이학석사학위논문

pH-Sensitive Antibiotics Delivery via Conjugation with Maleic Acid Amide Derivative Linkers

말레산 아미드 유도체와의 결합을 통한 pH-응답성 항생제 전달

2018년 8월

서울대학교 대학원

화학부 생화학 전공

김 슬 아

Abstract

pH-Sensitive Antibiotics Delivery via Conjugation with Maleic Acid Amide Derivative Linkers

Seulah Kim
Department of Chemistry
College of Natural Sciences
Seoul National University

Among various stimuli for triggering selective activity in drug delivery systems, pH change is one of the most widely utilized signals. The pH change is an endogenous stimulus of the biosystem. For instance, cancerous, inflammatory and abscess tissues show pH 5-6 in contrast with pH 7.4 in most normal tissues and physiological fluid. The weakly acidic pH conditions of the abnormal tissues are recognized for the development of smart drug delivery systems with pH-sensitive chemical bonds for the release and accumulation of anti-cancer or anti-inflammatory drugs. The chemical bonds responding to the pH variation which have been developed so far, however, are difficult to be used in the practical applications due to complex synthetic methods, slow degradation rates and low pH

sensitivity.

In this study, I developed a novel pH-sensitive drug delivery system based on a maleic acid amide derivative linker and β-cyclodextrin (β-CD). 1-methyl-2-(2'-carboxyethyl) maleic anhydride (MCM) moieties reacted with amine-containing drugs to form acid amide derivative bonds via a facile one-step conjugation, which are rapidly degradable at weakly acidic pH in biologically tolerable conditions. Owing to the characteristics, I expected that cargo drugs conjugated through the MCM-derived acid amide linkers can be released from the carriers in the weakly acidic environments of target tissues. In addition, the polypodal skeleton of the carrier backbone, β-CD, enables to conjugate many drugs, thus increasing the local concentration of the cargo drugs at the target site.

I selected two antibiotics, cephradine (CP) and moxifloxacin (MX), as model drugs because selective activation of the antibiotic activity at the infected or inflammatory tissues with weakly acidic pH conditions would be one promising solution for reducing side effects or resistance problems. pH-responsive release profiles of the antibiotics from the β-CD-MCM carrier were obtained at pH 7.4 and pH 5.5, mimicking normal and infected tissues, respectively, by using an HPLC instrument. Then, the antimicrobial activity of the antibiotics was measured *in vitro* against *S. aureus* and *B. fragilis*. Especially, time-dependent antimicrobial activity of the CP-conjugated drug carrier was measured in detail against *S. aureus*. Finally, *in vivo* efficacy of the CP-conjugated drug carrier was examined in abscess models in non-diabetic and diabetic mice. From the study, I suggested that the antibiotic-conjugated β-CD-MCM carrier has a strong potential as a selective antibiotic delivery system targeting weakly acidic inflammatory or abscess tissues.

Keywords : antibiotics delivery, pH-sensitive degradation, maleic acid amide, selective drug release, antimicrobial activity

Student Number : 2016-22868

Table of Contents

Abstract ····· i

Table of Contents ····· iv

List of Figures ····· vi

List of Tables ····· viii

I. INTRODUCTION ····· 1

II. MATERIALS AND METHODS ····· 6

2.1. Materials ····· 6

2.2. Synthesis ····· 7

2.2.1. Synthesis of MCM ····· 7

2.2.2. Synthesis of β-CD-MCM ····· 8

2.2.3. Antibiotics Conjugation with β-CD-MCM ··· 8

2.3. Measurement of pH-Sensitive Antibiotics Release ····· 9

2.4. *In Vitro* Antimicrobial Activity Test of β-CD-MCM-Antibiotics Conjugates ····· 10

2.4.1. Bacteria Culture ····· 10

2.4.2. MIC and MBC Determination for *S. aureus* ··· 10

2.4.3. MIC and MBC Determination for *B. fragilis* ··· 11

2.4.4. Time-Dependent Measurement of *S. aureus* Growth Treated with β-CD-MCM-Antibiotics Conjugates ····· 12

2.5. *In Vivo* Antimicrobial Activity Test of β-CD-MCM -Antibiotics Conjugates in Subcutaneous Abscess Mouse Models ····· 13

III. RESULTS ····· 15

3.1. Synthesis of β-CD-MCM Carrier and Drug Conjugation ····· 15

3.2. pH-Sensitive Release of Antibiotics from the Conjugates ····· 16

3.3. *In Vitro* Antimicrobial Activity of β-CD-MCM-Antibiotics Conjugates ····· 17

3.4. Time-Dependent Growth of *S. aureus* Treated with β-CD-MCM-Antibiotics Conjugates ····· 19

3.5. *In Vivo* Antimicrobial Efficacy of β-CD-MCM-Antibiotics Conjugates in Subcutaneous Abscess Mouse Models ····· 20

IV. DISCUSSION ····· 22

V. CONCLUSION ····· 30

VI. REFERENCES ····· 31

VII. FIGURES AND TABLES ····· 34

Abstract in Korean (국문초록) ····· 53

List of Figures

Figure 1. Synthetic schemes of MCM and β-CD-MCM.

Figure 2. Confirmation of β-CD-MCM synthesis.

Figure 3. Synthesis and confirmation of β-CD-MCM-CP and β-CD-MCM-MX.

Figure 4. pH-dependent release of antibiotics from the conjugates at pH 5.5 (acetate buffer, 100 mM) and 7.4 (phosphate buffer, 100 mM) measured by HPLC.

Figure 5. Proposed degradation mechanism of maleic acid amide at acidic pH.[16]

Figure 6. Release of CP and MX from β-CD-MCM-CP and β-CD-MCM-MX, respectively, at pH 5.5 and identification of the released CP and MX.

Figure 7. Cytotoxicity of β-CD, β-CD-MCM, β-CD-MCM-CP and b-PEI in NIH3T3 cells.[17]

Figure 8. Optical density (OD) at 600 nm with varying concentrations of CP and β-CD-MCM-CP at pH 7.4 and 5.5.

Figure 9. Photographs of 96-well microdilution plates for MIC determination and blood agar plates for MBC determination with both CP and β-CD-MCM-CP against *S. aureus*.

Figure 10. Scheme of animal experiments.

Figure 11. Abscess formation in non-diabetes and diabetes mice.

Figure 12. Abscess scores and bacterial counts in abscesses of non-diabetes and diabetes mice.

Figure 13. Schematic diagram of this study.

List of Tables

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) at pH 7.4 and 5.5.

Table 2. Criteria of abscess scores.

I. INTRODUCTION

Signal-responsive chemical moieties have been investigated for the development of smart materials. Chemical signals, including pH, glutathione and enzymes, as well as physical signals, including temperature and light have been used as triggers to induce characteristic changes in smart materials for drug delivery system.[1] Among them, pH signal which can indicate changes in certain physiological conditions has been widely applied for drug delivery system.[2] For example, stomach and intestine of gastrointestinal organs show pH 1.5-3.5 and 6.0-7.4, respectively.[3] Abnormal tissues such as cancerous and inflammatory tissues show pH 5-6.[4][5] These weakly acidic pH values are recognized by introducing pH-sensitive chemical bonds for the release and accumulation of anti-cancer or anti-inflammatory drugs. Moreover, the early endosomal pH value of 5-6 is frequently used as the signal for endosomal escape strategies in the field of drug delivery system.[6]

Various pH-sensitive linkers for conjugation of drugs to the drug delivery carriers have been developed, but some of them only showed slow and limited degradability at pH 5 - 6[7], and others required rather complex conjugation process including protection and deprotection of reactive nucleophilic functional groups.[8] For instance, imine[9] and acetal[10] bonds, which are representative acid-labile chemical bonds, are known to undergo degradation at pH 4-5. Siloxane[11] and β-thiopropionate[12] bonds can be degraded under pH 5.5, but the degradation rates are relatively slower than those of other pH-sensitive bonds. Hydrazone bonds are degraded in weakly acidic conditions but are synthetically difficult to be introduced in

drug conjugation system.[13] To compensate low pH-sensitivity, slow degradation rates and synthetic difficulties, maleic acid amide derivatives which undergo rapid hydrolytic degradation at pH 5-7 can be the best candidate for pH-sensitive linkers in drug delivery system.[14] Additionally, the maleic acid amide derivatives can be synthesized via a one-step ring-opening reaction between amines and the corresponding maleic anhydride derivatives under weakly alkaline conditions.[15] The facile synthesis of maleic acid amide derivatives under mild conditions is a strong advantage over other pH-sensitive linkages, which require quite complicated synthetic steps in conjugation of drugs or cross-linking of carrier backbones. Moreover, the responsive pH range and the degradation rate can be finely tuned by changing alkyl substituents on the *cis*-double bonds.[16]

Previously, a pH-responsive drug carrier with several residues of 1-methyl-2-(2'-carboxyethyl) maleic anhydride (MCM), also known as carboxylate dimethyl maleic anhydride (CDM) was proposed in my laboratory.[17] In this study, I utilized the MCM to form acid amide bonds with amine-containing drugs for pH-sensitive degradation. MCM is one of the maleic anhydride derivatives having some powerful advantages suitable for the aim of this study. Having two alkyl substituents of methyl and carboxyethyl on its *cis*-double bond, MCM-derived acid amide has a high pH-sensitivity induced by steric effect, showing rapid degradability in weakly acidic environments, whereas being relatively stable at normal physiological conditions. Moreover, the carboxyl group on one of its substituents in the MCM-derived acid amide enables additional modification with other functional groups, which broadens the possibility for further modification. Owing to the outstanding pH-sensitivity, being rapidly degradable at pH 5.5 whereas stable in physiological conditions, and

the possibility for additional functionalization, I regarded the MCM-derived acid amide as one of the most promising candidates for conjugating drugs with amine residues which target weakly acidic environments such as cancerous and inflammatory tissues.

As the backbone of the drug carrier, β-cyclodextrin (β-CD) was selected for biocompatibility, multiple drug conjugation and possible complex formation through host-guest interactions. A β-CD is an oligosaccharide which is composed of seven glucose molecules bound together in a ring via α-1,4-glycosidic linkages.[18] Since the components and linkages constituting the β-CD are identical with those of amylose, one of the main components for starch, the β-CD shows outstanding biocompatibility and biodegradability. Owing to its biologically inert properties, the β-CD has been widely used for drug carriers. Furthermore, a β-CD molecule has a capability for various structural modifications. Consisting of seven glucose molecules, a β-CD molecule has 7 primary and 14 secondary hydroxyl groups, providing great opportunities for multiple functionalization. Via esterification between the seven primary hydroxyl groups of the β-CD and one carboxyl group of the MCM-derived acid amide, a maximum of seven MCM-derived moieties can be grafted on the β-CD surface, increasing drug content within each β-CD-MCM carrier. By utilizing the β-CD-MCM as a multi-drug carrier, I expected the release of multiple drug molecules from each carrier would possibly contribute to enhancing the local concentration of drugs at the target site. In addition, the β-CD is well known for inclusion ability inside its hydrophobic cavity.[19] Small hydrophobic molecules such as adamantane and rotaxane can form complexes with β-CD via host-guest interaction, also broadening the possibility for further application in the future study.

In this study, I desired to develop a drug delivery system for antibiotics because among various types of drugs, there have been a few reports of selective delivery of anti-infectious or anti-inflammatory drugs. One of the reasons is probably because the pH-responsive properties of pre-existing drug delivery systems are not suitable for fast and selective release of drugs in the weakly acidic microenvironments full of rapidly metabolizing infectious microbes.[20] Furthermore, the toxicity of antibiotics is not so significant against human cells.[21] When systemically treated, however, many anti-infectious or anti-inflammatory drugs show severe side effects[22], and overuses of antibiotics often provoke antibiotic resistance problems.[23] Selective release of such drugs at the target sites would be one solution for overcoming the pharmacological problems.

Among anti-inflammatory drugs, those containing nucleophilic amines, such as drugs in cephalosporin and fluoroquinolone categories, would be possible candidate drugs for the conjugation with the MCM-based carrier. In this study, two antibiotic drugs, cephradine (CP), a representative first generation cephalosporin antibiotic with a primary amine[24], and moxifloxacin (MX), a fluoroquinolone antibiotic with a secondary amine[25], were used for pH-sensitive drug release via facile conjugation with the β-CD-MCM carrier.

The *in vitro* antimicrobial activity of the drug conjugates was examined against *Staphylococcus aureus* and *Bacillus fragilis*, two representative aerobic and anaerobic bacteria, causing skin abscess and intra-abdominal abscess, respectively. Especially, time-dependent antimicrobial activity of the CP-conjugated drug carriers was measured at pH 7.4 and pH 5.5, which mimicked normal and infected tissues, respectively. Then, *in vivo* efficacy of the CP-conjugates was

examined in an abscess-bearing diabetic mouse model, which is rather difficult to be treated by traditional surgery or simple antibiotic therapy.[26] Having weakly acidic environments of pH 5.5, abscess tissues infected by the bacteria were expected to selectively trigger rapid release of antibiotics from their conjugated complexes linked by pH-labile bonds, making high local concentration of antibiotics at the infection site. On the basis of the results, I suggested that the multi-functional antibiotic-conjugated carrier has a strong potential as a selective and rapid antibiotic delivery system targeting weakly acidic inflammatory or abscess tissues.

II. MATERIALS AND METHODS

2.1. Materials

MX was obtained from Aurobindo Pharma. Ltd. (Andhra Pradesh, India). CP, triethyl-2-phosphonopropionate and dimethyl-2-oxoglutarate were purchased from TCI (Tokyo, Japan). β-CD, triethylamine (TEA), diisopropylcarbodiimide (DIC), 4-dimethylaminopyridine (DMAP), p-toluenesulphonic acid (PTSA), sodium hydride (NaH) (60% in mineral oil), dimethylsulphoxide (DMSO) and Cytodex® were purchased from Sigma-Aldrich (St Louis, MO). Ammonium chloride (NH_4Cl), magnesium sulphate ($MgSO_4$), potassium hydroxide (KOH), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium bicarbonate ($NaHCO_3$), tetrahydrofuran (THF), dimethylformamide (DMF), hexane, ethylacetate (EA), methanol (MeOH), ethanol (EtOH), dichloromethane (DCM), hydrochloric acid (HCl), acetonitrile (ACN) and pyridine were purchased from Daejung (Seoul, South Korea). Anhydrous TEA, THF and DMF were obtained by distillation of the reagent-grade materials, and other reagents were used without further purification. *Staphylococcus aureus* subsp. *aureus* (ATCC 6538P) and *Bacteroides fragilis* (ATCC 25285) were obtained from KCTC (Korean Collection for Type Cultures, Taejon, Korea). Blood agar plates were purchased from Asan Pharmaceutical Co., Ltd. (Seoul, South Korea). 7-week-old Balb/c mice and C57BLKs-Jdb/db diabetes mice were purchased from Orient Co., Ltd. of the Charles River branch (Seoul, South Korea) and Central Lab. Animal Inc. (South Korea), respectively.

2.2. Synthesis

2.2.1. Synthesis of MCM

MCM was synthesized via a two-step reaction, following the method described in a previous report (Figure 1A).[27] In the first step, NaH (0.37 g, 9.2 mmol) was slowly added into a solution of triethyl-2-phosphonopropionate (1.64 g, 6.89 mmol) in anhydrous THF (30 mL) at 0°C under a nitrogen atmosphere. Dimethyl-2-oxoglutarate (1.00 g, 5.74 mmol) was slowly added to the solution after the hydrogen gas evolution had stopped. The reaction mixture was further stirred at 0°C. After the reaction completion was confirmed by TLC, a saturated aqueous solution of NH_4Cl was added dropwise. Following the THF removal by rotary evaporation, the resulting solid and water mixture was extracted with EA several times. The organic phase was combined, washed with distilled water (DW) and brine, dried over $MgSO_4$ and concentrated by rotary evaporation. Purified by silica gel chromatography with EA/hexane as eluent, the product was obtained as a colorless oil with 91% yield and confirmed by proton nuclear magnetic resonance (^{1}H NMR).

Subsequently, the product obtained in the previous step was dissolved in a 2 M KOH solution in EtOH and was allowed to reflux for 1 hr. DW was added, and the hot reaction mixture was cooled to ambient temperature. After evaporating EtOH, the aqueous phase was extracted with DCM several times and acidified to pH 2 using concentrated HCl. The aqueous phase was then extracted with EA several times. The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. MCM was obtained as a white solid with 74% yield and confirmed by ^{1}H NMR.

2.2.2. Synthesis of β-CD-MCM

MCM was coupled with β-CD following the previously reported procedure (Figure 1B).[17] To a solution of MCM (2.00 g, 1.09 mmol), β-CD (0.10 g, 0.092 mmol), DMAP (0.46 g, 0.37 mmol) and PTSA (0.70 g, 0.37 mmol) in DMF (10 mL) was added with DIC (0.33 g, 2.6 mmol). The reaction mixture went through overnight stirring at ambient temperature. The crude product was purified by dialysis with regenerated cellulose membrane (MWCO 1000, SPECTRUM®) in phosphate buffer (pH 9.0), HCl solution (pH 3.0) and DW, sequentially. Lyophilization was performed next. An off-white power, β-CD-MCM, was obtained with 85% yield and confirmed by ^{1}H NMR and MALDI-TOF spectra (Figure 2).

2.2.3. Antibiotics Conjugation with β-CD-MCM

CP was conjugated with the β-CD-MCM carrier utilizing the subsequent method (Figure 3A). A solution of β-CD-MCM (0.050 g, 0.024 mmol) and CP (0.050 g, 0.14 mmol) in anhydrous ACN (5 mL) was added with excess TEA at ambient temperature and stirred under a nitrogen atmosphere until becoming a completely clear solution. Completion of the reaction was monitored using HPLC to measure the residual CP. After 6 hr of stirring, an NaOH solution (12 equiv.) was added carefully to the aqueous solution of β-CD-MCM-CP to exchange excess ammonium cations with sodium cations. The reaction mixture was then evaporated to remove the solvent and dried in vacuum. β-CD-MCM-CP was obtained as a yellow solid with more than 90% yield and confirmed by ^{1}H NMR without further purification (Figure 3B). Drug loading efficiency and drug content were evaluated via HPLC analysis.

β-CD-MCM-MX was synthesized employing the same synthetic procedure as used for β-CD-MCM-CP, but using MX solution (0.058 g, 0.14 mmol) in anhydrous DMF instead of CP solution in ACN (Figure 3C). β-CD-MCM-MX was obtained as a yellow solid with approximately 90% yield and confirmed by ^{1}H NMR without further purification (Figure 3D). Drug loading efficiency and drug content were evaluated via HPLC analysis.

2.3. Measurement of pH-Sensitive Antibiotics Release

pH-dependent release test of CP and MX from their conjugates, β-CD-MCM-CP and β-CD-MCM-MX, respectively, was investigated *in vitro*. β-CD-MCM-CP and β-CD-MCM-MX were dissolved in both buffer solutions of a pH 7.4 phosphate buffer (100 mM) and a pH 5.5 acetate buffer (100 mM) at a concentration of 3 mg/mL. Each sample solution was incubated with stirring at 37°C. An Agilent HPLC equipped with a UV detector and a reverse-phase column (Agilent Eclipse XDB-C18 4.6 x 150 mm, 5 μm) was used for the HPLC-based measurement. At various time points from 0 to 5 hr, each sample was collected and diluted ten times with the same buffer and then injected into the HPLC system at a flow rate of 1.0 mL/min. For the β-CD-MCM-CP, a mixture (35:65 v/v) of MeOH:pH 8.0 phosphate buffer (50 mM) was used as eluent, and the release of CP was measured by UV absorbance at wavelengths of 254 and 270 nm (Figure 4A). In the case of β-CD-MCM-MX, a mixture (35:65 v/v) of ACN:pH 8.0 phosphate buffer (50 mM) was used as eluent, and the release of MX was measured by UV absorbance at wavelengths of 254 and 296 nm (Figure 4B).

2.4. *In Vitro* Antimicrobial Activity Test of β-CD-MCM-Antibiotics Conjugates

2.4.1. Bacteria Culture

Bacteria in an initial powder state were dispersed in sterilized water, and after an aliquot was put into a broth, it was cultured for 18 hr at 37°C with shaking. 10-20 μL of the culture medium was spread evenly on blood agar plates and cultured for 18-24 hr at 37°C. The resulting colonies were taken in a liquid medium and incubated repeatedly at 37°C. *Staphylococcus aureus* (ATCC 6538P), an aerobic bacterium, was cultured in cation-adjusted Mueller-Hinton broth (CAMHB) at 37°C in shaking incubator. *Bacteroides fragilis* (ATCC 25285), an anaerobic bacterium, was cultured in reinforced clostridial medium (RCM) at 37°C. An anaerobic environment was established by placing an oxygen scavenger and oxygen level indicator in a sealed container.

2.4.2. MIC and MBC Determination for *S. aureus*

Minimum inhibitory concentration (MIC) for *S. aureus* was determined using broth microdilution method. In the method, serial dilutions of antibiotics (free CP and β-CD-MCM-CP) were prepared in Dulbecco's phosphate-buffered saline (DPBS) to obtain a series of concentrations. 10 μL of 0.5 McFarland suspension of *S. aureus* in CAMHB was inoculated in plastic 96-well microdilution plates containing 20 μL of serially diluted antibiotics with 180 μL of CAMHB in each well (Figure 9A, 9B). To evaluate the pH-dependent antibiotic efficacy, broth solutions of pH 7.4 and 5.5 were used both.

Wells without antibiotics were included as controls in each plate. Bacterial viability was determined by measuring optical density (OD) at 600 nm after 12 hr of incubation at 37°C. The MIC was defined to be the lowest concentration that inhibited more than 90% of bacterial growth with respect to the growth of the controls.

After MIC determination, minimum bactericidal concentration (MBC) was investigated by spreading 10 μL of the suspensions from six wells in the microdilution plates, each with different concentrations of antibiotics around the MIC, on the surface of blood agar plates (Figure 9C). After subsequent incubation at 37°C for 24 hr, the MBC was defined to be the lowest concentration that showed no bacterial counts.

2.4.3. MIC and MBC Determination for *B. fragilis*

MIC for *B. fragilis* was determined using broth microdilution method. In the method, serial dilutions of antibiotics (free MX and β-CD-MCM-MX) were prepared in DPBS to obtain a series of concentrations. 10 μL of 0.5 McFarland suspension of *B. fragilis* in RCM was inoculated in plastic 96-well microdilution plates containing 20 μL of serially diluted antibiotics with 180 μL of RCM in each well. To evaluate the pH-dependent antibiotic efficacy, media of pH 7.4 and 5.5 were used both. Wells without antibiotics were included as controls in each plate. By measuring OD at 600 nm after 96 hr of incubation at 37°C in an anaerobic jar, bacterial viability was determined. The MIC was defined to be the lowest concentration that inhibited more than 90% of bacterial growth with respect to the growth of the controls.

After MIC determination, MBC was investigated by spreading

10 μL of the suspensions from six wells in the microdilution plates, each with different concentrations of antibiotics around the MIC, on the surface of blood agar plates. The plates were subsequently incubated at 37°C for 48-72 hr in an anaerobic jar. The MBC value was defined to be the lowest concentration that showed no bacterial counts.

2.4.4. Time-Dependent Measurement of *S. aureus* Growth Treated with β-CD-MCM -Antibiotics Conjugates

The growth of *S. aureus* treated with antibiotics (free CP and β -CD-MCM-CP) was recorded with optical density according to the time course using broth microdilution method. In the method, serial dilutions of antibiotics were prepared in DPBS to obtain a series of concentrations around the MIC value for *S. aureus*. 10 μL of 0.5 McFarland suspension of *S. aureus* in CAMHB was inoculated in plastic 96-well microdilution plates containing 20 μL of serially diluted antibiotics with 180 μL of CAMHB in each well. To evaluate the pH-dependent antibiotic efficacy, broth solutions of pH 7.4 and 5.5 were used both. Wells without antibiotics were included as controls in each plate. For measuring the growth over time, OD at 600 nm was automatically measured for 24 hr at 37°C using a multimode microplate reader (Tecan Group Ltd., Zurich, Switzerland). During the initial 6 hr, OD (600 nm) was measured every 30 min, and then the subsequent measurement of OD (600 nm) for 18 hr was conducted every 1 hr (n=3, duplicated).

2.5. *In Vivo* Antimicrobial Activity Test of β-CD-MCM-Antibiotics Conjugates in Subcutaneous Abscess Mouse Models

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Seoul National University Hospital (IACUC No. 150106) and were conducted accordance with the Guide for the Care and Use of Laboratory Animals of Seoul National University Hospital.

Staphylococcus aureus (ATCC 6538P) was subcultured on blood agar plates. The bacteria were grown overnight, harvested by centrifugation and then mixed in sterile saline with Cytodex® beads in the ratio of 1:1. 150 μL of the collected bacteria (9×10^8 CFU/mL) were inoculated to shaved dorsal skins of 7-week-old Balb/c mice (n=15) subcutaneously. On day 4, saline or each of the antibiotics (CP, β-CD-MCM-CP, mixture of β-CD-MCM and free CP (C+C) and β-CD-MCM carrier) was injected 25 mg/kg/day subcutaneously for 5 days. After 5 days of injection, mice were sacrificed (Figure 11A), and physical examination was performed according to the criteria of abscess scores (Table 2). Abscesses on the dorsal skins of the mice were isolated, moved to round-bottom tubes and then homogenized in 1 mL of sterile saline. 10 μL of the homogenates were diluted one thousand times with sterile saline and spread on blood agar plates. Calculation of colony forming units (CFUs) per mL for each abscess was followed after overnight incubation.

Antimicrobial activity test for the 7-week-old C57BLKs-Jdb/db diabetes mice (n=8) was conducted using the same experimental procedure for the non-diabetes mice, except that blood glucose levels were measured prior to the bacterial inoculation and

the sacrifice of the mice (Figure 11B). Statistical analysis was performed by the GraphPad Prism® 5 (GraphPad Software Inc., US). Each data set was compared by one-way ANOVA followed by Bonferroni test for multiple comparison, and the results were expressed as mean ± SEM. Values were considered statistically significant when $P < 0.05$.

III. RESULTS

3.1. Synthesis of β-CD-MCM Carrier and Drug Conjugation

A pH-sensitive drug carrier based on β-CD and MCM was previously reported in my laboratory.[17] First, MCM linker was synthesized by Horner-Wadsworth-Emmons reaction, a modified form of Wittig reaction, between triethyl-2-phosphonopropionate and dimethyl-2-oxoglutarate (Figure 1A). By carefully handling the reaction via slowly adding dimethyl-2-oxoglutarate to the triethyl-2-phosphonopropionate solution in an ice bath, products with *cis*-double bonds, which have thermodynamically unfavorable but desired structure in this reaction, were obtained successfully in a high yield. The carboxyl group of the MCM was coupled with the hydroxyl groups of the β-CD via carbodiimide-aided esterification to synthesize β-CD-MCM drug carrier (Figure 1B). Among seven primary and fourteen secondary hydroxyl groups in a β-CD molecule, the primary hydroxyl groups at the position of 6'-C in glucose show higher reactivity to electrophiles. By using a carbodiimide-coupling reagent, DIC, the seven primary hydroxyl groups of the β-CD favorably reacted with the carboxyl group of the MCM to form the β-CD-MCM carrier.

β-CD-MCM was then characterized by ^{1}H NMR and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Figure 2). By ^{1}H NMR, the number of MCM residues loaded on each β-CD molecule was calculated to be six on average, indicating 85% of primary hydroxyl

groups of β-CD participated in the conjugation. Even though not representing the exact distribution of various numbers of MCM residues with its main peak, the MALDI-TOF mass spectra exhibited the presence of β-CD-MCM molecules with different numbers of MCM residues varying from two to seven. Considering the mass spectra that no more than seven MCM residues were coupled with a β-CD molecule, I could confirm the regio-selective functionalization of the β-CD owing to the superior reactivity of the primary hydroxyl groups.[28]

I used cephradine (CP) and moxifloxacin (MX) as antibiotics containing primary and secondary amine residues, respectively. The amine moieties of CP and MX successfully reacted with anhydride groups of the β-CD-MCM carriers to form acid amide linkages in weakly alkaline conditions only by gentle stirring at room temperature. β-CD-MCM-CP and β-CD-MCM-MX conjugates were obtained by evaporation of the reaction solvent without further purification, and their conjugation was confirmed by ^{1}H NMR (Figure 3B, 3D). High conjugation efficiencies of CP (95%) and MX (90%) were quantitatively calculated via HPLC analysis, and the antibiotic contents were also calculated to be 24% (β-CD-MCM-CP) and 22% (β-CD-MCM-MX) by comparing the mass of CP and MX to the total mass of each corresponding conjugate with some residual sodium and TEA salts.

3.2. pH-Sensitive Release of Antibiotics from the Conjugates

The *in vitro* release profiles of CP and MX from β-CD-MCM-CP and β-CD-MCM-MX, respectively, were measured by HPLC in

buffer solutions at pH 5.5 and pH 7.4 to investigate the degradation kinetics (Figure 4). Rapid bursts of CP and MX were observed at pH 5.5 in both of the conjugated systems. At pH 5.5, approximately 90% and 85% of CP and MX, respectively, were released from their conjugates within 30 min, and the release of CP and MX was almost saturated at 95% after 1 hr and 2 hr, respectively. Release kinetics, however, were decreased at pH 7.4 in both conjugates. At pH 7.4, approximately 35% and 70% of CP and MX, respectively, were released from their conjugates for 5 hr. The secondary amide of β-CD-MCM-CP showed dramatic acceleration of degradation at pH 5.5 compared to pH 7.4, whereas the tertiary amide of β-CD-MCM-MX showed only limited difference between pH 5.5 and 7.4. The pH-dependent degradation can be possibly explained by the mechanism proposed by Kirby (Figure 5).[16]

Moreover, I confirmed the stabilities of CP and MX after two chemical procedures of conjugation and then release by ^{1}H NMR and electrospray ionization mass spectroscopy (ESI-MS) (Figure 6). Via the ^{1}H NMR spectra, the CP and MX, which were released from the corresponding conjugates at pH 5.5, proved to be identical with the initial CP and MX. In addition, each of the CP and MX had identical ESI mass spectra regardless of experiencing chemical changes.

3.3. *In Vitro* Antimicrobial Activity of β-CD-MCM-Antibiotics Conjugates

I determined minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) to evaluate antimicrobial activities of β-CD-MCM-CP and β-CD-MCM-MX. Since CP is well-known to be susceptible against Gram-positive aerobes, including *Staphylococci*,

Staphylococcus aureus (*S. aureus*) was selected as the target strain of the β-CD-MCM-CP.[29] For the β-CD-MCM-MX, a gram-negative anaerobe, *Bacteroides fragilis* (*B. fragilis*), was used as the target strain, providing versatility in this *in vitro* antimicrobial susceptibility test.[25] The evaluation of MIC and MBC followed the approved guideline of clinical and laboratory standards institute (CLSI).[30] In particular, the MIC and MBC were strictly defined as the lowest concentration of antibiotics showing viability less than 10% and showing no bacterial counts on blood agar plates, respectively. For antibiotic-conjugates, the concentration values represented the antibiotic concentrations (μg/mL) in the conjugates. Each value was measured at pH 7.4 and pH 5.5 mimicking normal conditions and inflammatory abscess conditions, respectively.

The MIC against *S. aureus* was determined by measuring optical density at 600 nm after 12 hr-treatment of free CP and β-CD-MCM-CP at pH 7.4 and pH 5.5, and the MBC was measured after additional incubation of 24 hr on blood agar plates (Table 1A). At pH 7.4, the β-CD-MCM-CP showed a higher value of MIC (2.0 μg/mL) than that of free CP (0.7 μg/mL), while showing the identical value of MBC (8.0 μg/mL) for both CP and β-CD-MCM-CP. At pH 5.5, however, free CP and β-CD-MCM-CP showed the identical values of MIC (0.7 μg/mL) and MBC (2.0 μg/mL), possibly owing to the rapid release of CP from the β-CD-MCM-CP at weakly acidic conditions. The β-CD-MCM-CP conjugate exhibited clear pH-responsive induction of toxicity against on *S. aureus*.

In the case of *B. fragilis*, the MIC was measured after 96 hr-treatment of free MX and β-CD-MCM-MX at pH 7.4 and pH 5.5, and the MBC was measured after additional incubation of 24 h on blood agar plates (Table 1B). *B. fragilis*, an anaerobe showing slow

growth rate, needed more time for enough growth than *S. aureus*. At pH 7.4, the β-CD-MCM-MX showed a lower value of MIC (0.3 μg/mL) than that of MX (0.5 μg/mL), while showing the identical MBC value (0.5 μg/mL) for both MX and β-CD-MCM-MX. At pH 5.5, the β-CD-MCM-MX showed lower values of both MIC (0.7 μg/mL) and MBC (1.0 μg/mL) than those of the free MX (MIC 1.0 μg/mL, MBC 1.5 μg/mL). Regardless of whether MX is conjugated or not, the antimicrobial effect was generally lower at pH 5.5 than at pH 7.4.

3.4. Time-Dependent Growth of *S. aureus* Treated with β-CD-MCM-CP Conjugates

Antimicrobial activities of free CP and β-CD-MCM-CP were examined in a more detailed manner by measuring time-dependent growth of *S. aureus* treated with both antibiotic reagents at pH 7.4 and pH 5.5. After treatment of free CP or β-CD-MCM-CP at different concentrations, OD (600 nm) of the *S. aureus* suspension was monitored during 24 hr (Figure 8). *S. aureus* was generally more viable at pH 7.4 than at pH 5.5. At pH 7.4, free CP could inhibit the bacterial growth for 12 hr at the MIC (0.7 μg/mL), and the bacteria started to grow up after 12 hr. The starting time point for the bacterial growth was delayed by increasing the antibiotic concentration over the MIC. The β-CD-MCM-CP could inhibit the bacterial growth for 20 hr at the MIC (2.0 μg/mL) and slow down the growth at the concentration below the MIC.

At pH 5.5, the growth curves of *S. aureus* treated with free CP and β-CD-MCM-CP were almost identical to each other. Over the MIC, both of the antibiotics could inhibit the bacterial growth

almost completely during 24 hr and slow down the growth at 0.5 μg/mL.

3.5. *In Vivo* Antimicrobial Efficacy of β-CD-MCM-Antibiotics Conjugates in Subcutaneous Abscess Mouse Models

In vivo efficacy of β-CD-MCM-CP was evaluated in subcutaneous abscess models, which were prepared using non-diabetic mice (Balb/c) and diabetic mice (C57BLKs-Jdb/db). The simplified scheme of the animal experiments is shown in Figure 10. CP and β-CD-MCM-CP were subcutaneously injected to the mice 5 days after the infection. CP was injected at a dose of 25 mg/kg/day for 5 days, and β-CD-MCM-CP containing the same amount of CP was injected in the same manner. The β-CD-MCM carrier and the simple mixture of β-CD-MCM and CP (C+C) at the same molar amount and ratio with the β-CD-MCM-CP were also injected as positive controls.

Figure 12A and 12B show the abscess grade scores and bacterial counts, respectively, in the abscesses which were isolated from the non-diabetic mice at the time point of 11 day after the infection. The abscess grade scores, which were evaluated according to the standard in Table 2, were not significantly different among groups. However, significant reduction of bacterial density (CFU/mL) was observed in the abscesses collected from all treated groups including positive control groups.

Figure 12C and 12D show the abscess grade scores and bacterial counts, respectively, in the abscesses collected from the non-insulin dependent diabetes mellitus (NIDDM) model mice. Unlike non-diabetic mice, there was significant difference in abscess scores.

Abscess scores of the CP and β-CD-MCM-CP groups were reduced significantly compared to the saline and carrier only groups. Similarly, significant reduction of the bacterial counts in the abscesses was observed in the groups treated with CP, β-CD-MCM-CP and C+C. However, there was no significant difference of both abscess scores and bacterial counts among CP, β-CD-MCM-CP and C+C groups. In this case, the carrier only showed no antimicrobial effect.

IV. DISCUSSION

I aimed to develop a pH-sensitive antibiotics delivery system specialized in selective drug release at weakly acidic target site. Considering that abscesses or inflammatory tissues, the target sites of antibiotics, have weakly acidic environments of pH 5-6 unlike normal tissues, utilizing the slightly low pH as an inducing stimulus was thought to be effective for the smart delivery system of antibiotics. To realize the pH-responsive system, I utilized β-CD-MCM as an antibiotic carrier, which had been previously reported in my laboratory, for enhanced pH-sensitivity and efficiency in antibiotic conjugation.

As a pH-sensitive linker, taking the major role in this study, a maleic acid amide derivative has outstanding features compared to other pH-labile linkages. A maleic acid amide derivative can be synthesized by a facile procedure of simple mixing between the corresponding maleic anhydride derivative and amine in weakly basic conditions.[15] Moreover, the maleic acid amide derivative shows rapid degradation in its responsive pH range[14], which can be finely tuned by changing the substituents on the *cis*-double bond.[16] The *cis*-β-carboxylate within the maleic acid amide goes through an internal attack on the carbonyl group of the amide via the formation of a five-membered ring. The bulkier the substituents on the *cis*-double bond, the more accelerated the internal attack of the *cis*-β-carboxylate becomes, resulting in enhanced degradability at the pH of weakly acidic environments. The degradability of various maleic acid amide derivatives responding to pH range between 3 and 7 was compared previously in my laboratory.[31] Among them, MCM-derived

acid amide was selected owing to the rapid degradable kinetics at pH 5.5, which is attributable to the steric effect of its methyl and carboxyethyl substituents, as well as relative stability at pH 7.4.[17] In addition, the carboxyl group in the carboxyethyl substituent enables the MCM-derived acid amide to be linked with a backbone molecule.

As the backbone of the antibiotic carrier, β-CD, an oligosaccharide family, was used. The seven primary and fourteen secondary hydroxyl groups in a β-CD molecule can be covalently bound to a number of MCM molecules. Thus, the amount of the drugs that can be introduced within a carrier can be increased by multiple conjugation of drug molecules with a β-CD molecule. In addition, the β-CD can form a complex inside its cavity with a certain compound such as adamantane and rotaxane by strong non-covalent host-guest interaction, which broadens its further application. Moreover, the outstanding biocompatibility and biodegradability make the β-CD an excellent carrier for the antibiotics delivery. Using the MCM and β-CD, I synthesized the antibiotic carrier β-CD-MCM successfully via a one-step esterification by using DIC as a carbodiimide coupling agent under catalysts of DMAP and PTSA (Figure 1B). By using both DMAP and PTSA as catalysts for esterification, the reaction could be facilitated without the formation of side products such as *N*-acylurea.

Among hundreds of antibiotic reagents, I selected cephradine (CP), a cephalosporin antibiotic with a primary amine, and moxifloxacin (MX), a fluoroquinolone antibiotic with a secondary amine, as model drugs against *S. aureus* and *B. fragilis*, respectively.[25][29] In conjugation reactions of these antibiotics, I expected high efficiencies considering the high reactivity of the anhydride group within the MCM to nucleophiles such as amines. For

the conjugates of both β-CD-MCM-CP and β-CD-MCM-MX, high conjugation efficiencies more than 90% were obtained as expected, indicating that the nucleophilic attack on the anhydride group occurs exhaustively even though the attack is interfered by steric hindrance.

I investigated pH-dependent degradation of the pH-sensitive MCM-derived maleic acid amide linker via measuring the kinetics of antibiotic release from both β-CD-MCM-CP and β-CD-MCM-MX at pH 5.5 and 7.4, the representative pH values of inflammatory tissues and normal tissues, respectively. At pH 5.5, a rapid burst of antibiotic release was observed within 30 min in both conjugated systems (Figure 4). However, the release kinetics of β-CD-MCM-CP and β-CD-MCM-MX were very different at pH 7.4. The β-CD-MCM-CP was relatively stable at pH 7.4 showing only 35% release of CP for 5 hr, whereas the β-CD-MCM-MX released 70% of the conjugated MX for 5 hr. Although detailed mechanism study is required for full elucidation of the results, the higher basicity of the secondary amine of MX than that of the primary amine of CP might stabilize the protonated form of the released antibiotic product and the corresponding intermediate, facilitating the degradation even at pH 7.4. On the basis of the different release kinetics depending on the pH conditions, I anticipated that the β-CD-MCM-CP, having a dramatic difference of kinetics in small pH changes, would be able to release the cargo antibiotics at the target sites more selectively, resulting in the enhanced efficiency of antibiotic delivery and reduced side effects of antibiotics such as hepatotoxicity.[32] In contrast, the β-CD-MCM-MX was considered to have a limited potential for the purpose of this study.

Structural stabilities of CP and MX during two chemical procedures, including conjugation in weakly alkaline conditions and

then release in mildly acidic conditions, were also investigated by ^{1}H NMR and ESI-MS (Figure 6B, 6C, 6E, 6F). Considering that the CP and MX, which went through the two chemical reactions, had identical spectra of ^{1}H NMR and ESI-MS to those of initial CP and MX, respectively, I confirmed that both of the antibiotics were stable during the two chemical procedures. In addition, the cytotoxicity of β-CD-MCM and β-CD-MCM-CP was previously confirmed in my laboratory through the MTT assays for NIH3T3 cells (Figure 7).[17] By verifying almost negligible cytotoxicity of the antibiotic carrier, β-CD-MCM, up to 100 μM in a eukaryotic cell line, I ensured the short-term safety of the carrier. However, long-term toxicity studies will be required for further application in clinical trials.

pH-dependent antimicrobial activity of β-CD-MCM-CP and β-CD-MCM-MX was evaluated *in vitro* on *S. aureus* and *B. fragilis*, respectively, by determining both MIC and MBC. Being treated on *S. aureus* for 12 hr, β-CD-MCM-CP showed almost identical antibiotic activity at pH 5.5, whereas showed three times lower activity at pH 7.4, compared to free CP (Table 1A). The free CP showed an identical MIC value of 0.7 μg/mL at both pH 7.4 and 5.5, while showing a higher MBC value at pH 7.4, possibly attributable to the poor stability of CP at physiological pH.[33] At pH 7.4, the conjugated β-CD-MCM-CP showed a higher value of MIC than that of free CP, due to slow and limited kinetics of CP release at physiological pH (Figure 4A). In the meantime, both CP and β-CD-MCM-CP had an identical value of MBC at pH 7.4, which can be explained that the factors for structural instability and slow release rate of CP contributed similarly in the MBC determination at physiological conditions. At pH 5.5, both CP and β-CD-MCM-CP showed identical values of both MIC and MBC, owing to the almost complete release

of CP from β-CD-MCM-CP within 30 min at weakly acidic conditions (Figure 4A). Comparing the pH-dependent efficacy of the β-CD-MCM-CP, both MIC and MBC were lower at pH 5.5 than those at pH 7.4, reflecting the dramatic difference in release kinetics between pH 5.5 and 7.4. On the basis of the results, the potential of the β-CD-MCM-CP conjugate was verified for the selective release of drugs at the weakly acidic target sites.

Time course of the bacterial growth curve, measured with optical density at 600 nm, also supported the pH-responsive release of CP from β-CD-MCM-CP (Figure 8). At pH 5.5, the growth curve of *S. aureus* treated with β-CD-MCM-CP was almost identical to that of bacteria treated with free CP, indicating that almost all of the conjugated CP molecules were released at the early stage of the incubation. However, at pH 7.4, β-CD-MCM-CP showed much less antimicrobial activity than free CP below the concentration of 1.0 μg/mL, which indicated the CP molecule as a conjugated form was not effective as an antibiotic pharmacophore at physiological conditions. Of course, some inhibition of bacterial growth by β-CD-MCM-CP was observed at pH 7.4, probably due to slowly released CP during the incubation. It should be noticed that the free CP could inhibit the bacterial growth almost completely at the concentration over the MIC until 24 hr at pH 5.5, but the inhibitory effect was finished after some time at pH 7.4. The disappearance of the antibiotic effect was probably caused by inactivation of beta-lactam ring of CP structure at neutral or alkaline pH.[33]

In the case of *B. fragilis*, an anaerobe requiring substantial time for enough growth, the antimicrobial tests were conducted after 96 hr of anaerobic incubation. Because of the elongated incubation time, accurate evaluation for the antimicrobial activity on *B. fragilis*

was impossible since the degradation extents of MCM-derived acid amide linkers reached an almost identical level regardless of pH conditions after 96 hr of incubation. Considering that penetration of MX to bacterial cytosol is far decreased at acidic pH, showing lower activity at acidic conditions, both MIC and MBC for the β-CD-MCM-MX were lower at pH 7.4 than pH 5.5.[34]

From the release profile and the *in vitro* antimicrobial activity experiments, I concluded that β-CD-MCM-MX might not be suitable as a candidate for the therapeutic agents targeting weakly acidic environments of infected tissues. Better stability of the conjugation at neutral pH conditions for preventing premature release as well as penetration strategies to the bacterial membrane at acidic pH would be required for the development of more effective antibiotic conjugates against anaerobic gram-negative bacteria.

I selected a subcutaneous abscess model to prove the effectiveness of the antibiotic-conjugated carrier system: β-CD-MCM-CP. Efficacy of antibiotics is generally lowered in abscesses due to low pH, bindings of proteins and bacterial enzymatic degradation in the microenvironments.[35] In diabetic patients, it is much more troublesome to treat abscesses than non-diabetic patients. Abscesses are frequently and largely predisposed to people with chronic diseases such as diabetes.[36] In addition, diabetic patients experience abnormal tissue perfusion which causes poor antibiotics penetration.[37] For these reasons, long-term and high-dose antibiotics are prescribed for patients with abscesses, particularly patients suffering from diabetes mellitus.[38] However, high-dose or long-term use of antibiotics often provokes adverse effects such as dyspepsia, diarrhea, and nausea.[39] Moreover, functions of liver, kidney and pancreas are quite declined in diabetic patients, which are all critical

for the antibiotic metabolism. Since non-metabolized or non-excreted antibiotics cause severe systemic toxicity[22][32], higher doses or longer treatment of antibiotics are more dangerous to diabetic patients possessing abscesses. Therefore, I prepared subcutaneous abscess models in non-diabetic and diabetic mice and intended to release effective antibiotics only at the target site, weakly acidic abscesses.

Figure 12 shows the *in vivo* antimicrobial activity of β-CD-MCM-CP with various controls. In non-diabetic mice, β-CD-MCM-CP showed significant reduction of bacterial counts in abscesses compared to saline. However, other controls, free CP, β-CD-MCM carrier and a mixture of β-CD-MCM and free CP (C+C), also showed similar reduction. I could not confirm that the reduction of bacterial counts was originated from the accumulation of CP to the abscesses. On the other hand, in diabetic mice, the non-antibiotic carrier showed no significant reduction of bacterial counts, whereas antibiotic-possessing groups, free CP, C+C and β-CD-MCM-CP, showed remarkable reduction. Moreover, β-CD-MCM-CP showed slightly higher antibiotic activity than others, both in abscess scores and bacterial counts, although the difference among free CP, C+C and β-CD-MCM-CP groups was not significant. From the results, I concluded that the antibiotic activity of β-CD-MCM-CP was not lower than free CP in both models and that marginal enhancement of the activity was observed in the diabetic model.

I suggested the reasons for the only marginal difference of *in vivo* efficacy among free CP, C+C and β-CD-MCM-CP groups: possible premature release of CP from the carrier at other sites, different pharmacokinetics and non-optimized drug doses, etc. Considering that pH-responsive degradation kinetics of maleic acid amide derivatives can be delicately controlled by changing the

substituents on the *cis*-double bonds[16][31], I expected to overcome the premature release problem. Probably, an acid amide bond based on carboxyethyl maleic anhydride missing one methyl substituent from the MCM structure would be a good candidate for the linker conjugating CP on the carrier. Of course, detailed pharmacokinetics, biodistribution and dose-optimization would be required for the development of such abscess-targeting antibiotic-conjugated carriers.

As a drug carrier, β-CD-MCM has attractive capabilities of facile conjugation with amine-containing drugs, multi-functionalization contributing to high drug content and rapid degradation in response to a small pH drop. Most of all, the enhanced sensitivity on weakly acidic pH makes the β-CD-MCM a powerful carrier with ability of selective drug release in abscesses or inflammatory tissues, thus increasing the local concentration at the target sites. The high drug content as a multi-drug carrier is also expected to further contribute to the high local concentration of the drugs. Moreover, additional modification of β-CD-MCM via host-guest interaction induced by the hydrophobic cavity of β-CD will lead to multifunctional properties such as increasing its molecular weight and size for elongated blood circulation through reducing the extent of renal clearance and possessing enhanced permeability and retention effect in the future. By verifying the effective antimicrobial activity of β-CD-MCM-CP *in vitro* and *in vivo*, which is attributable to the rapid degradability of MCM-derived acid amide linker at weakly acidic pH, the β-CD-MCM is expected to play an important role as a potential drug carrier for conjugation with the amine-containing drugs targeting weakly acidic environments.

V. CONCLUSION

In this study, I successfully demonstrated a pH-sensitive drug carrier, β-CD-MCM, with enhanced sensitivity and degradability at biologically tolerable acidic conditions. I clearly observed different release profiles of antibiotics from the conjugates between pH 7.4 and 5.5, the bio-mimetic pH conditions of normal and infected tissues, respectively. Therefore, owing to the burst-like degradation kinetics of the carrier at weakly acidic pH, selective local release of the drugs at the target sites such as abscesses or inflammatory tissues was expected, possibly increasing the local concentration of the drugs. Based on the antimicrobial activity, the developed pH-sensitive antibiotic-conjugated carrier system was proved to be a promising alternative of free antibiotics for the treatment of abnormal tissues representing weakly acidic environment. Especially, diabetic abscess would be a possible target of the pH-responsive antibiotic conjugate strategy. Optimization of linkers as well as selection of appropriate antibiotics and doses would help the development of novel antibiotic formulation targeting infected tissues in near future.

VI. REFERENCES

[1] E. Fleige, M. A. Quadir and R. Haag, *Adv. Drug Delivery Rev.*, 2012, **64**, 866-884.

[2] Y. Zhu and F. Chen, Chem. *Asian J.*, 2015, **10**, 284-305.

[3] G. Rajput, F. Majmudar, J. Patel, R. Thakor and N. B. Rajgor, *Sys Rev Pharm*, 2010, **1**, 36-44.

[4] M. Kanamala, W. R. Wilson, M. Yang, B. D. Palmer and Z. Wu, *Biomaterials*, 2016, **85**, 152-167.

[5] A. Riemann, A. Ihling, J. Thomas, B. Schneider, O. Thews and M. Gekle, *Biochim. Biophys. Acta*, 2015, **1853**, 299-307.

[6] J. Du, T. Sun, W. Song, J. Wu and J. Wang, *Angew. Chem. Int. Ed.*, 2010, **122**, 3703-3708.

[7] M. Prabaharan, J. J. Grailer, S. Pilla, D. A. Steeber and S. Gong, *Biomaterials*, 2009, **30**, 5757-5766.

[8] S. Lv, Z. Tang, D. Zhang, W. Song, M. Li, J. Lin, H. Liu and X. Chen, *J. Controlled Release*, 2014, **194**, 220-227.

[9] C. Ding, J. Gu, X. Qu and Z. Yang, *Bioconjugate Chem.*, 2009, **20**, 1163-1170.

[10] E. R. Gillies and J. M. J. Fréchet, *Chem. Commun.*, 2003, 1640-1641.

[11] S. A. Jacques, G. Leriche, M. Mosser, M. Nothisen, C. D. Muller, J. Remy and A. Wagner, *Org. Biomol. Chem.*, 2016, **14**, 4794-4803.

[12] J. Zou, F. Zhang, S. Zhang, S. F. Pollack, M. Elsabahy, J. Fan and K. L. Wooley, *Adv. Healthc. Mater.*, 2014, **3**, 441-448.

[13] Y. Bae, W. Jang, N. Nishiyama, S. Fukushima and K. Kataoka, *Mol. Biosyst.*, 2005, **1**, 242-250.

[14] D. B. Rozema, K. Ekena, D. L. Lewis, A. G. Loomis and J. A. Wolff, *Bioconjugate Chem.*, 2003, **14**, 51-57.

[15] J. Du, X. Du, C. Mao and J. Wang, *J. Am. Chem. Soc.*, 2011, **133**, 17560-17563.

[16] M. F. Aldersley, A. J. Kirby, P. W. Lancaster, R. S. McDonald and C. R. Smith, *J. Chem. Soc., Perkin Trans. 2*, 1974, **0**, 1487-1495.

[17] S. Kang, E. Park, Y. Kim, S. Lee, J. Kwon, H. Cho and Y. Lee, *J Drug Target*, 2014, **22**, 658-668.

[18] E. M. M. Del Valle, *Process Biochem.*, 2004, **39**, 1033-1046.

[19] K. Uekama, F. Hirayama and T. Irie, *Chem. Rev.*, 1998, **98**, 2045-2076.

[20] Y. Lee, S. Fukushima, Y. Bae, S. Hiki, T. Ishii and K. Kataoka, *J. Am. Chem. Soc.*, 2007, **129**, 5362-5363.

[21] K. Kümmerer, *Chemosphere*, 2009, **75**, 417-434.

[22] N. D. Stebbins, M. A. Ouimet and K. E. Uhrich, *Adv. Drug Delivery Rev.*, 2014, **78**, 77-87.

[23] J. Davies and D. Davies, *Microbiol. Mol. Biol. Rev.*, 2010, **74**, 417-433.

[24] N. H. Georgopapadakou and F. Y. LIU, *Antimicrob. Agents Chemother.*, 1980, **18**, 834-836.

[25] D. R. Snydman, N. V. Jacobus, L. A. McDermott, R. Ruthazer, Y. Golan, E. J. C. Goldstein, S. M. Finegold, L. J. Harrell, D. W. Hecht, S. G. Jenkins, C. Pierson, R. Venezia, V. Yu, J. Rihs and S. L. Gorbach, *Antimicrob. Agents Chemother.*, 2007, **51**, 1649-1655.

[26] J. Weigelt, K. Itani, D. Stevens, W. Lau, M. Dryden, C. Knirsch and the Linezolid CSSTI Study Group, *Antimicrob. Agents Chemother.*, 2005, **49**, 2260-2266.

[27] A. Naganawa, Y. Ichikawa and M. Isobe, *Tetrahedron*, 1994, **50**, 8969-8982.

[28] J. D. Brandner and R. L. Birkmeier, *J. Am. Oil Chem. Soc.*, 1960, **37**, 390-396.

[29] N. H. Georgopapadakou and F. Y. Liu, *Antimicrob. Agents Chemother.*, 1980, **18**, 834-836.

[30] National Committee for Clinical Laboratory Standards, Methods for Determining Bactericidal Activity of Antimicrobial Agents. Approved Guideline, CLSI document M26-A, NCCLS, Wayne, PA, USA, 1999.

[31] S. Kang, Y. Kim, Y. Song, J. Choi, E. Park, W. Choi, J. Park and Y. Lee, *Bioorg. Med. Chem. Lett.*, 2014, **24**, 2364-2367.

[32] J. F. Westphal, D. Vetter and J. M. Brogard, *J. Antimicrob. Chemother.*, 1994, **33**, 387-401.

[33] T. Yamana and A. Tsuji, *J. Pharm. Sci.*, 1976, **65**, 1563-1574.

[34] J. B. Dave, P. J. Vyas and C. N. Patel, *Chron. Young Sci.*, 2013, **4**, 24-31.

[35] G. D. Wright, *Adv. Drug Delivery Rev.*, 2005, **57**, 1451-1470.

[36] J. Casqueiro, J. Casqueiro and C. Alves, *Indian J. Endocrinol. Metab.*, 2012, **16**, 27-36.

[37] K. Skhirtladze, D. Hutschala, T. Fleck, F. Thalhammer, M. Ehrlich, T. Vukovich, M. Müller and E. M. Tschernko, *Am. Soc. Microbiol.*, 2006, **50**, 1372-1375.

[38] The Diabetes Control and Complications Trial Research Group, *N. Engl. J. Med.*, 1993, **329**, 977-986.

[39] S. R. Norrby, Eur. *J. Clin. Microbiol. Infect. Dis.*, 1991, **10**, 378-383.

VII. FIGURES AND TABLES

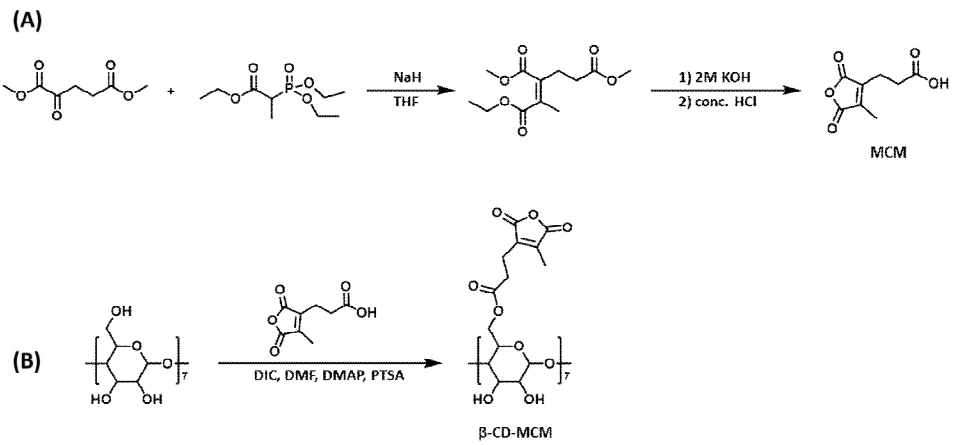

Figure 1. Synthetic schemes of MCM and β-CD-MCM.
(A) Synthesis of 1-methyl-2-(2'-carboxyethyl) maleic anhydride (MCM). (B) Synthesis of β-CD-MCM.

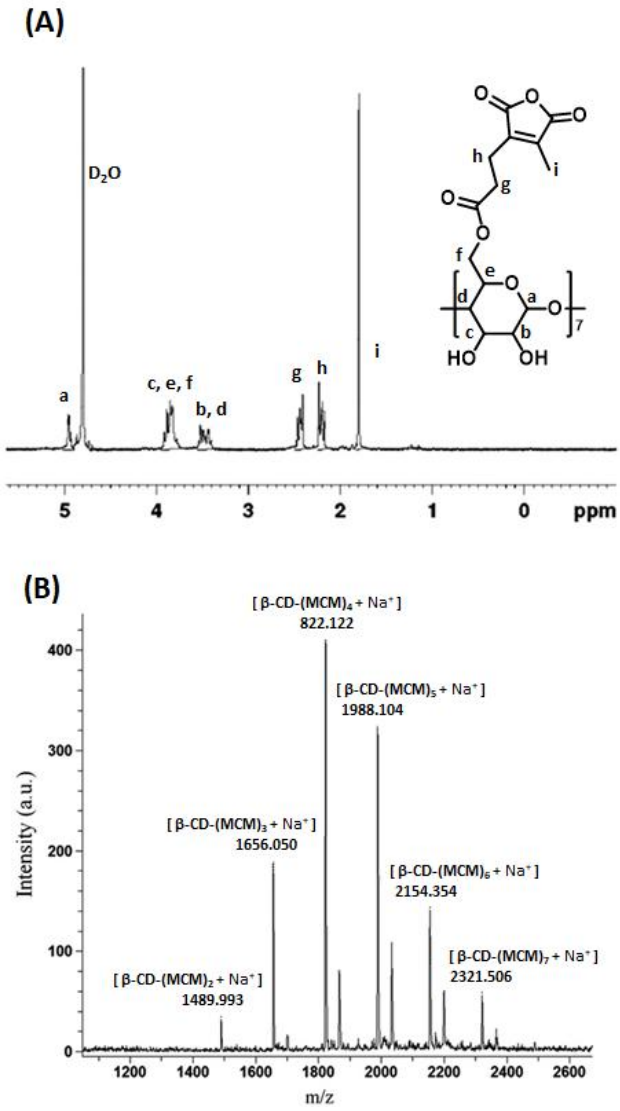

Figure 2. Confirmation of β-CD-MCM synthesis. (A) ^{1}H NMR (NMR solvent: D_2O). (B) MALDI-TOF spectra of β-CD-MCM.

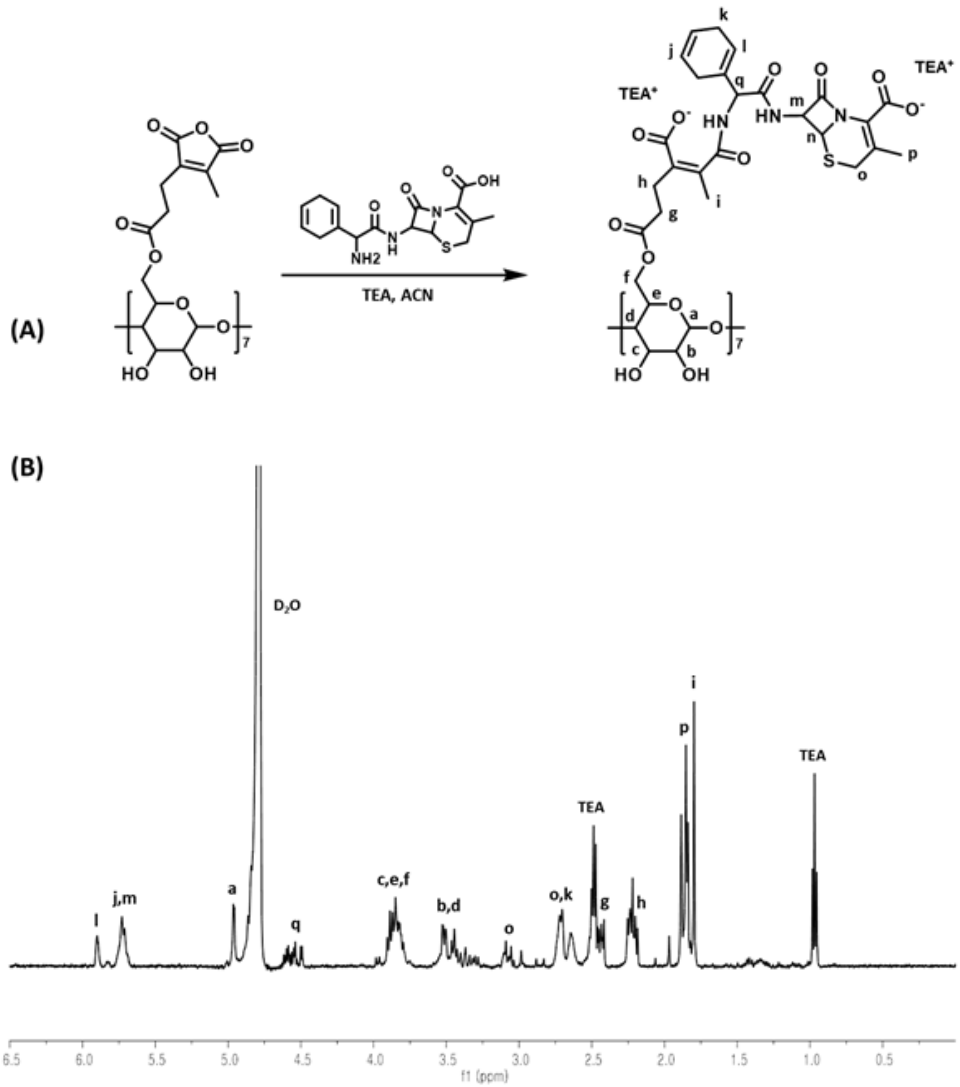

Figure 3. Synthesis and confirmation of β-CD-MCM-CP and β-CD-MCM-MX. (A) Synthetic scheme of β-CD-MCM-CP. (B) ^{1}H NMR (NMR solvent: D_2O + 0.6 wt% NaOD) spectra of β-CD-MCM-CP.

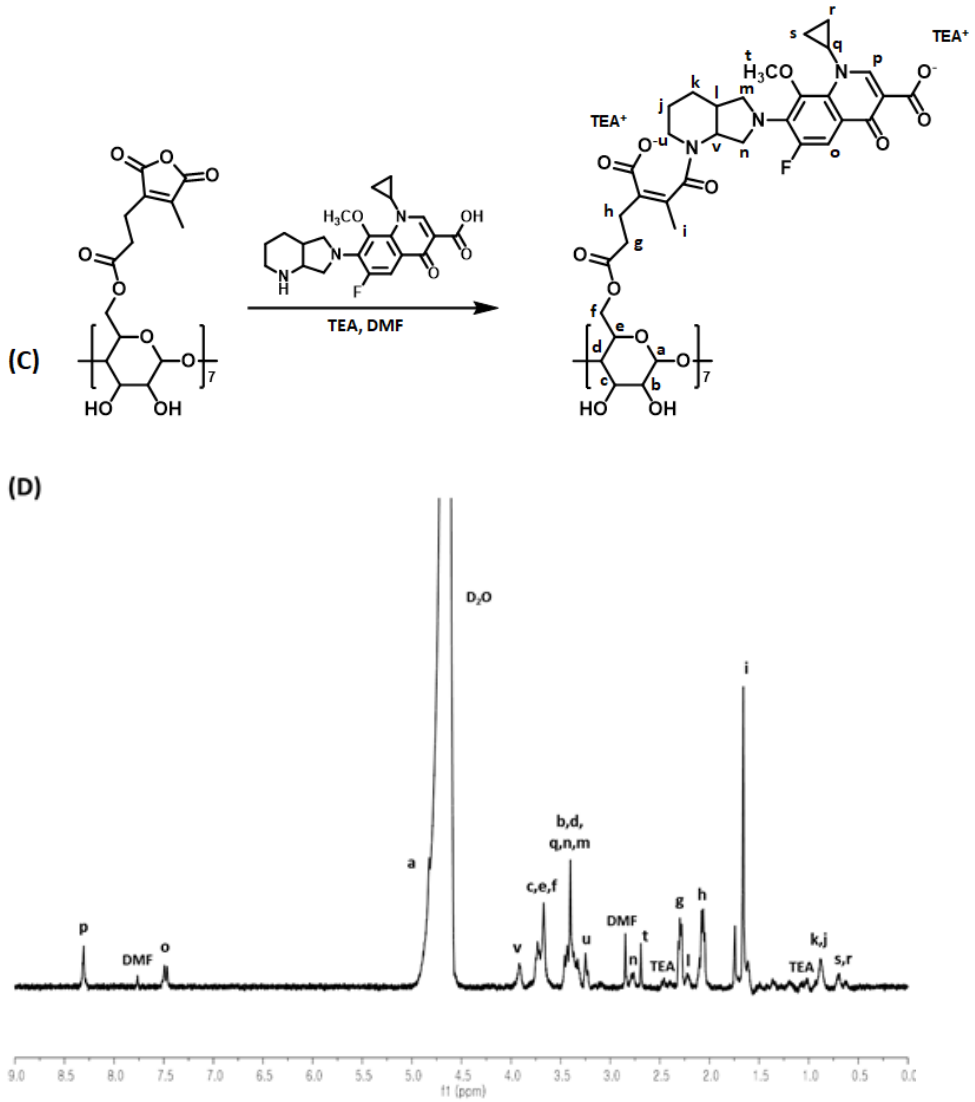

Figure 3. (***Continued***) (C) Synthetic scheme of β-CD-MCM-MX. (D) ^{1}H NMR (NMR solvent: D_2O + 0.6 wt% NaOD) spectra of β-CD-MCM-MX.

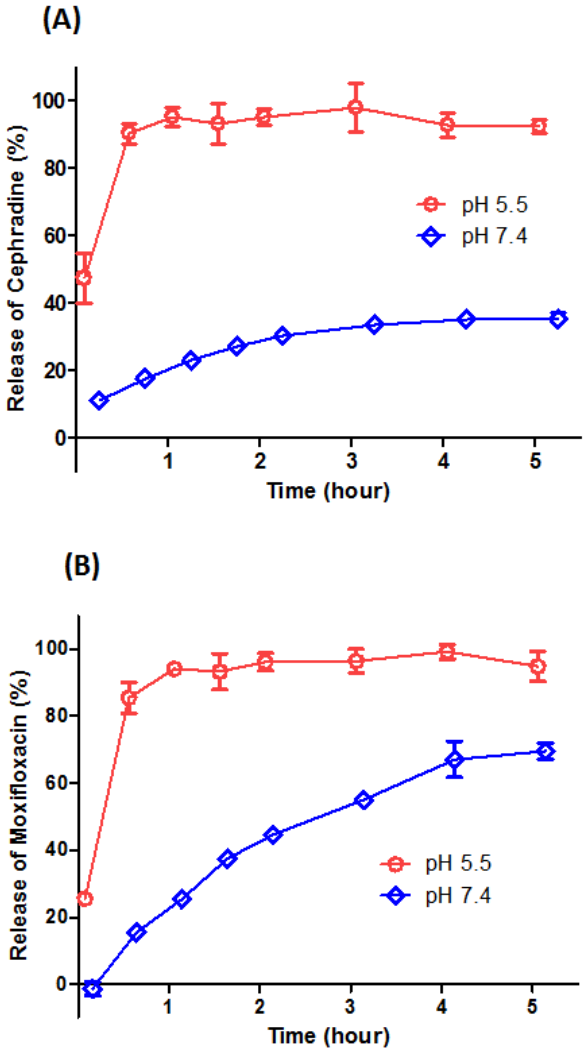

Figure 4. pH-dependent release of antibiotics from the conjugates at pH 5.5 (acetate buffer, 100 mM) and 7.4 (phosphate buffer, 100 mM) measured by HPLC. The error bar represents the standard deviation (n=3). (A) Cumulative release of CP from β-CD-MCM-CP. (B) Cumulative release of MX from β-CD-MCM-MX.

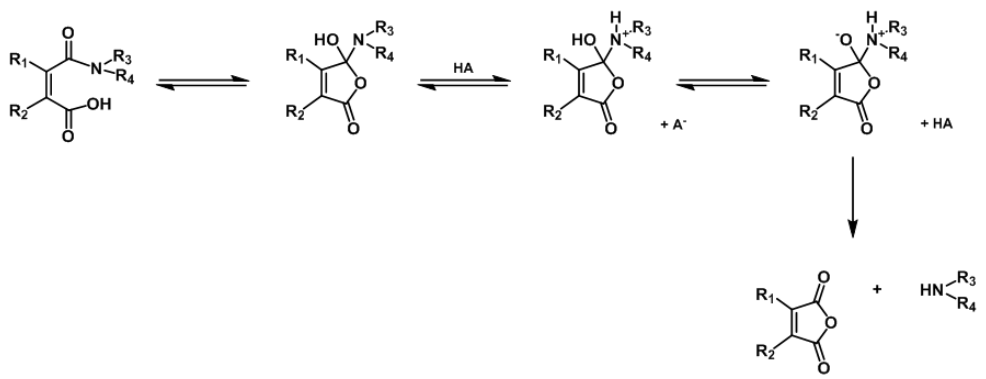

Figure 5. Proposed degradation mechanism of maleic acid amide at acidic pH.[16]

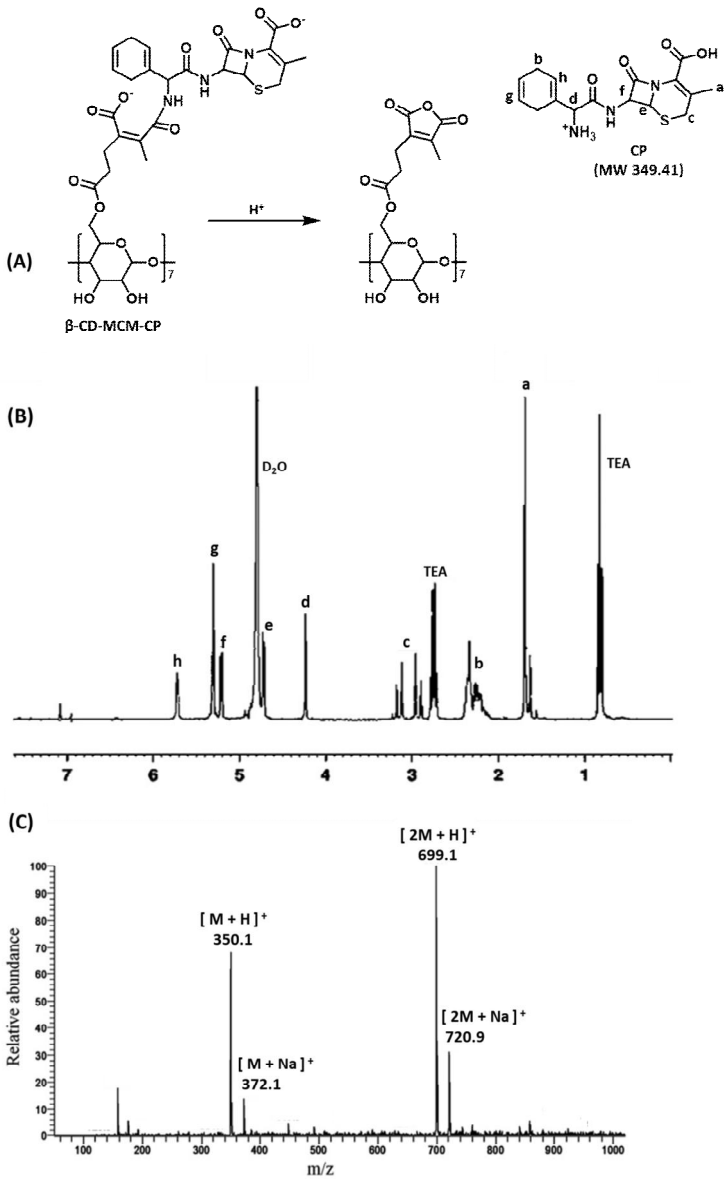

Figure 6. Release of CP and MX from β-CD-MCM-CP and β-CD-MCM-MX, respectively, at pH 5.5 and identification of the released CP and MX. (A) Release scheme of CP from β-CD-MCM-CP. (B) ^{1}H NMR (NMR solvent: D_2O) spectra of the released CP. (C) ESI mass spectra of the released CP.

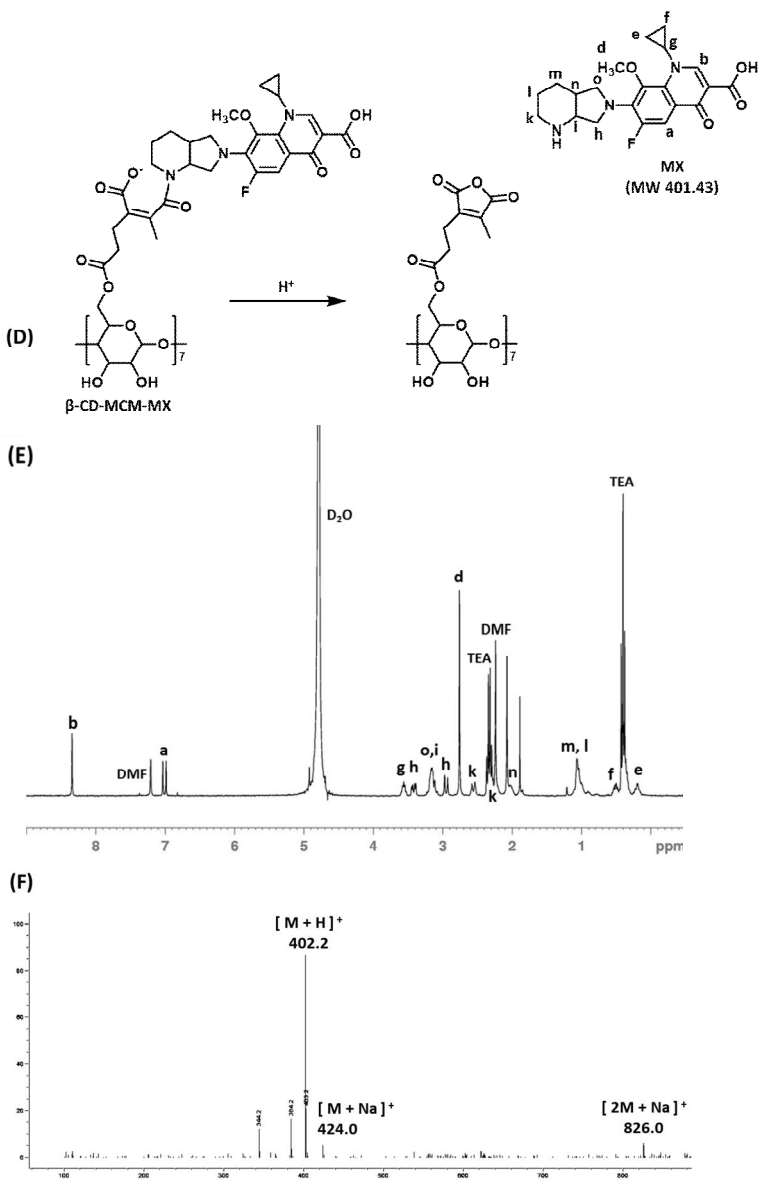

Figure 6. (*Continued*) (D) Release scheme of MX from β-CD-MCM-MX. (E) ^{1}H NMR (NMR solvent: D_2O) spectra of the released MX. (F) ESI mass spectra of the released MX.

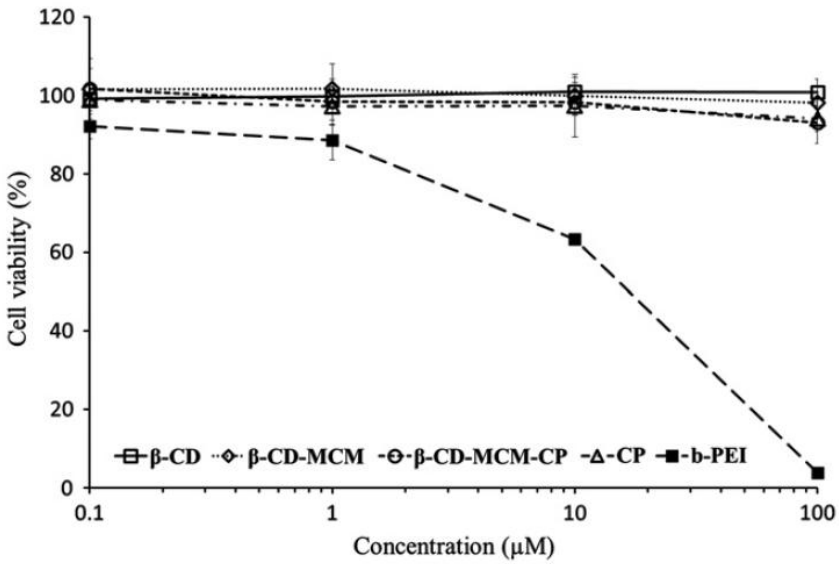

Figure 7. Cytotoxicity of β-CD, β-CD-MCM, β-CD-MCM-CP and b-PEI in NIH3T3 cells.[17] Each error bar represents the standard deviation (n=3).

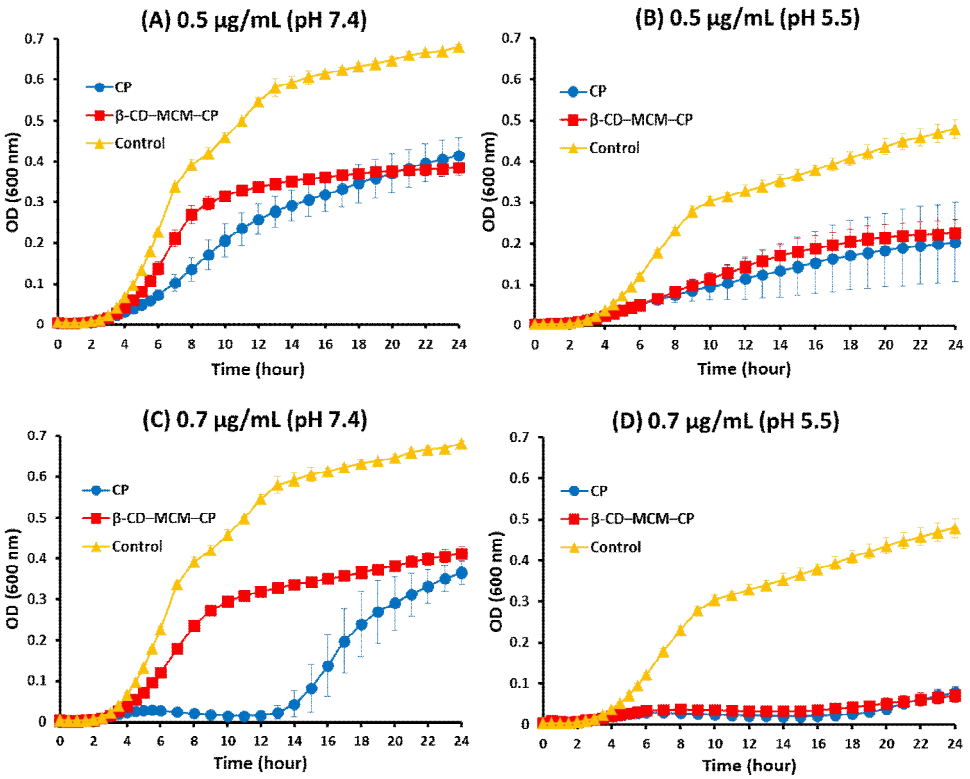

Figure 8. Optical density (OD) at 600 nm with varying concentrations of CP and β-CD-MCM-CP at pH 7.4 and 5.5. The control group does not contain any antibiotics. Each error bar represents the standard deviation (n=3). (A) OD (600 nm) at 0.5 μg/mL, pH 7.4. (B) OD (600 nm) at 0.5 μg/mL, pH 5.5. (C) OD (600 nm) at 0.7 μg/mL, pH 7.4. (D) OD (600 nm) at 0.7 μg/mL, pH 5.5.

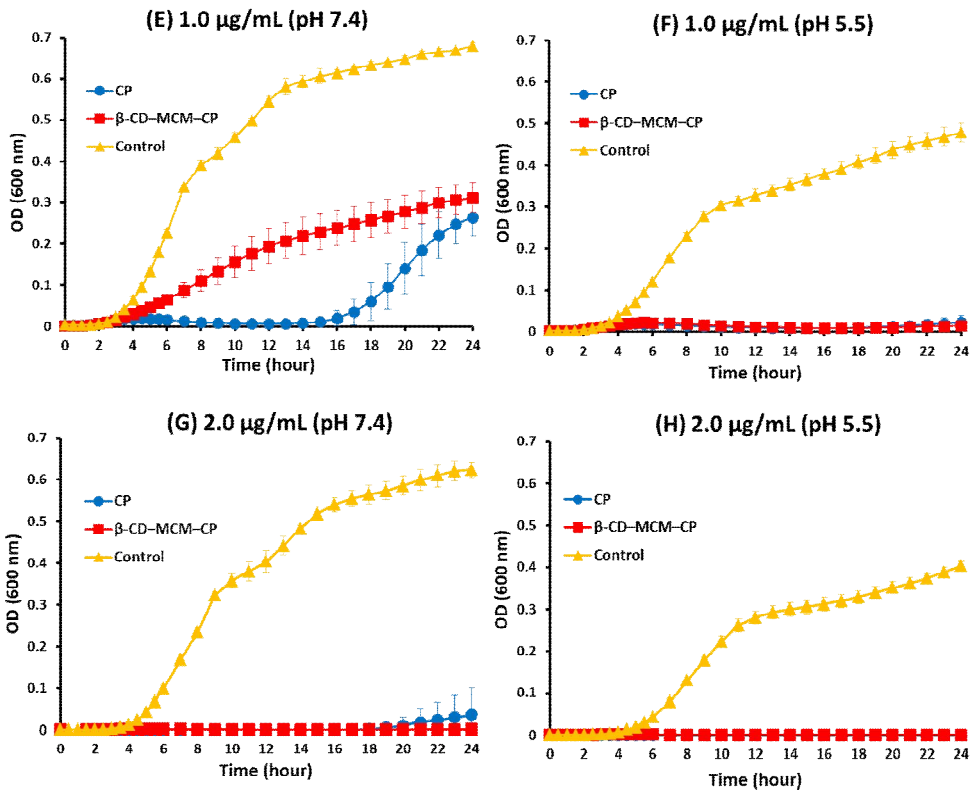

Figure 8. (*Continued*) (E) OD (600 nm) at 1.0 μg/mL, pH 7.4. (F) OD (600 nm) at 1.0 μg/mL, pH 5.5. (G) OD (600 nm) at 2.0 μg/mL, pH 7.4. (H) OD (600 nm) at 2.0 μg/mL, pH 5.5.

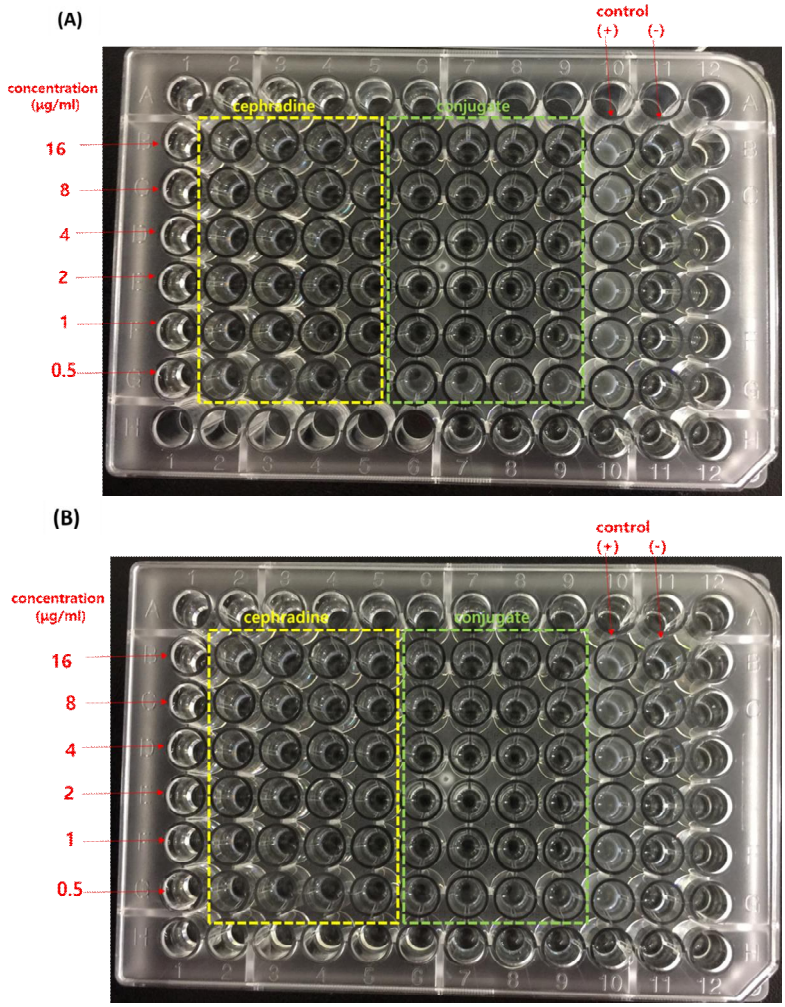

Figure 9. Photographs of 96-well microdilution plates for MIC determination and blood agar plates for MBC determination with both CP and β-CD-MCM-CP against *S. aureus*. Control (+) contanis only *S. aureus*, and control (-) contains neither bacteria nor antibiotics. (A) A 96-well microdilution plate after 12 hr of incubation at 37°C (pH 7.4). (B) A 96-well microdilution plate after 12 hr of incubation at 37°C (pH 5.5).

(C)

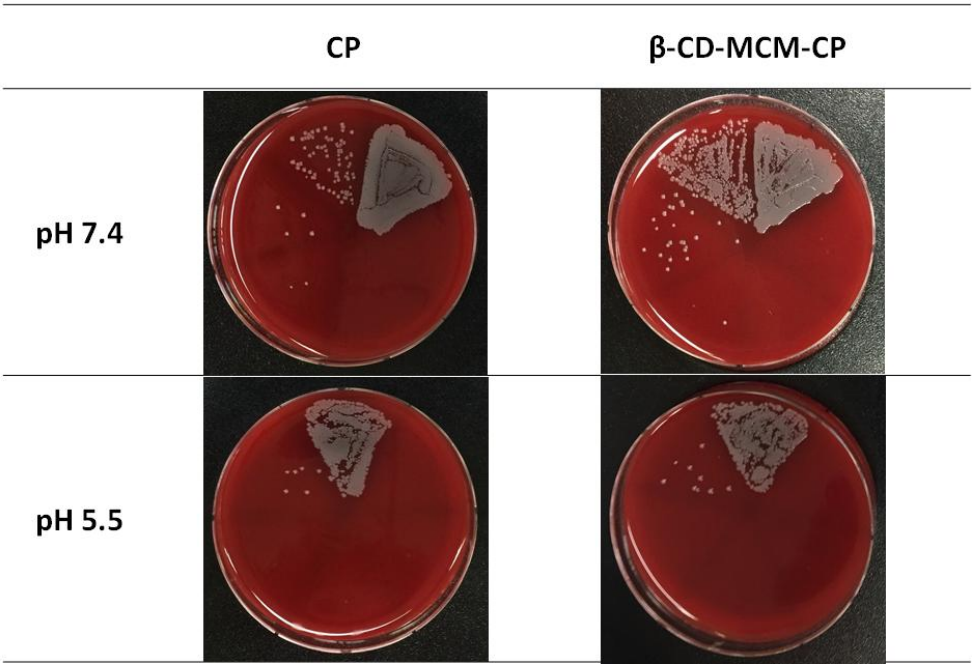

Figure 9. (*Continued*) (C) Blood agar plates with additional incubation of 24 hr at 37°C after MIC measurement. 10 μL of the antibiotic solutions with six different concentrations, indicated at the above Figure 9A and 9B, were applied on each agar plate.

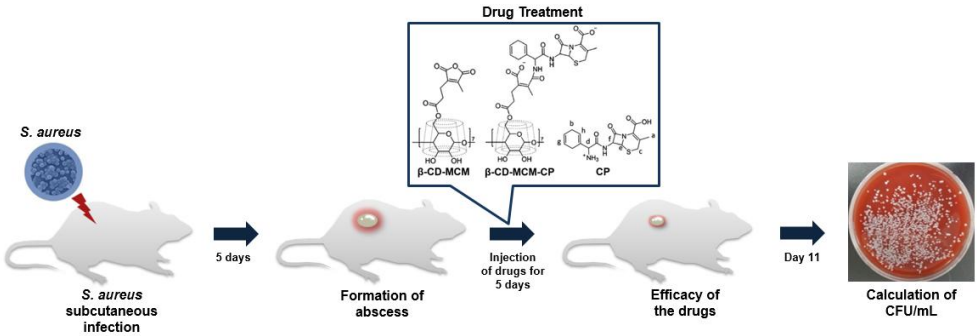

Figure 10. Scheme of animal experiments. Non-diabetes mice (Balb/c) and diabetes mice model (C57BLKs-Jdb/db) were used to evaluate efficacy of the drugs.

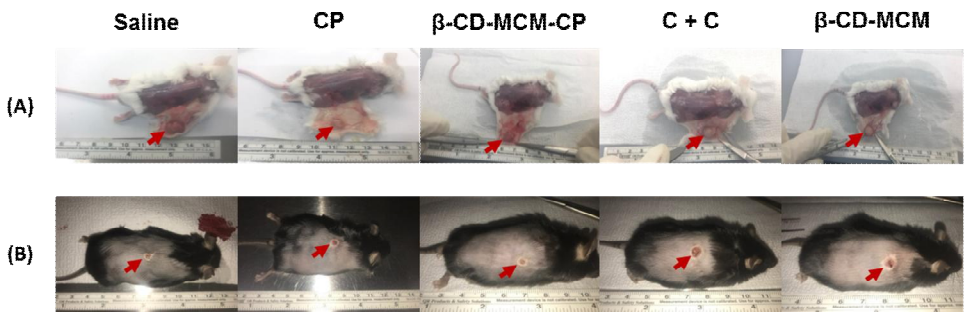

Figure 11. Abscess formation in non-diabetes and diabetes mice. Mice were sacrificed after 5 days of injection. Each red arrow indicates the abscess in the subcutaneous tissues of the animals. (A) Abscess formation in non-diabetes mice. (B) Abscess formation in diabetes mice.

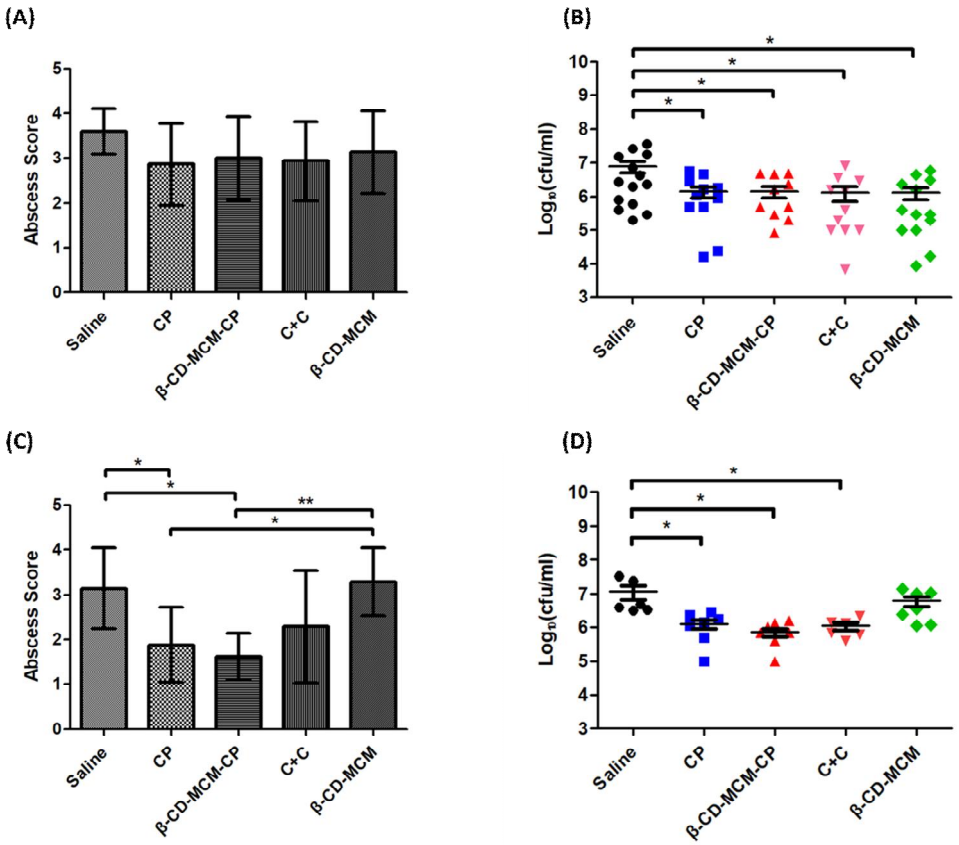

Figure 12. Abscess scores and bacterial counts in abscesses of non-diabetes and diabetes mice. Each data point in Figure 12B and 12D corresponds to one abscess from the subcutaneously infected mouse. (A) Abscess scores of non-diabetes mice. (B) Bacterial counts in the abscess of non-diabetes mice. (C) Abscess scores of diabetes mice. (D) Bacterial counts in the abscess of diabetes mice.

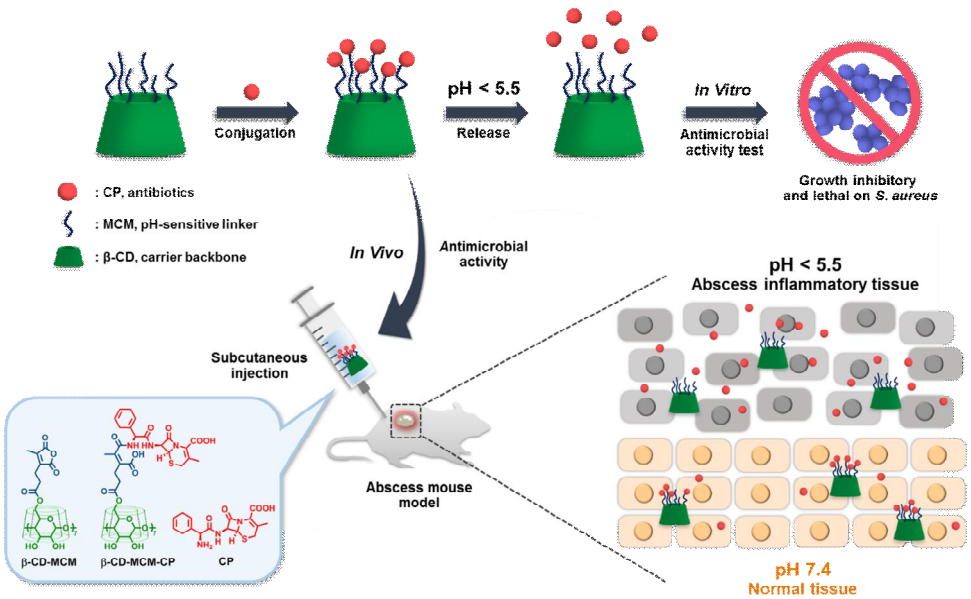

Figure 13. Schematic diagram of this study. The antibiotics conjugated with the pH-sensitive β-CD-MCM carriers show selective release at abscesses or inflammatory tissues which have weakly acidic environments. In contrast, the conjugates show limited release at normal tissues.

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) at pH 7.4 and 5.5. (A) MIC and MBC of CP and β-CD-MCM-CP (24% of drug content) against *S. aureus* after 12 hr of incubation. (B) MIC and MBC of MX and β-CD-MCM-MX (22% of drug content) against *B. fragilis* after 96 hr of incubation.

(A) MIC and MBC of CP and β-CD-MCM-CP (24% of drug content) against *S. aureus*.

	pH 7.4		pH 5.5	
S. aureus	**CP**	**β-CD–MCM-CP**	**CP**	**β-CD–MCM-CP**
MIC (μg/mL)	**0.7**	**2.0**	**0.7**	**0.7**
MBC (μg/mL)	**8.0**	**8.0**	**2.0**	**2.0**

(B) MIC and MBC of MX and β-CD-MCM-MX (22% of drug content) against *B. fragilis*.

	pH 7.4		pH 5.5	
B. fragilis	**MX**	**β-CD–MCM-MX**	**MX**	**β-CD–MCM-MX**
MIC (μg/mL)	**0.5**	**0.3**	**1.0**	**0.7**
MBC (μg/mL)	**0.5**	**0.5**	**1.5**	**1.0**

Table 2. Criteria of abscess scores. Abscesses of non-diabetes and diabetes mice were evaluated according to the criteria of grading system.

Grade	Description	Score
No Abscess	No skin necrosis. Abscess and inflammation were not observed.	0
Mild	No skin necrosis. Abscess and inflammation were observed slightly.	1
Moderate	No skin necrosis. Abscess and inflammation were observed definitely.	2
Severe	Skin necrosis occurred. Abscess and inflammation were observed definitely.	3
Very Severe	Skin was severely necrotic. Abscess ruptured. Pus was leaked.	4

국문초록

약물전달 시스템은 특정 신호를 인식하여 약물을 선택적으로 방출/축적 시키는 시스템으로, pH, 온도, 빛, 효소 등 다양한 종류의 자극을 목적에 맞게 선택하여 이용할 수 있다. 그 중 pH는 생체 내부의 특정 부분에서만 변화를 일으키는 내인성 자극으로 신호-응답성 약물전달 시스템 연구에 널리 이용되어 왔다. 예를 들어 생리적인 pH는 7.4를 나타내는 것에 반해, 암 조직이나 염증 조직, 농양과 같은 비정상 조직은 pH 5-6 정도의 약산성 환경을 갖는다. 이러한 비정상 조직이 갖는 약산성의 pH에만 특이적으로 반응할 수 있는 화학결합을 약물전달 시스템에 도입한다면, 항암제, 항생제와 같은 약물이 환부에서만 선택적으로 방출되거나 축적되도록 유도할 수 있다. 하지만 지금까지 개발된 pH-응답성 화학결합은 복잡한 합성 과정과 느린 분해 속도, 그리고 pH 변화에 대한 낮은 민감도 등의 한계점으로 인해 실질적으로 응용되기 어려운 실정이다.

본 연구에서는 약산성의 조건에서 분해되는 말레산 아미드 유도체와 생체적합성을 갖는 β-cyclodextrin (β-CD)을 결합하여 새로운 pH-응답성 약물전달체를 개발하였다. 여러 종류의 말레산 아미드 유도체 중에서도 1-methyl-2-(2'-carboxyethyl) maleic anhydride (MCM)로부터 유도된 말레산 아미드는 pH 변화에 대해 우수한 감응성을 나타낸다. 이러한 특징을 이용하고자, β-CD에 MCM을 결합시켜 약산성의 pH에서 빠르게 분해되는 약물전달체 (β-CD-MCM)를 합성하였다. β-CD-MCM은 말레산 무수물 형태에서 아민 계열의 약물과 한 단계의 반응을 통하여 말레산 아미드 결합을 용이하게 형성한다. MCM으로부터 유도된 말레산 아미드의 우수한 pH-감응성으로 인해, 약물전달체에 결합되어 있는 약물은 환부 조직의 약산성 환경 (pH 5.5)에서 빠르게 방출될 것으로 기대되었다. 또한, 여러 개의 작용기가 존재하는 β-CD를 전달체의 뼈대로 사용하여 약물의 적재량을 높임으로써, 환부에서 약물의

국소부위 농도가 증가되는 효과를 추가적으로 얻을 것으로 기대되었다.

본 실험에서는 두 종류의 아민 계열 항생제인 cephradine (CP)과 moxifloxacin (MX)을 모델 약물로 사용하였다. 체내에서 대사되거나 분비되지 않은 채로 잔류하는 항생제는 인체에 심각한 부작용을 초래하며, 항생제의 남용은 내성 문제를 빈번하게 발생시킨다. 따라서, 약물의 항생 작용을 염증 조직의 약산성 환경에서만 선택적으로 활성화시키는 것이 항생제의 부작용 및 내성 문제를 줄이는 해결 방안의 첫 걸음이 될 수 있을 것이라 생각하였기 때문이다. 본 약물전달체와 약물이 결합한 β-CD-MCM-drug로부터 pH에 따른 약물의 방출 정도를 HPLC 기기를 통해 확인하였고, 정상 조직과 염증 조직의 환경을 나타내는 pH 7.4와 pH 5.5에서 실험을 진행하였다. 이후, β-CD-MCM-drug의 *S. aureus*와 *B. fragilis*에 대한 항생 능력을 평가하고자 최소억제농도 (MIC)와 최소살균농도 (MBC)를 측정하였다. 보다 정교한 연구를 위해, 짧은 시간 간격으로 광학밀도를 측정하여 *S. aureus*의 성장 정도를 비교함으로써 pH에 따른 β-CD-MCM-CP의 선택적 약물 효능을 확인 할 수 있었다. 최종적으로, *in vivo* 실험을 통해 피하 농양을 갖는 일반쥐와 당뇨쥐에서 β-CD-MCM-CP의 효용성을 측정하였다. 본 연구 결과를 고려해보았을 때, 항생제와 결합한 β-CD-MCM 전달체는 염증 조직 또는 농양 조직의 약산성 환경을 표적하는 항생제 전달 시스템에서 큰 잠재력을 보여줄 것으로 사료된다.

주요어 : 항생제 전달, pH-응답성 분해, 말레산 아미드, 선택적 약물 방출, 항생 능력

학 번 : 2016-22868