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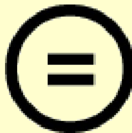

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Magnetically inducible CRISPR/Cas9 for genome engineering

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Magnetically inducible CIRSPR/Cas9 for genome engineering

Advisor: Jinwoo Cheon

A Dissertation

Submitted to the Graduate Program for Nanomedical Science

and the Graduate School of Yonsei University in partial

fulfillment of the requirements for the degree of Master of Science

Seulmi Kim

December, 2016

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This certifies that the master's thesis of Seulmi Kim is approved.

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December, 2016

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Abstract

Magnetically inducible CRISPR/Cas9 for genome engineering

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Magnetic nanoparticles have been one of the most efficient tools in biomedical applications because of the ability that can be remotely controlled. When magnetic nanoparticles are combined with external magnetic field, these nanoparticles can exert force that can be applied in receptor clustering or pulling. Furthermore, in the presence of alternative magnetic field, nanoparticle can generate heat because of the Brownian and Neel spin relaxation. By combining hyperthermia property of magnetic nanoparticle with CRISPR/Cas9 system, nanoparticle can become an actuator for precise genome engineering.

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In this study, three different thermo responsive linkers were synthesized on the surface of nanoparticle to activate CRISPR/Cas9 in response to the alternative magnetic field. And by comparing long term stability at 37 °C cell culture temperature, rationally designed magnetic nanoparticle was suggested that can be used in *in vivo* gene engineering applications.

Keywords: magnetic nanoparticle, hyperthermia, thermo responsive linker, CRISPR/Cas9, genome engineering, tetracycline inducible system

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1. Introduction

Since the discovery of restriction enzyme in 1970's, genome engineering has been a promising tool because of its ability to recognize and manipulate specific DNA sequences. With the advances of technologies, exquisite genome editing became possible. ZFNs (zinc finger nucleases) and TALENs (transcription activator-like effector nucleases) comprised of fusion proteins, DNA-binding and DNA-cleavage protein, have enabled new phase for human genomes because these systems can recognize 16 nucleotides of target DNA, which is the minimum number to be distinguished from others in humans. More recently, CRISPR/Cas9 system which utilizes guide-RNA for target recognition and cas9 protein for double-stranded DNA break[1] has offered a facile method for genome engineering.

However, means to regulate genomes in a spatiotemporally controlled manner still remain elusive. Various efforts including small molecule and optic-based methods have been reported. For instance, activation of nuclease functions using self-splicing intein[2] or dimerization of protein which induce conformational changes triggered by small molecules[3] was reported. Optogenetic systems can also control gene editing based on light-inducible heterodimerizing proteins such as CRY2 and CIB1[4]. Although these approaches have proved possibilities for precise and activatable genome engineering, their *in vivo* applications are still limited because small molecules are hard to be localized in a specific

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site and light is absorbed by tissues. Apart from these approaches, superparamagnetic nanoparticles combined with magnetic field provide several advantages. Because magnetic field in a range of 350-400kHz is barely absorbed by tissues, it is possible to penetrate deep into tissues. Furthermore, superparamagnetic nanoparticles can be localized in a target-specific site. And unlike tissues, these nanoparticles absorb magnetic field and transduce energy into heat by magnetic spin relaxation[5]. Remote activation of cells based on magnetic field-based approach to trigger gene editing, therefore, can suggest potential means for in vivo applications.

In this thesis, by synthesizing stimuli-responsive nanoparticles that can remotely activate CRISPR/Cas9, rationally designed magnetic nanoparticles are suggested that can be used in *in vivo* gene engineering applications.

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2. Experimental Method

Synthesis of zinc-doped iron oxide nanoparticle

15 nm zinc-doped iron oxide nanoparticles ($Zn_{0.4}Fe_{2.6}O_4$) were synthesized by thermal decomposition using previously reported method[6] with a slight modification. Iron(III) acetylacetonate and zinc(II) chloride were placed in a 250 mL round bottom flask in the presence of trioctylamine, oleylamine, and oleic acid. The reaction mixture was heated to 330 °C for 3 h, and then cooled to room temperature. To isolate $Zn_{0.4}Fe_{2.6}O_4$ nanoparticles from the reaction mixture, ethanol was added and centrifuged at 2000g for 5 min. The precipitated nanoparticles were redispersed in toluene and size and shape was measured using TEM (JEM-2100, JEOL, Japan) and Zetasizer Nano ZS (ZEN3600, Malvern Instruments, United Kingdom)

Ligand exchange of nanoparticle

15 nm $Zn_{0.4}Fe_{2.6}O_4$ nanoparticles which have organic surfactants on the surface were further modified to be dispersed in aqueous solutions. The nanoparticles were coated with silica using base-catalyzed sol-gel reaction. First, 1 mg of $Zn_{0.4}Fe_{2.6}O_4$ and 800 mg of IGEPAL-CO-520 were added to 12 mL of cyclohexane. Then, 100 μL of 30.0% ammonium hydroxide solution and TEOS (tetraethyl orthosilicate) 4 μL were added and reacted for 12

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h at room temperature. To synthesize amine-functionalized nanoparticles, reaction mixture was further reacted with 6 μL of APTMS ((3-aminopropyl) trimethoxysilane) for 12 h at room temperature. The reaction mixture was then magnetically purified by MACS column (Miltenyl Biotech, Germany) and washed with 0.1 M TMAOH (tetramethylammonium hydroxide) solution in methanol for the removal of remaining IGEPAL-CO-520. The amine-functionalized nanoparticles were then dispersed in DMSO (dimethyl sulfoxide). The surface modification of nanoparticle was confirmed by TEM and Zetasizer Nano ZS by measuring zeta potential.

Preparation of doxycycline-linked single-stranded DNA (ssDNA-doxy)

Single-stranded DNA_1 (5'-SH-$(CH_2)_6$-TATGATCTGTCACAGCTTGA-3', IDT, USA) was first reduced by TCEP (tris(2-carboxyethyl) phosphine) in a 1:100 molar ratio for 30 min at room temperature. The reaction mixture was purified by G-10 desalting column (GE healthcare, United Kingdom) with 10 mM phosphate buffer (pH 7.2). For attaching doxycycline to a thiolated single-stranded DNA, 6.0 mg of 9-aminodoxycycline hydrochloride was reacted with sulfo-SMCC 0.5 mg (sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate) in 100 mM phosphate buffer (pH 7.2). After 30 min, activated doxycycline was mixed with 150 nmol of thiolated single-stranded DNA which was reduced by TCEP for 3 h at room temperature. Doxycycline-linked-single-

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stranded DNA_1 ($ssDNA_1$-doxy) was then purified by G-10 desalting column with 10 mM phosphate buffer (pH 7.2).

Double-stranded DNA functionalization of nanoparticle

First, $ssDNA_1$-doxy was hybridized by combining $ssDNA_1$-doxy with it's complementary (5'-SH-$(CH_2)_6$-TCAAGCTGTGACAGATCATA-3', IDT, USA) single-stranded DNA_2 in the presence of TCEP in a 1:1 molar ratio. DNA hybridization was performed in TE buffer (tris-EDTA, 150 mM NaCl, 10 mM tris-HCl, 1 mM EDTA, pH 7.4). The solution was heated to 95 ℃, and then cooled to room temperature slowly in a water bath. Double-stranded DNA (dsDNA) which has both doxycycline and thiol group at each 5' end (SH-dsDNA-doxy) was purified by G-10 desalting column. To attach dsDNA to the surface of nanoparticle, 1 mg of amine-functionalized nanoparticle was reacted with 5 mg of iodoacetic anhydride in DMSO for 12 h at room temperature. The reaction mixture was then purified by MACS column with 10 mM phosphate buffer (pH 8.0). Before attaching dsDNA, poly(ethylene glycol) 2-mercaptoethyl ether acetic acid (MW = 5,000) which was reduced by TCEP and desalted by G-10 column was reacted with nanoparticle for 8 h at room temperature in a 1:2000 molar ratio. The reaction mixture was purified by MACS column. Then, SH-dsDNA-doxycycline which was freshly prepared was reacted with nanoparticle in 10 mM phosphate buffer (pH 8.0, 150 mM NaCl) for 12 h at room

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temperature in a 1:4000 molar ratio. The excess DNA was removed by MACS column, and the nanoparticle-dsDNA-doxy was dispersed in 10 mM phosphate buffer (pH 7.2, 150 mM NaCl).

Preparation of MNP@SiO_2-DOXY

For attaching doxycycline directly to the surface of nanoparticle to examine the stability of silica layer, 1 mg of amine-functionalized nanoparticle that was dispersed in DMSO was reacted with 5 mg of succinic anhydride for 12 h at room temperature to have carboxylic acid on the surface. After 12 h, reaction mixture was purified by MACS column to remove excess succinic anhydride with DMSO. Then, doxycycline was covalently attached to the nanoparticle via EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide) coupling. The carboxylic acid-functionalized nanoparticle 1 mg was first incubated with 0.767 mg of EDC and 2.174 mg of sulfo-NHS (N-hydroxysulfosuccinimide) for 15 min, and then reacted with 9-aminodoxycycline hydrochloride in 100 mM phosphate buffer (pH 7.2) for 2 h at room temperature in a 1:1000 molar ratio. After 2 h, unbound doxycycline was removed by MACS column.

Synthesis of doxycycline-linked maleimido hexanoamide derivative (MAL_{hex}-DOXY)

First, 6-Maleimidohexanoic acid chloride was prepared by dropping 0.946 mmol of

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oxalyl chloride to ice-cooled 0.189 mmol of 6-maleimidohexanoic acid in 1 mL of anhydrous dichloromethane solution with 4 μL DMF. After 10 min, the reaction mixture was allowed to reach room temperature and stirred overnight under argon. The volatiles were removed under vacuum with liquid nitrogen, and the residue was azeotroped three times with 2 mL of dichloromethane. Then, 0.945 mmol of 9-aminodoxycycline hydrochloride which was suspended in 1 mL of anhydrous dichloromethane with 3.78 mmol of DIEPA (*N,N*-diisopropylethylamine) was added to of 6-maleimidohexanoic acid chloride 0.189 mmol solution. The reaction mixture was stirred overnight and the volatiles were removed under vacuum with liquid nitrogen.

HPLC purification of MAL_{hex}-DOXY

Semipreparative reversed-phase HPLC (1260 Infinity LC, Agilent Technologies, USA) was conducted to purify doxycycline-linked maleimide from reactants and by-products. Eclipse XDB-C_{18} column (5 μm, 9.4 × 250 mm, Agilent Technologies, USA) was used using gradient of 5% B to 20% B over 1 min, 20% B to 53% B over 59 min, 53% B to 95% B over 2 min, and 95% B to 5% B over 3 min (A = dH_2O with 0.1% trifluoroacetic acid, B = acetonitrile, flow rate =1 mL min^{-1}, A_{320} nm) with an additional washing step. The desired product was collected and dried under vacuum with liquid nitrogen. Then, the purified HPLC product was confirmed by ^1H NMR (D_2O, 400 MHz) and UV-Visible

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spectrophotometer (JASCO V-760, Japan)

Functionalization of nanoparticle with doxycycline-linked Diels-Alder cycloadduct (MNP-DA-DOXY)

The 0.2 mg of carboxylic acid-functionalized nanoparticle was first reacted with 0.15336 mg of EDC for 15 min, and then reacted with furfurylamine in a 1:500 molar ratio for 2 h in DMSO to react with MAL-DOXY for diels-alder reaction. After 2 h, EDC and unbound furfurylamine were removed by MACS column with DMSO. Then, furan-functionalized nanoparticle was reacted with MAL-DOXY that was purified by HPLC in a 1:25000 molar ratio at 45 °C for 12 h in H_2O/DMSO (1:4) solution. Then, the reaction mixture was purified by Amicon Ultra-15 100K centrifugal filter (Merck Millipore) at 1400 g for 5 min. Then, the nanoparticle was further reacted with poly(ethylene glycol) bis(amine) (MW = 2,000) via EDC coupling. First, 0.2 mg of DA-DOXY-functionalized nanoparticle was reacted with 0.15336 mg of EDC for 15min, and then reacted with 10 mg of poly(ethylene glycol) bis(amine) for 2 h in H_2O/DMSO. The reaction mixture was then purified by Amicon Ultra-15 100K centrifugal filter at 1400 g for 5 min two times.

Synthesis of doxycycline-linked maleimido acetamide derivate (MAl_{acet}-DOXY)

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10 mg of 9-aminodoxycyline hydrochloride dissolved in anhydrous DMSO with two equivalents of DIPEA was reacted with 3 mg of N-succinimidyl maleimidoacetate for 3 h at room temperature. Then, the product was collected, dried under vacuum with liquid nitrogen, and confirmed by ^1H NMR (DMSO-d^6, 400 MHz).

HPLC purification of MAL_{acet}-DOXY

Semipreparative reversed-phase HPLC was conducted to purify MAL_{acet}-DOXY. Eclipse XDB-C_{18} column (5 μm, 9.4 × 250 mm) was used using gradient of 5% B to 20% B over 1 min, 20% B to 50% B over 29 min, 50% B to 90% B over 2 min, and 90% B to 5% B over 3 min (A = dH_2O with 0.1% trifluoroacetic acid, B = acetonitrile, flow rate =1 mL min^{-1}, A_{320} nm) with an additional washing step. The desired product was collected and dried under vacuum with liquid nitrogen.

Succinylation of 9-aminodoxycycline

1 mg of 9-aminodoxycycline hydrochloride that was dissolved in DMSO with 2 equivalents of DIPEA was reacted with 10 mg of succinic anhydride in DMSO for 12 h. Then, carboxylic acid-functionalized doxycycline was purified by HPLC

HPLC purification of carboxylic acid-functionalized doxycycline

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The carboxylic acid-functionalized doxycycline was purified by semipreparative reversed-phase HPLC using Eclipse XDB-C_{18} column (5 μm, 9.4 × 250 mm) with gradient of 5% B to 20% B over 1 min, 20% B to 40% B over 29 min, 40% B to 90% B over 2 min, and 90% B to 5% B over 3 min (A = dH_2O with 0.1% trifluoroacetic acid, B = acetonitrile, flow rate =1 mL min^{-1}, A_{400} nm). The product was collected, dried under vacuum with liquid nitrogen, and confirmed by ^1H NMR (DMSO-d^6, 400 MHz).

Functionalization of nanoparticle with doxycycline-linked 2,2'-Azobis(2-amidinopropane)

Carboxylic acid-functionalized nanoparticle was used to attach 2,2'-Azobis(2-amidinopropane) (AAPH) as a heat-responsive linker via EDC coupling. First, 0.2 mg of carboxylic acid-functionalized nanoparticle was reacted with 0.15336 mg of EDC in DMSO for 15 min, and then reacted with 2,2'-Azobis(2-amidinopropane) dihydrochloride that was dissolved in 10 mM phosphate buffer (pH 7.2) in a 1:2000 molar ratio for 2 h at room temperature. The reaction mixture was purified by MACS column. Then, AAPH-attached nanoparticle was reacted with carboxylic acid-functionalized doxycycline that was purified by HPLC in a 1:4000 molar ratio via EDC coupling. 78.5 μg of carboxylic acid- functionalized doxycycline was first reacted with 0.15335 mg of EDC for 15min, and then reacted with AAPH-attached nanoparticle for 2 h in DMSO. The unbound doxycycline

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was removed by MACS column with 10 mM phosphate buffer (pH 7.2).

Cell culture and lentiviral transduction of cell line

Human embryonic kidney 293T (HEK293T) cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen Carlsbad, CA, USA) supplemented with 0.1% penicillin/streptomycin and 10% fetal bovine serum. The tetracycline inducible sgRNA construct (Fg_H1t_UTG) was modified from previously described pFH1tUTG[7] and is available from Marco J. Herold upon request. Lentiviral particles were produced by transient transfection of HEK293T cells grown in 6-well dishes with 3.5 μg of vector DNA along with the lentiviral vector including inducible sgRNA (2 μg), pax2 packaging vector (1 μg), pMD2G envelop vector (0.5 μg) using FuGENE® HD Transfection Reagent (Roche Diagnostics, Basel, Switzerland). Virus-containing supernatants were collected at 48-72 h after transfection and passed through a 0.45 mm filter. For infection, 2×10^5 HEK293T cells were plated in each 6-well plate in 1 mL viral supernatant with 1 mL DMEM containing 10% fetal bovine serum and then cells were left for 72 h.

Transfection and doxycycline treatment

The constitutive cas9-expressing vector was transfected into infected HEK293T cells

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using FuGENE® HD Transfection Reagent. After 24 h of transfection, doxycycline that was freshly dissolved in sterile deionized water at a concentration of 1 mg ml^{-1} was added to culture medium for a final concentration of 1 μg ml^{-1}. After 48 h of treatment, cells were harvested and subjected to T7E1 assay.

Nanoparticle treatment

Magnetic nanoparticle which has an doxycycline on the surface with thermo responsive linker was treated to cell. First, nanoparticle which was previously dissolved in 10 mM phosphate buffer (pH7.2) was dissolved in culture media to a desired concentration. Then, the nanoparticle was filtered with syringe filter (0.22 μm, Millex-GP syringe filter, Merck Millipore)and treated to cell.

T7E1 assay

Genomic aDNA was isolated using a Genomic DNA purification Kit (Promega, Fitchburg, WI) according to the manufacturer's instructions. We performed nested PCR using 2 pairs of primer : SF3B1_outside_FP: 5'-TCA AGT GAT CCT CCT GCT CA -3'; SF3B1_outside_RP: 5'-TTG GCA TAT TCT GCA TCC AT -3'; SF3B1_inside_FP: 5'-GGA CTA GAG GTA CAC ACA CAG CC -3'; SF3B1_inside_RP: 5'-CTT CAA GAA AGC AGC CAA ACC -3'. The PCR amplicons were denatured by heating, annealed to

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form heteroduplex DNA, treated with 5 units of mismatch-sensitive T7 endonuclease 1 (New England Biolabs, Hitchin, UK) for 20 min at 37 °C, and finally analyzed by 2% agarose gel electrophoresis. Mutation frequencies were calculated as previously described based on the band intensities using Image J software and the following equation: mutation frequency (%) = 100×[1-(1-fraction of cleaved)1/2], where the fraction of cleaved is the total relative density of the cleavage bands divided by the sum of the relative density of the cleaved bands and uncut bands.

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3. Results and Discussion

To control activity of CRISPR/Cas9 system in a spatiotemporally controlled manner, we used magnetic nanoparticle and thermo responsive linker to release doxycycline from nanoparticle in response to alternative magnetic field to activate CRISPR/Cas9. In this thesis, by synthesizing magnetic nanoparticles which have three different thermo responsive linker, respectively, and testing them to CRISPR/Cas9 system in *in vitro*, rationally designed magnetic nanoparticles that can be used in *in vivo* applications are proposed. The basic concept of this thesis is shown in Figure 1. First, magnetic nanoparticle which has doxycycline conjugated-double-stranded DNA on the surface was synthesized and tested in CIRSPR/Cas9 system. Doxycycline-linked Diels-Alder cycloadduct which can undergo retro Diels-Alder reaction under the high temperature was also synthesized. Finally, doxycycline-linked azo compound was also tested in CRSPR/Cas9 system.

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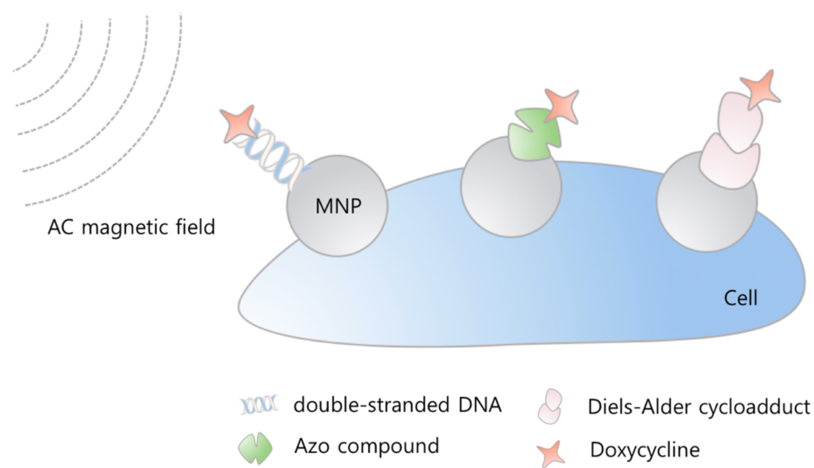

Figure 1. Basic concept scheme of this thesis. In the presence of alternative magnetic field, magnetic nanoparticle generates heat and release doxycycline. Three different thermo responsive linkers were synthesized and compared in CRISPR/Cas9 system.

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Double-stranded DNA-functionalized MNP

In this paper, 15 nm of zinc-doped iron oxide magnetic nanoparticle was used which was synthesized by following previously reported method with slight modification[6]. The synthesized nanoparticle was analyzed by TEM and Zetasizer Nano ZS to confirm the size and shape (Figure 2).

The average hydrodynamic size of ZnMNP measured by Zetasizer Nano ZS was 17.09 nm with 0.341 of polydispersity index (PDI). Considering that the average size of ZnMNP in TEM image is *ca*. 15 nm, average hydrodynamic size of ZnMNP is reasonable.

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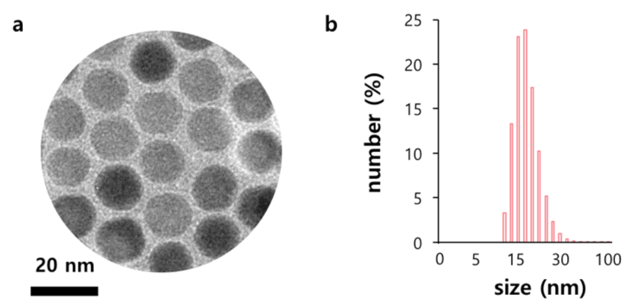

Figure 2. Zinc-doped iron oxide ($Zn_{0.4}Fe_{2.6}O_4$) magnetic nanoparticles (ZnMNP). (a) TEM image of ZnMNP. (b) Hydrodynamic size of ZnMNP measured by Zetasizer Nano ZS.

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Then, the nanoparticle which has organic surfactants, oleic acid and oleylamine, on the surface of nanoparticle was modified to be dispersed in aqueous solution by base-catalyzed sol gel reaction[8]. First, ZnMNP that is dispersed in toluene was reacted with TEOS (tetraethyl orthosilicate) in the presence of ammonium hydroxide for silica coating, and then reacted with APTMS ((3-aminopropyl) trimethoxysilane to synthesize amine-functionalized ZnMNP. Then, amine- functionalized nanoparticle was collected in DMSO (dimethyl sulfoxide) and analyzed by TEM and Zetasizer Nano ZS. According to the TEM image (Figure 3a), silica layer is observed to be *ca.* 1 nm, which is also confirmed by the change of hydrodynamic size. Before silica coating, average hydrodynamic size of ZnMNP that is dispersed in toluene was 17.01 nm. After the silica coating, however, average hydrodynamic size of ZnMNP is increased to 19.44 nm with 0.322 of PDI (Figure 3b).

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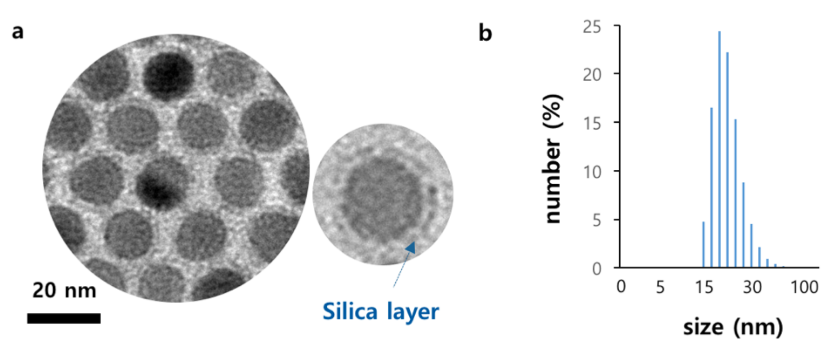

Figure 3. Surface modification of ZnMNP by base-catalyzed sol gel reaction. (a) TEM image of ZnMNP after silica coating and (b) Hydrodynamic size of ZnMNP after silica coating

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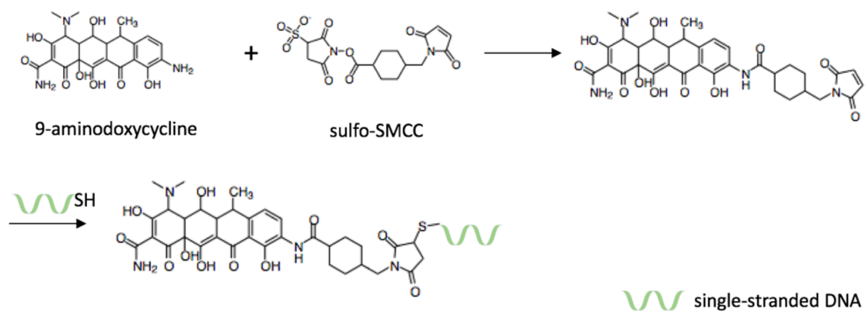

Figure 4. Synthetic scheme of doxycycline-linked single-stranded DNA.

9-Aminodoxycycline was reacted with sulfo-SMCC, and then reacted with single-stranded DNA which has thiol group on 5' end.

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The amine-functionalized ZnMNP dispersed in DMSO was reacted with iodoacetic anhydride, and then reacted with 20 mer double-stranded DNA which has thiol group on 5' end which was previously incubated with doxycycline-linked single-stranded DNA (Figure 4). To examine the release of doxycycline from double-stranded DNA on the surface of nanoparticle, the nanoparticle was heated to 37 °C (cell culture temperature), 50 °C, and 90 °C (positive control) by bulk heating, respectively in a water. The water bath was heated slowly (*ca*. 1 °C min^{-1}) and the released doxycycline was measured by UV-Vis spectrophotometer (Figure 5a). As you can see in Figure 5 a, the average release of doxycycline at 37 °C was 0.385%, 8.54% at 50 °C, and 100% at 90 °C. And then, the nanoparticle was exposed to 500 kHz AC magnetic field at 38 kAm^{-1} and the released doxycycline was measured (Figure 5b). As the exposure time increased, the release of doxycycline was also increased because of the heat generated from the nanoparticle. To confirm whether the nanoparticle can activate CRISPR/Cas9 system, we used tetracycline inducible sgRNA system[7] to be activated by magnetic nanoparticle. After the transfection of nanoparticle, cells were exposed to AMF (AC magnetic field) for 2 h and incubated at 37 °C for 48 h for the analysis by T7E1 assay. As you can see in Figure 6, magnetic nanoparticle groups activated sgRNA both in the presence of AMF and without AMF.

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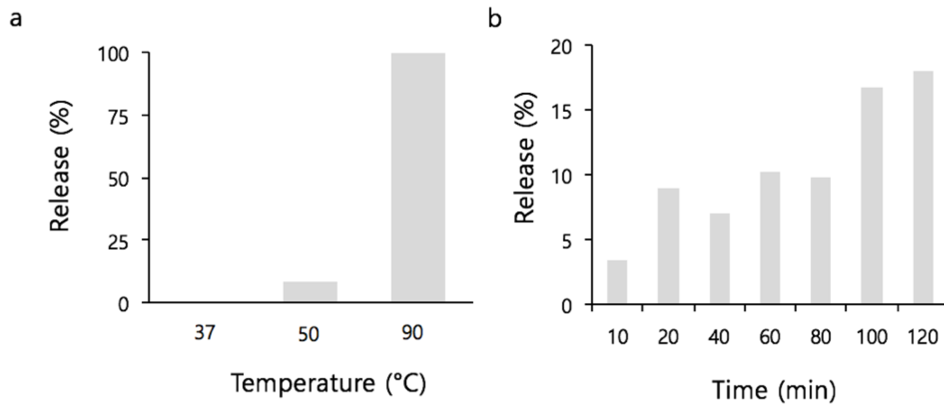

Figure 5. Release test of doxycycline-linked double-stranded DNA on the surface of nanoparticle. (a) double-stranded DNA-functionalized nanoparticle was heated to 37 °C, 50°C, and 90°C, respectively, by bulk heating. (b) double-stranded DNA-functionalized nanoparticle was heated by exposing to 500 kHz AC magnetic field at 38 kAm^{-1}.

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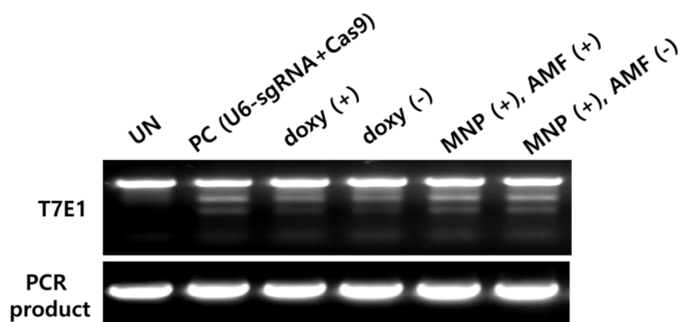

Figure 6. In vitro working test of doxycycline-linked dsDNA-functionalized magnetic nanoparticle.

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So, we tested nanoparticle which has doxycycline directly linked to the silica layer on the surface of nanoparticle without using double-stranded DNA as a thermo responsive linker for a longer period at 37 °C. As you can see in Figure 7a, release of doxycycline directly linked to the silica layer of nanoparticle was not increased. However, release of doxycycline linked to the nanoparticle via double-stranded DNA shows increase at 37 °C if we incubated for a longer period of time. As a result, nanoparticle functionalized with double-stranded DNA as a thermo responsive linker is hard to be controlled in *in vivo* applications if incubated for long periods of time.

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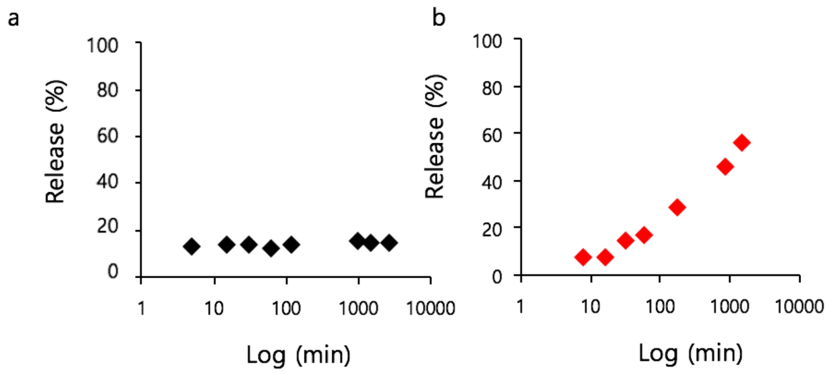

Figure 7. Release profile of doxycycline at 37 °C. (a) Doxycycline was directly linked to the silica layer on the surface of nanoparticle. (b) Doxycycline was linked through double-stranded DNA on the surface of nanoparticle.

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Doxycycline-linked azo compound-functionalized MNP

2,2'-Azobis(2-amidinopropane) (AAPH) was tested as a thermo responsive linker whether AAPH can be tightly controlled in response to AC magnetic field. AAPH was linked to the surface of nanoparticle to attach doxycycline (Figure 8). First, carboxylic acid-functionalized nanoparticle was reacted with AAPH via EDC coupling. And then, primary amine at the end of AAPH was reacted with succinic anhydride to be functionalized with carboxylic acid. Then, AAPH functionalized nanoparticle was reacted with 9-aminodoxycycline via EDC coupling. Then, AAPH-Doxy functionalized MNP was heated to 37 °C to check the leak, and also exposed to AMF respectively (Figure 9a). Then, doxycycline-linked AAPH was tested in *in vitro* to check whether the doxycycline conjugated AAPH can activate sgRNA. As you can see in Figure 9b, doxycycline-linked AAPH can activate CRISPR/Cas9 system. Unlike double-stranded DNA-functionalized nanoparticle, AAPH linker attached to the surface of nanoparticle shows relatively low release profile when incubated at 37 °C. Although the slope of released doxycycline was moderate at 37 °C, the accumulated doxycycline can reach 100 % if incubated at 37 °C for 2 days. Considering the *in vivo* application, AAPH linker is eventually hard to be used in biomedical applications that require tightly controllable tools, especially in genome engineering.

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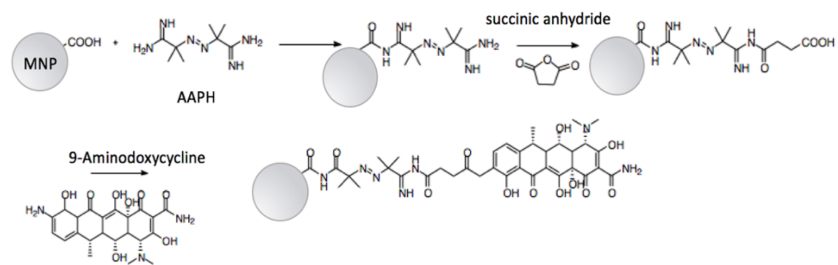

Figure 8. Synthetic scheme of doxycycline-linked azo-functionalized MNP

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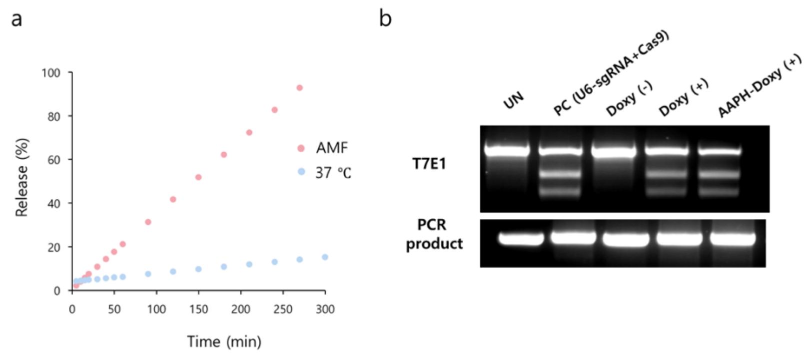

Figure 9. (a) Release profile of AAPH-Doxy-linked MNP. (b) In vitro working test of AAPH-Doxy linker.

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Diels-Alder cycloadduct-functionalized MNP

For a tightly controlled release system that are capable of release doxycycline in response to the AC magnetic field, we synthesized Diels-Alder (DA) cycloadduct on the surface of nanoparticle. Because the adducts of DA reaction undergo reverse reaction (retro-DA reaction) and generate the original reactants in high temperatures, doxycycline-linked DA cycloadduct was examined as a thermo responsive linker (Figure 10). To synthesize DA cycloadduct, 6-maleimidohexanoic acid was first reacted with oxalyl chloride, and then reacted with 9-aminodoxycycline in anhydrous CH_2Cl_2 (Figure 11). Then, doxycycline-linked maleimide derivative (MAL_{hex}-Doxy) was purified by HPLC and then analyzed by ^1H NMR and UV-Vis spectrophotometer.

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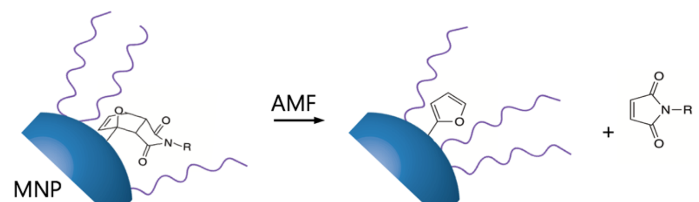

Figure 10. Scheme of Diels-Alder cycloadduct as a thermo responsive linker. After exposing to AC magnetic field, retro Diels-Alder reaction occurs.

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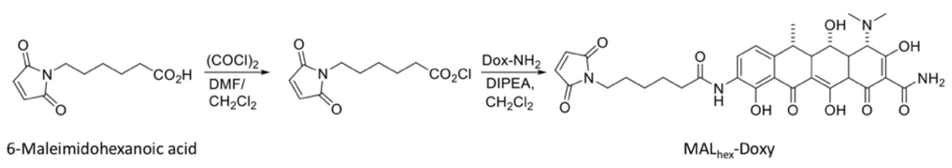

Figure 11. Synthetic scheme of MAL_{hex}-Doxy for Diels-Alder reaction.

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To synthesize DA cycloadduct, 6-maleimidohexanoic acid was first reacted with oxalyl chloride and then reacted with 9-aminodoxycycline in anhydrous CH_2Cl_2. Then, doxycycline-linked maleimide derivative (MAL_{hex}-Doxy) was purified by HPLC and then analyzed by ^1H NMR and UV-Vis spectrophotometer. Reaction product MAL_{hex}-Doxy was separated by HPLC and the product at retention time of 36.42 min was collected (Figure 12a). As you can see in Figure 12b, in the product peak (bottom), we can check singlet proton peak of maleimide (H_a) and doublet peak shift of doxycycline (H_b and H_c) because primary amine in 9-aminodoxycycline was changed to amide bond. Finally, we checked absorbance of MAL_{hex}-Doxy with two reactants 6-maleimidohexanoic acid and 9-aminodoxycycline (Figure 12c), and then by quantifying the absorbance we can confirmed that the maleimide and doxycycline was almost in a 1:1 ratio in the reaction product. To confirm whether the synthesized MAL_{hex}-Doxy can be reacted with furfurylamine to form DA cycloadduct, we monitored real time conversion of reactants into cycloadducts by 400 MHz ^1H NMR in D_2O/DMSO-d^6 (1:4) in a 1: 25 molar ratio. As the Diels-Alder reaction proceeds, ^1H NMR peak of H_a of DA adduct was changed to *ca.* 5 ppm (Figure 13a and b). And the proton peak was changed until 12 h (Figure 13c).

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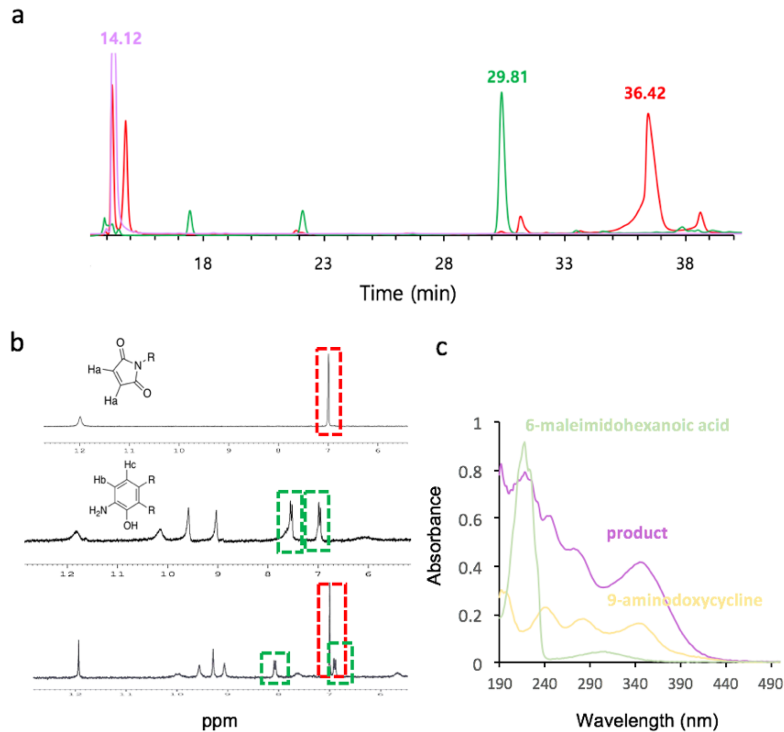

Figure 12. Synthesis of MAL_{hex}-Doxy. (a) HPLC separation data. Violet line indicates 9-aminodoxycycline, green line indicates 6-maleimidohexanoic acid, and red line indicates reaction product. (b) HPLC product of 36.42 min retention time was analyzed by 400 MHz ^{1}H NMR. Red square indicates singlet peak of maleimide (H_a) and green square indicates proton peak (H_b and H_c) of doxycycline. (c) UV-Vis absorption data.

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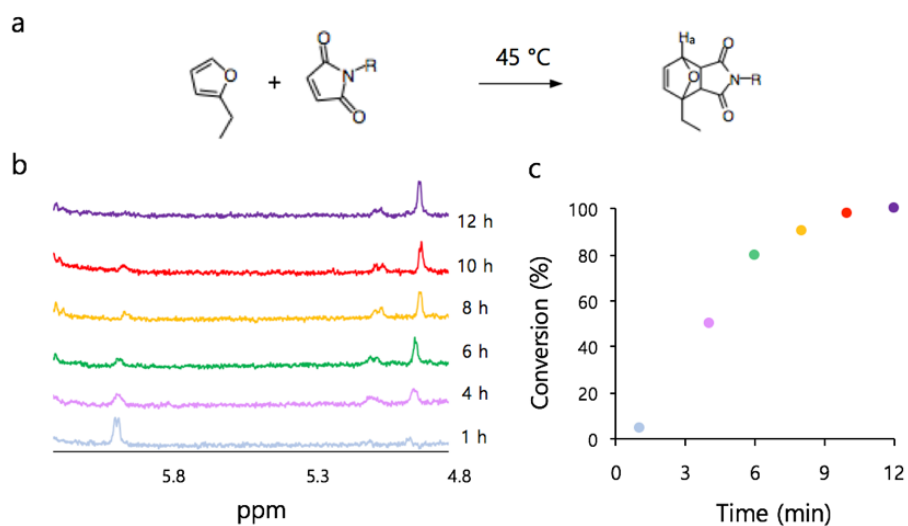

Figure 13. Real-time monitoring of Diels-Alder reaction. (a) Reaction scheme of Diels-Alder reaction. (b) ^{1}H NMR data. (c) quantitative conversion rate of Diels-Alder reaction in D_2O/DMSO-d^6 (1:4).

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Then, to functionalize DA_{hex}-Doxy cycloadduct to nanoparticle, carboxylic acid-functionalized nanoparticle was used to react with furfurylamine via EDC coupling. The unbound furfurylamine was magnetically purified by MACS column, and furfurylamine-attached ZnMNP was reacted with MAL_{hex}-Doxy at 45 °C for Diels-Alder reaction on the surface of nanoparticle by shaking the reaction mixture at 1000 rpm. Then, excess MAL_{hex}-Doxy was removed by Amicon Ultra-15 100K centrifugal filter (Merck Millipore) at 1400 g for 5 min. Then, the nanoparticle was further reacted with poly(ethylene glycol) bis(amine) (MW = 2,000) via EDC coupling to enhance colloidal stability of DA_{hex}-Doxy attached nanoparticle. The reaction mixture was then purified by Amicon Ultra-15 100K centrifugal filter at 1400 g for 5 min two times.

The nanoparticle which has DA_{hex}-Doxy on the surface was then heated to 25°C, 37 °C, and 60 °C, respectively by bulk heating, and exposed to AC magnetic field 500kHz, 38 kAm^{-1}(Figure 14a) to check whether the DA_{hex}-Doxy can undergo retro Diels-Alder reaction at different temperatures. When DA_{hex}-Doxy linked nanoparticle was exposed to AMF, their release profile was comparable with that of 60 °C bulk heating. So, we further tested release of DA_{hex}-Doxy-linked nanoparticle by heating the nanoparticle at 37 °C for long periods of time.

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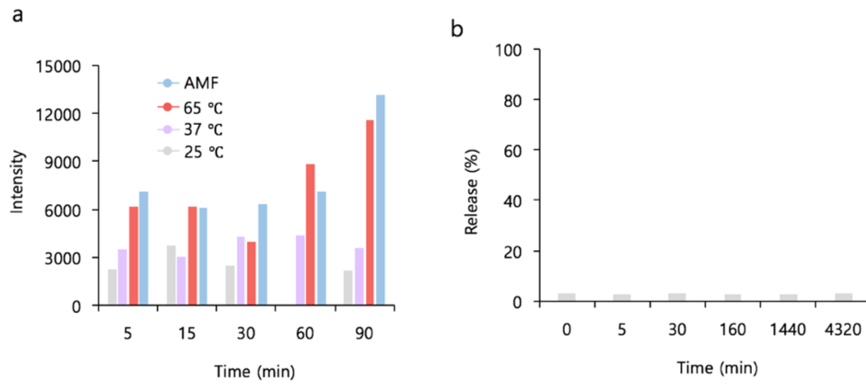

Figure 14. Release profile of DA_{hex}-Doxy-linked MNP. (a) At each experimental conditions, the release profile was compared. (b) Release profile of DA_{hex}-Doxy linked nanoparticle at 37 °C for long periods of time.

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As you can see in Figure 14b, release of DA_{hex}-Doxy linked nanoparticle at 37 °C shows negligible increase. So, we performed in vitro working test for the activation of CRISPR/Cas9 under the AC magnetic field (Figure 15). In vitro working test shows that, unlike other control groups, nanoparticle groups in the presence of AMF or without AMF both shows negative data.

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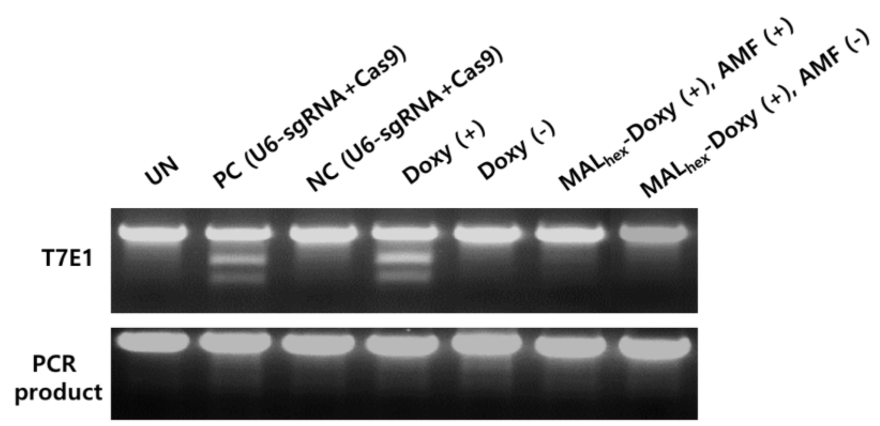

Figure 15. In vitro working test of DA$_{hex}$-Doxy linked MNP

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To figure out the reason, we changed the maleimidohexanoic acid to maleimidoacetate whether the hydrophobicity of carbon chain can affect nuclear localization of doxycycline. N-succinimidyl maleimidoacetate (AMAS) was used to attach doxycycline via Diels-Alder reaction. After the synthesis of MAL_{acet}-Doxy, reaction product was purified by HPLC, and then MAL_{acet}-Doxy linker was tested in *in vitro* (Figure 16). As shown in Figure 16, both MAL_{acet}-Doxy-attached nanoparticle cannot activate CIRSPR/Cas9 in the presence of AMF or without AMF, which indicates that carbon chain was not a major factor. Considering that the maleimide group is activated after the retro Diels-Alder reaction, which can react with thiol group in cysteine inside the cell, the released doxycycline can become hard to be translocated to the nucleus.

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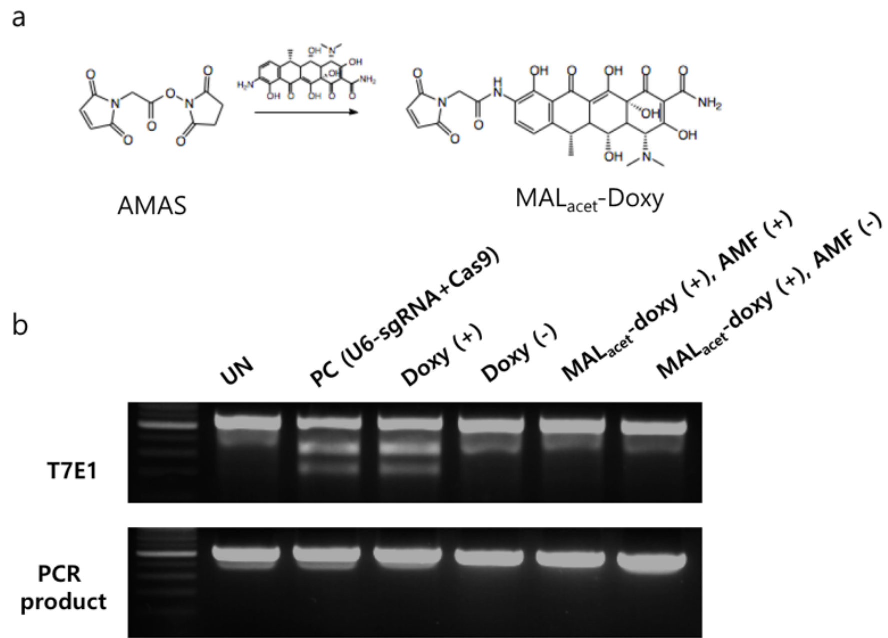

Figure 16. Synthesis of MAL_{acet}-Doxy and in vitro working test of MAL_{acet}-Doxy-linked MNP.

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In order to utilize DA-Doxy-attached nanoparticle in bio-applications, modified synthetic scheme is needed. For example, by using furfurylamine linked with doxycycline and attaching the maleimide group on the surface of nanoparticle, retro Diels-Alder reaction can be an efficient thermo responsive linker (Figure 17). Because maleimide group is attached to the surface of nanoparticle, the released doxycycline from retro Diels-Alder reaction has no functional groups that can react inside the cell which can block the function of doxycycline or interfere translocation of doxycycline.

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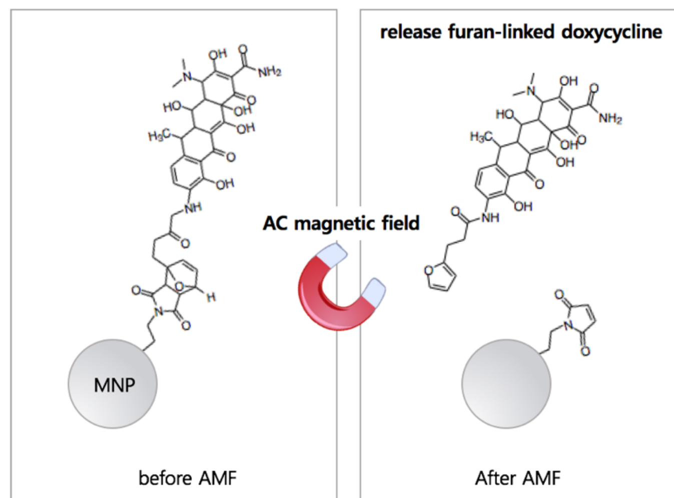

Figure 17. Release scheme of DA-Doxy-linked MNP synthesized by modified synthetic method. Maleimide was first attached to the surface of nanoparticle, which then undergoes Diels-Alder reaction with doxycycline-linked furan (left panel). In the presence of AC magnetic field, DA cycloadduct on the surface of nanoparticle undergoes retro Diels-Alder reaction, which then release furan-linked doxycycline (right panel).

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4. Conclusion

The present study has demonstrated magnetic nanoparticle and thermo responsive linker that can be controlled to release doxycycline in response to AC magnetic field. By comparing three different thermo responsive linker based on the release profile when exposed to external stimuli, their potential possibility in *in vivo* application was examined. Double-stranded DNA (20 bp) linked to doxycycline was first examined. On the surface of nanoparticle, doxycycline-linked double-stranded DNA shows negligible release at 37 °C if the nanoparticle was heated in a heating rate of *ca*. 1 °C min^{-1}. However, when double-stranded DNA-functionalized nanoparticle was incubated at 37 °C for a longer period of time for *in vitro* feasibility test, dramatic increase of released doxycycline was observed. Then, 2,2'-Azobis(2-amidinopropane) (AAPH)-functionalized nanoparticle was examined. In the presence of thermal energy, AAPH was also cleaved to release doxycycline. Although doxycycline was released from the nanoparticle in the presence of AC magnetic field, according to their release profile, AAPH also released doxycycline when the nanoparticle was incubated at 37 °C, which is saturated if incubated for 2 days. For a tightly controlled release system, we synthesized Diels-Alder cycloadduct on the surface of nanoparticle which can undergo retro Diels-Alder reaction to release doxycycline in response to thermal energy. Unlike other thermo responsive linker, DA cycloadduct shows

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negligible release of doxycycline from the nanoparticle that is incubated at 37 °C for a long period of time. Although in vitro working test of DA-Doxy-functionalized nanoparticle shows negative data, DA-Doxy linker can be adjusted to activate CRISPR/Cas9 when retro Diels-Alder reaction occurs. Because doxycycline was linked to maleimide, the released doxycycline may have reacted with thiol group of cysteine inside the cell, before they are translocated to the nucleus to activate CRISPR/Cas9. As a result, DA-Doxy-functionalized nanoparticle can be used in bio-applications by a simple modification of synthetic method. By attaching doxycycline to furan and maleimide to nanoparticle, released doxycycline can be applied to activate CRISPR/Cas9, because there are no functional groups that can react inside the cell which can block the function of doxycycline or interfere translocation of doxycycline.

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국문요약

자기적 성질을 통해 유전자의 발현이 조절가능한 CRISPR/Cas9

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자성 나노입자는 외부자기장의 존재하에 비침습적으로 반응할 수 있다는 성질을 가지며, 이로 인해서 생명활동을 조절하고자 하는 연구 분야에서 다양하게 응용되고 있다. 자성 나노입자가 나타내는 여러가지 성질 중에서도, 자성 나노입자는 교류자기장과 결합하였을 때 열을 발생시킬 수 있으며 이를 이용한 연구가 온열치료 분야에서 활발하게 진행되고 있다.

본 연구에서는, 나노입자에 교류자기장을 가했을 때 방출되어 나오는 열을 통해서 유전자의 발현을 시공간적으로 조절하고자 하며, 특히 유전공학에서 각광 받고 있는 CRISPR/Cas9 모델에 적용하여 이를 보여주고자 한다.

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나노입자로부터 방출된 열은 나노입자 표면에 존재하는 열에민감한 링커로 전달되고, 입자로부터 방출된 열로 인해서 표면에 결합하고 있던 링커가 끊어짐으로써 유전자 발현을 조절할 수 있는 물질을 방출하게 된다. 이 때, 열에 민감한 링커를 사용하여 유전자의 발현을 미세하게 조절하기 위해서는, 동물의 체온 조건에서는 장시간 동안 끊어지지 않고 비활성 상태를 유지하다가 자극이 들어왔을 때에만 활성상태로 전환되어야 한다. 따라서. 세 가지 다른 링커에 결합한 나노입자를 각각 합성하고, CRISPR/Cas9 모델에서 각각의 나노입자가 가진 가능성을 타진함으로써 자기적 성질을 통해 유전자의 발현을 조절할 수 있음을 제시하고자 한다.

핵심되는 말 : 자성 나노 입자, 온열 치료, 유전자 조절, CRISPR/Cas9