Published in final edited form as:

ACS Nano. 2017 March 28; 11(3): 2452–2458. doi:10.1021/acsnano.6b07600.

Efficient Gene Editing through Direct Cytosolic Delivery of CRISPR/Cas9-Ribonucleoprotein

Rubul Mout, Moumita Ray, Gulen Yesilbag Tonga, Yi-Wei Lee, Tristan Tay, Kanae Sasaki, and Vincent M. Rotello*

Department of Chemistry, University of Massachusetts, 710 North Pleasant Street, Amherst, MA 01003, USA

Abstract

Genome editing through the delivery of CRISPR/Cas9-ribonucleoprotein (Cas9-RNP) reduces unwanted gene targeting and avoids integrational mutagenesis that can occur through gene delivery strategies. Direct and efficient delivery of Cas9-RNP into the cytosol followed by translocation to the nucleus remains a challenge. Here we report a remarkably high efficient (~90%) direct cytoplasmic/nuclear delivery of Cas9 protein complexed with a guide RNA (sgRNA) through the co-engineering of Cas9 protein and carrier nanoparticles. This construct provides effective (~30%) gene editing efficiency and opens up new opportunities in studying genome dynamics.

Keywords

CRISPR/Cas9; gene editing; CRISPR/Cas9 engineering; nanoparticle; CRISPR delivery; genome engineering

Bacterially derived CRISPR system (Clustered Regularly Interspaced Short Palindromic Repeat) is a versatile tool for genome editing,^{1,2} transcriptional control of genes,^{3,4} and visualizing genome dynamics.⁵ Due to its genome editing efficiency, CRISPR/Cas9 system holds promises for curing human genetic diseases, as demonstrated through correction of a variety of disease-causing mutations in cultured cells⁶ and in animal models.⁷ These studies used gene delivery strategies to generate Cas9 inside cells. However, the required CRISPR genes stay in the host cells once delivered, causing unwanted gene editing and thus posing a major concern for CRISPR/Cas9 based gene therapy.^{8–10} Additionally, the constitutive

*Corresponding author. rotello@chem.umass.edu.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at <http://pubs.acs.org>.

Author contributions

R.M. conceived the idea; designed the experiments; G.Y.T. synthesized and characterized the particle; R.M. constructed and characterized the assemblies; R.M. and M.R. cloned the Cas9En variants, expressed and purified proteins, *in vitro* transcribed the sgRNA, delivered into cells and performed editing experiments; R.M. and Y.-W. L. performed the confocal time-lapse imaging; T.T. and K.S. helped in protein expression and purification; V.M.R. supervised the project; R.M. and V.M.R. wrote the manuscript with revisions from other authors.

Competing financial interests

The authors declare competing financial interests: details are available in the online version of the paper.

expression of Cas9 gene in the host may elicit immunogenic response, making CRISPR gene therapy less practical for therapy.^{9,11}

Delivery of Cas9 protein along with a guide RNA (sgRNA) (Cas9-ribonulceoprotein, or Cas9-RNP) provides an alternative strategy for CRSIPR process, offering a transient way of editing genes. Although a few strategies for Cas9 protein delivery have been reported,^{12–15} these strategies suffer from endosomal entrapment of both Cas9 and sgRNA. Mechanical methods including electroporation^{16,17} and membrane deformation¹⁸ provide direct delivery, however they require specialized processing and are generally impractical for *in vivo* therapeutic applications.

Here, we report a highly efficient editing strategy based on co-delivery of Cas9 protein and sgRNA into cells. This approach uses gold nanoparticles to co-assemble with engineered Cas9 protein and sgRNA into nanoassemblies. These vectors deliver protein and nucleic acid efficiently to the cytoplasm, with concomitant transport to the nucleus. Using this approach we achieved up to ~90% delivery efficiency in a range of cell types, with subsequent gene editing efficiency up to 30%.

RESULTS

Co-engineering of Cas9 protein and gold nanoparticles

We first engineered Cas9 protein for self-assembly with the cationic arginine gold nanoparticles (ArgNPs) (Figure 1), whose preparation and properties we have described earlier.¹⁹ Cas9 is a highly positively charged protein, so a glutamate peptide tag (E-tag)²⁰ was inserted at the N-terminus of *Streptococcus pyogenes* (*Sp*) Cas9 protein. We engineered a series of Cas9 proteins having a variable E-tag (En) length, where n= 0, 5, 10, 15, and 20. Notably, a Cas9 protein with no modification (Cas9E0) possesses a net 24 positive charges, however, insertion of the E-tag provided a patch of local negative charges that presumably enabled interaction with the positively charged ArgNPs.²¹ In addition, a nuclear localization signal (NLS) was inserted to the C-terminus to provide nuclear targeting of Cas9.

Fabrication of Cas9En-ArgNP nanoassemblies

Having engineered and purified Cas9En proteins, we focused on fabricating self-assemblies between Cas9En and ArgNPs. When the E-tagged Cas9 protein or Cas9-RNP were mixed with ArgNPs, they formed self-assembled nanoassemblies (Figure 1). These nanoassemblies are designed to fuse to cell membranes upon contact, releasing encapsulated Cas9En or Cas9-RNPs directly into the cell cytoplasm (Figure 1), and eventually to the nucleus. The desired self-assemblies were fabricated by mixing Cas9En or Cas9En-RNPs and ArgNPs at varying molar ratio in cell culture DMEM media.²⁰ Following this step, we characterized the assemblies after incubating the mixture at room temperature for 10 min. Transmission electron microscopic (TEM) results indicated the formation of nanoassemblies. As the length of E-tag increased, larger sized assemblies were observed reaching 475 ($\pm$ 60) nm in diameter (Figure 2). The resulting large size of the assemblies compared to the individual ArgNPs (~10 nm hydrodynamic diameter), Cas9En (~7.5 nm hydrodynamic diameter), and sgRNA (5.5 nm) (Figure S1) indicated the incorporation of a large number of nanoparticles

and proteins into the self-assembled structures. Interestingly, high resolution TEM image indicated the dense packing of granular proteins into the nanoassemblies (Figure 2b). Cas9E20-RNPs also formed similar nanoassemblies with ArgNPs, however, additional aggregates were observed (Figure 2a). The optimal working molar ratio for assembly fabrication was found to be 2:1 (ArgNP:Cas9En), as determined from subsequent delivery experiments. These results collectively indicated that the length of E-tag, hence the multivalency, plays a crucial role in the self-assembly formation between engineered Cas9En and ArgNPs.

Direct cytoplasmic delivery of Cas9 using nanoassemblies

We next investigated the protein delivery capability of these nanoassemblies. We fabricated assemblies of ArgNPs with Cas9En or Cas9En-RNPs and incubated with HeLa cells in cultured media. Cas9En were labelled with fluorescein isothiocyanate (FITC) to monitor the cellular uptake efficiency. Delivery efficiency was evaluated after 3 h of incubation using confocal laser scanning microscopy (CLSM). Cytoplasmic delivery efficiency of Cas9En gradually increased as the E-tag length increased from E0 to E20, achieving up to Cas9E20 delivery in 90% of the cells (Figure 3a, b; Figure S2). Delivered Cas9En readily dispersed into cytoplasm, and reached the nucleus, a requirement for gene editing (Figure 3c). Confocal microscopy Z-stacking further supported the cytoplasmic and nuclear localization of the delivered payload (Figure S3, supplementary movie 1)). Additionally, delivered Cas9En proteins stayed in the cells for at least 30h, without hampering the cell growth and viability (Figure S4). It is possible that poor cytoplasmic delivery of Cas9En with shorter length of E-tag, i.e. Cas9E0, Cas9E5, and Cas9E10, may be attributed to their inadequate nanoassembly formation with ArgNPs (Figure 2a). Likewise, Cas9E20-RNP was also delivered into cells, although to a lesser extent compared to Cas9E20 alone (Figure 3a). Interestingly, Cas9En with a shorter E-tag (E0 and E5) was found to bind the cell membrane, presumably due to the presence of unbound positively charged Cas9 protein in the assembly solution, which alone is capable of binding to the cell membrane (Figure S5). The delivery was further validated in other cell lines including human embryonic kidney cells (HEK-293T), and mouse macrophage (Raw 264.7) cells (Figure S6). Collectively, these results demonstrated the importance of co-engineering of the protein and ArgNPs for effective Cas9 delivery.

We performed time lapse video imaging to study the intracellular release-dynamics of Cas9En-RNP. We recorded the video at 30s intervals immediately after the addition of the nanoassemblies into HeLa cells. FITC labelled Cas9E20 delivery was nearly complete in cultured cells after 3h of post incubation (Figure S7, supporting movie 2). Real time tracking of a delivery event revealed a remarkably fast intracellular delivery, requiring only minutes for complete cytoplasmic/nuclear delivery after the initial contact by a nanoassembly (Figure 3d, supporting movie 3). Notably, a slight delay (1–2 min) in Cas9E20 transport into the nucleus from the cytosol was observed, presumably due to active nuclear transport of NLS-tagged Cas9En (Figure 3d, supporting movie 3). The instantaneous release of Cas9E20 into the whole cell further suggested that the payload may be directly released from the cell membrane and did not go through endocytosis.

Mechanism of CRISPR/Cas9 delivery

We investigated the mechanistic details of nanoassembly mediated Cas9-ribonucleoproteins delivery into cells. Nanoparticle mediated biomolecular delivery can occur through either an endocytic or a membrane fusion mechanisms (Figure 1d).²² We pretreated HeLa cells with inhibitors of endocytosis (chlorpromazine and wortmannin) or cholesterol-dependent membrane fusion (methyl-beta-cyclodextrin (MBCD)),²³ to investigate whether similar mechanisms are involved in Cas9-RNP delivery. After the inhibitor treatment, HeLa cells were incubated with the nanoassemblies, and monitored the delivery by CLSM. As shown in Figure 4, MBCD treatment inhibited FITC-Cas9E20 delivery into cells (2% delivery), compared to chlorpromazine (CPM) (83%) and wortmannin (79%), and untreated controls (90%). These studies collectively suggested that the nanoassembly-mediated Cas9-RNP delivery occurred preferably through a cholesterol dependent membrane fusion-like process, but not *via* cellular endocytosis. Thus, our approach provided a direct transfer of Cas9-RNP protein across the cell membrane into the cytoplasm resulting in a remarkably high delivery efficiency.

Gene editing in CRISPR/Cas9 delivered cells

Having efficiently delivered engineered Cas9En protein or Cas9En-RNP into cells, we evaluated the gene editing capability of this construct. We assembled Cas9E15-RNP with ArgNPs targeting human *AAVS1* gene and delivered these nanoassemblies into the HeLa cells.²⁴ In these experiments, the nanoassemblies were incubated with the cells for 3 h. in serum-free media; suitable conditions for *in vitro* and *ex vivo* applications. Genome editing efficiency was evaluated after 48 h, using indel (insertion and deletion) analysis.²⁷ As evident from Figure 5, targeting *AAVS1* gene resulted up to 29% of indel efficiency. As expected, delivering Cas9E15-RNP alone, or the untreated controls did not result in gene editing. To validate the usability of our method for any gene, we further targeted the human *PTEN* gene with an appropriate sgRNA.²⁵ Likewise, targeting *PTEN* gene resulted up to 30% of indel efficiency. The gene editing was further validated in HEK-293T and Raw 264.7 cell lines (Figure S8). These results collectively showed the efficient genome editing capability of our methodology.

CONCLUSIONS

In summary, we present here an engineering approach to drastically enhance the cytoplasmic/nuclear delivery of Cas9-RNPs, with concomitantly effective gene editing. This system provides a direct platform for multiple *in vitro* applications, and will greatly facilitate research in many other areas of rapidly growing genome engineering including spatiotemporal control of gene transcription and imaging chromatin dynamics. Additionally, this system provides a starting point for the creation of transient gene editing therapeutics without the requirement for gene delivery.

METHODS

Engineering E-tagged Cas9En

Glutamic acid tag (E-tag) was inserted to N-terminus of SpCas9 through site directed mutagenesis (SDM). Briefly, following primers were used for the insertion of E-tag (inserted nucleotides are underlined in the primers) into the N-terminus Cas9 using pET28b-Cas9 expression vector (Addgene plasmid id= 47327)²⁶ as the template. Note that the C-terminus of Cas9 contained a nuclear localization signal (NLS) and a 6xHis tag.

Cas9E0- F: ATGGACAAGAAGTACTCCATTGGGCTCGATATCGGC

Cas9E0- R: GGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGG

Cas9E5- F: ATGGACAAGAAGTACTCCATTGGGCTCGATATCGGC

Cas9E5- R:

CTCTTCCTCCTCCATGGTATATCTCCTTCTTAAAGTTAAACAAAATTATT
TCTAGAGGGG

Cas9E10- F:

ATGGAGGAAGAAGAGGAAGAGGAGGAGGAAGAGATGGACAAGAAGTACT
CCATTGGGCTCGAT

Cas9E10- R:

GGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGG

Cas9E15- F: (Cas9E10 as template)

ATGGAAGAGGAGGAAGAAGAGGAAGAAGAGGAAGAGGAGGAGGAAGAG
ATGGAC

Cas9E15- R:

GGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGG

Cas9E20- F: (Cas9E15 as template)

ATGGAAGAAGAGGAGGAAGAAGAGGAGGAAGAAGAGGAAGAAGAGGAA
GAGGAGGAG

Cas9E20- R:

GGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGG

Recombinant proteins (Cas9En) were expressed in *E. coli* BL21 Rosetta strain using standard protein expression protocol. Briefly, protein expression was carried out in 2xYT media with an induction condition of 0.75 mM IPTG and 18 °C for 16 hours. At this point, the cells were harvested and the pellets were lysed by using 1% Triton-X-100/DNase-I treatment. Triton-X-100 treatment was performed for ~30 min followed by DNase-I treatment for 15 min. Lysed cells were then spun down at 14,000 rpm for 30 min. The supernatant was collected and to it an additional 150 mM salt was added.²⁰ Proteins were purified using HisPur cobalt columns. Proteins were finally preserved in PBS buffer containing 300 mM salt. The purity of native proteins was determined using 12% SDS-PAGE gel.

sgRNA design and synthesis

sgRNAs were *in vitro* transcribed from dsDNA template (containing the protospacer and the tracrRNA sequence) using AmpliScribe-T7-Flash Transcription kit according to manufacturer's protocol. dsDNA was PCR amplified from a template plasmid carrying the tracrRNA sequence.²⁷ The following primers were used for the PCR amplification.

sgRNA-R: AAAAAAGCACCGACTCGGTGCCACT (common for all)

sgAAVSI-F:

GAAATTAATACGACTCACTATAGGctccctcccaggatcctctcGTTTTAGAGCTAGAA
ATAGCA

sgPTEN-F:

GAAATTAATACGACTCACTATAGGgagatcgtagcagaacaaaGTTTTAGAGCTAG
AAATAGCA

In vitro transcribed sgRNAs were purified using RNA purification kit (Zymo research RNA Clean & Concentrator).

Nanoparticle synthesis and characterization

Arginine-functionalized gold nanoparticles (ArgNPs) were prepared according to our previous methods.¹⁹ The arginine-functionalized thiol ligand was synthesized first. Following this, ArgNPs were prepared by conventional place-exchange reaction of 2-nm sized 1-pentanethiol-protected gold nanoparticles (Au-C5) with HS-C11-TEG-NH-Arginine. The resultant ArgNPs were dissolved in distilled water, purified by dialysis, and whose characterization was reported previously.^{19,20} Complete synthesis of a batch of ArgNPs takes around 1–2 weeks, however can be synthesized in large batches that may be stable and used for years for delivery purpose. The manufacturing cost of our gold nanoparticle is roughly ~10–20 cents (USA) per sample of 'delivery'.

Nanoassembly fabrication

Cas9En-RNP:ArgNPs nanoassemblies were prepared through a simple mixing procedure. Cas9En and sgRNAs (1:1 molar ratio)²⁸ were assembled in 1×PBS for 30 min at room temperature first, then ArgNPs (50 μM stock in 5 mM PB, pH 7.4) were added to 100 μL of 1×PBS in another vial, followed by adding the preassembled Cas9En-RNP at appropriate working molar ratio [usually at 2:1 ratio (ArgNP, 125 nM)/(Cas9En-RNP, 62 nM), which corresponds to ~10 μg of Cas9En protein and ~2 μg of sgRNA per cultured dish]. The working molar ratio was determined by screening different ratios in the subsequent delivery experiments. The nanoassemblies were incubated at room temperature for another 10 min. DMEM was added to the nanoassemblies to make the final volume up to 1000 μL. The nanoassemblies were then incubated either at 37 °C for 30 min for TEM, or directly added to cells grown overnight in confocal dish for delivery experiments.

Cell culture

240,000–300,000 cells were grown in a confocal dish (glass bottom culture dishes, MatTek) in DMEM (with 10% FBS, and 1% antibiotics) for overnight at 37 °C under 5% CO₂. Cells were washed with 1×PBS (twice) before incubation with nanoassemblies.

Delivery

Assembled Cas9En-RNP:ArgNPs nanoassemblies (preassembled in 100 µL PBS for 10 min, plus 900 µL DMEM media) were immediately transferred to each dish of confluent grown cells. Cells were then incubated at 37 °C and 5% CO₂ for 3h. At this point cells were washed with 1×PBS buffer and immediately processed for investigating delivery efficiency that was determined by Confocal microscopy (Zeiss LSM 510 Meta microscope, or Nikon A1 laser scanning microscope). Z-stacking was performed using Nikon A1, at every 125 nm interval.

It is noteworthy that the nanoassemblies can be incubated with cells for a longer period of time (~24h) without affecting cell growth/viability, an important issue for in vitro and ex vivo editing.

Estimation of cytoplasmic/nuclear delivery efficiency

Since flow cytometry cannot distinguish between cytoplasmic/nuclear delivery and endosomally-entrapped delivery, we used confocal microscopy to estimate the delivery efficiency. Around 400 cells were counted for each Cas9En, 3h after the delivery as described above.

Time lapse video imaging

Live-delivery imaging was performed using Nikon A1 confocal laser scanning microscope. Briefly, as soon as the nanoassemblies were added to the cultured HeLa cells in a live-cell imaging chamber containing humidified 5% CO₂ at 37 °C, the images were acquired at every 30s interval for 3h using 60× oil immersion lens.

Cholesterol depletion

Endocytic and membrane fusion inhibitors were used to block the Cas9-RNP delivery. Cells were pretreated with wortmannin (150ng/mL), chlorpromazine (1.5µg/mL), and methyl-β-cyclodextrin (MBCD, 7.5mg/mL) in DMEM media for 1 h at 37 °C and 5% CO₂.²³ In the meantime, nanoassemblies were prepared. Inhibitor-treated cells were washed with 1×PBS twice, then the nanoassembly solutions were applied for Cas9 delivery. Confocal microscopy experiments were performed after 3h of nanoassembly incubation to image and estimate cytoplasmic/nuclear delivery.

Indel analysis

After Cas9E15-RNP delivery for 3h, cells were washed and replaced with DMEM media (with 10% FBS, and 1% antibiotics), then allowed to grow for another 48 h. At this point cells were harvested to extract genomic DNA using QuickExtract genomic DNA isolation

kit (Epicentre biotechnologies). Indel assays were performed using T7 endonuclease-I according to standard protocol.²⁷

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the NIH (GM077173), NSF (CHE-1307021) and a UMass OTCV grant.

References

1. Doudna JA, Charpentier E. The New Frontier of Genome Engineering with CRISPR-Cas9. *Science*. 2014; 346:1258096. [PubMed: 25430774]
2. Cox DBT, Platt RJ, Zhang F. Therapeutic Genome Editing: Prospects and Challenges. *Nat Med*. 2015; 21:121–131. [PubMed: 25654603]
3. Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, Hsu PD, Habib N, Gootenberg JS, Nishimasu H, Nureki O, Zhang F. Genome-scale Transcriptional Activation by an Engineered CRISPR-Cas9 Complex. *Nature*. 2015; 517:583–588. [PubMed: 25494202]
4. Kiani S, Chavez A, Tuttle M, Hall RN, Chari R, Ter-Ovanesyan D, Qian J, Pruitt BW, Beal J, Vora S, Buchthal J, Kowal EJ, Ebrahimkhani MR, Collins JJ, Weiss R, Church G. Cas9 gRNA Engineering for Genome Editing, Activation and Repression. *Nat Methods*. 2015; 12:1051–1054. [PubMed: 26344044]
5. Chen B, Gilbert Luke A, Cimini Beth A, Schnitzbauer J, Zhang W, Li G-W, Park J, Blackburn Elizabeth H, Weissman Jonathan S, Qi Lei S, Huang B. Dynamic Imaging of Genomic Loci in Living Human Cells by an Optimized CRISPR/Cas System. *Cell*. 2013; 155:1479–1491. [PubMed: 24360272]
6. Schwank G, Koo B-K, Sasselli V, Dekkers Johanna F, Heo I, Demircan T, Sasaki N, Boymans S, Cuppen E, van der Ent CK, Nieuwenhuis EE, Beekman JM, Clevers H. Functional Repair of CFTR by CRISPR/Cas9 in Intestinal Stem Cell Organoids of Cystic Fibrosis Patients. *Cell Stem Cell*. 2013; 13:653–658. [PubMed: 24315439]
7. Yin H, Xue W, Chen S, Bogorad RL, Benedetti E, Grompe M, Kotliansky V, Sharp PA, Jacks T, Anderson DG. Genome Editing with Cas9 in Adult Mice Corrects a Disease Mutation and Phenotype. *Nat Biotechnol*. 2014; 32:551–553. [PubMed: 24681508]
8. Nelson CE, Gersbach CA. Engineering Delivery Vehicles for Genome Editing. *Annu Rev Chem Biomol Eng*. 2016; 7:637–662. [PubMed: 27146557]
9. Hsu PD, Lander ES, Zhang F. Development and Applications of CRISPR-Cas9 for Genome Engineering. *Cell*. 2014; 157:1262–1278. [PubMed: 24906146]
10. Oude Blenke E, Evers MJ, Mastrobattista E, van der Oost J. CRISPR-Cas9 Gene Editing: Delivery Aspects and Therapeutic Potential. *J Control Release*. 2016; 244:139–148. [PubMed: 27498021]
11. Li L, He ZY, Wei XW, Gao GP, Wei YQ. Challenges in CRISPR/Cas9 Delivery: Potential Roles of Nonviral Vectors. *Hum Gene Ther*. 2015; 26:452–462. [PubMed: 26176432]
12. Zuris JA, Thompson DB, Shu Y, Guilinger JP, Bessen JL, Hu JH, Maeder ML, Joung JK, Chen Z-Y, Liu DR. Cationic Lipid-Mediated Delivery of Proteins Enables Efficient Protein-Based Genome Editing *in vitro* and *in vivo*. *Nat Biotechnol*. 2015; 33:73–80. [PubMed: 25357182]
13. Liu J, Gaj T, Yang Y, Wang N, Shui S, Kim S, Kanchiswamy CN, Kim J-S, Barbas CF III. Efficient Delivery of Nuclease Proteins for Genome Editing in Human Stem Cells and Primary Cells. *Nat Protoc*. 2015; 10:1842–1859. [PubMed: 26492140]
14. Ramakrishna S, Kwaku Dad A-B, Beloor J, Gopalappa R, Lee S-K, Kim H. Gene Disruption by Cell-Penetrating Peptide-Mediated Delivery of Cas9 Protein and Guide RNA. *Genome Res*. 2014; 24:1020–1027. [PubMed: 24696462]

15. Sun W, Ji W, Hall JM, Hu Q, Wang C, Beisel CL, Gu Z. Self-Assembled DNA Nanoclews for the Efficient Delivery of CRISPR-Cas9 for Genome Editing. *Angew Chem Int Ed Engl.* 2015; 54:12029–12033. [PubMed: 26310292]
16. Schumann K, Lin S, Boyer E, Simeonov DR, Subramaniam M, Gate RE, Haliburton GE, Ye CJ, Bluestone JA, Doudna JA, Marson A. Generation of Knock-in Primary Human T Cells using Cas9 Ribonucleoproteins. *Proc Natl Acad Sci USA.* 2015; 112:10437–10442. [PubMed: 26216948]
17. Kim S, Kim D, Cho SW, Kim J, Kim J-S. Highly Efficient RNA-guided Genome Editing in Human Cells via Delivery of Purified Cas9 Ribonucleoproteins. *Genome Res.* 2014; 24:1012–1019. [PubMed: 24696461]
18. Han X, Liu Z, Jo Mc, Zhang K, Li Y, Zeng Z, Li N, Zu Y, Qin L. CRISPR-Cas9 Delivery to Hard-to-transfect Cells via Membrane Deformation. *Sci Adv.* 2015; 1:e1500454. [PubMed: 26601238]
19. Yang XC, Samanta B, Agasti SS, Jeong Y, Zhu ZJ, Rana S, Miranda OR, Rotello VM. Drug Delivery using Nanoparticle-Stabilized Nanocapsules. *Angew Chem Int Ed.* 2011; 50:477–481.
20. Mout R, Tonga GY, Wang LS, Ray M, Roy T, Rotello VM. Programmed Self-Assembly of Hierarchical Nanostructures through Protein-Nanoparticle Co-Engineering. *ACS Nano.* submitted (nn-2016-07258p).
21. Mout R, Tonga GY, Ray M, Moyano DF, Xing Y, Rotello VM. Environmentally Responsive Histidine-carboxylate Zipper Formation Between Proteins and Nanoparticles. *Nanoscale.* 2014; 6:8873–8877. [PubMed: 24960536]
22. Jiang Y, Tang R, Duncan B, Jiang Z, Yan B, Mout R, Rotello VM. Direct Cytosolic Delivery of siRNA using Nanoparticle-stabilized Nanocapsules. *Angew Chem Int Ed Engl.* 2015; 54:506–510. [PubMed: 25393227]
23. Saha K, Kim ST, Yan B, Miranda OR, Alfonso FS, Shlosman D, Rotello VM. Surface Functionality of Nanoparticles Determines Cellular Uptake Mechanisms in Mammalian Cells. *Small.* 2013; 9:300–305. [PubMed: 22972519]
24. Sadelain M, Papapetrou EP, Bushman FD. Safe Harbours for the Integration of New DNA in the Human Genome. *Nat Rev Cancer.* 2011; 12:51–58. [PubMed: 22129804]
25. Xue W, Chen S, Yin H, Tammela T, Papagiannakopoulos T, Joshi NS, Cai W, Yang G, Bronson R, Crowley DG, Zhang F, Anderson DG, Sharp PA, Jacks T. CRISPR-mediated Direct Mutation of Cancer Genes in the Mouse Liver. *Nature.* 2014; 514:380–384. [PubMed: 25119044]
26. Gagnon JA, Valen E, Thyme SB, Huang P, Ahkmetova L, Pauli A, Montague TG, Zimmerman S, Richter C, Schier AF. Efficient Mutagenesis by Cas9 Protein-mediated Oligonucleotide Insertion and Large-scale Assessment of Single-guide RNAs. *PLoS One.* 2014; 9:e98186. [PubMed: 24873830]
27. Ran FA, Hsu P, Wright J, Agarwala V, Scott DA, Zhang F. Genome Engineering Using the CRISPR-Cas9 System. *Nat Protoc.* 2013; 8:2281–2308. [PubMed: 24157548]
28. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier EA. Programmable Dual-RNA-guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science.* 2012; 337:816–821. [PubMed: 22745249]

Figure 1. Rational engineering of Cas9 protein and arginine nanoparticles (ArgNPs) for intracellular delivery of Cas9 protein or Cas9-RNP via membrane fusion

(a) Engineering Cas9 to carry an N-terminus E-tag and a C-terminus nuclear localization signal (NLS). (b) Chemical structure of ArgNPs. (c) Schematic showing nanoassembly formation by Cas9En-RNP and ArgNPs. (d) Delivery of Cas9En via membrane fusion mechanism. Fusion of nanoassemblies to the cell membrane may facilitate direct release of the protein payload into cytoplasm, bypassing endosomes.

Figure 2. Nanoassembly formation between ArgNPs and Cas9En or Cas9En-RNP is dictated by E-tag length

(a) TEM images of nanoassemblies. As the length of E-tag increased, larger nanoassemblies formed that are favorable for intracellular delivery of Cas9En (Figure 3). (b) High magnification image of nanoassemblies showing the inner structure containing protein and nanoparticle granules.

Figure 3. Endosomal entrapment-free direct cytoplasmic/nuclear delivery of engineered Cas9En or Cas9En-RNP is dictated by E-tag length

(a) Cytoplasmic/nuclear delivery of FITC labelled Cas9En increased as the length of E-tag increased, reaching maximum at E20. (b) Percentage cytoplasmic/nuclear delivery efficiency of Cas9En as measured by confocal microscopy. (c) Distribution of delivered Cas9E20 protein inside the cell, showing preferential accumulation of the protein in the cytoplasm and nucleus. (d) Real time tracking of a delivery event. Time lapse imaging of Cas9En-RNP delivery showed FITC labelled Cas9E20 was rapidly released into the cytosol and subsequently to the nucleus following a nanoassembly (red arrow) made contact with the cell surface (also see supporting movie 3). Zero second (00 s) represents the beginning of the delivery event.

Figure 4. A cholesterol dependent membrane fusion-like delivery mechanism is involved in nanoassembly-mediated Cas9En-RNP delivery

(a) cholesterol depletion (MBCD treatment) completely inhibited FITC-Cas9E20 delivery, whereas endocytic inhibitors (b) chlorpromazine (CPM) and (c) wortmannin did not block the delivery significantly. (d) Percentage of Cas9E20 cytoplasmic/nuclear delivery after various inhibitors treatment.

Figure 5. Efficient gene editing resulted from Cas9En-RNP delivery

(a) Delivery of Cas9E15-RNP to target *AAVS1* and *PTEN* gene in HeLa cells resulted efficient gene editing, as determined by indel (insertion and deletion) assay: Lane 1: Cas9E15-RNP:ArgNPs, 2: Cas9E15-RNP, and 3: cells only. Indel efficiency is given in percentage.