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RESEARCH PAPER

Sugar-Grafted Cyclodextrin Nanocarrier as a "Trojan Horse" for Potentiating Antibiotic Activity

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ABSTRACT

Purpose The use of "Trojan Horse" nanocarriers for antibiotics to enhance the activity of antibiotics against susceptible and resistant bacteria is investigated.

Methods Antibiotic carriers (CD-MAN and CD-GLU) are prepared from β-cyclodextrin grafted with sugar molecules (D-mannose and D-glucose, respectively) via azide-alkyne click reaction. The sugar molecules serve as a chemoattractant enticing the bacteria to take in higher amounts of the antibiotic, resulting in rapid killing of the bacteria.

Results Three types of hydrophobic antibiotics, erythromycin, rifampicin and ciprofloxacin, are used as model drugs and loaded into the carriers. The minimum inhibitory concentration of the antibiotics in the CD-MAN-antibiotic and CD-GLU-antibiotic complexes for Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains, and a number of Gram-positive *Staphylococcus aureus* strains, including the methicillin-resistant strains (MRSA), are reduced by a factor ranging from 3 to >100. The CD-MAN-antibiotic complex is also able to prolong the stability of the loaded antibiotic and inhibit development of intrinsic antibiotic resistance in the bacteria.

Conclusions These non-cytotoxic sugar-modfied nanocarriers can potentiate the activity of existing antibiotics, especially against multidrug-resistant bacteria, which is highly advantageous in view of the paucity of new antibiotics in the pipeline.

KEY WORDS antibacterial · antibiotic nanocarrier · glucose · mannose · β-cyclodextrin

ABBREVIATIONS

A. baumannii	*Acinetobacter baumannii*
CD-GLU	D-glucose-grafted cyclodextrin (CD)
CD-MAN	D-mannose-grafted cyclodextrin (CD)
CIP	Ciprofloxacin
E. coli	*Escherichia coli*
ERY	Erythromycin
MIC	Minimum inhibitory concentration
MTT	3-[4,5-Dimethyl-thiazol-2-yl]-2, 5-diphenyltetrazolium bromide
P. aeruginosa	*Pseudomonas aeruginosa*
PBI	3,4,9,10-perylenetetracarboxylic 3,4:9,10-dianhydride
RIF	Rifampicin
S. aureus	*Staphylococcus aureus*

INTRODUCTION

Antibiotics have been of critical importance in the fight against infectious diseases caused by bacteria since 1920s. However, the extensive use of antibiotics has given rise to increasing frequency of antibiotic resistance due to bacterial evolution from mutation and horizontal transfer of genetic materials from other resistant bacteria (1). Currently, about 70% of bacteria that cause infections in hospitals are resistant to at least one of the widely used antibiotics (2). These resistant

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bacteria are a growing serious public health concern and have resulted in increased economic burden of infection control. For example, methicillin-resistant *Staphylococcus aureus* (MRSA) strains-induced infections resulted in ~ $9.7 billion extra medical cost in United States in 2005 (3). A recent review has bleakly predicted that the antibiotic resistance problem will continue into the foreseeable future, and even if antibiotic use could be reduced, resistant clones would remain persistent (4). In addition, the antibiotic pipeline is running almost dry, but the number of new synthetic antibiotics annually approved by Infectious Diseases Society of America (IDSA) for marketing continues to decrease since 1983, and there were only two new approved antibiotics from 2009 to 2013 (5). Thus, it would be highly advantageous if existing antibiotics can be modified to be more effective against resistant bacteria.

The use of antibiotics in combination with nanoparticulate systems for combating resistant bacteria has gained increasing interest in past decades. This strategy capitalizes on the unique physiochemical properties of nanoparticles and their interactions with biological systems. Metal nanoparticles with intrinsic bactericidal properties, such as gold, silver, iron oxide, magnesium oxide and zinc oxide, have been used as antibiotic delivery systems (6). A typical example is gold nanoparticles combined with erythromycin, which reduced the minimum inhibitory concentration (MIC) of erythromycin against *Yersinia enterocolitica* and *Escherichia coli* (*E. coli*) by about a factor of 8 as compared to erythromycin alone (7). The gold nanoparticles were shown to breach the bacterial cell wall thereby increasing the uptake of the antibiotic. Organic nanoparticles, including polymeric nanoparticles, DNA based nanostructures, liposomes and dendrimers, have also been used for loading antimicrobial agents through physical adsorption, encapsulation, or chemical conjugation (8,9). For example, carboxymethyl chitosan (CMCS) nanoparticles were used to deliver ciprofloxacin into bacterial cells by altering bacterial cell membrane and the MIC of CMCS nanoparticle-loaded antibiotic on *E. coli* was reduced by a factor of 2 as compared to the free form (10). A number of prevailing strategies using nanoparticles in combination with antibiotics to enhance the efficacy of the antibiotics depend on the ability of the nanoparticles to disrupt the bacterial cell membrane. However, a recent article has shown that nanoparticles which damage bacterial cell membranes promote the horizontal conjugative transfer of multidrug-resistance genes and may increase antibiotic resistance (11). Therapeutic strategies based on nanoparticle-antibiotic complexes that can inhibit bacterial infection as well as prevent development of bacterial resistance are still lacking.

Cyclodextrins (CDs) are cyclic oligosaccharides containing six or more glucopyranose units linked via α-(1,4) bonds (12). A CD molecule is considered as a nanoparticle (outer diameter from 1.46 nm onwards) with hydrophilic outer wall and hydrophobic inner cavity, which can be used for loading a variety of hydrophobic agents via hydrophobic association to form inclusion complexes (host-guest complexes) (13). This inclusion complex leads to advantageous changes to the physiochemical properties of guest molecules, such as improvement of solubility, modification of chemical activity and protection against degradation by enzymatic substances. As a result of these unique physiochemical properties, CDs have been widely used in many applications, including cosmetics, personal care, food, pharmaceutics and chemical industries. In addition, CDs are also appealing as cores for the design of multivalent glycoconjugates due to the selective functionality of their hydroxy moieties. For example, n-heptyl α-D-mannoside, a nanomolar FimH antagonist, was tethered on β-CD to develop heptavalent β-CD to capture and aggregate living bacteria and reduce bacterial adhesion in mouse bladder (14). In this work, β-CD, which is the most widely-used CD in its family because of its availability, lowest price and acceptance by USFDA as generally regarded as safe (GRAS) for use in food (15), was selected as an antibiotic carrier. The β-CD was grafted with sugar molecules, D-mannose or D-glucose, to form the CD-MAN and CD-GLU carriers, respectively. We hypothesize that since mannose and glucose are carbon sources for bacteria and can readily permeate into bacterial cells through sugar transporters on the cell membrane (16), the grafted sugar moieties on the CD carrier will serve as chemoattractant for the bacteria. The subsequent interactions between the bacteria and the CD carrier may enhance the uptake of the antibiotic and rapidly increases its cytoplasmic concentration and efficacy. Furthermore, the carrier may also protect the antibiotic from hydrolysis and degradation and preserve its activity for a longer period than its free form. The antibacterial efficacy of CD-MAN and CD-GLU loaded antibiotics (CD-MAN-antibiotic and CD-GLU-antibiotic complexes) was evaluated with *Staphylococcus aureus* (*S. aureus*), *E. coli*, *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Acinetobacter baumannii* (*A. baumannii*) bacteria. The possibility of the bacteria developing resistance to the sugar-modified CD-antibiotic complexes and cytotoxicity of the carrier-complexes were also evaluated.

MATERIALS AND METHODS

Materials

Beta-cyclodextrin (β-CD, 97%), triphenylphosphine (Ph_3P, 98.5%), iodine (99.8%), sodium azide (99.5%), copper(I) bromide (CuBr, 98%), *N*, *N*′, *N*″, *N*″-pentamethyldiethylenetriamine (PMDETA, 99%), D-mannose (99%) and D-glucose (99.5%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was

obtained from Alfa Aesar Co. (Ward Hill, MA, USA). Spectra-Por dialysis membranes (molecular weight cut-off: 1000) were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA). *E. coli* DH5α, *S. aureus* ATCC 25923, Human HEK 293T embryonic kidney cells, murine RAW 264.7 macrophage and murine 3T3 fibroblast cells were purchased from American Type Culture Collection (Manassas, VA, USA). Human NCM460 colonic epithelial cells were purchased from INCELL Corporation (San Antonio, TX, USA). *P. aeruginosa* PAO1 was purchased from National Collection of Industrial Food and Marine Bacteria (NCIMB, Bucksburn, Aberdeen, Scotland). *P. aeruginosa* PAO397 was obtained by blocking the multidrug efflux pumps of *P. aeruginosa* PAO1 as reported in an earlier publication (17). The MRSA strains, DM23605 and DR9369, were clinical isolates provided by the Singapore General Hospital (SGH, Singapore), while the MRSA strain ATCC BAA-44 was purchased from American Type Culture Collection. *A. baumannii* S1, a clinical isolate, was provided by the Network for Antimicrobial Resistance Surveillance (Singapore). 1-(2′-Propargyl)-D-mannose (mannose-alkyne) and 1-(2′-propargyl)-D-glucose (glucose-alkyne) were synthesized using procedures similar to that described in the literature (18).

Synthesis of D-mannose- or D-glucose-grafted β-CD (CD-MAN or CD-GLU)

Heptakis(6-deoxy-6-azido)-β-cyclodextrin (CD-N_3) was prepared using procedures described in an earlier publication (19). Briefly, Ph_3P (40.1 g, 153 mmol) was dissolved in dry *N*,*N*-dimethylformamide (DMF, 160 mL) and iodine (40.5 g, 160 mmol) was added to this solution over 10 min. The solution was then heated to 70°C and dry β-CD (11.6 g, 10.2 mmol) was added. The reaction was allowed to proceed at 70°C for 24 h under N_2 protection. After completion of the reaction, the solution was partially concentrated under reduced pressure followed by addition of sodium methoxide (3 M, 60 mL). The reaction mixture was then poured into excess methanol. The precipitate was collected by filtration and purified by Soxhlet extraction with methanol for 72 h followed by drying under reduced pressure to obtain heptakis(6-deoxy-6-iodo)-β-cyclodextrin (CD-I). Then, CD-I (2.99 g, 1.57 mmol) was dissolved in dry DMF (50 mL) and sodium azide (1 g, 15.4 mmol) was added. The reaction suspension was heated to 70°C and kept at this temperature for 36 h under N_2 atmosphere. After reaction, DMF was partially removed and the reaction suspension was poured into excess doubly distilled water. The resultant precipitate was collected by filtration, washed with water and freeze-dried to obtain the CD-N_3 product.

CD-MAN was synthesized via azide-alkyne click reaction between CD-N_3 and mannose-alkyne. Briefly, CD-N_3 (1.33 g, 1.0 mmol), mannose-alkyne (1.64 g, 7.5 mmol) and PMDETA (156.6 μL, 130 mg, 0.75 mmol) were dissolved in DMF (15 mL) in a 50 mL flask. The solution was degassed by purging with argon for 30 min and CuBr (107.6 mg, 0.75 mmol) were then added. The reaction mixture was further purged with argon for 10 min. The flask was then sealed tightly and the reaction was allowed to proceed under continuous stirring at 60°C for 24 h. After reaction, the solution was poured into excess ethyl ether. The precipitate was collected after filtration and re-dissolved in doubly distilled water. The aqueous solution was then subjected to dialysis against doubly distilled water for 3 days followed by freeze-drying to obtain the final CD-MAN product. CD-GLU was prepared using similar procedures with glucose-alkyne instead of mannose-alkyne.

Preparation of CD-MAN or CD-GLU Loaded Antibiotic (CD-MAN-antibiotic or CD-GLU-antibiotic) Complex

Three different antibiotics, erythromycin (ERY, inhibitor of protein synthesis), rifampicin (RIF, inhibitor of transcription) and ciprofloxacin (CIP, inhibitor of DNA replication), were used to prepare CD-MAN-antibiotic complexes. Typically, the antibiotic (0.01 mmol) and CD-MAN (0.01 mmol) were mixed and triturated with ethanol-water mixture (200 μL, ethanol/water = 100 μL/100 μL). The mixture was kneaded for 45 min and then dissolved in doubly distilled water (20 mL). The solution was passed through a 0.2 μm Nylon membrane filter followed by freeze-drying to obtain the CD-MAN-antibiotic complex. CD-antibiotic and CD-GLU-antibiotic complexes were prepared using similar procedures with β-CD and CD-GLU, respectively, instead of CD-MAN.

The content of antibiotics in CD-MAN-antibiotic complexes was determined as follows: CD-MAN-antibiotic (1 mg) was suspended in methanol (10 mL) and subjected to ultrasonication for 30 min. The suspension was kept overnight for the loaded antibiotic to be released from the complex. After the precipitation of the undissolved CD-MAN by centrifugation at 8000 rpm for 10 min, the concentration of the antibiotic in the supernatant was analyzed to calculate the content of antibiotics in the complexes. The concentration of ERY was analyzed by high-performance liquid chromatography (HPLC). HPLC assay was performed with an Agilent Technologies 1200 series liquid chromatographic system at a 45°C (oven temperature). The mobile phase was 15 wt% methanol, 45 wt% acetonitrile and 40 wt% ammonium phosphate 0.01 M solution, and the flow rate was 1 mL/min. The injection volume was 50 μL and the effluent was monitored at 215 nm. For the HPLC analysis of CIP, the mobile phase was 5 vol% methanol, 5 vol% acetonitrile and 90 vol% acetic acid aqueous solution (50 mL/L). The injection volume was 50 μL and the effluent was monitored at 280 nm. The oven temperature was set at 50°C and flow rate was 1 mL/min. The concentration of RIF was analyzed by the colorimetric

method described in the literature (20). The content of antibiotics in CD-antibiotic and CD-GLU-antibiotic complexes was analyzed using similar methods as described above.

Antimicrobial Susceptibility Assay

In this work, the antibacterial activity of the antibacterial agents (free antibiotic, CD-MAN-antibiotic, CD-GLU-antibiotic and CD-antibiotic complexes) was evaluated using MIC assay. MIC is defined as the lowest concentration of an antimicrobial agent that will prevent the visible growth of a microbe after overnight culture (21). MIC is commonly used as a research tool to evaluate the antimicrobial activity of a new agent *in vitro* and used by diagnostic laboratories to determine resistance. The MIC was determined by a standard turbidimetric method. Bacteria were cultured overnight in growth medium (nutrient broth (NB) for *E. coli*, tryptic soy broth (TSB) for *S. aureus* and lysogeny broth (LB) for *P. aeruginosa* and *A. baumannii*). The bacteria-containing growth medium was then centrifuged for 10 min at 2700 rpm to remove the supernatant. The bacterial cells were washed with phosphate buffer saline (PBS, pH 7.4) and resuspended in Mueller-Hinton broth (MHB) at a concentration of 2×10^5 colony forming unit (CFU)/mL, as estimated from the optical density of the suspension at 600 nm (OD_{600}). OD_{600} of 0.1 is equivalent to ~10^8 CFU/mL based on calibration from spread plate counting. One hundred μL of two-fold serially diluted antibacterial agent (free antibiotic, CD-antibiotic, CD-MAN-antibiotic or CD-GLU-antibiotic complex) solution in MHB and 100 μL of the bacterial-containing MHB were added in each well of a 96-well plate. Initial screening was carried out with 1024 to 0.125 mg/L of the antibacterial agent in MHB. If the MIC was determined to be out of this range, further screening was carried out with the concentration range adjusted accordingly. The plate was then incubated in an orbital shaker at 37°C for 20 h with a shaking speed at 200 rpm/min. The MIC of the antibacterial agent was determined as the minimum concentration at which there was no visible change in the turbidity of the medium in the well. The corresponding MIC of the antibiotic in the complex was calculated from the antibiotic content in the complex. All the tests were carried out at least in triplicate.

Bacterial Uptake and Accumulation Assays

A hydrophobic fluorescent dye, 3,4,9,10-perylenetetracarboxylic 3,4:9,10-dianhydride (PBI), was loaded in place of antibiotic into CD-MAN and β-CD to form CD-MAN-PBI and CD-PBI complexes, respectively, using similar procedures as those described above for CD-MAN-antibiotic or CD-antibiotic complexes. The resultant CD-MAN-PBI and CD-PBI contained 10.1% and 20.3% (wt%) of PBI, respectively, as measured by a fluorometric method reported earlier (22). The uptake of CD-MAN-PBI and CD-PBI by *E. coli*, *P. aeruginosa* and *S. aureus* was qualitatively evaluated using fluorescence microscopy. Overnight bacterial culture broth was diluted to a concentration of 1×10^8 CFU/mL with PBS. A piece of medical grade silicone film (1×1 cm^2) was placed in a 24-well plate and covered by 1 mL of bacterial suspension at 37°C for 4 h to allow bacterial attachment. After the bacterial attachment process, the film was washed thrice with PBS to remove the non-adherent bacteria. Then, 1 mL of MHB medium containing either PBI (10 μg) or CD-MAN-PBI (99 μg, corresponding to 10 μg of PBI) or CD-PBI (49.3 μg, corresponding to 10 μg of PBI) was added to cover the silicone film with adherent bacterial cells and incubated at 37°C for 4 h. After incubation, the film was washed thrice with PBS followed by observation under a fluorescence microscope.

The accumulation of CD-MAN-PBI and CD-PBI within the bacterial cells was evaluated in a similar manner as that described in an earlier report with a few modifications (23). One mL of bacterial suspension containing 1×10^5 CFU in MHB was incubated with PBI (10 μg) or CD-MAN-PBI (99 μg) or CD-PBI (49.3 μg) for 0.5 and 4 h, respectively. After incubation, the bacterial cells were collected by centrifuging at 2700 rpm for 10 min and washed thrice with PBS. The bacteria were then lysed using 1 mL of bacterial lysis solution to release the PBI which had accumulated in the bacterial cells. After bacterial lysis, the suspension (200 μL) was transferred to the wells of a black microtitre tray (Corning, Amsterdam, The Netherlands) and fluorescence intensity was measured by a fluorescence microplate reader.

Antibiotic Resistance Assay

Since bacteria can develop resistance to antibiotics, an important consideration is whether bacteria will develop resistance to the sugar-modified CD-antibiotic complexes. An antibiotic resistance assay using *S. aureus* ATCC 25923 was carried out to assess its potential to develop resistance to free ERY, CD-ERY and CD-MAN-ERY. To encourage *S. aureus* to develop resistance to free ERY, TSB medium (1 mL) containing 0.25 times MIC of free ERY was inoculated with overnight culture (10 μL) of *S. aureus*. The bacterial culture was passaged every 2 days by inoculating the culture (10 μL) into fresh TSB medium containing 0.25 times MIC of free ERY. At the end of 8 and 16 days of incubation, the bacterial suspension after appropriate dilution was spread onto TSB agar to determine total bacterial counts. The bacterial suspension was also spread onto TSB agar containing 2 times MIC of free ERY to determine the portion of bacteria that had developed resistance to free ERY. Bacterial cultures in TSB medium alone were also tested to confirm no spontaneous mutations that might give rise to ERY-resistant bacteria. The portion of bacteria that developed resistance to CD-ERY or CD-MAN-ERY complex was determined using similar procedures with CD-ERY and CD-MAN-ERY, respectively, instead of free

ERY. A schematic illustration of the procedures of the antibiotic resistance assay is shown in Fig. S1 (Supplementary Material). The antibiotic resistance assay was also carried out using *E. coli* DH5α to assess its potential to develop resistance to free RIF, CD-RIF and CD-MAN-RIF. The procedures used were similar to those using *S. aureus* ATCC 25923 but with NB as the medium instead of TSB.

Bacterial Lysis Assay

The possibilty of lysis arising from the interactions of the CD-MAN nanocarrier with bacterial cell membrane was assessed from detection of bacterial nucleic acids after incubation with CD-MAN. Ten mL of PBS suspension of bacteria (2×10^8 CFU/mL) was mixed with 10 mL of PBS solution of CD-MAN (0.6 g/L). The bacterial suspension was incubated at 37°C with 150 rpm of constant shaking. At hourly intervals, 2 mL of the suspension was then collected and filtered through a 0.2 μm Nylon membrane filter to remove the bacterial cells. The absorbance of the filtrate at 260 nm (OD_{260}) was measured on a Shimadzu UV-1601 spectrophotometer to detect any nucleic acids leached from the bacteria. Bacterial lysis buffer (sodium dodecyl sulfate in PBS, 2.0 wt%) and PBS were used as positive and negative control, respectively.

Stability Assay

The stability of the antibacterial agents was assessed by conducting the bacterial growth inhibition assay over a period of time. Two hundred μL of MHB medium containing 1×10^5 CFU/mL of bacterial cells (*E. coli* DH5α, *P. aeruginosa* PAO1 and *S. aureus* ATCC 25923) and the antibacterial agent (free ERY or CD-MAN-ERY complex) at MIC or 2 times MIC were added in a well of a 96-well plate. The plate was incubated at 37°C for 21 days. The bacterial culture was replenished by adding fresh MHB every 2 days and the volume of culture medium in each well was maintained at 200 μL. The OD_{600} of the bacterial culture was measured on a BIO-TEK microplate reader (Model Powerwave XS) at regular intervals.

Cytotoxicity Assay

The cytotoxicity of CD-MAN-ERY complex was investigated using two methods, the MTT assay and Tali™ scan analysis, which are based on different principles. The MTT assay was carried out with murine 3T3 fibroblasts and RAW 264.7 macrophages, while the Tali™ scan analysis was carried out with human NCM460 colonic epithelial and HEK 293T embryonic kidney cells. 3T3 fibroblasts were seeded in DMEM growth culture medium at a density of 1×10^4 cells per well in a 96-well plate and incubated at 37°C for 24 h. The growth culture medium was then replaced with one containing the antibacterial agent (free ERY, CD-MAN-ERY complex or CD-MAN carrier) at different concentrations, and the cells were incubated at 37°C for 24 and 72 h. Control experiments were carried out without the antibacterial agents. The culture medium in each well was then removed and the medium (90 μL) and MTT solution (10 μL, 5 mg/mL in PBS) were added to each well. After 4 h of incubation at 37°C, the medium was removed and the formazan crystals were dissolved with dimethylsulfoxide (DMSO, 100 μL) for 15 min. The optical absorbance of the DMSO solution was measured at 560 nm on a BIO-TEK microplate reader. The cell viability is expressed as a percentage relative to that obtained in the control experiment.

For the Tali™ scan analysis, NCM460 and HEK 293T cells were both seeded on 24 wells plates at a seeding density of 1×10^5 cells per well. After overnight incubation, the original media solutions were replaced with CD-MAN-ERY or free ERY suspension in DMEM at different concentrations. The treated cells were further incubated for 24 h, washed once with PBS containing 0.05% trypsin and then incubated for 5 min. Fresh medium was added to neutralize the trypsin. After flushing the wells several times with fresh medium, the cell suspensions were pelleted down by centrifugation at 2000 g for 5 min. The supernatant was then removed and the remaining cell pellet was re-suspended in medium. Subsequently, chilled propidium iodide (PI, 1 μL, 50 μM) was added as a dye to the suspension, and the suspension was vortexed lightly and incubated for 5 min. The mixture (25 μL) was loaded onto a Tali™ Cellular Analysis Slide (Life Technologies, Invitrogen, USA) and analyzed with the Tali™ Image Based Cytometer (Invitrogen) for cell viability.

Characterization

Fourier transform infrared (FT-IR) spectra were obtained in the transmission mode on a Bio-Rad FT-IR spectrophotometer (Model FTS135). UV-visible (UV–vis) absorption spectra were recorded on a Shimadzu UV-1601 spectrophotometer. Hydrodynamic diameter (D_h) of CD-MAN, CD-GLU and their antibiotic complexes in aqueous solution, and zeta potential of these samples in PBS buffer were measured using a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA, USA) with a 633 nm He-Ne laser at a scattering angle of 173°. PBS buffer was used to stabilize the pH and ionic strength of sample solution during zeta potential measurement. Electrospray ionization mass spectra (ESI-MS) were obtained on a Bruker MicroTOF-Q system (Bruker Corporation, Karlsruhe, Germany).

Statistical Analysis

The results were reported as mean ± standard deviation (SD). Statistical assessment was made with Tukey post hoc test using

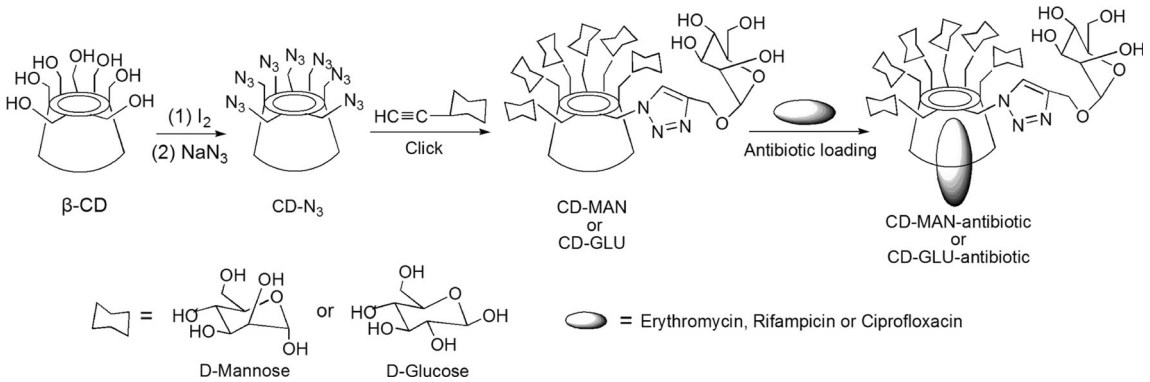
one-way analysis of variance (ANOVA). Statistical significance was accepted at $P < 0.05$.

RESULTS AND DISCUSSION

Characterization of Antibiotic Nanocarriers

The preparation of D-mannose-modified β-CD (CD-MAN) and D-glucose-modified β-CD (CD-GLU) is illustrated schematically in Scheme 1. CD-MAN (or CD-GLU) was synthesized via the azide-alkyne click reaction between CD-N_3 and mannose-alkyne (or glucose-alkyne). Azide-alkyne click reaction has gained much attention as a promising approach to synthesize new materials with almost complete conversion of reagents to a single product since it was developed in 1940s (24). In recent years, azide-alkyne click reaction has been extensively used for the synthesis of biomaterials with high conversion efficiency under mild conditions (25). The chemical structure of CD-MAN and CD-GLU were analyzed by FT-IR and UV–vis spectroscopy. In the FT-IR spectrum of β-CD (Fig. 1a), absorption bands at 3370, 1156 and 1037 cm^{-1} corresponding to the stretching vibrations of –OH, C-O and C–O-C groups, respectively, of the β-CD are observed. In the spectrum of CD-N_3 (Fig. 1b), the band at 2108 cm^{-1} is attributed to the stretching vibrations of the azide group (26). The successful conjugation of CD-N_3 and mannose-alkyne was confirmed by the FT-IR spectrum of CD-MAN (Fig. 1c), in which the characteristic band of the azide group at 2108 cm^{-1} disappeared after the azide-alkyne click reaction. The successful synthesis of CD-GLU was similarly confirmed by the disappearance of the stretching vibration absorption band of the azide group in the FT-IR spectrum of CD-GLU (Fig. 1d). In the UV–vis spectra of CD-MAN, β-CD and D-mannose (Fig. 2a), no absorption was observed for β-CD and D-mannose between 200 and 600 nm, while a strong absorption peak at around 216 nm was found in the spectrum of CD-MAN. A similar absorption was found in the UV–vis spectrum of CD-GLU, which was not present in that of D-glucose (Fig. 2b). The strong absorption at around 216 nm can be attributed to the 1,2,3-triazole rings (27) of CD-MAN and CD-GLU generated by the alkyne-azide click reaction. The molecular weight of CD-MAN and CD-GLU were analyzed by ESI-MS. The molecular weight of the as-prepared CD-MAN and CD-GLU was found to be 2860.1 for both compounds, which is consistent with the theoretical value of 2860 for $C_{105}H_{161}O_{70}N_{21}$ ($M + Na^+$), confirming the successful preparation of CD-MAN and CD-GLU.

The most well-known feature of CD is its ability to load hydrophobic agents into its inner cavity to form solid inclusion complexes. The effect of inclusion of antibiotics (erythromycin (ERY), rifampicin (RIF) and ciprofloxacin (CIP)) in the sugar-grafted CD carriers on the hydrodynamic diameter (D_h) of the carriers was investigated. The D_h and zeta potential of CD-MAN and CD-GLU before and after inclusion with antibiotics are shown in Fig. 3 and Table S1. There was no significant difference between the zeta potential of CD-MAN, CD-GLU and their antibiotic complexes. On the other hand, changes were observed in the D_h. The mean D_h of CD-MAN and CD-GLU is 3.9 and 3.8 nm, respectively, which is slightly bigger than that of unmodified β-CD (1.54 nm) (13). After antibiotic inclusion, the mean D_h of CD-MAN-antibiotic (CD-MAN-CIP, CD-MAN-RIF and CD-MAN-ERY) and CD-GLU-antibiotic (CD-GLU-CIP and CD-GLU-ERY) complexes increased to 9.8-24.4 nm, depending on the carrier and antibiotic. The increase in D_h may have resulted from some degree of aggregation of CD-MAN-antibiotic and CD-GLU-antibiotic in aqueous medium due to the hydrophobic nature of the antibiotics (ERY, RIF and CIP), similar to the aggregation of CD-loaded ERY complex (CD-ERY) reported in an earlier work (28). While the free antibiotics have low solubility in aqueous medium (≤1 mg/mL), the CD-MAN-antibiotic and CD-GLU-antibiotic complexes dissolve readily in such medium. The observed changes in the D_h of the carriers before and after antibiotic inclusion, as well as the change in the solubility of the antibiotic before and after loading into the carriers, indicate that CD-MAN-antibiotic and CD-GLU-antibiotic complexes were successfully prepared. The content of antibiotics in the complexes (wt%) is: ERY/CD-MAN-ERY = 15.02%. ERY/CD-ERY = 23.09%. ERY/CD-GLU-



Scheme 1 Schematic illustration of the preparation procedures for CD-MAN (or CD-GLU) and CD-MAN-antibiotic (or CD-GLU-antibiotic) complex.

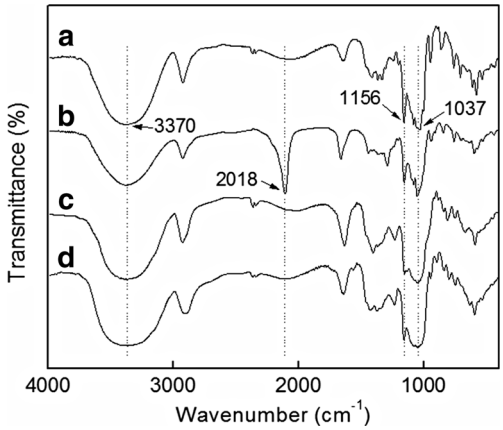


Fig. 1 FT-IR spectra of (**a**) β-CD, (**b**) CD-N_3 (**c**) CD-MAN and (**d**) CD-GLU.

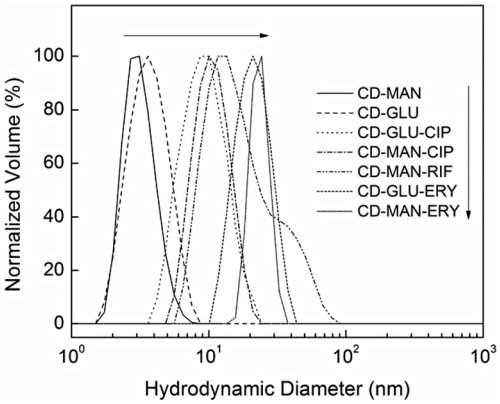


Fig. 3 Distribution of hydrodynamic diameter of CD-MAN, CD-GLU and their antibiotic complexes in aqueous medium.

ERY = 15.10%. RIF/CD-MAN-RIF = 15.38%. RIF/CD-RIF = 20.30%. CIP/CD-MAN-CIP = 8.10%. CIP/CD-GLU-CIP = 7.90%. The loading efficiency (defined as antibiotic loaded into carrier/initial amount of antibiotic) ranged from 48 to 78%, depending on the carrier and antibiotic.

Antibacterial Activity of CD-MAN Loaded Antibiotics

In this study, MIC assay was used to evaluate the antibacterial activity of CD-MAN-antibiotic complex. Four clinically important bacteria were selected for our investigations, and both susceptible and resistant strains were tested. The resistance and susceptibility breakpoints for the bacteria were based on data published by The European Committee on Antimicrobial Susceptibility Testing (29). ERY was chosen as a model antibiotic because it is the most important macrolide antibiotic and has been widely used to treat infectious diseases caused by both Gram-positive and Gram-negative bacteria for more than 60 years. However, it has been reported that common bacteria have generally been resistant to ERY since 1970s (30). As shown in Table Ii, ERY in CD-MAN-ERY complex has the lowest MIC for all the test bacteria as compared with that in CD-ERY and free ERY. *S. aureus* was selected as a model Gram-positive bacterium because it is one of the most prevailing causes of serious infections, and methicillin-resistant *S. aureus* (MRSA) is responsible for several most difficult-to-treat infections in humans (31). The MIC of ERY in CD-MAN-ERY for the ERY-susceptible strains (29), MRSA DM23605 and ATCC 25923, (MIC of free ERY = 0.5 mg/L for both) was reduced by 3.3 and 6.7 times, respectively. For the highly ERY-resistant strains, ATCC BAA-44 and MRSA DR9369, (MIC of free ERY > 1024 mg/L for both), the ERY in CD-MAN-ERY shows a reduction of MIC by a factor of >10 and >100, to 76.8 and 4.8 mg/L, respectively. These results indicate that the antibacterial activity of ERY was potentiated by forming a complex with CD-MAN even though the carrier itself (CD or CD-MAN) has no antibacterial property, as indicated by its MIC of > 1024 mg/L for all the bacteria tested.

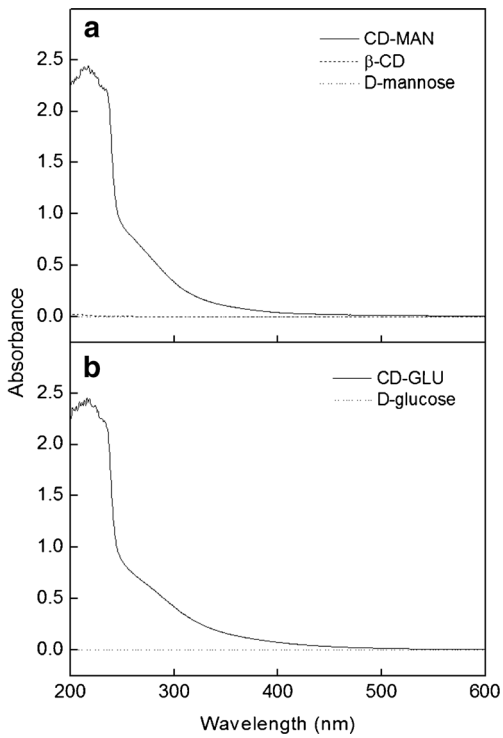


Fig. 2 UV–vis spectra of 1 mM aqueous solution of (**a**) CD-MAN, β-CD and D-mannose, and (**b**) CD-GLU and D-glucose.

Some of the Gram-negative bacteria, such as *E. coli*, *P. aeruginosa* and *A. baumannii*, are among the most challenging multidrug-resistant (MDR) microorganisms (32). For the Gram-negative bacteria, the MIC of ERY in CD-MAN-ERY for *E. coli* DH5α and *P. aeruginosa* PAO1 decreased to 9.6 and 19.2 mg/L, respectively, which is 3.3 times lower than that of free ERY (32 and 64 mg/L, respectively). In addition to ERY, rifampicin (RIF), a widely-used hydrophobic antibiotic of the rifamycin group, was also loaded into CD-MAN to test its antibacterial efficacy. As shown in Table Iii, the RIF in CD-MAN-RIF complex also shows a lower MIC for *S. aureus*, *E. coli* and *P. aeruginosa* as compared to free RIF. These results

indicate that as an antibiotic carrier, CD-MAN is able to improve the antibacterial activity of the loaded hydrophobic antibiotics against both Gram-positive and Gram-negative bacteria, possibly by rapidly increasing their intracellular concentrations.

In addition to CD-MAN-antibiotic complexes, CD-GLU-antibiotic complexes were also prepared and investigated (Scheme 1). The MIC of ERY in CD-GLU-ERY for *S. aureus* ATCC 25923 and *P. aeruginosa* PAO1 is similar to that of ERY in CD-MAN-ERY (Table Ii), which is not surprising since both glucose and mannose are carbon sources for *S. aureus* and *P. aeruginosa*. A similar result was obtained for CIP, a fluoroquinolone antibiotic, when loaded in CD-MAN or CD-GLU against *P. aeruginosa* PAO1 (Table Iiii). Further investigation with CIP was carried out against *A. baumannii*. Both CD-GLU-CIP and CD-MAN-CIP complexes was able to reduce its MIC for *A. baumannii* S1 (Table Iiii) by a factor of ~6. This result is consistent with the postulate that the sugar-modified carriers deliver the antibiotic more effectively into the bacterial cells. It should be noted that the above-mentioned results were obtained in simple media. The efficacy of this carrier system may be different when applied in different biological environments (such as via intravenous injections or eye drops) since biomolecules present in a particular environment may be capable of displacing the loaded antibiotic. Furthermore, the pH of biological environments may also affect the antibiotic release kinetics since an antibiotic can have different degree of ionization at different pH, and unionized drugs usually form more stable complexes with CD carrier than their ionic counterparts (33). The three antibiotics used in this work have different ionization constant (pKa): ERY 8.8, RIF 1.7 and 7.9, CIP 6.1 and 8.7. Depending on the antibiotic, the effect of pH in the range of physiological interest on antibiotic release may or may not be significant.

It has been reported that there are several mechanisms involved in the development of bacterial resistance to antibiotics, namely 1) changes in the antibiotic-binding sites on RNA/proteins to prevent the antibiotic action, 2) hydrolysis of antibiotics by enzymes, 3) active efflux to decrease the intracellular concentration of antibiotics, and 4) alteration in the permeability of the outer membrane of Gram-negative bacteria to antibiotics (34). Among these mechanisms, the activity of the efflux system can confer bacterial resistance to macrolides. To investigate whether the CD-MAN-ERY complex will potentiate the antibiotic activity in efflux-proficient bacteria, we compared the antimicrobial activity of ERY in an efflux-proficient strain of *P. aeruginosa* (PAO1) and its efflux-deficient derivative, PAO397 (Table I). The MIC of free ERY for *P. aeruginosa* PAO397 (8 mg/L) was eight times lower than that for *P. aeruginosa* PAO1 (64 mg/L), indicating that the efflux system has a significant contribution to ERY resistance in *P. aeruginosa* PAO1. In the efflux-proficient PAO1 strain, the

Table I MIC of antibiotic in CD-MAN-antibiotic, CD-GLU-antibiotic, CD-antibiotic complex, and free antibiotic for different bacterial strains

(i) Bacterium	Gram	Number of strains	Strain name	MIC (mg/L)		
				ERY in CD-MAN-ERY (CD-GLU-ERY)[a]	ERY in CD-ERY	Free ERY
S. aureus[b]	Positive	4	MRSA DM23605	**0.15**	**0.23**	**0.5**
			ATCC 25923	**0.075** (**0.075**)[a]	**0.12**	**0.5**
			MRSA DR9369	4.8	7.36	>1024
			ATCC BAA-44	76.8	>1024	>1024
E. coli	Negative	1	DH5α	9.6	29.4	32
P. aeruginosa	Negative	2	PAO1	19.2 (19.2)[a]	29.4	64
			PAO397 (PAO1 without efflux pumps)	4.8	7.36	8
(ii) Bacterium	Gram	Number of strains	Strain name	MIC (mg/L)		
				RIF in CD-MAN-RIF	RIF in CD-RIF	Free RIF
S. aureus[b]	Positive	1	ATCC 25923	**0.0012**	**0.0033**	**0.016**
E. coli	Negative	1	DH5α	1.23	1.62	4
P. aeruginosa	Negative	1	PAO1	4.92	12.99	32
(iii) Bacterium	Gram	Number of strains	Strain name	MIC (mg/L)		
				CIP in CD-MAN-CIP	CIP in CD-GLU-CIP	Free CIP
P. aeruginosa[b]	Negative	1	PAO1	**0.088**	**0.088**	**0.25**
A. baumannii[b]	Negative	1	S1	**0.044**	**0.044**	**0.25**

[a] The value in brackets means the MIC of ERY in CD-GLU-ERY complex for the corresponding bacterial strain

[b] Values indicating susceptibility to the antibiotic are highlighted in bold. For *S. aureus*, MIC of >2 mg/L of ERY is considered resistant and MIC of ≤1 mg/L is considered susceptible to ERY; MIC of ≤0.06 mg/L of RIF is considered susceptible and >0.5 mg/L is considered resistant to RIF. For *P. aeruginosa*, MIC ≤0.5 mg/L of CIP is considered susceptible and MIC > 1 mg/L is considered resistant to CIP. For *A. baumannii*, MIC ≤1 mg/L of CIP is considered susceptible and MIC > 1 mg/L is considered resistant to CIP. There are no corresponding resistance and susceptibility breakpoints for ERY for *E. coli* and *P. aeruginosa*, or RIF for *E. coli* and *P. aeruginosa* (29)

MIC for ERY was reduced by 3.3 times using CD-MAN-ERY as compared to free ERY. By comparison, the corresponding MIC reduction for the efflux-deficient *P. aeruginosa* PAO397 strain was only 1.7 times. This suggests that the CD-MAN carrier can potentiate the activity of the loaded antibiotic even in efflux-proficient bacterial strains. The mechanism by which the carrier is able to accomplish this is not clear at present, but it is plausible that effective delivery of the antibiotic into the bacterial cytoplasm rapidly increases its intracellular concentrations and thus optimizes its activity.

Previous studies have reported that antibiotics in CD-antibiotic complexes have a lower MIC (usually by a factor of 2 to 8) than the free form for some bacterial strains because of the inhibition of enzymatic hydrolysis and improved intestinal permeability of the antibiotic after inclusion in β-CD or β-CD derivatives (methylated β-cyclodextrin and hydroxypropyl-β-cyclodextrin). However, the MIC of these CD-loaded antibiotics are still similar to their free form for other bacterial strains (35). Similarly, in the present study, it was found that although the ERY loaded in the CD carrier exhibited lower MIC for some of the tested bacterial strains, its efficacy against *S. aureus* ATCC BAA-44, *E. coli* DH5α and *P. aeruginosa* PAO397 is not different from that of free ERY (Table Ii). On the othr hand, with the grafting of mannose and glucose moieties on the CD carrier, ERY in CD-MAN-ERY and CD-GLU-ERY complexes was able to reduce the MIC for the bacteria indicated in Table I by a factor of 3 to >100, with the exception of efflux-deficient *P. aeruginosa* PAO397 for reasons mentioned above. While it is not entirely clear how the CD carrier increases the intracellular concentration of the antibiotic, it is known that CDs can pass through the bacterial outer membrane via a specific transport system (porins) (36). In addition, D-mannose, like D-glucose, is a carbon source for many bacteria which have transporters and active uptake systems for mannose on the cell membrane. Thus, bacteria, through their sugar sensing mechanisms, will be attracted to the sugar on the CD carriers. We postulate that in the subsequent interactions between the bacterial cell and CD carrier, the antibiotic will be released from the carrier, perhaps by actions of bacterial enzymes, resulting in high local concentration of the antibiotic which enhances its entry into the cell. Thus, in the presence of β-CD-degradable enzymes like α-amylase (37), a faster antibiotic release from CD-MAN-antibiotic and CD-GLU-antibiotic complexes can be expected. The presence of free D-mannose on the efficacy of the CD-MAN carrier was investigated by determining the MIC of CD-MAN-ERY on *S. aureus* ATCC25923 in MHB medium containing different concentrations of free D-mannose (1 g/L and 10 g/L, respectively). It was found that the MIC values are the same as that without D-mannose. Thus, an excess amount of free D-mannose did not competitively affect the interactions of the bacterial cells with CD-MAN-ERY.

To further investigate the mechanism for the enhanced antibacterial activity of the CD-MAN-antibiotic complex, a hydrophobic fluorescent dye probe PBI was loaded into CD-MAN to evaluate the ability of CD-MAN loaded drugs to permeate into the bacterial cells. The uptake of hydrophobic fluorescent dyes by bacteria is commonly applied as a method to gauge bacterial resistance to hydrophobic antibiotics (23). The fluorescent images of bacteria after incubation with free PBI, and PBI loaded into CD (CD-PBI) and CD-MAN (CD-MAN-PBI) are illustrated in Fig. 4. As shown in Fig. 4a"', *E. coli* DH5α incubated with CD-MAN-PBI exhibited the strongest fluorescence intensity as compared to those incubated with the other two agents (Fig. 4a, a' and a"). Similar results for *P. aeruginosa* PAO1 and *S. aureus* ATCC 25923 were also observed in Fig. 4b-b"' and c-c"', respectively. In addition, the accumulation assay results (Fig. S2) show that the accumulation of PBI from CD-MAN-PBI in these bacteria after 4 h is significantly higher than that from CD-PBI and free PBI, which is consistent with the results in Fig. 4. These results suggest that antibiotic loaded in CD-MAN carriers is delivered into the bacterial cells more effectively than either the antibiotic loaded in the CD carrier or the free form antibiotic. A recent study has reported that the strongest resistance of *S. aureus* to free ERY (MIC > 1024 mg/L, as shown as by MRSA DR9369 and ATCC BAA-44 strains in Table Ii) is the result of decreased uptake of the antibiotic into the bacterial cells (38). Thus, the ability of CD-MAN to effectively deliver the loaded antibiotic into bacterial cells is of crucial importance in potentiating its efficacy against these highly resistant bacteria.

Antibiotic Resistance Assay

Antibiotic resistance assays using *S. aureus* ATCC 25923 and *E. coli* DH5α were carried out to assess their potential to develop resistance to free antibiotic, CD-antibiotic and CD-MAN-antibiotic complexes. Table IIi shows the resistant *S. aureus* ATCC 25923 count after the antibiotic resistance assay. After incubation with CD-MAN-ERY, CD-ERY and free ERY, respectively, for 8 days, no resistant bacteria were found for these agents. When the incubation period was increased to 16 days, about 1 in 26700 and 1 in 25300 bacterial cells developed resistance to free ERY and CD-ERY, respectively. However, no resistant bacteria against CD-MAN-ERY were found after incubation for 16 days. Table IIii shows the corresponding antibiotic resistance assay results for *E. coli* DH5α. After incubation with CD-MAN-RIF, CD-RIF and free RIF, respectively, for 8 days, no resistant bacteria for all the agents was found. When the incubation period was increased to 16 days, *E. coli* developed resistance to CD-RIF and free RIF. The corresponding number of resistant bacteria exposed to CD-RIF and free RIF is 1 in 6210 and 1 in 6250, respectively, indicating that antibiotic loaded into CD is not able to inhibit the evolution of bacterial resistance. However, no resistant bacteria against CD-MAN-RIF were found after

incubation for 16 days. Detailed results of the antibiotic resistance assay are given in Table S2 and S3. We postulate that CD-MAN-ERY and CD-MAN-RIF did not promote the development of intrinsic resistance in *S. aureus* ATCC 25923 and *E. coli* DH5α, unlike CD-ERY, CD-RIF and the free form antibtiotics, because enhanced delivery of the antibiotics from the sugar-modified carriers into the bacterial cytosol resulted in rapid attainment of its effective intracellular concentration.

The bacterial lysis assay results are shown in Fig. 5. The higher OD_{260} of the bacterial suspension incubated with CD-MAN in PBS as compared to incubation with only PBS can be attributed to the absorbance of CD-MAN carrier, as shown in Fig. S3. However, the OD_{260} of the bacterial suspension (*S. aureus*, *E. coli* and *P. aeruginosa*) changed very little with time when incubated with either a high-dose of CD-MAN carrier in PBS (0.3 g/L) or just PBS (negative control). On the other hand, when the bacterial suspension was incubated with bacterial lysis buffer (positive control), a significant increase in OD_{260} was observed within the first hour (Fig. 5). These results indicate that the CD-MAN carrier does not cause bacterial cell lysis and the principle of enhancing the delivery of antibiotics into bacteria using such carriers does not rely on damaged cell membrane. It has been reported that nanoparticles which damage bacterial cell membranes promote the horizontal conjugative transfer of multidrug-resistance genes and may increase antibiotic resistance (11). Hence, this problem may not arise with carriers such as CD-MAN which do not cause cell lysis.

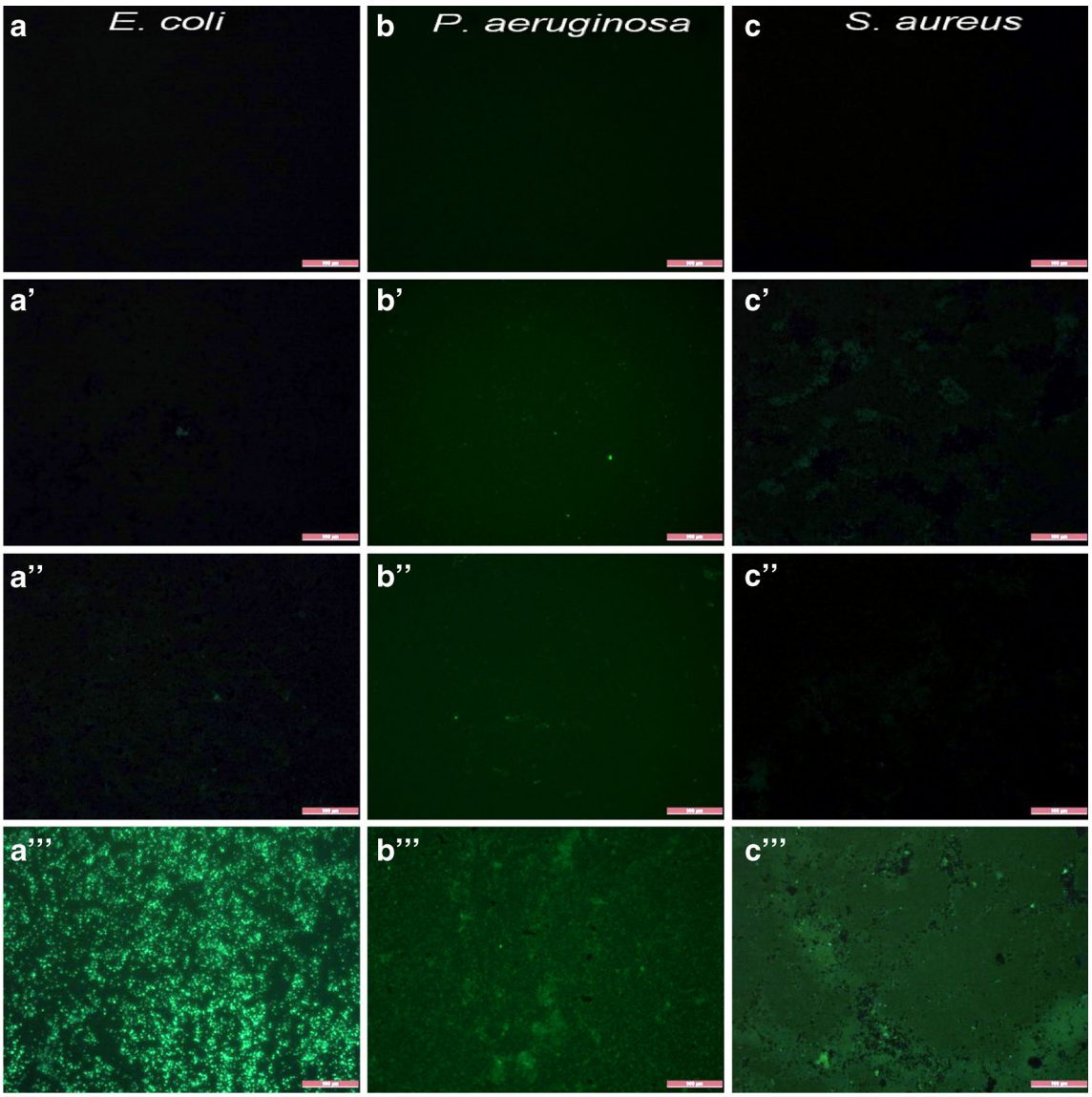


Fig. 4 Fluorescence microscopy images showing accumulation of a hydrophobic fluorescent dye probe (3,4,9,10-perylenetetracarboxylic 3,4:9,10-dianhydride, PBI) in *E. coli* DH5α (**a-a'''**), *P. aeruginosa* PAO1 (**b-b'''**) and *S. aureus* ATCC 25923 (**c-c'''**) after incubation for 4 h in culture medium only (**a-c**), culture medium containing free PBI (**a'-c'**), culture medium containing CD-PBI (**a''-c''**) and culture medium containing CD-MAN-PBI (**a'''-c'''**). Scale bar is 100 μm.

Stability

The stability of the antibiotic in the CD-MAN-antibiotic complex was evaluated by bacterial growth inhibition assay over an extended period. As shown in Fig. 6a, *E. coli* when cultured in growth medium without antibiotic grew well and the bacterial concentration increased (as indicated by the increasing OD_{600}) with increasing time. When culture medium with free ERY and CD-MAN-ERY at their respective MIC was used, the growth of *E. coli* can be inhibited for only 1 day (Fig. 6a and a'). The loss of ERY's antibacterial activity can be attributed to its decomposition by hydrolysis in the medium (39). On the other hand, when culture medium with 2 times the respective MIC of free ERY and CD-MAN-ERY was used, the growth of *E. coli* was inhibited for 3 and 18 days, respectively (Fig. 6a and a'). Similarly, with *P. aeruginosa* (Fig. 6b and b') and *S. aureus* (Fig. 6c and c'), bacterial growth was surpressed even after 18 days with 2 times the respective MIC of CD-MAN-ERY whereas free ERY at the corresponding MIC lost its efficacy much earlier. These results indicate that ERY in CD-MAN-ERY is more stable and its antibacterial activity is preserved for a much longer period than the free form.

Cytotoxicity

The cytotoxicity of the free ERY, CD-MAN-ERY complex and CD-MAN carrier was evaluated on a variety of mammalian cell lines. Murine 3T3 fibroblasts and RAW 264.7 murine macrophages were evaluated using the MTT assay. 3T3 fibroblasts were used as model cells to study cytotoxicity of CD-MAN-ERY complex *in vitro* as fibroblasts play a critical role in fibrous encapsulation and wound healing due to their ability to synthesize proteins of extracellular matrix (40). The viability of 3T3 fibroblasts incubated with free ERY of concentration ranging from 8 to 512 mg/L (i.e. 1/8 to 8 times MIC for *P. aeruginosa* PAO1) for 24 and 72 h was higher than 85% in all cases (Fig. 7a). The viability of the cells incubated with CD-MAN-ERY complex of concentration ranging from 8 to 2048 mg/L (containing 1.2 to 307.2 mg/L of ERY, corresponding to 1/16 to 16 times MIC for *P. aeruginosa* PAO1) was also higher than 85% (Fig. 7b). Similarly, the viability of the cells incubated with CD-MAN carrier of concentration ranging from 125 to 2000 mg/L was higher than 85% (Fig. S4). There is no significant difference in cell viability when the cells were incubated with either CD-MAN-ERY complex or CD-MAN carrier, compared to that with free ERY and the control experiment (cells incubated in growth medium only), indicating that CD-MAN-ERY possesses no significant cytotoxicity to 3T3 fibroblasts and the complexation with CD-MAN does not increase the cytotoxicity of ERY.

Table II Resistant bacterial count after antibiotic resistance assay

(i) *S. aureus* ATCC 25923		
Culture medium	After 8 days	After 16 days
TSB + 0.25 × MIC[a] CD-MAN-ERY	0	0
TSB + 0.25 × MIC[a] CD-ERY	0	1 in 26700
TSB + 0.25 × MIC[a] free ERY	0	1 in 25300
(ii) *E. coli* DH5α		
Culture medium	After 8 days	After 16 days
NB + 0.25 × MIC[b] CD-MAN-RIF	0	0
NB + 0.25 × MIC[b] CD-RIF	0	1 in 6210
NB + 0.25 × MIC[b] free RIF	0	1 in 6250

[a] Refer to Table I for MIC value of free ERY and ERY in CD-MAN-ERY and CD-ERY. ERY in CD-MAN-ERY and CD-ERY constitutes 15 wt% and 23 wt%, respectively

[b] Refer to Table I for MIC value of free RIF and RIF in CD-MAN-RIF and CD-RIF. RIF in CD-MAN-RIF and CD-RIF constitutes 15 wt% and 20 wt%, respectively

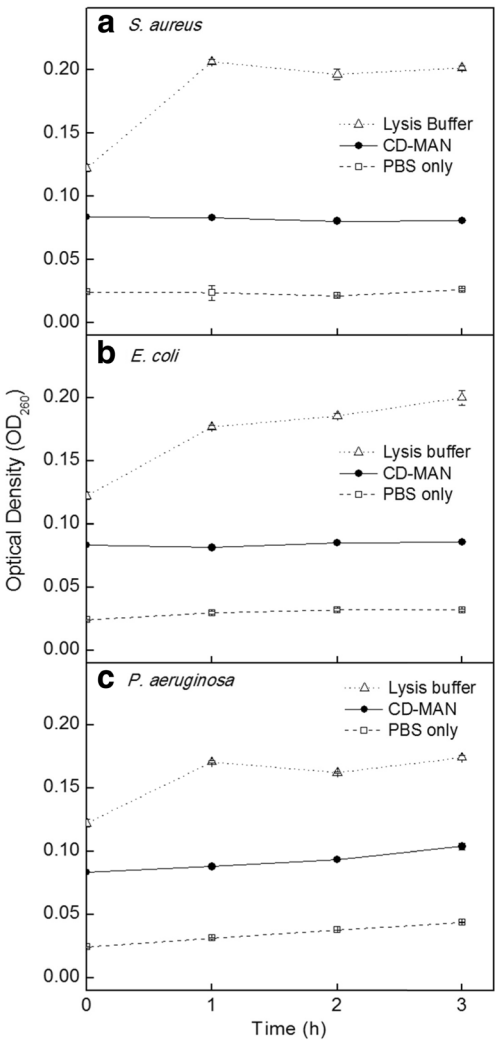


Fig. 5 Comparison of OD_{260} of (**a**) *S. aureus* ATCC 25923, (**b**) *E. coli* DH5α and (**c**) *P. aeruginosa* PAO1 suspension after incubation with CD-MAN in PBS. Bacterial lysis buffer and PBS were used as positive and negative control, respectively.

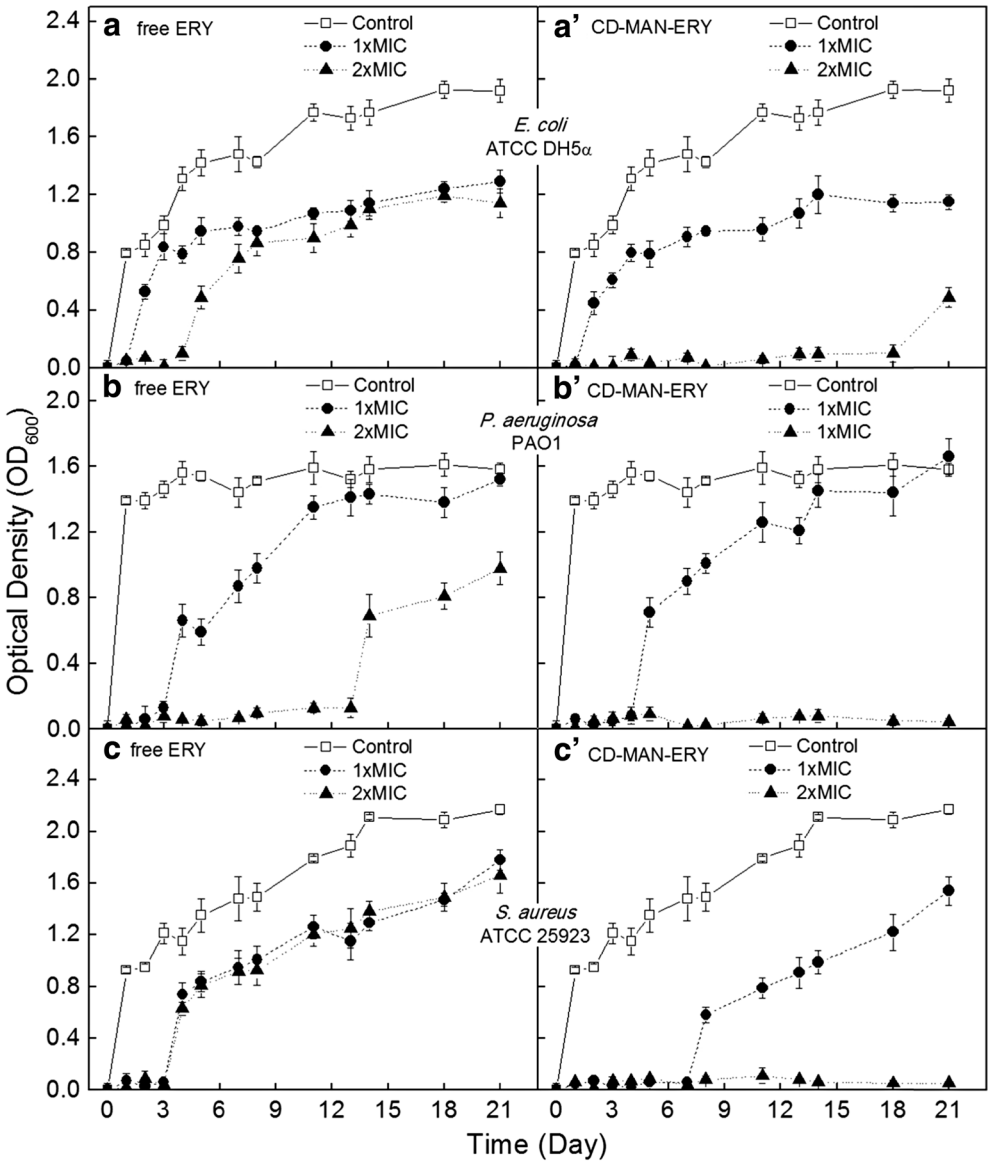


Fig. 6 Comparison of antibacterial activities of free ERY versus CD-MAN-ERY using growth curves for (**a**,**a'**) *E. coli* DH5α, (**b**,**b'**) *P. aeruginosa* PAO1 and (**c**,**c'**) *S. aureus* ATCC 25923. Growth of bacteria (as indicated by OD_{600}) was monitored over 21 days when cultured in medium containing free ERY (**a-c**) or CD-MAN-ERY (**a'-c'**) at MIC or 2 times MIC. Control experiments were carried out in the culture medium without ERY or CD-MAN-ERY.

MTT assay was also carried out with RAW 264.7 macrophages, which express mannose receptors on their cell surface. The results showed that after incubation with CD-MAN-ERY complex at a concentration 2048 mg/L for 24 h, the viability

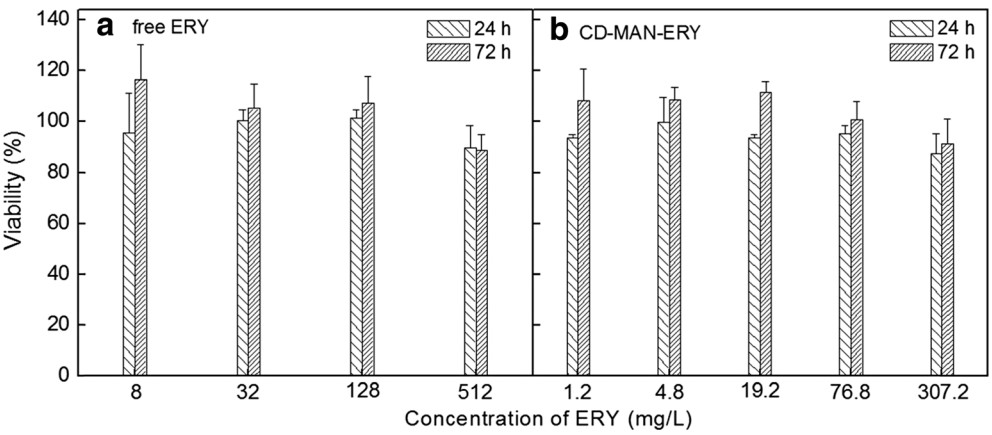


Fig. 7 Effect of (**a**) free ERY and (**b**) CD-MAN-ERY on the viability of 3T3 fibroblasts after 24 and 72 h. Viability is expressed as a percentage relative to the result obtained with the control (3T3 fibroblast cells incubated in growth medium only).

of macrophages was ~88%, and there was no significant difference in cell viability as compared to the control experiment (cells incubated in growth medium only), indicating that CD-MAN-ERY possesses no significant cytotoxicity to RAW 264.7 macrophages. In addition to murine 3T3 fibroblasts and RAW 264.7 macrophages, human NCM460 colonic epithelial and HEK 293T embryonic kidney cells were also used for the evaluation of cytotoxicity of CD-MAN-ERY with Tali™ scan analysis. As shown in Fig. S5, more than 85% of NCM460 and HEK 293T cells remained viable after incubation with 5 to 400 mg/L of free ERY for 24 h. Similarly, more than 80% of NCM460 and HEK 293T cells remained viable after incubation with CD-MAN-ERY, even at a very high dosage (2000 mg/L, containing 300 mg/L of ERY, corresponding to ~16 times of MIC for *P. aeruginosa* PAO1) for 24 h. These findings are very similar to those obtained from the MTT assay, and confirm that the CD-MAN-ERY has no significant cytotoxicity to mammalian cells over the concentration range tested.

CONCLUSIONS

Mannose and glucose molecules were grafted on modified β-cyclodextrin via alkyne-azide click reaction. The resultant CD-MAN and CD-GLU were used as nanocarriers for loading hydrophobic antibiotics (ERY, RIF and CIP) and delivering these antibiotics into Gram-positive (*S. aureus* including MRSA) and Gram-negative (*E. coli*, *P. aeruginosa* and *A. baumannii*) bacteria. These sugar-modified nanocarriers enhanced the delivery and accumulation of the loaded antibiotic in the bacterial cells, and as a result, the minimum inhibitory concentration of the loaded antibiotics against the tested bacteria were reduced by a factor ranging from 3 to >100 compared to the free antibiotics. In addition, the CD-MAN-antibiotic complex is able to inhibit the development of antibiotic resistance of *S. aureus and E. coli*, and prolong the stability of the loaded antibiotic and its activity to inhibit bacterial growth. In view of the paucity of new antibiotics in the pipeline, it is highly advantageous that these non-cytotoxic sugar-modfied nanocarriers can potentiate the activity of existing antibiotics, especially against multidrug-resistant bacteria. However, *in vivo* evaluation of the performance of these sugar-modified nanocarriers is necessary since the *in vitro* assays cannot duplicate the complicated biological environment, and the performance of these carriers in oral or intravenous applications may be very different from that in topical application.

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