Electronic Supplementary Material (ESI) for Nanoscale.

A Non-cationic Nucleic Acid Nanogel for the Delivery of CRISPR/Cas9 Gene Editing Tool

Fei Ding[a], Xiangang Huang[a], Xihui Gao[a], Miao Xie[a], Gaifang Pan[a],

Qifeng Li[b], Jie Song[c], Xinyuan Zhu[a], Chuan Zhang[a]*

[a]School of Chemistry and Chemical Engineering, State Key Laboratory of Metal Matrix Composites, Shanghai Key Laboratory of Electrical Insulation and Thermal Ageing, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai, 200240, China.

[b]Department of Pediatric Neurosurgery, Xinhua Hospital, Shanghai Jiao Tong University, School of Medicine, Shanghai, 200092, China

[c]Department of Instrument Science and Engineering, School of Electronic Information and Electrical Engineering, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai, 200240, China

*Correspondence and requests for materials should be addressed to C. Z. (chuanzhang@sjtu.edu.cn)

Materials and methods

Materials. The solvents and reagents for DNA synthesis were purchased from DNAchem technology Co., Ltd. (Beijing, China). The Cas9 and sgRNA were purchased from Inovogen biotechnology Co., Ltd. (Chongqing, China). The RNase A and DNase Ⅰ purchased from ThermoFisher Scientific (USA). All other chemical reagents were purchased from Tansoole Co., Ltd. (Shanghai, China). All oligonucleotide sequences are listed in the table below.

Table S1. DNA and RNA sequences used in this study

DNA or RNA		Sequences	Note
DBCO-DNA		5'-DBCO-TTT AAT ACG ACT-3'	
DNA Linker 1		5'-ACT ATG CGT GAA TCC GTT AGA GTC GTA TTA AA-3'	
DNA Linker 2		5'-CTA ACG GAT TCA CGC ATA GTA GTC GTA TTA AA-3'	
RNA Transcription Template	**sgRNA**	5'-GTT TTT TTT AAT ACG ACT CAC TAT AGG AGT CGT ATT AAA GGG CAC GGG CAG CTT GCC GGG TTT TAG AGC TAG AAA TAG CAA GTT AAA ATA AGG CTA GTC CGT TAT CAA CTT GAA AAA GTG GCA CCG AGT CGG TGC TTT TTT T-3'	T7 Promoter EGFP Targeting DNA-*g*-PCL binding
	cgRNA	5'-GTT TTT TTT AAT ACG ACT CAC TAT AGG AGT CGT ATT AAA GGG TAA CCG TGC GGT CGT ACG TTT TAG AGC TAG AAA TAG CAA GTT AAA ATA AGG CTA GTC CGT TAT CAA CTT GAA AAA GTG GCA CCG AGT CGG TGC TTT TTT T-3'	T7 Promoter non-targeting sequence DNA-*g*-PCL binding
SURVEYOR assay primer	**T7EI-F**	5'-CTT GAA GTC GAT GCC CTT CA-3'	
	T7EI-R	5'-TGA ACC GTC AGA TCG CCT-3'	

Measurements.

Ultraviolet-Visible absorption (UV-Vis). The DNA samples were quantified by measuring the UV-Vis absorption of sample at 260 nm (UV-1800, Shimadzu corporation).

Dynamic light scattering (DLS). DLS measurements were performed on a Zetasizer Nano ZS90 instrument (Malvern Instruments Ltd.). The scattering angle was kept at 173o and the laser wavelength was 633 nm during the experiment.

Atomic force microscopic (AFM). AFM images were obtained using FM-Nanoview 1000 AFM (Suzhou Flyingman Precision Instrument Co., Ltd.) in tapping mode to collect height information.

Transmission electron microscope (TEM). TEM imaging was conducted by using a Tecnai G2 Spirit Biotwin transmission electron microscope (FEI, USA) operated at 120 KV.

Methods

Synthesis of DNA-grafted polycaprolactone (DNA-*g*-PCL). The preparation of DNA-*g*-PCL followed our previous report.[1] In brief, a mixture of dibenzocyclooctyl-modified DNA (DBCO-DNA) (100 OD, 775 nmol) and ethyl-poly(α-N_3-εCL) (112.2 μg, ~ 775 nmol -N_3) in 600 μL dimethyl sulfoxide (DMSO) was shaken at 50 ^oC for 48 h. Then the solution was dialyzed against water to remove DMSO. The DNA-*g*-PCL was further purified using 50 kDa molecular weight cutoff centrifugal filtration units (Amicon Ultra-4 Ultracel-50K, Millipore) to remove the unreacted DBCO-DNA in solution.

Preparation of Cas9/sgRNA-embedded nucleic acid nanogel (nanogel-Cas9/sgRNA). In brief, the stock solution of DNA-*g*-PCL brushes (30 μM) and Cas9/sgRNA complexes were mixed together at 30 : 1 molar ratio (brush DNA:Cas9/sgRNA) in 1×TAE/Mg^{2+} buffer (40 mM Tris, 2 mM EDTA•Na•H_2O, 20 mM acetic acid, 12.5 mM $(CH_3COO)_2Mg•4H_2O$) at room temperature for 10 min. Subsequently, the mixtures were crosslinked by DNA linkers (30 μM) at 1:6 molar ratios (linker:brush DNA) in 1×TAE/Mg^{2+} buffer and stirred for 30 min, then stored at 4 ^oC.

Characterizations of nanogel-Cas9/sgRNA. Each DNA sample was analyzed by

agarose 0.8 % (weight/weight) gel electrophoresis at 4 °C (100 V, constant voltage) in 1×TAE/Mg^{2+} buffer. And the nanogel-Cas9/sgRNA was imaged by AFM and TEM to reveal its morphology and structure. The size of nanogel-Cas9/sgRNA was measured by DLS and acquired on a Zetasizer Nano ZS.

Agarose gel electrophoresis. Each sample (50 μL) was mixed with loading dye and analyzed by 0.8 % agarose (weight/weight) at 4 °C (100 V, constant voltage) in 1×TAE/Mg^{2+} buffer. After electrophoresis, the gels were firstly visualized by ChemiDoc MP (Bio-red, USA) using Cy5 and FAM channel, then stained by ethidium bromide (EB) and visualized again under UV exposure.

Nanogel-Cas9/sgRNA stability assay. The stability of nanogel-Cas9/sgRNA in physiological condition was characterized by agarose electrophoresis. The nanogel-Cas9/sgRNA were incubated with culture medium containing 10 % (v/v) fetal bovine serum (FBS) at 37 °C for different incubation time. Then, the incubated samples were loaded on 0.8 % agarose gel for electrophoresis at 4 °C in 1×TAE/Mg^{2+} buffer.

RNase A resistance assay of nanogel-Cas9/sgRNA. The nanogel-Cas9/sgRNA complexes were incubated with different concentrations of RNase A for 1 h at 37 °C. Then, the samples were analyzed by 0.8 % agarose gel electrophoresis at 4 °C in 1×TAE/Mg^{2+} buffer.

Gene editing capability of nanogel-Cas9/sgRNA in presence of RNase A. The nanogel-Cas9/sgRNA were incubated with different concentration of RNase A for 1 h at 37 °C. Then the samples were incubated with EGFP genomic DNA substrate for 0.5 h at 37 °C. Finally, the obtained mixtures were analyzed by 2.8 % agarose gel electrophoresis at 4 °C in 1×TAE/Mg^{2+} buffer.

DNase Ⅰ induced degradation of EGFP genomic substrate. Briefly, the EGFP genomic DNA substrates were incubated with different concentrations of DNase Ⅰ for 0.5 h at 37 °C. Then, the treated samples were analyzed by 2.5 % agarose gel electrophoresis at 4 °C in 1×TAE/Mg^{2+} buffer .

DNase Ⅰ mediated release of Cas9/sgRNA from nanogel-Cas9/sgRNA. The nanogel-Cas9/sgRNA were incubated with 0.4 U/mL DNase Ⅰ for different incubation time at 37 ^{o}C. Then, the samples were analyzed by 0.8 % agarose gel electrophoresis at 4 ^{o}C in 1×TAE/Mg^{2+} buffer.

Gene editing capability of nanogel-Cas9/sgRNA in the presence of DNase Ⅰ. The nanogel-Cas9/sgRNA were incubated with 0.4 U/mL DNase Ⅰ for different incubation time at 37 ^{o}C. Then, the samples were incubated with EGFP genomic DNA substrates for 0.5 h at 37 ^{o}C. Finally, the obtained mixtures were analyzed by 2.8 % agarose gel electrophoresis at 4 ℃ in 1×TAE/Mg^{2+} buffer.

Gene editing capability of nanogel-Cas9/sgRNA in the presence of both DNase Ⅰ and RNase A. The nanogel-Cas9/sgRNA were incubated with 0.4 U/mL DNase Ⅰ and 0.1 U/mL RNase A for different incubation time at 37 ^{o}C. Then, the samples were incubated with EGFP genomic DNA substrate for 0.5 h at 37 ^{o}C. Finally, the obtained mixtures were analyzed by 2.8 % agarose gel electrophoresis at 4 ℃ in 1×TAE/Mg^{2+} buffer.

Cell culture. HeLa cells (a human cervical carcinoma cell line) were purchased from China Type Culture Collection (CTCC) and cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10 % FBS and antibiotics (50 units/mL penicillin and 50 units/mL streptomycin) at 37 ^{o}C in a humidified atmosphere containing 5% CO_2. EGFP-HeLa cells (sostenuto express a destabilized form of EGFP) were purchased from Inovogen Technology Co., Ltd and cultured in DMEM containing 10 % FBS and antibiotics (50 units/mL penicillin and 50 units/mL streptomycin) at 37 ^{o}C in a humidified atmosphere containing 5% CO_2.

Cellular uptake studies of nanogel-Cas9/sgRNA. Flow cytometry (FCM, LSRFortessa, Becton Dickinson) was used to characterize the cellular uptake behavior of nanogel-Cas9/sgRNA. HeLa cells (~ 2×10^4 cell per well) were plated into 24-well

culture plates and cultured at 37 °C for 24 h, followed by removing culture medium and washing with PBS. Then the HeLa cells were incubated with nanogel-Cas9/sgRNA, FAM-labelled DNA linkers (equivalent FAM concentration: 1 μM), and Cas9/sgRNA (Cy5-labelled Cas9, equivalent Cy5 concentration: 0.5 μM) in Opti-MEM at 37 °C for 2 h. After removing the medium, the cells were washed thrice with cold PBS and detached by trypsin. The suspending cells were centrifugated for 5 min at 1000 rpm to remove the supernatant. After repeating the operation twice, the cells were resuspended with 300 μL PBS for flow cytometry analysis and the data were analyzed by FlowJo software.

Colocalization analysis of DNA linkers and the embedded Cas9/sgRNA inside the cells. HeLa cells were plated in 24-well culture plates with a clean coverslip (~ 2×10^4 cells per well) and cultured for 24 h. After removing the culture medium and washing with PBS, the cells were subsequently incubated with dye-labelled nanogel-Cas9/sgRNA where DNA linkers were labelled with FAM (equivalent FAM concentration: 1 μM) and Cas9/sgRNA was labelled with Cy5 (equivalent Cy5 concentration: 0.5 μM) at 37 °C in Opti-MEM for 2 h. Then, the medium was replaced with fresh medium and further incubated for 2 h, 10 h, 22 h, respectively. After that, the solutions were removed and the cells were carefully washed thrice with ice-cold PBS, fixed with 4 % formaldehyde for 30 min at room temperature in each well, washed thrice with ice-cold PBS again. Then, the cells were stained by Hoechst for 15 min, followed by washing thrice with ice-cold PBS. The slides were mounted and observed by confocal laser scanning microscope (CLSM, Leica TCS SP8 STED 3X).

Biocompatibility of DNA-*g*-PCL determined by MTT assay. The biocompatibility of the DNA-*g*-PCL was investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The HeLa cells were plated in 96-well culture plates (~ 2×10^4 cells per well), and cultured for 24 h. Then, cells were incubated with various grafted-DNA concentrations at 37 °C for 48 h. 20 μL of MTT solution (5 mg/mL) was added to each well and the plates were incubated for 4 h. And then the

MTT formazan crystals were dissolved with 250 μL DMSO at room temperature. Finally, and the absorbance was measured in a microplate reader (BioTek® Synergy H4) at a wavelength of 490 nm.

***In vitro* EGFP gene editing.** Flow cytometry and confocal laser scanning microscope were used to assess the efficiency of EGFP gene editing. For FCM study, EGFP-HeLa cells (~ 2×10^4 cells per well) were plated into 24-well culture plates and cultured at 37 ^oC for 24 h, followed by removing culture medium, washing with PBS. Then, the cells were incubated with PBS, Cas9/sgRNA, nanogel-Cas9/cgRNA, and nanogel-Cas9/sgRNA (with Cas9/sgRNA or Cas9/cgRNA concentration: 300 nM) in Opti-MEM at 37 ^oC for 8 h. After being replaced with DMEM, the cells were incubated for another 48 h. Subsequently, the medium was removed and the cells were washed thrice with PBS and harvested by trypsin treatment. The suspending cells were centrifugated for 5 min at 1000 rpm to remove the supernatant. The washing process was repeated twice. Then, the cells were resuspended with 300 μL PBS for flow cytometry analysis and the data were analyzed by FlowJo software. For CLSM study, EGFP-HeLa cells (2×10^4 cells per well) were plated in 24-well culture plates with a clean coverslip put in each well and cultured for 24 h, followed by removing culture medium. Thereafter, the EGFP-HeLa cells were incubated with PBS, Cas9/sgRNA, nanogel-Cas9/cgRNA, nanogel-Cas9/sgRNA (with Cas9/sgRNA or Cas9/cgRNA concentration: 300 nM) in Opti-MEM at 37 ^oC for 8 h. The cells were carefully washed thrice with ice-cold PBS, fixed with 4 % formaldehyde for 30 min at room temperature, and then rewashed thrice with ice-cold PBS, subsequently stained by Hoechst for 15 min. After washing thrice with ice-cold PBS, the slides were mounted and observed by a laser scanning confocal microscope.

SURVEYOR assay to detect genomic modifications. EGFP-HeLa cells (~ 2×10^4 cells per well) were plated into 24-well plate and cultured at 37 ^oC for 24 h, followed by removing the culture medium, washing with PBS. Then the EGFP-HeLa cells were incubated with PBS, Cas9/sgRNA, nanogel-Cas9/cgRNA, nanogel-Cas9/sgRNA (Cas9/sgRNA or Cas9/cgRNA concentration : 300 nM) in Opti-MEM at 37 ^oC for 8

h. After being replaced with DMEM, the cells were incubated for another 48 h. Then the medium was removed and the genomic DNA of EGFP-HeLa cells was extracted by GeneJET Genomic DNA Purification Kit (Thermo Scientific) according to manufacturer's instructions. The sgRNA-targeted genomic region was amplified by PCR method. Touchdown PCR program ((98°C for 10 s; 66-56°C with -1 °C/cycle for 15s, 72°C for 60 s) for 10 cycles and (98°C for 10 s, 56°C for 15 s, 72 °C for 60 s) for 25 cycles) was used to reduce the non-specific amplifications. After purification of the amplicons by gel extraction, 200 ng of the purified DNA was mixed with 20 μL cleavage reaction buffer (1×NEBuffer 2, NEB). With annealing from 95°C to room temperature, heteroduplex DNA were formed in the mixture solution. Finally, 1μL T7EI (10 U/μL, NEB) was added in each sample and incubated at 7 °C for 15 min.[2] The digestions of DNA substrates were analyzed using 2.8 % agarose gel electrophoresis (w/w). Efficiencies of insertions or deletions (Indels) were calculated using ImageJ.

DNA sequencing to detect genomic mutations. Purified PCR amplicons of the T7EI assay were cloned into Zero Blunt TOPO DNA sequencing vectors (Life Technologies, USA). The cloned plasmids were purified by GeneJET Plasmid Miniprep Kit (Thermo Scientific, USA) and sequenced by Sangon Biotech (Shanghai, China) with T7 promoter primer.[3]

Supplementary Figures

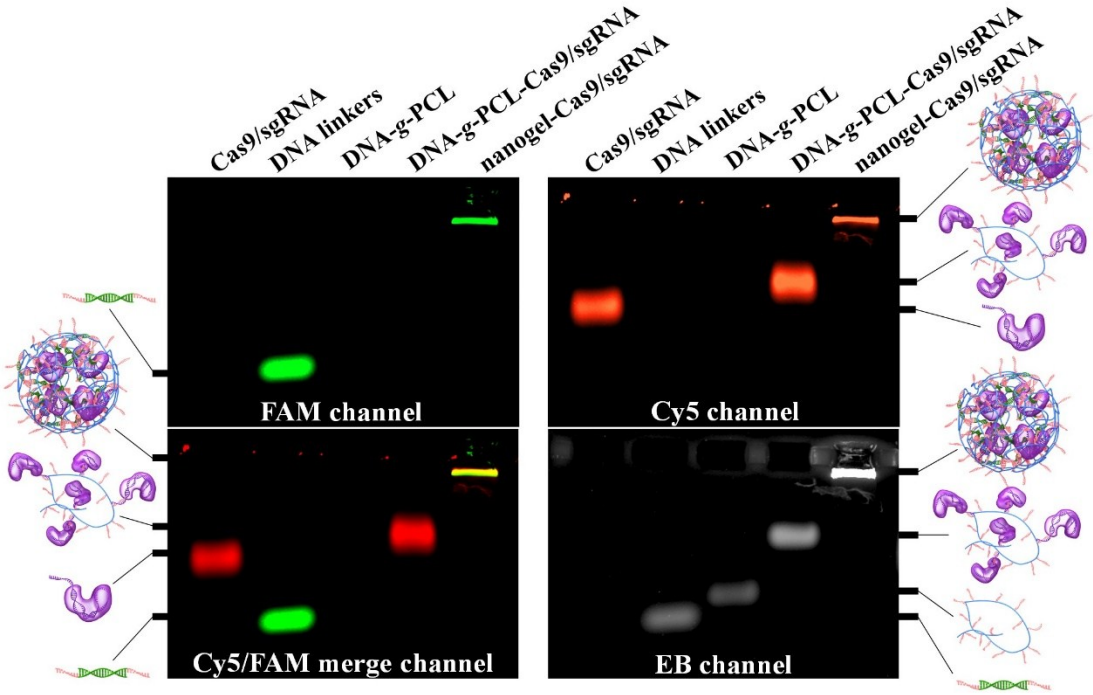

Figure S1. Characterization of nanogel-Cas9/sgRNA complex by 0.8 % agarose gel electrophoresis. Note that, Cas9 was labelled with Cy5 and the DNA linkers were labelled with FAM.

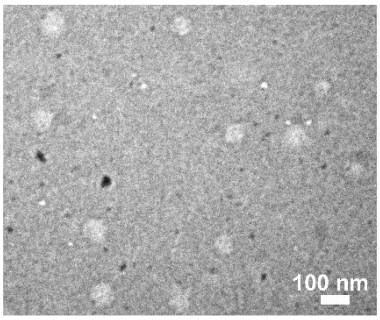

Figure S2. TEM image of nanogel-Cas9/sgRNA stained with 2% uranyl acetate.

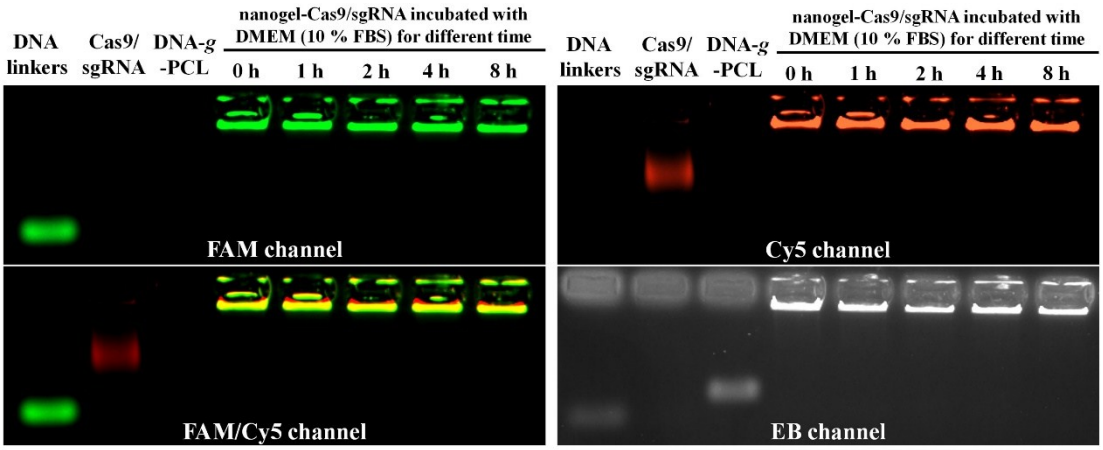

Figure S3. The stability of nanogel-Cas9/sgRNA under physiological condition. The samples were incubated with DMEM (10 % FBS) for different time and then analyzed by 0.8 % agarose gel electrophoresis. Note that, Cas9 was labelled with Cy5 and the DNA linkers were labelled with FAM.

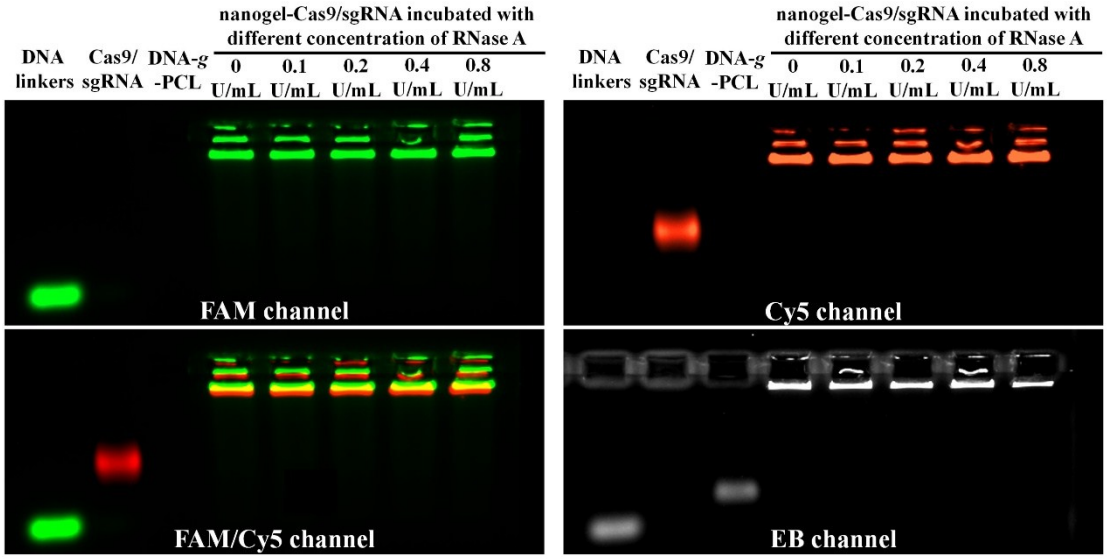

Figure S4. The nuclease resistance of nanogel-Cas9/sgRNA against the RNase. The samples were incubated with different concentration of RNase A at 37 ^{o}C for 1 h and analyzed by 0.8 % agarose gel electrophoresis. Note that, Cas9 was labelled with Cy5 and the DNA linkers were labelled with FAM.

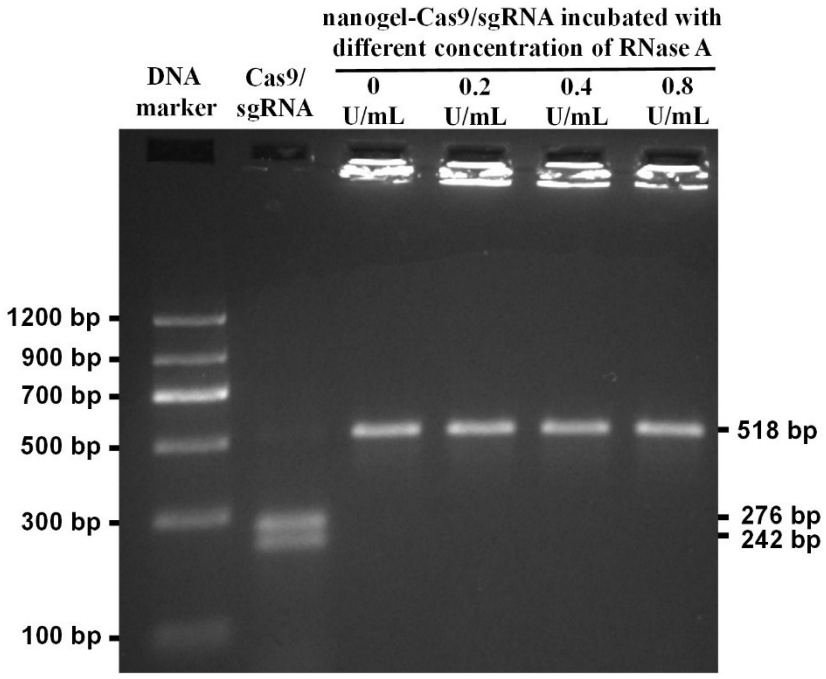

Figure S5. Gene editing efficiency of nanogel-Cas9/sgRNA after being incubated with different concentration of RNase A at 37 °C for 1 h. After incubation, the targeted DNA substrates were added and incubated for another 30 min. As shown in 2.8 % agarose gel in 1×TAE/Mg^{2+} buffer, nanogel-Cas9/sgRNA did not show gene editing activity in short time owing to the protection effect of the nanogel architecture.

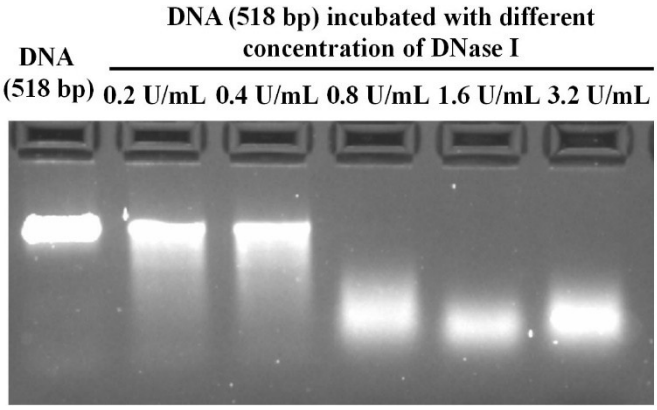

Figure S6. The stability of DNA substrates (518 bp) in the presence of different concentration of DNase Ⅰ at 37 °C for 30 min. After incubation, samples were checked by 2.5% agarose gel electrophoresis in 1×TAE/Mg^{2+} buffer.

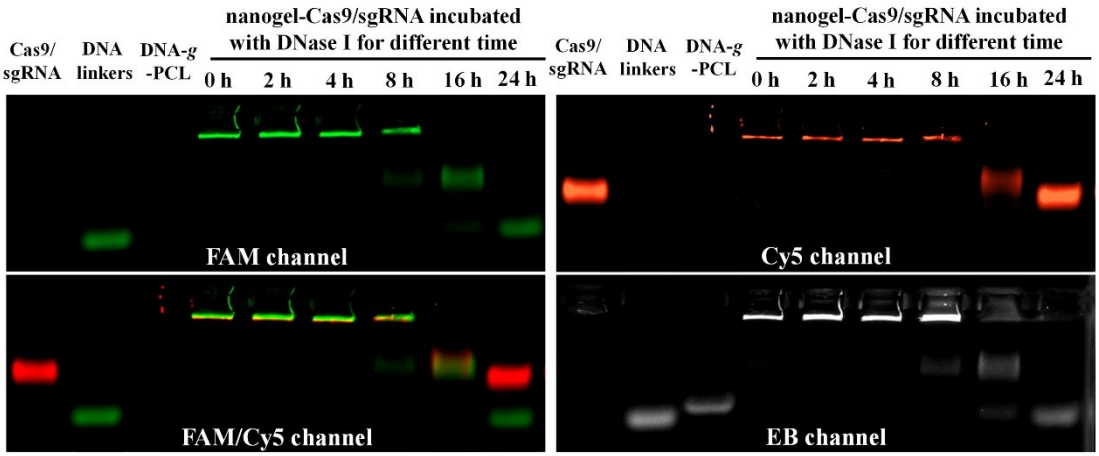

Figure S7. Enzyme-mediated Cas9/sgRNA release from nanogel-Cas9/sgRNA. The samples were incubated with DNase Ⅰ (0.4 U/mL) at 37 °C for different time and then analyzed by 0.8 % agarose gel in 1×TAE/Mg^{2+} buffer. Note that, Cas9 was labelled with Cy5 and the DNA linkers were labelled with FAM.

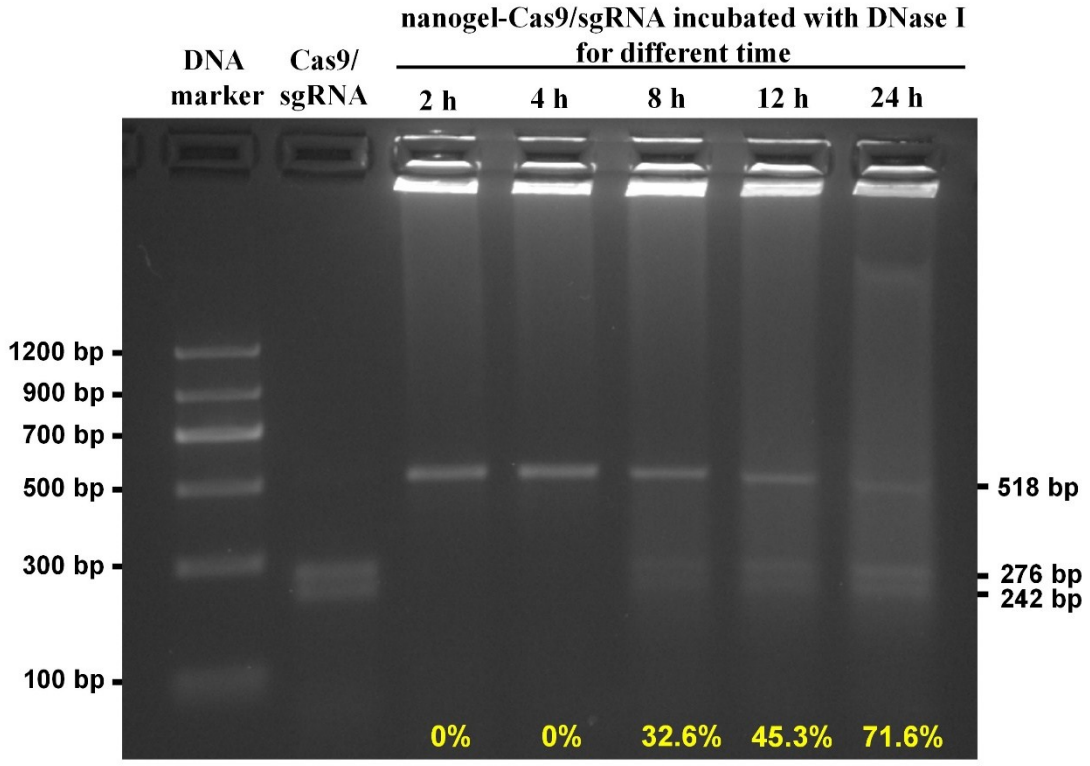

Figure S8. Gene editing efficiency of nanogel-Cas9/sgRNA after being incubated with DNase I (0.4 U/mL) for different time. After incubation, the targeted DNA substrates were added and incubated for another 30 min. The samples were then analyzed by 2.8 % agarose gel in 1×TAE/Mg^{2+} buffer.

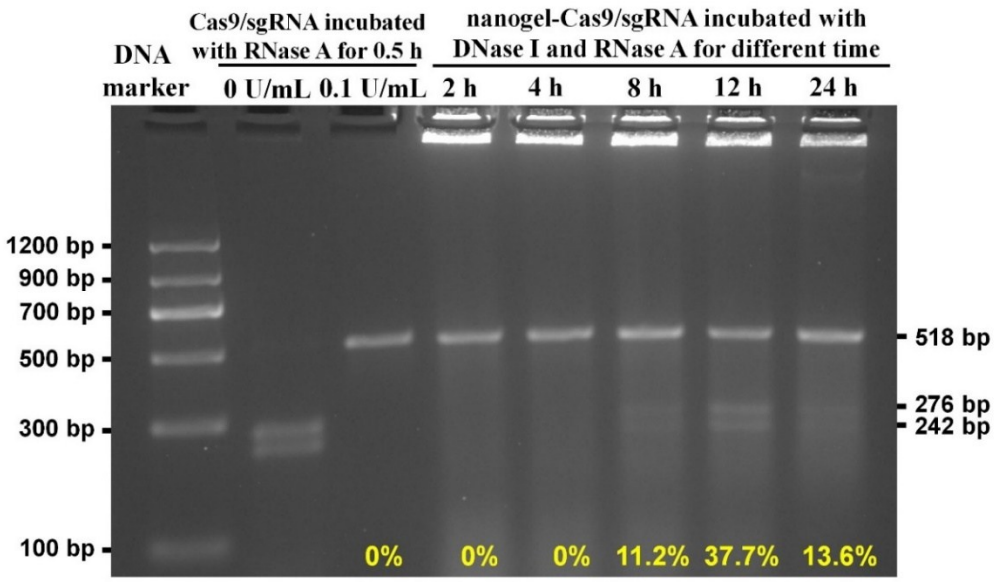

Figure S9. Gene editing efficiency of nanogel-Cas9/sgRNA after being incubated with DNase I (0.4 U/mL) and RNase A (0.1 U/mL) for different time. After incubation, the targeted DNA substrates were added and incubated for another 30 min. The samples were then analyzed by 2.8 % agarose gel in 1×TAE/Mg^{2+} buffer.

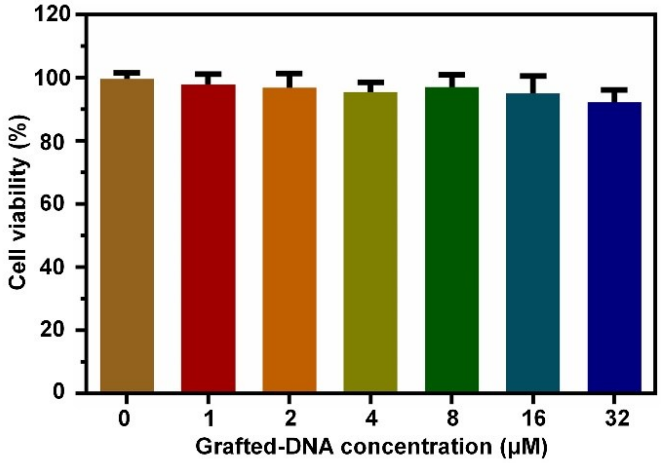

Figure S10. Biocompatibility of the DNA-*g*-PCL analyzed by MTT assay.

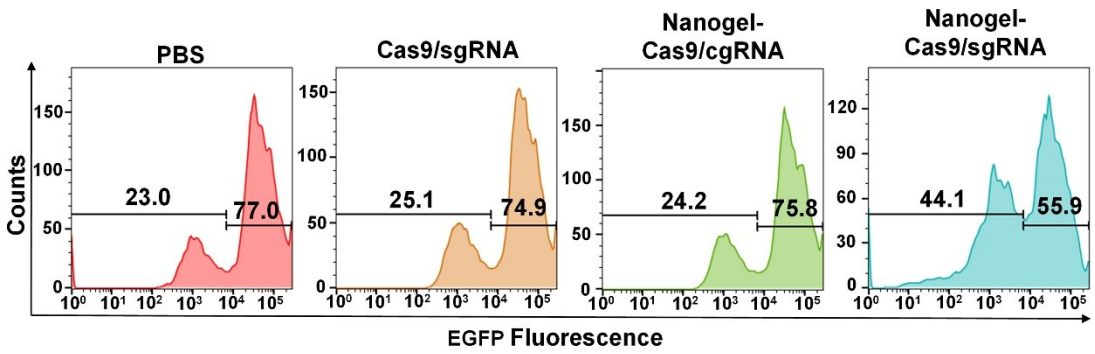

Figure S11. FCM analysis of the EGFP-HeLa cells treated with different formulations at Cas9/sgRNA concentration of 300 nM.

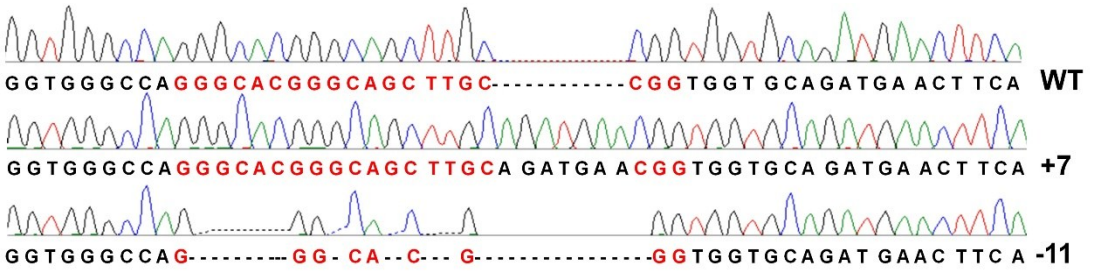

Figure S12. DNA sequencing of Cas9/sgRNA targeted genomic locus in EGFP-HeLa cells. Target sequence complementary to the sgRNA is highlighted with red color. Mutations were detected in 2 out of 20 sequenced clones. Number of insertions/deletions as compared to the wild type sequence is shown on the right.

References

1. F. Ding, Q. Mou, Y. Ma, G. Pan, Y. Guo, G. Tong, C. H. J. Choi, X. Zhu and C. Zhang, *Angew. Chem. Int. Ed.*, 2018, **57**, 3064-3068.
2. F. A. Ran, P. D. Hsu, J. Wright, V. Agarwala, D. A. Scott and F. Zhang, *Nat. Protoc.*, 2013, **8**, 2281.
3. W. Sun, W. Ji, J. M. Hall, Q. Hu, C. Wang, C. L. Beisel and Z. Gu, *Angew. Chem. Int. Ed.*, 2015, **54**, 12029-12033.