Electronic Supplementary Material (ESI) for Nanoscale.

Improving the Knock-In Efficiency of MOFs-Encapsulated CRISPR/Cas9 system through Controllable Embedding Structures

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1. Experimental

1.1. Materials:

Zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6H_2O$, 99%), 2-methylimidazole (2-MIM, 99%), branched polyethyleneimine (PEI, MW = 10000), paraformaldehyde (PFA, paraformaldehyde, powder, 95%), DAPI (4',6-diamidino-2-phenylindole, for nucleic acid staining), glycine, triton X-100, agarose and cell proliferation reagent (WST-1) were purchased from Sigma-Aldrich. Nuclease free water was obtained from IDT. Sulfo-Cyanine5.5 NHS ester (Cy5.5) was purchased from Lumiprobe. The PXN cutdown plasmid (GFP-Cas9-paxillin_gRNA, P1) that encoded with the gRNA sequence targeting paxillin (5'-GCACCTAGCAGAAGAGCTTG-3') into pSpCas9(BB)-2A-GFP backbone, the template plasmid (AICSDP-1:PXN-EGFP, P2) were kind gifts from Prof. Johanna Ivaska at Turku Bioscience Centre (Biocity, Finland). The primary antibodies against paxillin (Recombinant Anti-Paxillin antibody [Y113] (ab32084)) were bought from Abcam. The secondary antibodies Tetramethylrhodamine (TRITC) conjugated Goat Anti-Rabbit IgG (H+L) were bought from Novex.

1.2. Characterization

The morphology of the synthetic NPs was evaluated by a TEM (JEOL 1400 Plus, USA). Particle sizing was performed using dynamic light scattering with a Zetasizer Nano ZS (Malvern Instruments Ltd., UK). The nanocarrier surface zeta potential was measured with a Zetasizer Nano ZS by using disposable folded capillary cells (DTS1070, Malvern, UK). The confocal fluorescence images were acquired with a confocal laser scanning microscopy (Zeiss, LSM880) under ambient conditions. 3D reconstruction and 3D Pearson's Correlation Coefficient (PCC) calculation were performed using the Fiji software. The BD LSRFortessa flow cytometer was employed to analyze cellular uptake and transfection.

1.3. Plasmid (P1 and P2) amplification and isolation

P1 and P2 plasmids were amplified in Escherichia coli and isolated from a bacteria suspension using a TIANprep Mini Plasmid Kit (TIANGEN Biotech, Beijing, China). Plasmid concentration measurements at a wavelength of 260 nm were performed on a NanoDrop 2000c (Thermo Fisher Scientific, USA). The A260/A280 ratio was measured to assess the purity. Finally, the two plasmids were dissolved in DNase free water and stored in -20°C for further use.

1.4. Synthesis of based ZIF-8 biocomplexes with different architectures

In the typical synthesis process, different amount of P1 was dispersed into 2 µL zinc nitrate solution (67.5 $mg \cdot mL^{-1}$) then stirred for 5 min before added into 8 µL 2-methyl imidazole solution (2-MIM, 463.75 $mg \cdot mL^{-1}$). The mixture was aged for 1 h, and the formed P1Z biocomplexes was collected by centrifugation at 8000 rpm, and then washed, sonicated, and centrifuged three times to remove loosely adsorbed plasmids.

The similar synthesis process, where P1 was dispersed into 8 µL 2-MIM solution then stirred for 5 min, followed by adding 2 µL zinc acetate solution. The mixture was aged for 1 h, and the formed ZP1 biocomplexes was collected by centrifugation at 8000 rpm, and then washed, sonicated, and centrifuged three times to remove loosely adsorbed plasmids.

P1P2Z nanostructures were synthesized with the similar process. The P1 and P2 mixed with 1:1 ratio and 2 µL zinc nitrate solution were well mixed and stirred for 5 min at room temperature before added into 8 µL 2-MIM solution slowly. 1 h later, the final product was obtained after centrifugation at 8000 rpm for 5 min, washed and dispersed into water.

1.5. Calculation of P1 and P1P2 loading efficiency

Plasmids that were not loaded into the particles were collected from the supernatant. These non-loaded plasmids were then measured via UV-vis spectroscopy (NanoDrop 2000c, ThermoFisher) at wavelengths of 260 and 280 nm. From the DNA concentration, loading efficiency (LE%) was determined by using the formula below:

$LE\% = (Ai-Af)/Ai \times 100\%$

1.6. Agarose gel electrophoresis of binding affinity

For DNA electrophoresis assay: 8 mg of agarose powder was added to 100 mL TBE (Tris-borate-ethylenediaminetetraacetic acid, 0.5%) in a microwavable flask and microwaved for several minutes until the agarose was completely dissolved. After the agarose solution cooled down, then the agarose was poured into a gel tray with the well comb and waited for 20-30 min until it had completely solidified.

10 µL aqueous solution of P1Z and ZP1 nanostructures containing the different amount of P1 were mixed with 2 µL of 6× DNA loading buffer, respectively. Then, the DNA ladder, free P1 and the nanostructures were electrophoresed on 0.8% agarose gel containing 0.1% GelRed (10 µL, GelRed Nucleic Acid Stain 10000×) at 130V for 100 min. DNA/RNA bands were visualized by a UV Trans Illumination and photographed with a ChemiDoc imaging system (Bio-Rad). The binding affinity was expressed by the weight ratio of P1 and the pure ZIF-8 vectors in the system.

1.7. Protection assay from EcoRV Digestion

For digestion reaction: The nuclease-free water (16 μL) and 10x buffer R (2 μL) were mixing together and then adding 1 μL pure P1, released P1 from P1Z and ZP1, following by adding EcoRV (2 μL). The solution for P1 without EcoRV was taken as control group. Every solution was mixed gently and spun down for a few seconds. Afterward, they were incubated overnight at 37 °C water bath.

After that, the samples (10 μL) mixed with 6× loading buffer (2 μL) were loaded into comb wells carefully. The Fast Ruler High Range DNA ladder was loaded into the first lane of the gel. The gel was placed into the gel box and run it at 130 V for 100 min. Finally, the gel was photographed with a ChemiDoc imaging system (Bio-Rad) to visualize the DNA fragments.

1.8. pH-responsive release of P1 from P1Z and ZP1 nanostructures

The in vitro release of encapsulated plasmid from nanocarriers and the effect of pH on the release profiles were determined by suspending the P1Z and ZP1 in 20 μL PBS buffer with different pH (7.4 and 5.5), respectively. All suspensions were placed in a shaker (GrantGLS400). The amount of released plasmid was determined by removing the supernatant after centrifugation (8000 rpm, 5 min) and replacing it with a new buffer at the collecting time points. Then the release profile was measured using NanoDrop 2000c Fluorospectrometer (ThermoFisher, USA) and all the measurements were performed in triplicate.

1.9. Cell Viability Assay

The used cell line, U2OS were cultured in a 37 ℃ incubator under 5% CO_2 and 90% humidity with complete growth medium: DMEM supplemented with 10% v/v heat inactivated FBS, 0.5% penicillin/streptomycin solution, 1% nonessential amino acid, and 1% L-glutamine.

To study cytotoxicity, WST-1 assay was performed according to the manufacture's protocol. U2OS cells were seeded into 96-well plates (2×10^4 cells/well) one day before. Then, the cell growth medium was replaced with a fresh medium containing different concentrations of P1Z and P1P2Z, which positive control was 100 uL DMEM with 10% dimethyl sulfoxide. After 48 h incubation, the WST-1 reagent was added to each well and the cells were incubated for 2 h at 37 °C with 5% CO_2. The absorbance was measured by a Varioskan Flash Multimode Reader (Thermo Scientific Inc., Waltham, MA, USA) at 440 nm. Triplicates were used for the experiment and averaged absorbance readings were plotted.

1.10. Cell Uptake Study

The U2OS cells were seeded and incubated overnight in confocal dishes (2×10^5 cells/dish) and 6-well plates (2×10^5 cells/well). Then the medium was replaced with the fresh medium containing P1Z and incubated for another 1, 2, 4 and 6 h at 37 °C. For CLSM (Zeiss, LSM880, Germany), the cells in confocal dishes were rinsed with PBS twice, fixed with 4% PFA, and stained with DAPI (5 $\mu g \cdot mL^{-1}$). Detection of DAPI was performed with 405 nm laser excitation and laser 633 was utilized for Cy5.5 exciting. For quantitative analysis, the cells in the 6-well plate were collected by trypsin and dispersed in 1% PFA PBS solution. Then the acquisition of cellular uptake was determined by flow cytometer BD LSRFortessa by using the Alexa Fluor 700 channel. All measurements were carried out in triplicate.

1.11. Cellular endo-/lysosomal escape assay

The U2OS cells were seeded in confocal dishes (2×10^5 cells/dish) and incubated overnight for attachment. The plasmids-loaded nanocarrier (Cy5.5 labelled P1Z) was dispersed into DMEM and added to each dish. LysoTracker@Green probe was used to stain endosomes according to the manufacture protocol. At the determined time point (1, 2, 4 and 6 h), the cells were fixed with 4% PFA and further stained with DAPI. Then the endosomal escape ability of the nanocarrier were measured with CLSM.

1.12. In Vitro Gene Transfection Study

The U2OS cells were seeded into 24-well plate (5×10^4 cells/well) and cultured overnight. The P1Z were dispersed into 1 mL DMEM culture medium and added to corresponding wells, in which the P1 and ZIF were used as control. After incubating for the determined time at 37 °C, the cells were analyzed by EVOS fluorescence microscopy (AMG) using a GFP filter.

1.13. Analysis of Gene Editing Efficiency

Fibronectin staining: Fibronectin can work as an extracellular matrix ligand for the integrins; thus, GFP-tagged paxillin should be localized mainly into these focal adhesion structure in cultured U2OS cells. A solution of fibronectin was prepared in PBS (10 $\mu g \cdot mL^{-1}$) and this solution was used for staining the confocal plate at overnight incubation at 4 °C. Next day the confocal glass was washed with PBS, and then the cells that were incubated a week prior with plasmid loaded P1P2Z were plated carefully with a pipette on to the fibronectin-coated glass and incubated at room temperature for 2 h. After attachment, the cells were fixed with 4% PFA for 5 min at room temperature and washed with PBS three times. DAPI solution (5 $\mu g \cdot mL^{-1}$) was then added into the cells for 5 min. Then it was washed three times with PBS.

Western blotting: To analyze protein expression of PXN and PXN-EGFP, U2OS were washed with PBS and disrupted in RIPA lysis buffer containing complete EDTA-free protease inhibitor and phosphatase inhibitor. The sample was

separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membrane was blocked with the blocking buffer at room temperature for 2 h, then incubated at 4°C overnight in 3% BSA/TBST with the appropriate primary antibodies (1:1000). The membrane was incubated at room temperature with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (1:5000) for 1 hour. Finally, the western blotting was developed with chemiluminescence and analyzed.

Immunofluorescence test: After the PFA fixation, the cells were treated with glycine (1 M in PBS, 30 min) to quench background autofluorescence coming from the PFA and then permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Permeabilization removes the cellular membrane lipids which helps the antibodies accessibility to the interior of the cell for better targeting of the paxillin protein. Cells were stained with primary antibodies at 1:100 dilution of the Rabbit recombinant monoclonal Paxillin antibody in PBS and kept at +4 ℃ overnight. The cells were then washed twice with PBS and incubated with secondary antibodies, Tetramethylrhodamine (TRITC) conjugated Goat Anti-Rabbit IgG (H+L) (Novex) at dilution of 1:500 (45 min, RT). After the incubation, cells were washed three times with PBS and later observed by using a confocal microscope (Zeiss, LSM 880). Helium-Neon 543 nm laser line was used for TRITC, Argon 488 nm laser line for paxillin-GFP and 405 nm diode laser line for DAPI.

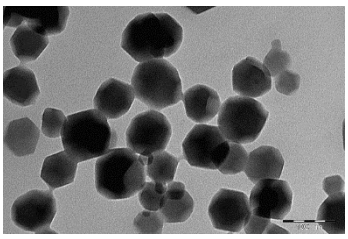

Figure S1. ZIF-8 nanoparticles without plasmid modulation.

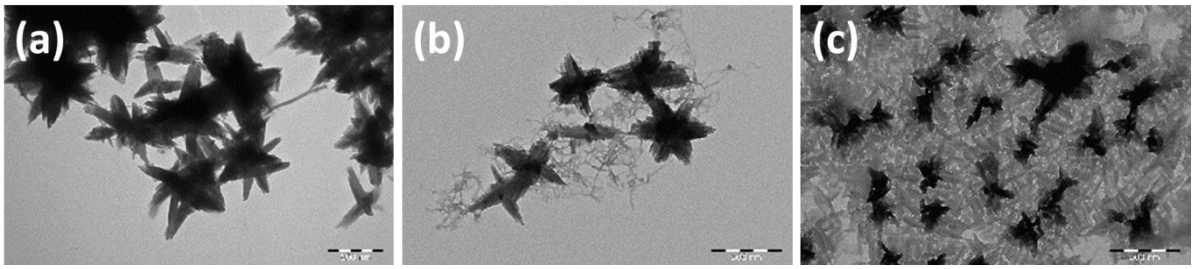

Figure S2. TEM of P1Z with 10, 20 and 30 µl lyophilized P1.

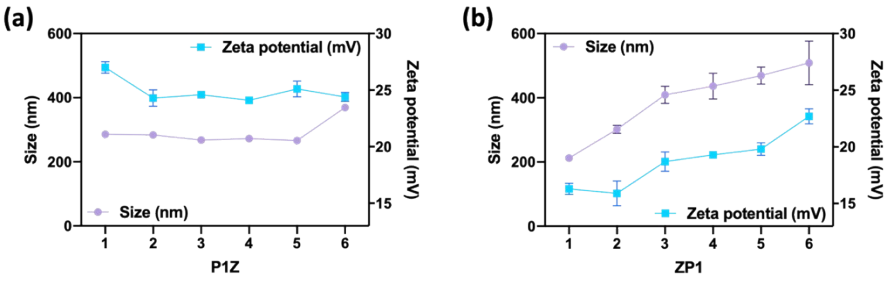

Figure S3. DLS and Zeta potential of P1Z (a) and ZP1 (b).

Table S1. the encapsulation capacity of P1 in different embedding patterns.

Structures	P1Z (1 μL)	P1Z (2 μL)	P1Z (3 μL)	P1Z (4 μL)	P1Z (5 μL)	P1Z (6 μL)
Encapsulation	100%	97%	93%	87%	80%	63%
Structures	ZP1 (1 μL)	ZP1 (2 μL)	ZP1 (3 μL)	ZP1 (4 μL)	ZP1 (5 μL)	ZP1 (6 μL)
Encapsulation	100%	95%	84%	60%	47%	39%

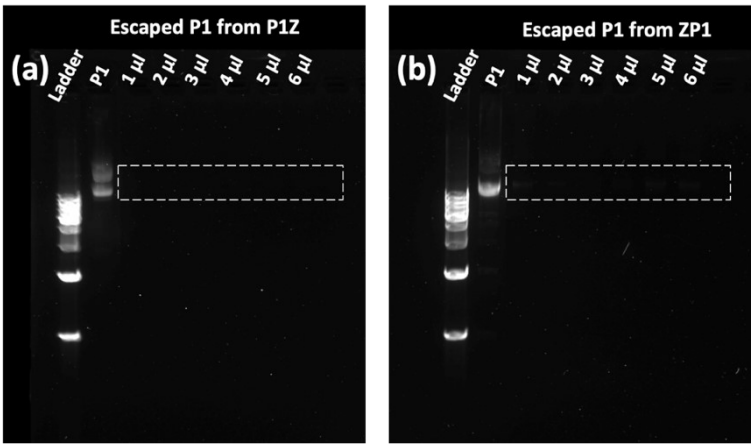

Figure S4. The binding affinity of P1 in the P1Z (a) and ZP1 (b) nanostructures under agarose gel electrophoresis.

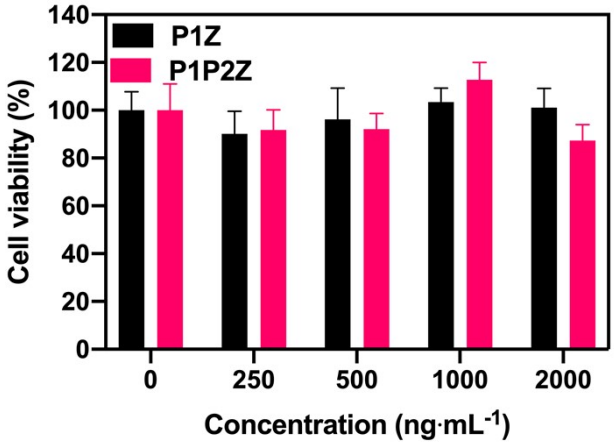

Figure S5. Cell viability of U2OS cells treated with P1Z and P1P2Z system at different concentrations from 250 to 2000 ng·mL^{-1} for 48 h.

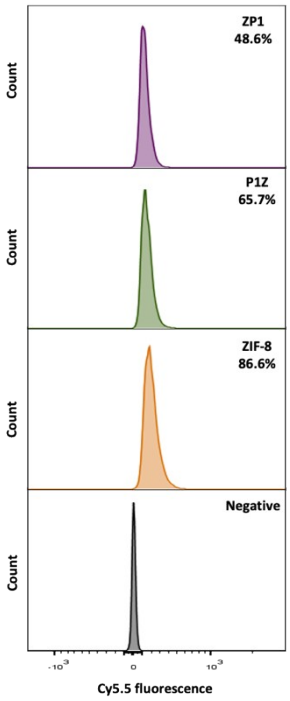

Figure S6. Cellular uptake of ZIF-8, P1Z and ZP1 nanocarriers after 6 h incubation detected by flow cytometry.

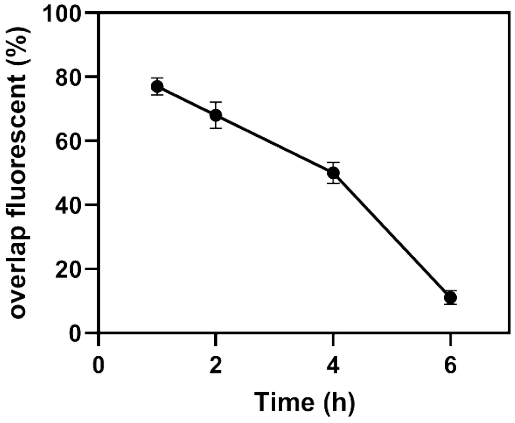

Figure S7. The Pearson's Correlation Coefficient (PCC) is calculated via Image J software for overlap fluorescence analysis to quantitatively analyze the endo-/lysosomal escape capacity of nanostructures.

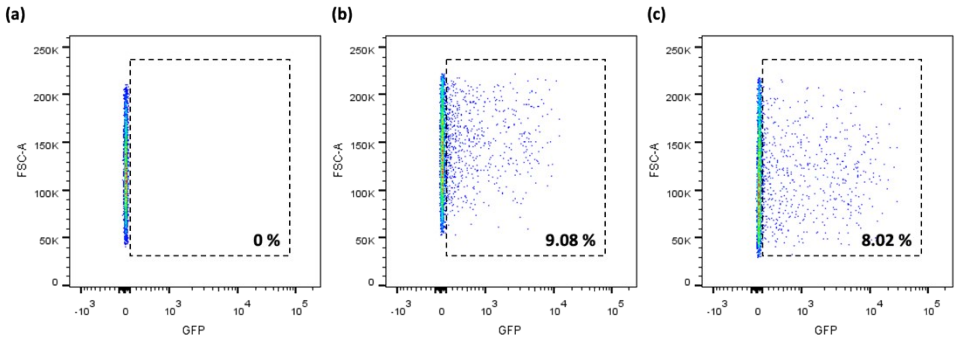

Figure S8. Transfection efficiency of U2OS cells treated with negative control (a), Lipofectamine (b) and P1Z (c) after 48h incubation determined by flow cytometry.

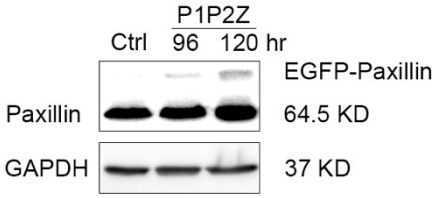

Figure S9. EGFP-Paxillin protein expression was analyzed by performing Western blotting analysis. The cells were treated with P1P2Z for 96 and 120 h. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

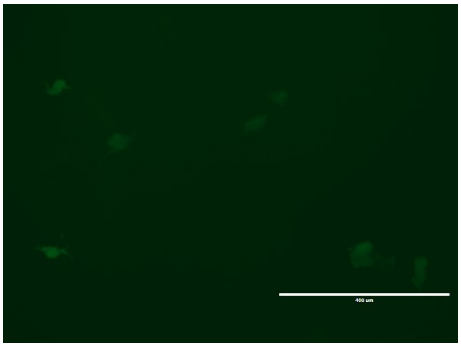

Figure S10. Fluorescence microscopy image of U2OS cells treated with P1P2Z after 7 days incubation.