forward 5'ACGACGGCAACTACAAGACC-3', EGFP reverse 5'-TTGTACTCCAGCTTGTGCCC-3'PCR regimen involved the following: 95°C for 20 s, 40 cycles at 95 °C for 1 s and 60 °C for 20 s. Gene expression was calculated using the Sequence Detection System software, provided by the manufacturer with a 7900HT Fast Real-Time PCR system (Applied Biosystems).

Surveyor assay. GeneArt genomic cleavage detection kit was used to detect specific locus cleaved in the genomic DNA. The assay uses genomic DNA extracted from EGFP transfected CHO treated with free Cas 9/sgRNA or CC-ZIFs and harvested for 4 days after treatment. According to manufacturer's instructions, the Loci where the gene-specific double-strand breaks occur are amplified by PCR. PCR primers: EGFP forward T7EI-forward 5'-GGAGTTCCGCGTTACATAACTTACG-3', T7EI-reverse 5'AACCTCGACTAAACA CATGT AAAGCATG-3'. PCR program (95 ℃) for 10 minutes, (95 ℃ for 30 s; 55 ℃ for 30 s, 72℃ for 30 s) for 40 cycles and (72 ℃ for 7 minutes) one cycle. The amplicons contain the mismatch occurred by the used gRNA were subsequently detected and cleaved by detection enzyme and the resultant bands were analyzed by 2 % agarose gel electrophoresis and indel formation efficiencies were calculated using Image J.

Statistical analysis. Comparisons between different treatments were made using one-way ANOVA. Statistical significance was defined as highly significant ***P< 0.001, statistically significant **P<0.01, not significant *P<0.1.

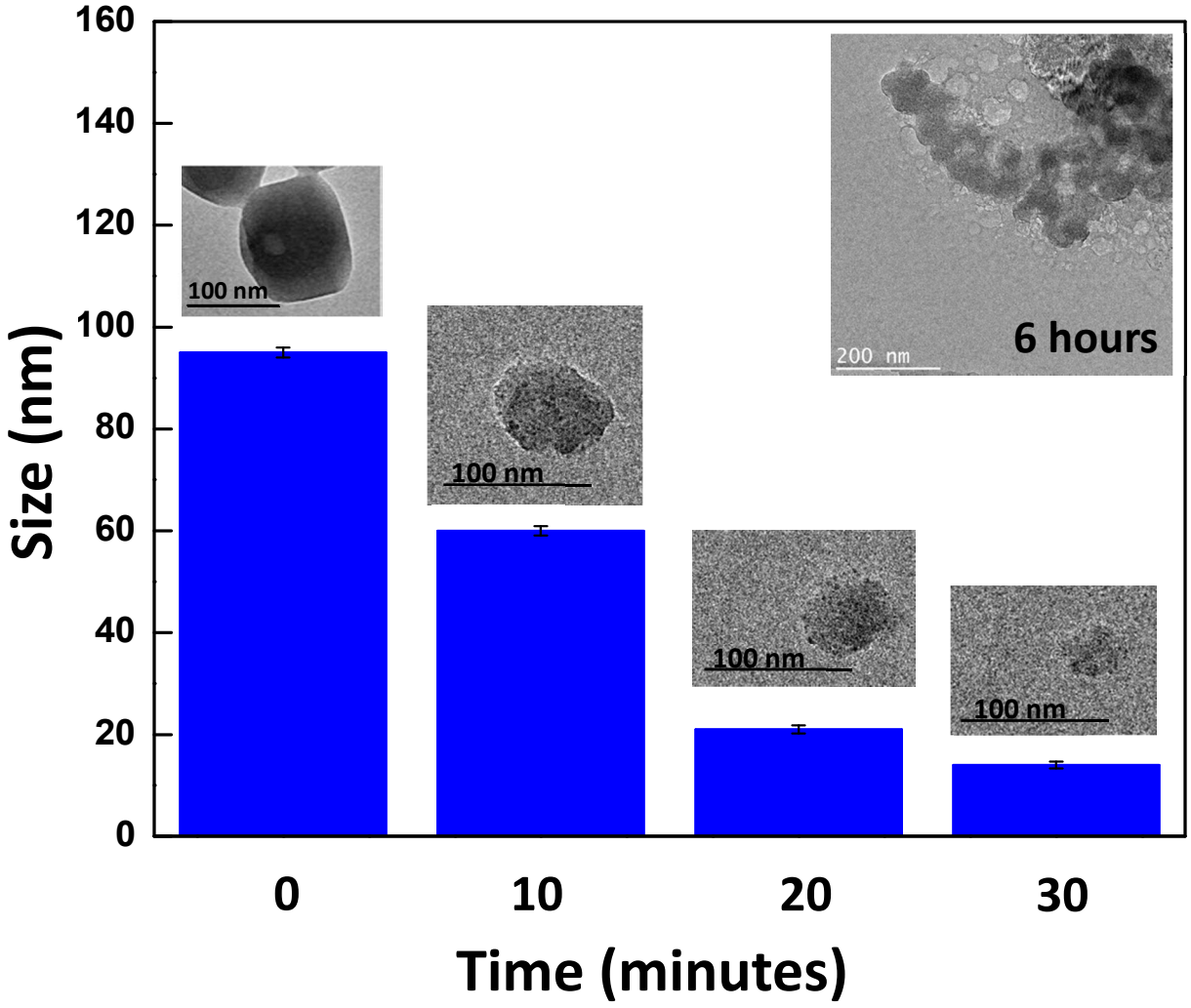

Figure S1. DLS analysis and TEM images of CC-ZIFs at neutral conditions (0 time) and then at acidic conditions (pH 5.5) over 6 hours.

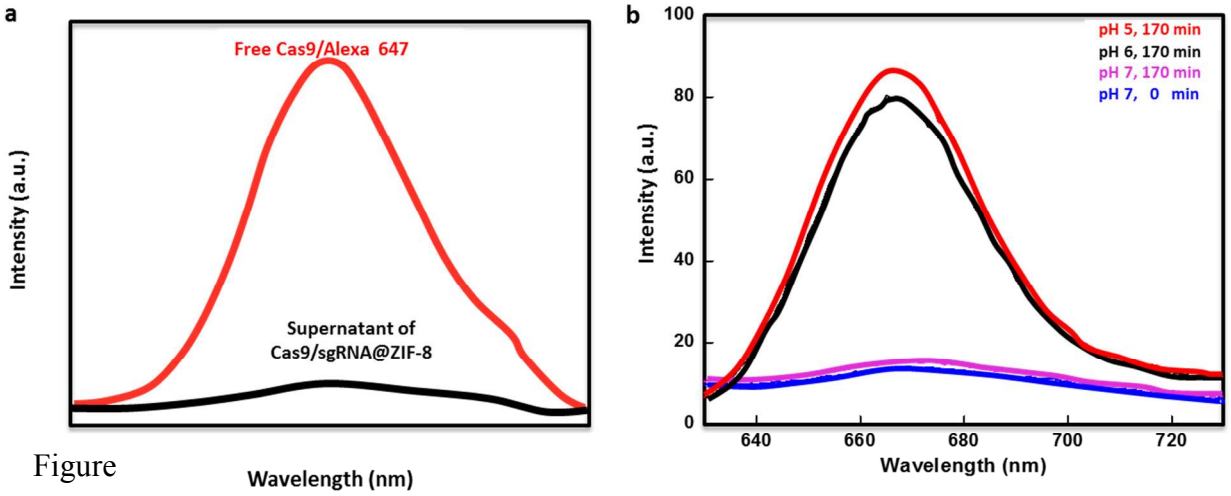

Figure S2. (a) Fluorescence emission spectra of the supernatant of CC-ZIFs after washing with water. Cas 9 was labeled with AF 647 to monitor its loading and release. (b) The fluorescence spectra of the supernatant over 180 minutes were measured to demonstrate the release of AF-CC-ZIFs under acidic conditions.

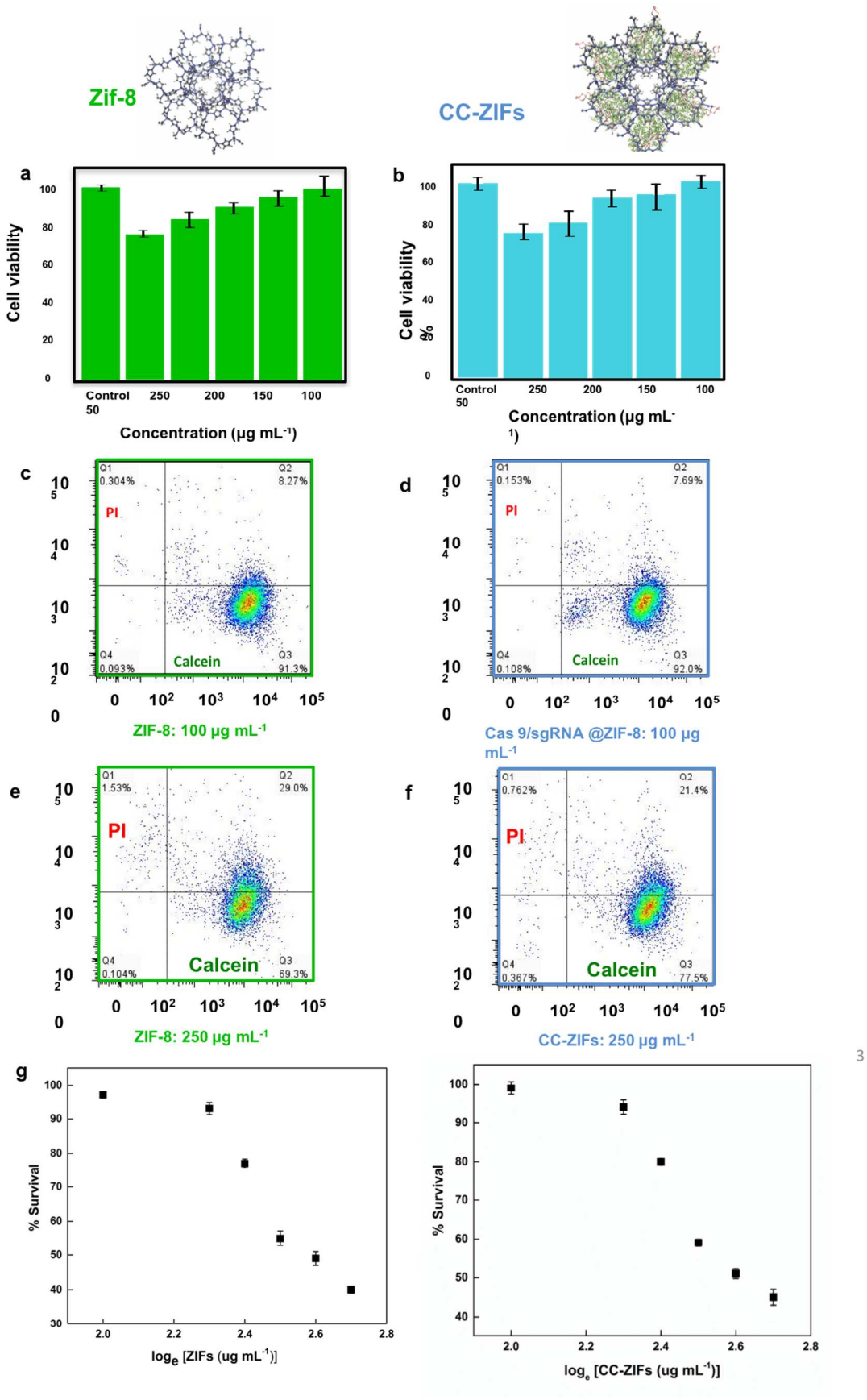
Zif-8
CC-ZIFs
a
Cell viability
Control
250
200
150
100
50
Concentration (μg mL-1)
b
Cell viability
Control
250
200
150
100
50
Concentration (μg mL-1)
c
Q1 0.304%
Q2 8.27%
PI
Q4 0.093%
Calcein
Q3 91.3%
ZIF-8: 100 μg mL-1
d
Q1 0.153%
Q2 7.69%
PI
Q4 0.108%
Calcein
Q3 92.0%
Cas 9/sgRNA @ZIF-8: 100 μg mL-1
e
Q1 1.53%
Q2 29.0%
PI
Q4 0.104%
Calcein
Q3 69.3%
ZIF-8: 250 μg mL-1
f
Q1 0.762%
Q2 21.4%
PI
Q4 0.367%
Calcein
Q3 77.5%
CC-ZIFs: 250 μg mL-1
g
% Survival
loge [ZIFs (ug mL-1)]
% Survival
loge [CC-ZIFs (ug mL-1)]

Figure S3. Biocompatibility was measured by cell counting kit-8 assay (CCK-8) for (a) ZIF-8 and (b) CC-ZIFs. CHO cells were plated on 96 wells plate and treated with different concentrations of ZIF-8 for 24 h. Bars represent mean ± SD (n=3). Flow cytometric viability assay using LIVE/DEAD Viability/Cytotoxicity kit for (c) ZIF-8 at 100 μg mL^{-1}, (d) CC-ZIFs 100 μg mL-1, (e) ZIF-8 at 250 μg mL^{-1} and (f) CC-ZIFs at 250 μg $mL^{-1}$and (g) LD50 of ZIFs and CC-ZIFs.

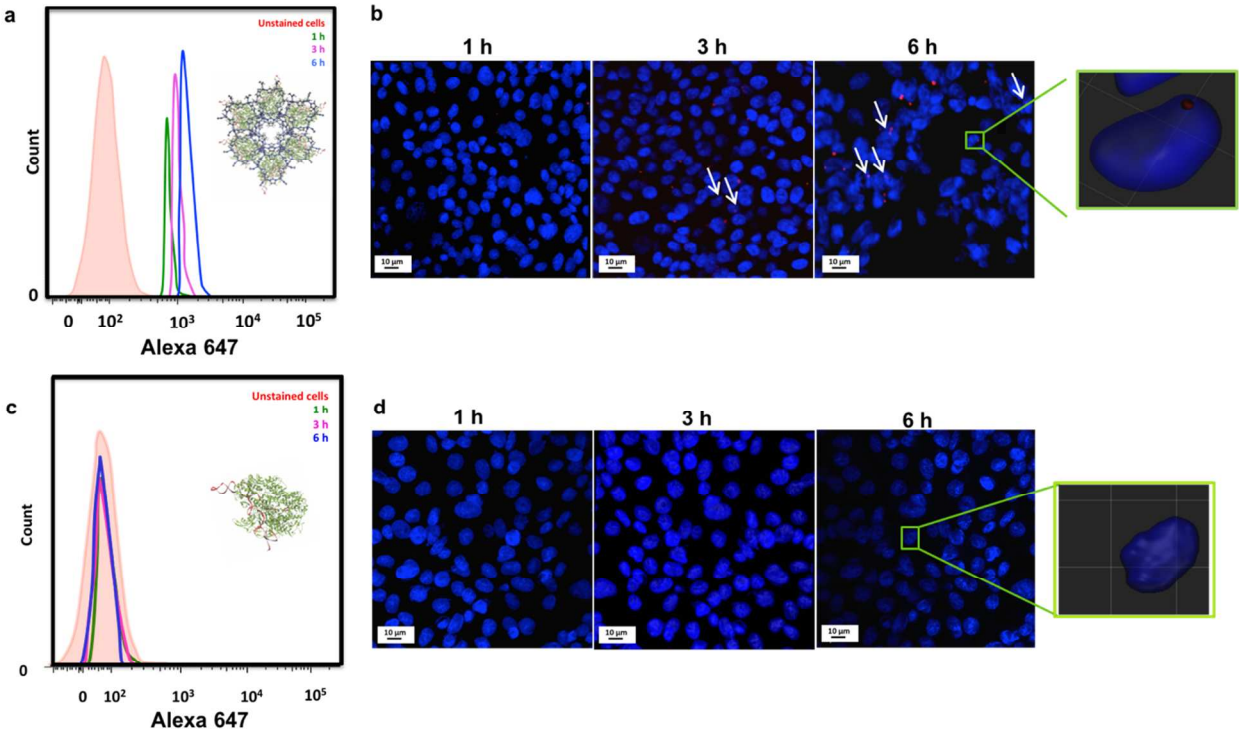

Figure S4. (a) Flow cytometry analysis of the uptake of AF-CC-ZIFs by CHO cells. Cas 9 was labeled with alexa 647 and the uptake was monitored over 6 h. (b) CLSM images of the uptake of CC-ZIFs. (c) Flow cytometry analysis of the uptake of free AF-Cas 9/sgRNA by CHO cells. (d)) CLSM images of the uptake analysis of free AF-Cas 9/sgRNA. Nuclei were stained with hoechst 33342. Free Cas 9/sgRNA and CC-ZIFs (240 nM) were incubated with CHO cells for 1 h, 3 h and 6 h.

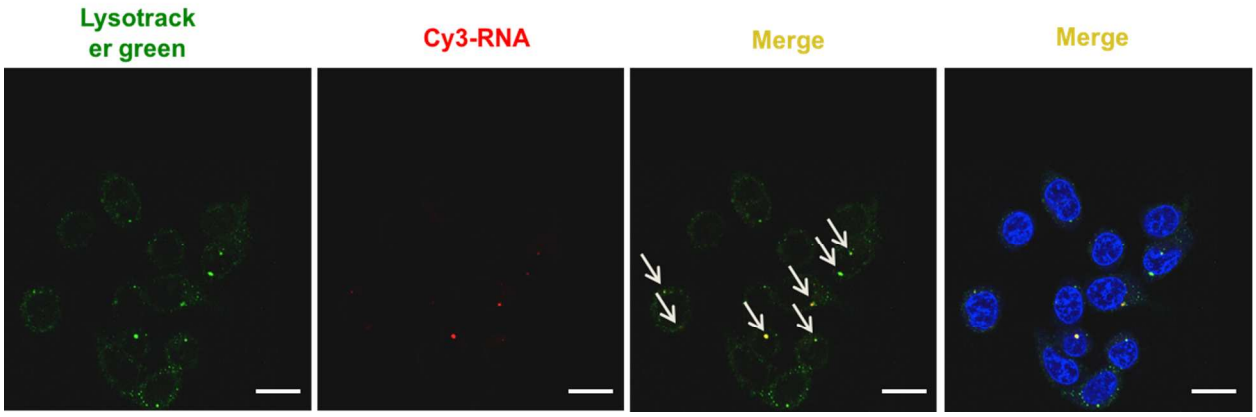

Figure S5. (a) CLSM images of CHO cells after incubation with Cy3 labeled RNA loaded liposomes for 6 hours. Lysotracker Green (green) was used to stain the acidic organelles (endosomes). The merged images are used to confirm that Cy3 labeled RNA is still entrapped in endosome after 6 hours of uptake. Scar bar: 20 μm.

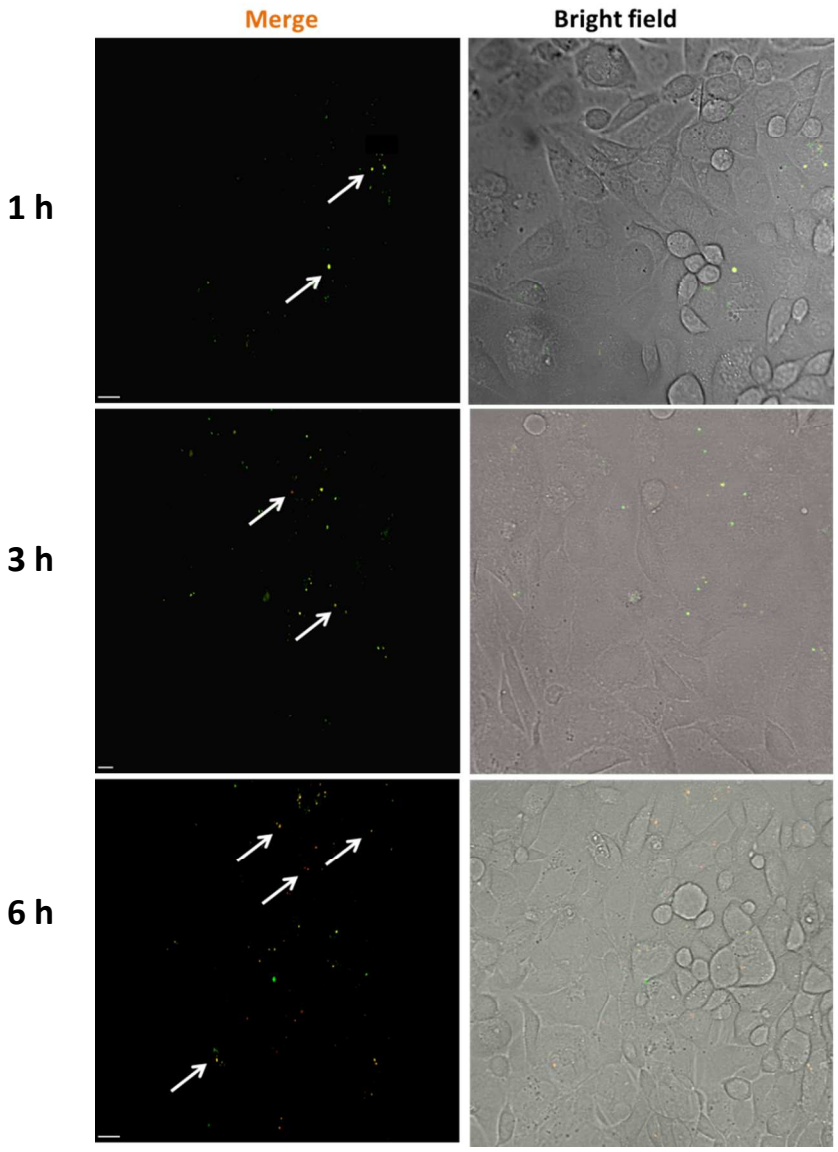

Figure S6. CLSM images (20x) of CHO cells after incubation with AF-CC-ZIFs (red) for 1, 3 and 6 hours. Lysotracker Green (green) was used to stain the acidic organelles (endosomes). The merged images are used to confirm that AF-Cas 9/sgRNA is released and escaped into the nucleus within 6 hours of uptake. Scar bar: 20 μm.

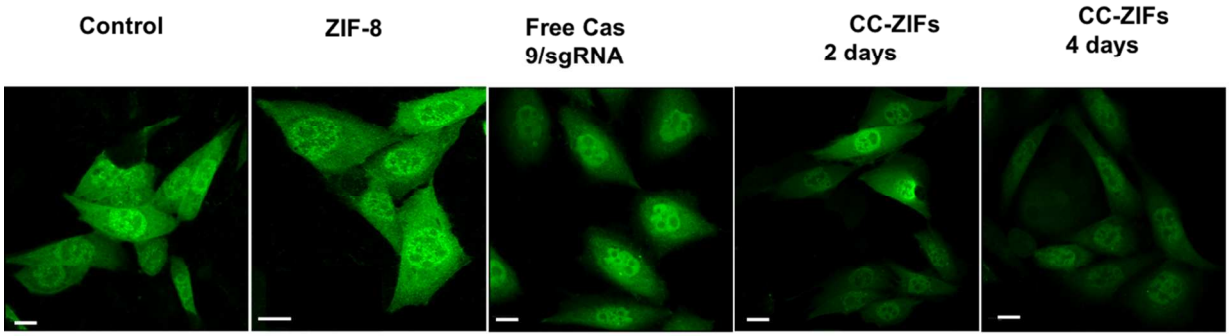

Figure S7. EGFP transfected CHO cells treated with free Cas 9/sgRNA, ZIF-8 and CC-ZIFs. Cells were washed after 3 hours and incubated in fresh RPMI media for 2 and 4 days. Scar bar: 10 nm

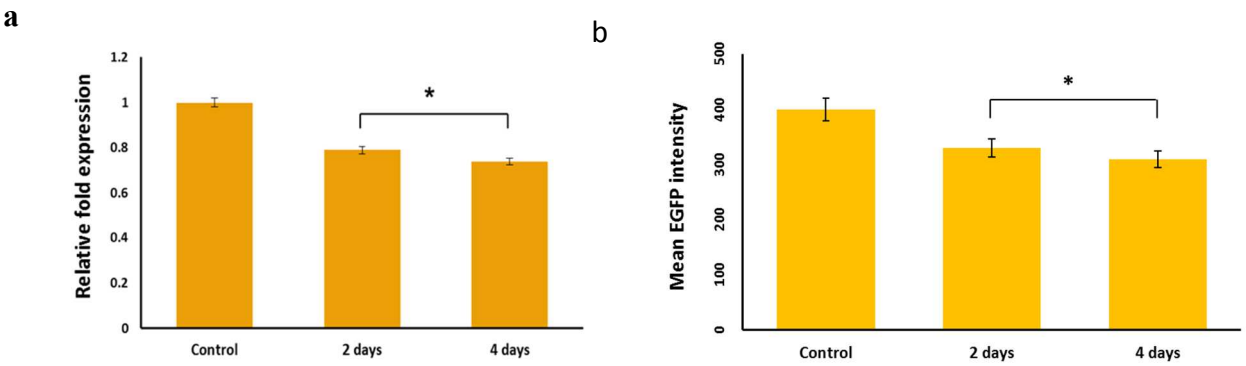

Figure S8. Genome editing by CC-lipofecatamine. Quantitation of EGFP expression by qPCR (a) and flow cytometry (b) at concentration of 240 nM. CHO cells were incubated with CC-lipofectamine for 3 h, then, they were washed and incubated in media for 2 and 4 days. Statistical analysis was determined using unpaired t-test (***P< 0.001, **P<0.01, *P<0.1).