Self-Assembly of DNA–Minocycline Complexes by Metal Ions with Controlled Drug Release

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Supporting Information

ABSTRACT: Here we reported a study of metal ions-assisted assembly of DNA–minocycline (MC) complexes and their potential application for controlling MC release. In the presence of divalent cations of magnesium or calcium ions (M²⁺), MC, a zwitterionic tetracycline analogue, was found to bind to phosphate groups of nucleic acids via an electrostatic bridge of phosphate (DNA)-M²⁺-MC. We investigated multiple parameters for affecting the formation of DNA-Mg²⁺-MC complex, including metal ion concentrations, base composition, DNA length, and single- versus double-stranded DNA. For different nitrogen bases, single-stranded poly(A)₂₀ and poly(T)₂₀ showed a higher MC entrapment efficiency of DNA-Mg²⁺-MC complex than poly(C)₂₀ and poly(G)₂₀. Single-stranded DNA was also found to form a more stable DNA-Mg²⁺-MC complex than double-stranded DNA. Between different divalent metal ions, we observed that the formation of DNA-Ca²⁺-MC complex was more stable and efficient than the formation of DNA-Mg²⁺-MC complex. Toward drug release, we used agarose gel to encapsulate DNA-Mg²⁺-MC complexes and monitored MC release. Some DNA-Mg²⁺-MC complexes could prolong MC release from agarose gel to more than 10 days as compared with the quick release of free MC from agarose gel in less than 1 day. The released MC from DNA-Mg²⁺-MC complexes retained the anti-inflammatory bioactivity to inhibit nitric oxide production from pro-inflammatory macrophages. The reported study of metal ion-assisted DNA-MC assembly not only increased our understanding of biochemical interactions between tetracycline molecules and nucleic acids but also contributed to the development of a highly tunable drug delivery system to mediate MC release for clinical applications.

KEYWORDS: DNA nanostructure, minocycline, DNA-minocycline assembly, controlled drug release, anti-inflammation

1. INTRODUCTION

DNA is a “Popstar” molecule in biochemistry that carries genetic information to guide the construction of biomolecular complexes in living systems. In the past three decades, self-assembled DNA nanostructures have emerged as promising biomaterials to organize molecules on the nanoscale. The use of double-helical DNA molecules for nanoscale engineering began with Seeman’s construction of an artificial branched DNA “Holliday” junction. Recent breakthroughs in scaffolded DNA origami and single-stranded DNA bricks have empowered the design and fabrication of a virtually unlimited reservoir of spatially addressable one-dimensional (1D), 2D, and 3D nanostructures, including structures with complex curvatures, polyhedral meshes, and periodic DNA crystals. DNA scaffold-directed assembly holds great promise to organize bioactive therapeutics with precise control of spatial patterning. Self-assembled DNA nanostructures can serve as delivery platforms that are integrated with various functions ranging from molecular recognition and computations; dynamically structural switch to carry molecular payloads; and selectively release. Single- or double-stranded DNA (ssDNA or dsDNA) can also bind to small drug molecules and deliver them into cells for therapy, such as doxorubicin, daunorubicin, and epirubicin.

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Tetracycline molecules are broad-spectrum antibiotic drugs that are used to clinically treat various bacterial infections and are also applied to the treatment of rheumatoid arthritis and in the prevention of malaria. The structural analysis has revealed that tetracycline molecule can bind to ribosomal 3OS subunit stabilized by magnesium salt bridge between hydroxyl groups of tetracycline and phosphate groups of rRNA, and thus inhibited ribosome activity. This structural information suggests a possibility that tetracycline molecules may interact with nucleic acids and could be assembled to tetracycline/nucleotide hybrid nanostructures. Tetracycline was also reported to bind with Calf thymus DNA in the presence of Cu2+. Minocycline (MC), a semisynthetic tetracycline analogue, is a clinically available antibiotic and anti-inflammatory drug that has a broader antibiotic spectrum than other members of the tetracycline group. In addition, MC also demonstrates potent antioxidant and antiapoptotic properties. Due to its multifaceted activities, MC has been shown to target a number of debilitating neurological diseases, including traumatic spinal cord injury, brain injury, stroke, intracerebral hemorrhage, Parkinson’s disease, Alzheimer’s disease, multiple sclerosis, and amyotrophic lateral sclerosis. However, the effectiveness of MC treatment for these disorders has been compromised by the inability to locally deliver high concentrations of this drug. To mediate its local delivery in tissue, people have developed various MC-hybrid particles for encapsulation and release of MC, such as hydrophobic poly(lactic-co-glycolic acid) (PLGA) microspheres and dextran sulfate-MC nanoparticles. Here, we reported a study of metal ions (M2+)–assisted self-assembly of DNA-M2+–MC hybrid structures. In the presence of divalent cations of magnesium and calcium, MC self-assembled with various DNA nanostructures to form coaggregated particles. The DNA-Mg2+–MC particles were further used to control MC release from agarose hydrogel and to maintain its anti-inflammatory effect.

2. MATERIALS AND METHODS

2.1. Materials. Analytical grade or molecular biology reagents and distilled (DI) water were used in all experiments unless otherwise specified. Magnesium acetate (MgAc, Mg(CH3COO)2·4H2O), calcium acetate (CaAc, Ca(CH3COO)2·H2O), Tris base, EDTA, DNA grade water, and agarose were obtained from Fisher Scientific (Pittsburgh, PA, USA). All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA), and their sequences were listed in the Supporting Information Table S1. DNA oligonucleotides were dissolved in DNA grade water, and the concentrations of DNA stock solutions were quantified by 260 nm absorbance using NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). Minocycline hydrochloride, adenosine, Griess reagent, lipopolysaccharide, and paraffin were purchased from Sigma-Aldrich (St. Louis, MO, USA). A 10 mg/mL amount of MC solution was prepared using DI water and were stocked at 4 °C in dark. A 10 mg/mL amount of MC solution was prepared using DI water and were stocked at 4 °C. Tetracycline/nucleotide hybrid nanostructures. Tetracyline may interact with nucleic acids and could be assembled to anti-inflammatory drug that has a broader antibiotic spectrum than other members of the tetracycline group. 25–27 In addition, MC also demonstrates potent antioxidant and antiapoptotic properties.28–30 Due to its multifaceted activities, MC has been shown to target a number of debilitating neurological diseases, including traumatic spinal cord injury, brain injury, stroke, intracerebral hemorrhage, Parkinson’s disease, Alzheimer’s disease, multiple sclerosis, and amyotrophic lateral sclerosis, etc. However, the effectiveness of MC treatment for these disorders has been compromised by the inability to locally deliver high concentrations of this drug. To mediate its local delivery in tissue, people have developed various MC-hybrid particles for encapsulation and release of MC, such as hydrophobic poly(lactic-co-glycolic acid) (PLGA) microspheres and dextran sulfate-MC nanoparticles. Here, we reported a study of metal ions (M2+)–assisted self-assembly of DNA-M2+–MC hybrid structures. In the presence of divalent cations of magnesium and calcium, MC self-assembled with various DNA nanostructures to form coaggregated particles. The DNA-Mg2+–MC particles were further used to control MC release from agarose hydrogel and to maintain its anti-inflammatory effect.

2.2. Preparation of Buffer Solutions. Buffer solutions were prepared using DI water and were stocked at 4 °C in dark. A 50 × TA stock solution contains 2 M Tris base and 1 M acetic acid. The pH value was adjusted to 7.0 using acetic acid. A 10 × TA solution was diluted from 50 × TA with the final concentration of 0.4 M Tris. A 1 × TA buffer was diluted from 10 × TA with the final concentration of 0.04 M Tris. A 1 × TA buffer with 32 mM Mg2+ was prepared by adding 5 mL of 10 × TA stock and 16 mL of 100 mM MgAc into DI water for the total volume of 50 mL. Solutions of 1 × TA-Mg2+ containing 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 mM Mg2+ were prepared by the serial dilution (2-fold) of 1 × TA with 32 mM Mg2+ using the 1 × TA solution. Buffer of 1 × TAE-Mg2+ contains 40 mM Tris, 20 mM acetic acid, 2 mM EDTA, and 12.5 mM MgAc, and the pH value was adjusted to 8.0. TA buffer solutions with Ca2+ were prepared similarly using CaAc instead of MgAc as described above. The 2 × Hank’s balanced salt solution (HBSS) was prepared as previously described that contains 0.8 g/L KCl, 0.12 g/L KH2PO4, 0.4 g/L MgSO4-7H2O, 16 g/L NaCl, 0.096 g/L Na2HPO4, 0.7 g/L NaHCO3, and 0.28 g/L CaCl2. The pH value was adjusted to 7.0. The 1 × HBSS solution was prepared by adding 50 mL of 2 × HBSS into 50 mL of DI water for a total volume of 100 mL.

2.3. Preparation of DNA Sample. ssDNA samples were directly prepared from stock solutions by diluting them into 1 × TA-Mg2+ buffer. dsDNA samples were preincubated in 1 × TA-Mg2+ buffer at room temperature for a half-hour prior to the addition of MC. Two-strand multidomain DNA hydrogel was prepared as previously reported. Briefly, 10 mg/mL solutions of Hydro-1 and Hydro-2 were prepared in 1 × TA with 10 mM MgAc separately. The two solutions were then mixed together at a 1:1 volume ratio that was thermally annealed to form a 10 mg/mL two-strand multidomain DNA structure. DNA origami rectangular tiles were prepared as previously described. Briefly, 20 nM M13mp18 DNA was incubated with a 5-fold molar excess of staple strands in 1 × TAE-Mg2+ buffer. The mixture was heated to 95 °C and then cooled to 4 °C with a temperature gradient (Supporting Information Table S2). The origami tiles were purified using Amicon filters (MWCO, 100 kDa; 500 μL) to remove excess staple strands. The concentration of DNA origami tiles was quantified by measuring the absorbance at 260 nm, assuming an extinction coefficient of ~109,119,009 M−1 cm−1.

2.4. Preparation of DNA-Mg2+/Ca2+-MC Complexes. A 4 mg/mL DNA solution was first mixed with a 4 mg/mL MC solution in 1 × TA buffer with 1:1 volume ratio to produce a 2 mg/mL mixture solution of DNA and MC. Then, an equal volume of 2 × Mg2+ or 2 × Ca2+ solution prepared in 1 × TA buffer was added into the mixture solution of DNA and MC to produce a 1 mg/mL DNA-MC mixture at desired Mg2+ concentration. The mixture was incubated on the orbital shaker (50 rpm; ArmaLab, Bethesda, MD, USA) at room temperature for 30 min in the dark.

2.5. Quantification of the Entrapment Efficiency of DNA and MC in DNA-Mg2+/Ca2+-MC Complexes. A 50 μL aliquot of DNA-Mg2+/Ca2+-MC complexes was prepared as described in section 2.4. The solution was then centrifuged at 10,000 rpm under room temperature for 5 min. DNA-Mg2+/Ca2+-MC complexes were precipitated on the bottom of the centrifuge tube. A 32 μL aliquot of supernatant solution was collected and diluted by 10-fold into 1 × TA buffer. The absorbance spectrum of the diluted supernatant was measured using a 96-well Cytation 3 plate reader (Biotek, Winoski, VT, USA). The concentrations of DNA and MC were characterized by the absorbance at 260 and 355 nm, respectively. The MC absorbance at 355 nm was affected little by magnesium concentrations and was used to characterize MC concentration (Supporting Information Figures S1 and S2). The detailed calculation of MC and DNA entrapment efficiency within DNA-Mg2+/Ca2+-MC complexes was described in the Supporting Information. The accuracy of the absorbance analysis of MC supernatant was also verified by the particle dissolution of DNA-Mg2+-MC complexes (Supporting Information Figure S3).

2.6. Atomic Force Microscope Imaging. A 2 μL aliquot of supernatant solution was deposited onto a freshly cleaved mica surface (Ted Pella, Redding, CA, USA) and was left to adsorb for 2 min. Then, 80 μL of 1 × TA buffer was added to the sample and 2 μL of 100 mM Ni2+ was added to enhance DNA adsorption on mica. Then, the buffer solution was removed, and the surface was rinsed using DI water for three times. After the mica surface was dried under
clean air, the samples were scanned with SCANASYST-Air probe (Bruker, Billerica, MA, USA) using "Scanasyst in air mode" of Multimode 8 atomic force microscope (AFM; Bruker).

2.7. MC Release from DNA-Mg^{2+}/Ca^{2+}-MC Complexes Encapsulated in Agarose Gel. DNA-Mg^{2+}/Ca^{2+}-MC complexes were encapsulated in 1.5% agarose gel for releasing MC in 1×HBSS buffer. Please see Supporting Information for detailed procedures. The released MC in milligrams per milliliter was calculated from a standard curve of MC concentrations (Supporting Information Figure S2).

The kinetical fitting of MC release curves was performed using GraphPad Prism 7. A simplified Weibull model was used to fit the exponential profile of cumulative release curves as shown in eq 1:47,48

\[
Q_{\text{cumulative}}/\% = (1 - e^{-kT}) \times 100
\]

where \(Q_{\text{cumulative}}\) is the cumulative drug release percentage, \(k\) is the release constant, and \(T\) is the release period.

2.8. MC Bioactivity Test. Anti-inflammatory bioactivity of MC was tested on RAW264.7 murine macrophages. The cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Lipopolysaccharide (LPS) at 250 pg/mL was used to stimulate macrophages for the upregulation of nitric oxide (NO), a pro-inflammatory mediator.49 Fresh MC (0.5 μg/mL) or released MC (diluted to 0.5 μg/mL) was added to the LPS-stimulated cultures. After 48 h, NO production was determined by the measurement of accumulated nitrite (an indicator of NO) in the culture medium using Griess reagent,42 which produced an increased absorbance at ∼540 nm (Supporting Information Figure S4). All data were presented as mean ± standard deviation (S.D.). Pairwise comparisons were conducted by using one-way ANOVA and Tukey test. \(p < 0.05\) was considered statistically significant.

3. RESULTS AND DISCUSSION
MC has four rings that are similar to tetracycline (Figure 1A) with multiple \(pK_a\) values for different functional groups: \(pK_a \sim 2.8\) for the hydroxyl group at C3, \(pK_a \sim 5\) for the dimethylamine group at C7, \(pK_a \sim 7.8\) for the hydroxyl group at C12, and \(pK_a \sim 9.5\) for the dimethylamine group at C4.50 MC is zwitterionic within the pH range of 5–7.8, with
one negative charge from the deprotonation of the hydroxyl group at C3 and one positive charge from the protonated dimethyl amine at C4.50 Based on the previous studies of tetracycline interacting with rRNA,22,23 it is suggested that Mg2+ might form a salt bridge between the hydroxyl group on MC and the phosphate group on nucleotides, serving as a metal bridge to assemble MC and nucleic acids together (Figure 1B). A weak π−π interaction between DNA bases and the aromatic ring of MC may also exist to stabilize DNA-MC assemblies. To test this hypothesis, we first titrated Mg2+ concentrations to effect the assembly of a ssDNA with MC in solution. As shown in Figure 1C, the mixture of poly(A)20 and MC is a yellow solution without any observed precipitant at 0 mM Mg2+. As gradually increasing Mg2+ concentration from 0 to 16 mM, the solution started to become turbid and yellow precipitants were observed at higher Mg2+ concentrations. The spectrum analysis indicated that there was a decreased absorbance for both MC (355 nm) and DNA (260 nm) in solution supernatant with the addition of Mg2+ (Figure 1D), suggesting less MC and DNA were left in the solution layer. It indicated that MC and DNA were coaggregated into precipitants assisted by magnesium ion. Mechanistic information given by fluorescence spectrum and infrared (IR) analysis supported our hypothesis of a Mg2+ bridged DNA-Mg2+-MC assembly (Supporting Information Figures S5 and S6). As shown in Figure 1E, AFM imaging of DNA-Mg2+-MC complexes showed that tiny nanoparticles (~100 nm, top image) were first formed in the presence of magnesium. These tiny nanoparticles further aggregated into large particles of a few micrometers size (bottom image). The formation of DNA-Mg2+-MC was also characterized by dynamic light scattering (Supporting Information Figure S7).

To evaluate the capability of DNA for interacting with MC, we first evaluated the effect of ssDNA length on the formation of DNA-Mg2+-MC complexes. As shown in Figure 2A, MC entrapment efficiency of DNA-Mg2+-MC complexes reached maximum at ~4 mM Mg2+ for all poly(A) strands with length varying from 11 nt (55%) and 15 nt (62%) to 20 nt (74%). For poly(A) longer than 20 nt, the MC entrapment efficiency did not increase significantly (Figure 2B). As shown in Figure 2C, the molar ratio of MC to monomer nucleotide in DNA-Mg2+-MC complexes depending on the length of ssDNA. (A) Titration of Mg2+ concentrations for MC entrapment efficiency of different lengths of poly(A) ranging from 11 to 50 nt and adenosine. (B) MC entrapment efficiency of different lengths of poly(A) ranging from 11 to 50 nt at 4 mM Mg2+. (C) Ratios of MC to nucleotide monomer in DNA-Mg2+-MC complexes. All experiments were performed for 1 mg/mL DNA and 1 mg/mL MC in pH 7, 1 × TA buffer. Error bars: the range of data for three replicates.

Figure 2. Formation of DNA-Mg2+-MC complexes depending on the length of ssDNA. (A) Titration of Mg2+ concentrations for MC entrapment efficiency of different lengths of poly(A) ranging from 11 to 50 nt and adenosine. (B) MC entrapment efficiency of different lengths of poly(A) ranging from 11 to 50 nt at 4 mM Mg2+. (C) Ratios of MC to nucleotide monomer in DNA-Mg2+-MC complexes. All experiments were performed for 1 mg/mL DNA and 1 mg/mL MC in pH 7, 1 × TA buffer. Error bars: the range of data for three replicates.

Figure 3. Formation of DNA-Mg2+-MC complexes for poly(A)20, poly(T)20, poly(C)20, and poly(G)20 with the evaluation of (A) MC entrapment efficiency, (B) DNA entrapment efficiency, and (C) ratios of MC to nucleotide monomer. All of the experiments were performed for 1 mg/mL DNA and 1 mg/mL MC in pH 7, 1 × TA buffer with 4 mM Mg2+. Error bars: the range of data for three replicates. *, p < 0.05 compared with poly(A)20 or poly(T)20 (n = 3).
DNA-MC particles was ∼0.8–0.9 for these poly(A) strands, except for poly(A)15 with a ratio >1. This indicated that one nucleotide could bind with almost one MC on average in ssDNA-Mg2+-MC complexes.

We compared different bases of A, T, C, and G for their ability to form DNA-Mg2+-MC complexes. As shown in Figure 3A, poly(A)20 (∼74%) and poly(T)20 (∼70%) entrapped more MC in DNA-MC particles than poly(C)20 (∼62%) and poly(G)20 (∼64%). However, in DNA-Mg2+-MC complexes, more DNA were entrapped in the solid precipitants for poly(C)20 (>90%) and poly(G)20 (∼100%) than poly(A)20 (∼45%) and poly(T)20 (∼45%) (Figure 3B). As a result, the molar ratio of MC to monomer nucleotide in DNA-MC complexes was ∼0.4 for poly(C)20 and poly(G)20, only half of the ratio (∼0.8–0.9) for poly(A)20 and poly(T)20 (Figure 3C).

One possible reason is that poly(C)20 and poly(G)20 present partially folded structures in solution (e.g., cytosine-cytosine pair in i-motif structures and G4 interactions in G-quadruplex) and thus bind less MC than relatively linear poly(A)20 and poly(T)20. In addition, we performed isothermal titration of DNA concentrations for entrapping MC (Supporting Information Figure S8) and pH titrations from 4 to 8 (Supporting Information Figure S9).

We also tested dsDNA for forming the DNA-Mg2+-MC complex. As shown in Figure 4A, the MC entrapment efficiency reached maximal at 4 mM Mg2+ (∼60%) for the poly(A-T)20-Mg2+-MC complex, and then quickly decreased when Mg2+ concentrations further increased. In contrast, poly(A)20 entrapped more MC (>70%) than poly(A-T)20 in the assembled complex at the same Mg2+ concentration, and the MC entrapment efficiency did not show significant decrease at higher Mg2+ concentrations of 8 and 16 mM. Figure 4B showed that the DNA entrapment efficiency of poly(A-T)20 was also lower than that of poly(A)20 in DNA-Mg2+-MC complexes formed by different DNA nanostructures, including (1) poly(A)20 and poly(A-T)20, (2) poly(A)40 and poly(A-T)40, (3) multidomain ssDNA and two-strand multidomain DNA hydrogel, and (4) M13mp18 ssDNA and rectangular DNA origami. All of the experiments were performed for 1 mg/mL DNA and 1 mg/mL MC in pH 7, 1 × TA buffer. Error bars: the range of data for three replicates.
Mg\textsuperscript{2+}-MC complexes. A similar pattern of decreased DNA entrapment was also observed for poly(A-T)\textsubscript{20} at higher Mg\textsuperscript{2+} concentrations of 8 and 16 mM. However, the overall molar ratio of MC-to-monomer nucleotide in assembled complexes was similar for all ssDNA and dsDNA strands (Figure 4C). dsDNA is more rigid in structure than flexible ssDNA, where divalent magnesium ion binds tighter with dsDNA than ssDNA. The Mg\textsuperscript{2+}-dsDNA interaction can shield the negative charge of backbone phosphate and stabilize double-helix structures. However, this also reduces the electrostatic interaction of the MC-Mg\textsuperscript{2+}-phosphate (DNA) bridge. This reduction is significant at high magnesium concentrations (e.g.,

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Figure 6. Formation of DNA-Mg\textsuperscript{2+}-MC complexes for Mg\textsuperscript{2+} and Ca\textsuperscript{2+} with the evaluation of (A) MC entrapment efficiency of poly(A)\textsubscript{20}-MC complexes depending on Mg\textsuperscript{2+} or Ca\textsuperscript{2+} concentrations and (B) MC entrapment efficiency of poly(A-T)\textsubscript{20}-MC complexes depending on Mg\textsuperscript{2+} or Ca\textsuperscript{2+} concentrations. (C) Reduction of MC entrapment efficiency of poly(A)\textsubscript{20}-Mg\textsuperscript{2+}-MC complexes due to the addition of Na\textsuperscript{+}. Condition: NaCl competition was performed for 1 mg/mL poly(A)\textsubscript{20} and 1 mg/mL MC in pH 7, 1× TA buffer with 4 mM Mg\textsuperscript{2+}. Error bars: the range of data for three replicates.

Figure 7. Cumulative MC releases from various DNA-Mg\textsuperscript{2+}-MC complexes for (A) poly(A)\textsubscript{20} encapsulated in agarose gel, poly(T)\textsubscript{20}, poly(C)\textsubscript{20}, and poly(G)\textsubscript{20} as well as free MC; (B) poly(A)\textsubscript{20}, poly(A)\textsubscript{40}, and M13mp18 DNA; (C) ssDNA of poly(A)\textsubscript{20} and poly(A)\textsubscript{40} and dsDNA of poly(A-T)\textsubscript{20} and poly(A-T)\textsubscript{40}; (D) DNA-Mg\textsuperscript{2+}-MC complex and DNA-Ca\textsuperscript{2+}-MC complex. All DNA-M\textsuperscript{2+}-MC complexes were performed with 1 mg/mL DNA and 1 mg/mL MC in pH 7, 1× TA buffer with 4 mM Mg\textsuperscript{2+} or Ca\textsuperscript{2+}. DNA-M\textsuperscript{2+}-MC complexes were entrapped within 1.5% agarose gel and were released in 1× HBSS buffer. Error bars: the range of data for three replicates.
entrapped 95% of MC in the assembled complex when Ca2+ entrapment decreased at higher Mg2+ concentrations (>4 mM) and thus is unable to interact with MC e

Figure 5, we further compared various DNA nanostructures for entrapping MC in DNA-Mg2+-MC complexes. ssDNA of poly(A)20 or 40 entrapped more MC (>70%) than rigid dsDNA. We also found that monovalent cations such as Na+ destabilized the DNA-Mg2+-MC complex. As shown in Figure 6C, with the addition of more Na+ into the solution, less MC was entrapped within poly(A)20-Mg2+-MC complexes due to the disruption of the Mg2+ bridge under the competition of Na+. On the other hand, at 160 mM NaCl (higher than physiological NaCl concentration of 154 mM), poly(A)20-Mg2+-MC still entrapped more than 50% of MC in precipitant particles. In addition, the body fluid contains Ca2+ and Mg2+ ions that can stabilize MC release. Therefore, we used HBSS buffer as the release medium to simulate body fluid.

Toward local MC delivery, we encapsulated DNA-Mg2+-MC complexes in 1.5% (m/v) agarose gel that could be applied to immobilizing the complexes at the injury/disease site. As shown in Figure 7A, when free MC was loaded in agarose hydrogel, more than 90% of loaded MC was released at day 1, due to the fast diffusion of MC through the macroporous hydrogel. In contrast, agarose hydrogel loaded with ssDNA-Mg2+-MC complex released MC substantially slower: ~40% and 60% MC were released at day 1 and day 2, respectively, followed by a slow MC release from day 3 to day 10. To analyze the release kinetics, a simplified Weibull model was used to fit the exponential profile of cumulative release curves. Poly(A)20-Mg2+-MC and poly(T)20-Mg2+-MC had similar release constants (k value) of ~0.43 ± 0.02 and ~0.44 ± 0.02, respectively, which were smaller than the release constants of poly(C)20-Mg2+-MC (k ~ 0.56 ± 0.01) and poly(G)20-Mg2+-MC (k ~ 0.50 ± 0.01). This result was consistent with our observation of weaker poly(C)20/poly-(G)20-Mg2+-MC interactions as shown in Figure 3. Next, we evaluated how the length of ssDNA affected the MC release from the complexes. As shown in Figure 7B, the release of MC from poly(A)20-Mg2+-MC (longer ssDNA) was significantly slower (k ~ 0.20 ± 0.01) than that from poly(A)20-Mg2+-MC (shorter ssDNA) (k ~ 0.43 ± 0.02). The release of MC from 7249 nt M13mp18-Mg2+-MC complex was even slower (k ~ 0.16 ± 0.01). Figure 7C showed that the release of MC from dsDNA-Mg2+-MC complexes was faster than that from...
ssDNA-Mg2+-MC complexes, possibly due to the unstable dsDNA-Mg2+-MC interactions as discussed in Figure 4. For different divalent ions, the MC release from poly(A)20Ca2+-MC (k ~ 0.14 ± 0.01) was significantly slower than that from poly(A)20Mg2+-MC (k ~ 0.43 ± 0.02). This could be attributed to the enhanced electrostatic interactions of the phosphate (DNA).Ca2+-MC bridge (Figure 7D). Conversely, the dsDNA poly(A-T)20Ca2+-MC complex was unstable, resulting in substantially and significantly faster MC release (k ~ 0.70 ± 0.01) than that from the ssDNA complex of poly(A)20Ca2+-MC.

To evaluate the anti-inflammatory activity of released MC, RAW 264.7 murine macrophages were treated with LPS to induce the pro-inflammatory phenotype of macrophages, which were marked by the upregulation of NO production. It has been reported that MC, as an anti-inflammatory drug, is able to inhibit the activation of pro-inflammatory macrophages and reduce NO production. As shown in Figure 8A, significantly more accumulated nitrite (an NO indicator) was detected in LPS-stimulated macrophages (nitrite, ~7.2 ± 0.3 μM) compared with untreated control (nitrite, <1 μM). The addition of fresh MC significantly reduced the level of accumulated nitrite in LPS-induced macrophages to ~3.4 ± 0.2 μM, confirming its anti-inflammatory effect. Fresh MC and MC released from poly(A)20Mg2+-MC at day 4 or poly(A)40Mg2+-MC at day 7 showed similar anti-inflammatory bioactivity for suppressing NO production. As shown in Figure 8B, the DNA-Mg2+-MC incorporated agarose gel was demonstrated to suppress NO production of macrophages. DNA-Mg2+-MC gels with different release periods (from 3 days to 7 days) were also tested for anti-inflammation against LPS-induced macrophages (Supporting Information Figure S10). These results demonstrated that the released MC from DNA-Mg2+-MC complexes retained bioactivity similar to that of fresh MC. To be noted, M13mp18 viral ssDNA and its scaffolded origami structures were found to strongly induce the inflammatory response of macrophages and were not used to mediate the release of MC (Supporting Information Figure S11). M13mp18 DNA or M13mp18-scaffolded DNA nanostructures have been previously reported for activating a potent inflammatory cytokine response that was similar to the response from the exposure to bacterial or viral nucleic acids.

4. CONCLUSION
We have studied the self-assembly and coaggregation of DNA molecules with MC stabilized by the divalent metal ion bridge of Mg2+ or Ca2+. By comparing the interactions of different DNA nanostructures with MC, we concluded that ssDNA was more efficient (e.g., poly(A)20 or poly(A)40) than dsDNA for entrapping MC within DNA-Mg2+-MC complexes, which was attributed to the more flexible structures of ssDNA than dsDNA. We also observed that ssDNA of poly(A)20 and poly(T)20 entrapped more MC with a higher ratio of MC to monomer nucleotide than ssDNAs of poly(C)20 and poly-(G)20. Between different cations, the formation of DNA-Ca2+-MC complex was found to be more stable and efficient than DNA-Mg2+-MC complex due to a stronger Ca2+ bridge. For localized MC release, DNA-Mg2+/Ca2+-MC complexes were encapsulated in agarose gel, in which the release of MC lasted from a few days to more than 10 days, depending on varied DNA nanostructures. The released MC maintained its activity to inhibit pro-inflammatory response in macrophages. The ideal dose and duration of MC release is dependent on specific clinical applications. For example, the treatment of chronic inflammation needs a stable release profile (near zero-order release), which may prefer the release profile of poly(A)40-Mg2+-MC complex. On the other hand, for applications such as spinal cord injury, it requires an initial high-dose release for neuroprotection followed by low-dose release to target chronic inflammation. Therefore, poly(A)20-Mg2+-MC may be more suitable for spinal cord repair.

MC is a tetracycline analogue that has broad clinical applications, due to its antibiotic, anti-inflammatory, and antiapoptotic activities. This study reported a metal ion-assisted interaction between MC and DNA structures that increased our understanding of biomolecular interactions between DNA and small-molecule drugs. A similar effect may also be observed for DNA binding with other tetracycline-like molecules24 or metal-assisted assembly of DNA with chemotherapeutic drug doxorubicin. Compared with other polymers, DNA molecules have the unique advantages of highly tunable chemical composition, chain length, and self-assembled structures that can modulate their interaction with therapeutic agents. In future developments, one could employ the power of switchable DNA nanostructures to develop a highly tunable drug delivery system enabling versatile and controlled release of MC or other drug molecules. The self-assembled DNA-Mg2+-MC complexes could also potentially be used for anti-inflammatory and cytoprotective treatments in clinical applications, such as spinal cord therapy.39,43

**ASSOCIATED CONTENT**

**Supporting Information**
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.9b08126.

Detailed methods; absorption spectra for MC under different Mg2+ concentrations (Figure S1); standard MC concentration vs absorbance curve (Figure S2); test of DNA molecules for inducing inflammatory macrophages to release NO (Figure S3); ssDNA sequences used in experiments (Table S1) and thermal annealing program for preparing DNA nanostructures (Table S2); other characterizations (PDF)

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**Notes**
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