Combating Antibiotic-Resistant Gram-Negative Bacteria Strains with Tetracycline-Conjugated Carbon Nanoparticles

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Nontoxic carbon nanoparticle samples prepared by both bottom-up and top-down approaches do not inhibit Gram-negative bacterial growth, indicating excellent biocompatibilities. However, cell growth inhibitory efficacies increase considerably when the carbon nanoparticles are conjugated with the antibiotic tetracycline. In tetracycline-resistant bacteria, these efficacies can approach tenfold higher activities when compared to tetracycline alone. No structural abnormality such as membrane disruptions is evident in the tested bacterial strains; this is in contrast with other nanocarbon systems such as graphene oxides, carbon nanotubes, and amine-functionalized carbon nanoparticles which do exhibit membrane disruptions. In comparison, the tetracycline-conjugated carbon nanoparticles induce membrane perturbations (but not membrane disruptions), inhibiting bacterial efflux mechanisms. It is proposed that when tetracycline is conjugated to the surface of carbon nanoparticles, it functions to direct the nanoparticles to membrane-associated tetracycline efflux pumps, thereby blocking and subsequently inhibiting their function. The conjugation between biocompatible carbon nanoparticles and subtherapeutic but well-established antibiotic molecules may provide hybrid antibiotic assembly strategies resulting in effective multidrug efflux inhibition for combating antibiotic resistance.

1. Introduction

The development of modern antimicrobial therapies has saved countless lives and allowed modern medicine to treat infectious diseases that would otherwise cause serious illness and/or mortality. However, the excessive use of antibiotics via irrelevant and unregulated access,^[1] and their use in food^[2] and household^[3] products has decreased the efficacy of these therapies. As a result, pathogenic bacteria have acquired genes that provide resistance to many current antimicrobials,^[4] and, consequently, these antibiotic-resistant bacteria have become a growing threat to public health.^[5] Early-generation antibiotics are now less effective or even completely ineffective against many bacterial pathogens. Therefore, there is an urgent need for the continuous modifications of existing antibacterial scaffolds, as well as the discovery of new antibiotic classes.^[6] Indeed, the World Health Organization (WHO) considers the development of antibiotic resistance

as the most serious issue currently facing medical science; thus, new effective antibiotics are urgently needed.^[7] The rise of antibacterial resistance and the challenges of the conventional approaches have led to a very small portfolio of new therapies.^[8] Potential approaches to extend the useful therapeutic life of the available antibiotics include cycling different classes of antibiotics,^[9] repurposing of withdrawn drugs,^[10] and combination of multiple drugs simultaneously.^[4] However, these strategies also possess subsequent drawbacks related to efficacy costs of these therapies^[9] and potential patient side effects.^[11]

Recent progress in our understanding of the structural changes and associated mechanisms of bacterial resistance offer considerable opportunity to address the problem at source, rather than simply generating additional future resistances.^[4–6,8] Among new approaches, systems containing nanoparticles such as antimicrobial polymers,^[12] peptides,^[13] Ag,^[14] Au,^[15] and graphene oxide^[16] have been studied as alternative to classical antibacterial compounds due to their effectiveness against a broad spectrum of bacteria and biocompatibility arising from their size and functional groups. Similarly, in the field of biomedicine,^[17] carbon nanoparticles (CNPs) have been investigated as sensitive and efficient bioprobes owing to their excellent biocompatibility, drug carrying, and direct imaging

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capabilities. Most biocompatible CNPs are functionalized with oxygen-containing functional groups on the particle surface, demonstrate stable fluorescence (FL) properties, and provide accessibility to their surface for further structural modifications/elaborations. Therefore, CNPs may have potential for the construction of a range of novel and effective antimicrobial agents.

Herein, we report the assembly of tetracycline (TC)-conjugated carbon nanoparticle systems, prepared using both bottom-up and top-down approaches that demonstrate consistent antibacterial results regardless of the synthetic strategies utilized in their assembly. Similar to other CNP systems reported previously,^[18] the prepared CNPs demonstrated excellent biocompatibilities. Notably, the TC-conjugated CNPs displayed substantially increased antibacterial activities over TC alone and appear to function by inhibition of drug extrusion processes mediated by efflux pumps in tetracycline (multidrug)-resistant bacteria strains including *Klebsiella pneumoniae*, *Acinetobacter baylyi*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Proteus mirabilis*.

Efflux pumps are found in almost all bacterial species. The active efflux of antibiotics and the drug extrusion by multidrug efflux pumps serves as one of the important mechanisms of multidrug resistance.^[19] Efflux pumps not only expel a broad range of antibiotics, owing to their poly-substrate specificity^[19] but drive an additional resistance mechanism by also reducing cellular antibiotic influx processes.^[20] Accordingly, the over-expression of multidrug efflux pumps is increasingly associated with clinically relevant drug resistance.^[21] The active efflux of antibiotics has been identified

as having a predominant role in the resistance to certain individual drugs such as tetracycline. Furthermore, previously published literature suggests that efflux mechanisms are likewise responsible for nanoparticle-based antibacterial resistance in Gram-negative bacteria.^[22] Thus, efflux pump inhibitors represent an attractive alternative to address the increasing multidrug resistance.^[23]

The use of biocompatible and fluorescently traceable CNPs may provide an excellent platform for studies of macromolecular systems that attempt to address the problems of the multidrug-resistant bacteria. Instead of inhibiting these multidrug efflux mechanisms, promotion of efflux can be facilitated using therapeutically ineffective antibiotics which are conjugated onto the carbon nanoparticles. This drives the nanoparticle assemblies toward the cell membrane modulating the efflux pumps function.

2. Results and Discussion

2.1. Carbon Nanoparticles and Their Surface Functionalities

We prepared two types of CNPs using both bottom-up and top-down approaches, allowing the direct comparison of their respective biological properties and efficacies. The bottom-up CNPs (CNP-BUP) were prepared using propylene glycol and citric acid in a hydrothermal reactor^[24] and the top-down CNPs (CNP-TDn) were prepared by a modified Hummer's method.^[25] The as-synthesized samples contained residual reaction products such as large agglomerates from the CNP-BUP

Figure 1. TEM and particle size distribution (inset) of a) CNP-BUP, b) CNP-TDn, c) high-resolution XPS C1s spectra of CNP-BUP and CNP-TDn (survey scan results are available in the Supporting Information). d) Schematic of bare and tetracycline-conjugated CNPs. e) IR spectra. f) UV-vis absorption spectra, g) FL emission spectra at $\lambda_{\text{ex}} = 405$ nm. h) TCSPC FL decays of the CNPs and their conjugates in comparison with TC.

and graphene oxide sheets from the CNP-TDn methods (Figure S1, Supporting Information). Consequently, the as-synthesized crude sample CNPs were further purified via centrifugation, filtration, and dialysis to obtain monodispersed samples. As presented in Figure 1a,b, the majority of the particles were measured between 2 and 15 nm with large particles up to 25 nm derived from the CNP-TDn, determined using transmission electron microscopy (TEM). Atomic force microscopy (AFM; Figure S2, Supporting Information) analysis also revealed particles between 2 and 15 nm, which correlates well with the TEM data. X-ray photoelectron spectroscopy (XPS) survey scan results (Figure S3, Supporting Information) indicated that both CNPs have similar carbon and oxygen atomic ratio at $\approx 7:3$. High-resolution C1s and O1s (Figure 1c; Figure S3, Supporting Information) further revealed the various oxygenic groups including C–O, C=O, and COOH. The IR spectra (Figure 1e) also suggested highly oxidized carbon functionalities including C–O, C=O, and COOH.^[26]

2.2. Tetracycline and Carbon Nanoparticle Conjugates

As a broad-spectrum second-generation antibiotic, tetracycline has been widely used to treat various bacterial diseases. Unfortunately, the development of resistance to TC has been rapid, and its clinical usefulness has been actively declining over recent years.^[27] Thus, TC systems are ideal antibiotic models to compare and contrast the efficacies of TC versus TC–CNP conjugate systems. The TC moieties were attached to the surface of the prepared CNPs by direct conjugation of the carboxyl groups on the CNPs with the available hydroxyl group of the TC via esterification (Figure 1d).^[28] The IR spectra obtained for the TC–CNPs were similar to the IR spectrum of the untethered TC (Figure 1e; full range IR spectra are available in the Supporting Information), but a stretch of C=O of carbonyl at 1770 cm^{-1} and stretches of C–O at 1250 and 1065 cm^{-1} , which are assignable to the ester formed during the conjugation, were noted from the TC–CNP conjugates only. The emergence of the ester functionalities within the TC–CNP conjugates is symptomatic of covalent attachment the TC attachment onto the surface of the CNPs.^[29]

2.3. Optical Properties of Carbon Nanoparticles and Tetracycline Conjugates

UV–visible (UV–vis) absorbance of the bare CNPs (Figure 1f) was comparable to other types of nonfunctionalized CNPs reported previously, with no distinctive absorbance peaks.^[30] UV–vis absorbance values of the untethered TC and the TC–CNPs were similar to each other, indicating no notable changes in the chemistry of the TC chromophore. However, a close inspection of the UV–vis spectra reveals that the two absorption maxima arising from TC (at 276 and 356 nm) were shifted to 274 and 359 nm after the TC and CNP conjugation. This may be rationalized by changes in the electrostatic environment experienced between the tethered TC moieties and the surface of the CNPs.^[31] These interactions between the TC and CNPs likely result in changes in the absorption transition dipole

moments, which reflect in the absorption maxima shifts due to the covalent attachment of the TC.^[31,32]

FL emission spectra from the bare CNPs were also similar to other types of nonfunctionalized CNPs, displaying typical nonsymmetrical blue FL emissions.^[30] The FL emissions of the TC–CNP conjugates showed green dominant emission, while the FL emission from the untethered TC showed yellow emission. The bathochromic FL emission shift from the bare CNPs and hypsochromic shift from TC in Figure 1e are highly likely to have originated from the changes in the CNPs' surface states, further confirming the conjugation of the TC on the surface of the CNPs.^[33] FL decays of the bare CNPs and the TC–CNPs were examined using time-correlated single photon counting (TCSPC) techniques. The decays in Figure 1h displayed notably delayed FL decays from the TC–CNPs. This is also due to the covalently tethered TC changing the surface states of the CNPs.^[34]

2.4. Antibacterial Efficacy of Tetracycline Nanoparticle Conjugates

The antibacterial activities of the conjugates were initially evaluated by an agar diffusion assay system as it serves as a guide to deduce the antibacterial potency of the samples.^[35]

Therefore, the antibacterial efficacy of the conjugates was evaluated by determination of their minimum inhibitory concentration (MIC) against known antibiotic-resistant *Enterobacteriaceae* strains including, *K. pneumoniae*, *A. baylyi*, *P. aeruginosa*, *P. vulgaris*, and *P. mirabilis* using the well-established iodonitrotetrazolium chloride colorimetric assay.^[35] Several strains of Gram-positive bacteria (*Bacillus megaterium*, *Bacillus cereus*, *Staphylococcus aureus*, *Staphylococcus pyogenes*) displayed only minor antibacterial activities. Therefore, our discussion is focused on the response of the Gram-negative bacteria to the TC–CNP conjugates. However, for comprehensiveness, the results from the Gram-positive bacteria are available in the Supporting Information.

No bacterial inhibitory activities were observed from either the CNP-BUP or CNP-TDn when tested alone on both Gram-positive and Gram-negative species, representative of the high degree of biocompatibility of the bare, unmodified CNPs (Table 1; Tables S5 and S6, Supporting Information), and are consistent with a previous report on the antibacterial activities of CNPs of this type.^[24] However, as presented in Table 1 and Figure 2, *K. pneumoniae*, *A. baylyi*, and *P. vulgaris* were inhibited strongly in comparison with *P. aeruginosa* and *P. mirabilis*. The lowest MIC (2×10^{-6} M equivalent TC concentration) was exhibited by the TC–CNP-BUP conjugate against *A. baylyi*, whereas the MIC of TC alone in this cell line was 21×10^{-6} M, an approximate tenfold potentiation in activity. The growth inhibition of *K. pneumoniae* and *P. vulgaris* was also notably more pronounced; a five times lower MIC being recorded from the TC–CNP conjugates compared with the concentration of unmodified TC required to produce the same inhibitory efficacy.

The *P. aeruginosa* and *P. mirabilis* assays resulted in substantially higher MIC values than the other bacteria tested across the CNP conjugates. However, the strength of the growth

Table 1. Antibacterial activities of CNPs and TC–CNPs expressed in minimum inhibitory concentration, $\times 10^{-6}$ M. The molarity of the TC–CNP conjugates is based on the equivalent amount of TC attached onto the CNPs obtained from extinction coefficient (this method was validated using direct conjugation technique using Fuchsine red; see the Supporting Information for further information). Each value represents the mean MIC $\pm$ standard deviation ($\times 10^{-6}$ M) measured from triplicates. *P*-values are calculated using one-sample *t*-test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001. †No inhibitory activities were recorded within the TC concentrations tested.

	<i>Klebsiella pneumonia</i>	<i>Acinetobacter baylyi</i>	<i>Pseudomonas aeruginosa</i>	<i>Proteus vulgaris</i>	<i>Proteus mirabilis</i>
CNP-BUp	–	–	–	–	–
CNP-TDn	–	–	–	–	–
TC + CNP-BUp	17.2 $\pm$ 1.0**	17.8 $\pm$ 1.8**	19.9 $\pm$ 0.8***	17.2 $\pm$ 1.6**	†
TC + CNP-TDn	21.4 $\pm$ 1.9**	19.8 $\pm$ 2.0**	19.2 $\pm$ 1.4**	19.4 $\pm$ 1.9**	†
TC–CNP-BUp	3.8 $\pm$ 0.8**	2.0 $\pm$ 1.2	15.5 $\pm$ 0.7***	3.8 $\pm$ 1.6*	50.9 $\pm$ 1.0***
TC–CNP-TDn	2.7 $\pm$ 1.0*	2.7 $\pm$ 0.8*	11.6 $\pm$ 0.9**	2.7 $\pm$ 1.9	39.1 $\pm$ 0.9***
Tetracycline	20.8 $\pm$ 1.2**	20.8 $\pm$ 1.4**	20.8 $\pm$ 1.2**	20.8 $\pm$ 1.0***	†
Chloramphenicol (Positive control)	7.7 $\pm$ 1.8**	30.9 $\pm$ 1.3***	30.9 $\pm$ 1.1***	3.9 $\pm$ 0.7**	15.5 $\pm$ 0.6***
Culture medium (Negative control)	–	–	–	–	–

inhibition by the TC–CNPs was subsequently potentiated in contrast with the growth inhibition by TC on its own or the positive control. The strains evaluated in this work have been previously identified as a tetracycline resistant, with their resistance attributable to effective molecular efflux pumps.^[36] Another set of control experiment screened mixtures of TC and the bare CNPs (TC+CNP-BUp and TC+CNP-TDn; Table 1 and Figure 2). The antibacterial activities obtained from the mixtures were comparable to the results from the TC only control. This strongly suggests that no synergistic effects occur across the strains evaluated via the mixture of TC and the bare CNPs.^[37] Subsequently, we hypothesize that the carbon nanoparticle portion of the conjugate assembly may sterically (or otherwise) inhibit the efflux pumps' effectiveness within the bacteria cell membrane, while the tethered TC may work as both a transport agent, targeting the conjugate to the efflux pump, and the active antibacterial agent.^[21,38]

2.5. Bacteria Cell Membrane Interactions with Tetracycline Conjugates

To investigate the proposed inhibitory mechanism, cryogenic TEM (Cryo-TEM) experiments were conducted. Although the assemblies demonstrated the strongest inhibition in *A. baylyi*, while *P. mirabilis* showed the strongest resistance to the molecules, we chose *K. pneumonia* as a model system as they are not only the largest cell in physical size among the strains tested, but also their TC efflux pump systems are well documented.^[11,39] Antimicrobial-resistant *K. pneumonia* have increased tolerance to high proportions of antibiotics including aztreonam, trimethoprim/sulfamethoxazole, ceftazidime, ciprofloxacin, tetracycline, tobramycin, piperacillin/tazobactam, cefepime, amikacin, and imipenem.^[36] The bacterial cells from the negative control showed healthy cells with intact cellular membranes (Figure 3a). The cells in the positive control also

Figure 2. Minimum inhibitory concentration (MIC) in TC molar concentration. Data represent the mean MIC value measured from triplicates while the error bars represent the standard deviation of the mean. *P*-values are calculated using one-sample *t*-test, **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. †No inhibitory activities were recorded within the TC concentrations tested.

Figure 3. Cryogenic TEM images of a) negative control and b) positive control. *K. pneumoniae* incubated with 4×10^{-6} M TC solution (equivalent TC concentration to that incorporated into the TC–CNP conjugates). *K. pneumoniae* incubated with c) TC–CNP-BUP and d) TC–CNP-TDn at MIC. Portions of outer/cytoplasmic membranes are highlighted. Convolved cytoplasmic membranes can be observed in panels (c) and (d) due to the membrane perturbation.

showed good membrane integrity from both the outer and cytoplasmic membranes and cytoplasm within the cells (Figure 3b). However, the cells incubated with the TC–CNP conjugates displayed obvious signs of morphologic changes within the cell structures. Similar to the control cells, no structural abnormalities, such as cell envelope perforations, were observed in any of the Cryo-TEM experiments, suggesting that little or no interactions exist between the bacterial outer membrane and TC–CNPs that would result in disruptions within the cell. This is in contrast with previous reports which used peptide-coated antimicrobial nanoparticles^[13] and other nanocarbon systems including graphene oxide sheets,^[16] carbon nanotubes,^[40] and carbon nanoparticles.^[41] Instead, the cells demonstrate envelope disruptions and cytoplasmic membrane perturbations/convolutions when they are incubated with the TC–CNPs as presented in Figure 3c,d. Since the bare CNPs demonstrated no toxic responses in the MIC assay, it can be acknowledged that this represents interactions formed between the TC–CNP conjugates and cellular efflux pumps within the bacterial cells.

2.6. Tetracycline–CNP Conjugate Distributions

To take advantage of the FL emission of the CNPs and investigate the observations from Cryo-TEM further, we conducted

confocal laser scanning microscopy (CLSM) experiments using TC–CNP conjugates within *K. pneumoniae* and incorporated SynaptoRed C2 to stain the membrane. As presented in Figure 4a,c, a high density of the bacterial cells showed distinguishable incorporation of the TC–CNP-BUP and TC–CNP-TDn conjugates on the cytoplasmic membrane at MIC. Based upon the FL emission spectroscopic analysis of TC (Figure 1g, yellow line), we expected to observe yellow FL emission from the TC control cells. However, no FL emission signals were detectable from the TC control experiment (Figure S8, Supporting Information), further indicating that the bacterial cells extruded the TC.^[19] Furthermore, no detectable emissions from TC were found from the other control experiments (TC+CNPs). Interestingly, evenly distributed FL emission signals from the bare CNPs were observed across the cells (Figure S8b,c, Supporting Information). The FL intensity profiles further suggest the uniform distributions of the bare CNPs within the bacterial cells. On the other hand, the FL intensity profiles obtained from TC–CNPs indicate the localization of the TC–CNPs toward the cytoplasmic cell membrane. At concentrations twice the MIC ($\text{MIC} \times 2$) of the TC–CNPs (Figure 4b,d), further internalization and specific localization of the TC–CNPs were observed. The Z-projection images indicate that the internal membrane-localized TC–CNPs appear to agglomerate and span the cell envelope. The FL intensity profiles of $\text{MIC} \times 2$ showed

Figure 4. Confocal laser scanning microscope images of *K. pneumoniae* incubated with TC-CNP-BUP at a) MIC, b) MIC $\times$ 2, TC-CNP-TDn at c) MIC, d) MIC $\times$ 2 for 1 h. Scale bars, 5 μm . The *K. pneumoniae* cell membrane was stained with SynaptoRed (Ch1. red) prior to the TC-CNP (Ch2. green) exposure. The intensity of each channel was presented in the spectra of the cross section in the Z-projection images, and the bright red and green spectra represent the intensity from SynaptoRed and TC conjugates, respectively. The control and *K. pneumoniae* incubated with TC at MIC $\times$ 2 were presented in the Supporting Information. Schematics of *K. pneumoniae* incubated with e) TC versus f) TC-CNPs. Efflux in the TC-CNPs was inhibited due to the CNPs.

that the TC–CNPs were internalized throughout the cytosol, in contrast with the concentration equivalent to the MIC. No structural abnormalities from the bacterial cells were observed throughout the CLSM experiments, regardless of the TC–CNP concentrations tested. These observations not only correlate well with the Cryo-TEM results but further indicate that no direct interactions occurred between the bacterial outer membranes and TC–CNPs.

3. Conclusions

This study shows that the conjugation between biocompatible carbon nanoparticles and longstanding, but well-established antibiotic molecules may deliver new antibiotic-carrier hybrid molecular assemblies resulting in effective multidrug efflux inhibition for combating antibiotic-resistant bacteria. The results obtained from the physiochemical and optical characterizations reported herein were comparable across the CNP samples. The biocompatibility of carbon-based nanoparticles is widely known as nontoxic,^[17] and CNPs have demonstrated no toxic and/or inhibitory effects on the bacteria.^[24] Corresponding to the previous reports, the bare CNPs prepared in this study also showed no activities against the bacteria assessed regardless of the synthetic routes. The results from the bacteria cells incubated with TC alone indicated very low inhibitory activities owing to the efficient efflux system (Figure 4e). On the other hand, the TC–CNP conjugates showed substantial inhibitory activities due to the covalently tethered TC molecule. As presented in Figure 4f, we hypothesize that the conjugated TC moieties on the surface of the CNPs enable the cells to recognize them as TC inducing the TC transport and associated efflux mechanisms. This process, locating the TC–CNP assembly proximal to the cytoplasmic membrane, blocks the efflux process effectively affecting cell viability.

It is reported that carbon nanoparticles exhibit good photothermal properties and their photothermal properties can be utilized to treat tumor cells.^[42] Additionally, metallic nanoparticles,^[43] supramolecular complexes,^[44] and graphene^[45] have demonstrated their effectiveness against multidrug-resistant bacteria utilizing their thermoplasmonic properties. Therefore, combination of the antibacterial-functionalized carbon nanoparticles and photothermal lysis techniques may also be applied to increase the efficacy.

We anticipate that chemical conjugation of biocompatible nanoparticles and well-established subtherapeutic antibiotic molecules may provide novel strategies to combat multidrug-resistant bacteria where efflux systems are suspected.

4. Experimental Section

Carbon Nanoparticle Preparations: The CNP-BUP sample particles were prepared by the decomposition of citric acid in propane-1,2-diol (propylene glycol) in a hydrothermal reactor. A mass of 0.78 g of citric acid anhydrous (Alfa Aesar) was mixed with 30 mL of propane-1,2-diol (Alfa Aesar); then, the mixture was transferred to a 100 mL PTFE-lined hydrothermal reactor. The reactor was kept for 24 h at 180 °C. After the reaction, the crude reaction product was filtered by a 0.22 µm nylon filter and dialyzed against deionized (DI) water at a molecular weight

(MW) cut-off of 2000 Da for a week (10 L H₂O per day). The CNP-TDn sample was obtained by centrifugation and filtration of a graphene oxide sample. A mass of 3 g of graphite flakes (Sigma–Aldrich) was dispersed in a mixture of sulfuric acid (Ajax Finechem) and orthophosphoric acid (Ajax Finechem) at 7.5:1 ratio. The mixture was stirred for 1 h at 50 °C, followed by spontaneous addition of 1 g of potassium permanganate (Sigma–Aldrich). Care must be taken with the addition of KMnO₄ due to the evolution of sulfate and phosphate gases, resulting from the exothermic reaction between acids and reducing agent. The mixture was stirred for additional 7 h at 50 °C followed by the addition of 3.2 mL hydrogen peroxide (35%, Chem-Supply) to quench the reaction. Ice cubes prepared by 1 L deionized water was added to the mixture and stirred until the reaction mixture was cooled to room temperature. The mixture was washed and neutralized by copious centrifugations. Finally, the CNP-TDn was obtained by collecting clear supernatant after 2 h centrifugation at 4696 × g followed by filtration using a 0.22 µm nylon filter. Both CNP-BUP and CNP-TDn were freeze-dried prior to further use.

Preparation of Tetracycline–CNP Conjugates: A mass of 1 mg freeze-dried CNP sample was dispersed in 10 mL of DMF, and the solution containing CNPs was heated and stirred for 15 min at 60 °C under nitrogen reflux. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl (EDC, 0.12 mmol, Sigma–Aldrich) and N-hydroxybenzotriazole (HOBt, 0.12 mmol, Sigma–Aldrich) were added to the solution, and the system was left for another 15 min. Finally, 0.1 mmol of tetracycline-HCl (Sigma Aldrich) dissolved in 5 mL of DMF were added to the CNP-containing solution and reacted for 23.5 h at 60 °C. The excess tetracycline, reagents, and by-products were removed by dialysis at an MWCO of 2000 Da (Sigma–Aldrich). The final products were freeze-dried and kept at –20 °C before further use.

Disk Diffusion Assay: Antibacterial activity of the CNP samples was determined using a modified disk diffusion assay.^[46] About 100 µL of the test bacteria was grown in 10 mL of fresh nutrient broth media until they reached a count of $\approx 10^8$ cells mL^{–1}. A volume of 100 µL of bacterial suspension was spread onto nutrient agar plates. The plates containing bacteria were allowed to stand at 4 °C for 2 h prior to the incubation with the test antibacterial agents. To test antibacterial activities, sterilized 6 mm filter paper disks (Whatman number 54) were impregnated with 10 µL of test solutions and allowed to dry. The dried disks were placed onto the inoculated agar plates. A sterile control (nutrient broth without bacterial inoculum), a negative control (sterile deionized water), and a positive control (control antibiotic: chloramphenicol and tetracycline, 0.01 mg mL^{–1}, Sigma–Aldrich) were included on each plate. All the agar plates were incubated in 37 °C for 24 h, and then the diameters of the inhibition zones were measured in millimeters using a caliper. Each assay was performed in triplicates and the mean of the MIC ± standard deviations was reported in the study.

Liquid Dilution Screening Assay: A modified serial dilution assay^[47] was used to confirm the MIC of the CNP samples alone and TC–CNP conjugates. A volume of 100 µL of sterile nutrient broth and 100 µL of working bacterial culture was added to the wells in a 96-well plate and incubated at 37 °C for 24 h prior to the test. In a separate 96-well plate, a volume of 100 µL of stock test solutions was added to the first row followed by placing 100 µL of twofold serially diluted test solutions into the next row wells making seven serially diluted concentrations of each test solution across the well plate. Finally, 100 µL of bacterial culture prepared was added to the well containing the test solutions, and the plates were incubated at 37 °C for 24 h. A sterile control (nutrient broth without bacterial inoculum), a negative control (1% DMSO in sterile deionized water), and a positive control (control antibiotic: chloramphenicol and tetracycline, 0.01 mg mL^{–1}, Sigma–Aldrich) were included on each plate. In addition, the standard bacterial culture was streaked prior to the addition to the test solutions to ensure that the culture was pure and free of microbial contamination. To identify bacterial growth, 50 µL of *p*-iodonitrotetrazolium violet (INT solution at 0.4 mg mL^{–1}, Sigma–Aldrich) was added to the wells and incubated at room temperature for 2 h before recording the change of color. Bacterial growth in the wells was identified by a distinctive red/pink color, whereas

clear wells indicated inhibition by the samples and their concentrations tested. Each assay was performed in triplicates and the mean of the MIC $\pm$ standard deviations was reported in the study.

Cryo-TEM: Solutions containing TC–CNPs were introduced to *K. pneumonia* to the MIC of each TC–CNP conjugates. The positive control *K. pneumonia* samples were prepared using the equivalent amount of tetracycline from the TC–CNP conjugates at 4×10^{-6} M. The samples were incubated for 1 h at room temperature followed by washing with PBS. Lacey carbon films on 300 Mesh copper grids (Electron Microscopy Sciences, Hatfield, USA) were used as sample bacteria carriers and the grids were washed for 5 min in ethyl acetate and glow-discharged for 30 s at 20 mA in a Balzers MED 020. The *K. pneumonia* samples were prepared using an FEI Vitrobot MK II plunge freezer in liquid ethane at -180°C . The freezing conditions were 25°C at 100% relative humidity. A volume of 4 μL of the cell containing medium was transferred onto the grid followed by a 5 s blotting. The cryo-fixed samples were stored under liquid nitrogen until further characterization.

Confocal Laser Scanning Microscopy: To stain the membrane of *K. pneumonia*, the cells were stained using SynaptoRed C2 solution (Sigma–Aldrich) to a final concentration of 4×10^{-6} M for 1 min at room temperature followed by washing with PBS. Solutions containing TC–CNPs were subsequently introduced and incubated for 1 h at room temperature. *K. pneumonia* samples were washed three times for 5 min each, and 1 μL of final aliquot was fixed onto a sterile microscope coverslip using a 1 μL inoculation loop and was dried. The sample fixed coverslip was attached onto a sterile microscope slide using nail polish.

Instrumentals: TEM images were captured on a Hitachi HT7700 TEM at 10 kV, and the particle size distributions were obtained using Gatan Microscopy Suite software package (v GMS 3). AFM spectra were captured on an NT-MDT NTEGRA Spectra AFM using a semicontact mode probe (NT-MDT CSG01-A). XPS spectra were acquired using a Kratos Axis ULTRA X-ray photoelectron spectrometer incorporating a 165 mm hemispherical electron energy analyzer. Calculations of atomic concentrations and peak fittings of the high-resolution data were carried out using the CasaXPS software package (v2.2.73). Fourier transform infrared (FTIR) spectra were recorded on a PerkinElmer Spectrum Two IR spectrometer equipped with a diamond-attenuated total reflectance (ATR) attachment. ATR corrections were applied using PerkinElmer Spectrum software package (v10.03.09) after acquisitions. UV–vis absorption spectra for dispersed samples were measured on an Agilent 8453 UV–vis spectrometer. Fluorescence spectra were measured by a Thermo Lumina fluorescence spectrometer. Fluorescence decays were recorded by a using 374 nm picosecond pulse laser excitation source in an Edinburgh Photonics FLS920 photoluminescence spectrometer. Fluorescence lifetimes were estimated from the measured fluorescence decay curves and the laser profiles using nonlinear least-squares iterative fittings on F900 software package (v7.2.1). Quantum yields were measured by comparing quantum yield standards at 360 nm excitation (quinine sulfate, 0.54).^[48] Cryo-TEM images were obtained on an FEI Tecnai F30 FEG-TEM G2 fitted with a Gatan K2 Summit direct electron detector operating at 300 kV. Samples were imaged at -174°C under low-dose conditions using a Gatan 626 cryo-holder. The images were processed and analyzed using ImageJ software package (v1.52i). CLSM images were acquired on a Carl Zeiss LSM 710 confocal laser scanning microscope employing a Carl Zeiss α Plan-Apochromat 100 $\times$ /1.46 Oil DIC M27 objective. The confocal images were processed and analyzed using Carl Zeiss ZEN software package (v2.5.75.0).

Statistical Analysis: Statistical analysis was performed using OriginPro software package (V9.0.0). Results were presented as mean $\pm$ standard deviation. Experiments were performed at least in triplicate ($n = 3$). *P*-values were calculated using one-sample *t*-test and the *p*-value less than 0.05 was considered to be significant.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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- [1] R. M. Anderson, *Nat. Med.* **1999**, 5, 147.
- [2] W. Witte, *Science* **1998**, 279, 996.
- [3] S. B. Levy, M. Bonnie, *Nat. Med.* **2004**, 10, S122.
- [4] A. H. Holmes, L. S. P. Moore, A. Sundsfjord, M. Steinbakk, S. Regmi, A. Karkey, P. J. Guerin, L. J. V. Piddock, *Lancet* **2016**, 387, 176.
- [5] R. Laxminarayan, P. Matsoso, S. Pant, C. Brower, J. A. Røttingen, K. Klugman, S. Davies, *Lancet* **2016**, 387, 168.
- [6] E. D. Brown, G. D. Wright, *Nature* **2016**, 529, 336.
- [7] a) P. Collignon, J. H. Powers, T. M. Chiller, A. Aidara-Kane, F. M. Aarestrup, *Clin. Infect. Dis.* **2009**, 49, 132; b) V. N. Yewale, in *Antimicrobial Resistance: Global Report on Surveillance*, World Health Organization, Geneva, Switzerland **2014**, p. 171.
- [8] M. A. Fischbach, C. T. Walsh, *Science* **2009**, 325, 1089.
- [9] L. L. Marcusson, N. Frimodt-Møller, D. Hughes, *PLoS Pathog.* **2009**, 5, e1000541.
- [10] D. Brown, *Nat. Rev. Drug Discovery* **2015**, 14, 821.
- [11] M. F. Chellat, L. Raguž, R. Riedl, *Angew. Chem., Int. Ed.* **2016**, 55, 6600.
- [12] C. Yang, X. Ding, R. J. Ono, H. Lee, L. Y. Hsu, Y. W. Tong, J. Hedrick, Y. Y. Yang, *Adv. Mater.* **2014**, 26, 7346.
- [13] a) N. Srinivas, P. Jetter, B. J. Ueberbacher, M. Werneburg, K. Zerbe, J. Steinmann, B. Van der Meijden, F. Bernardini, A. Lederer, R. L. Dias, P. E. Misson, H. Henze, J. Zumbunn, F. O. Gombert, D. Obrecht, P. Hunziker, S. Schauer, U. Ziegler, A. Kach, L. Eberl, K. Riedel, S. J. DeMarco, J. A. Robinson, *Science* **2010**, 327, 1010; b) S. J. Lam, N. M. O'Brien-Simpson, N. Pantarat, A. Sulistio, E. H. Wong, Y. Y. Chen, J. C. Lenzo, J. A. Holden, A. Blencowe, E. C. Reynolds, G. G. Qiao, *Nat. Microbiol.* **2016**, 1, 16162; c) C. D. Fjell, J. A. Hiss, R. E. Hancock, G. Schneider, *Nat. Rev. Drug Discovery* **2012**, 11, 37.
- [14] a) Z. M. Xiu, Q. B. Zhang, H. L. Puppala, V. L. Colvin, P. J. Alvarez, *Nano Lett.* **2012**, 12, 4271; b) S. Chernousova, M. Eppe, *Angew. Chem., Int. Ed. Engl.* **2013**, 52, 1636.
- [15] a) J. Bresee, C. M. Bond, R. J. Worthington, C. A. Smith, J. C. Gifford, C. A. Simpson, C. J. Carter, G. Wang, J. Hartman, N. A. Osbaugh, R. K. Shoemaker, C. Melander, D. L. Feldheim, *J. Am. Chem. Soc.*

- 2014, 136, 5295; b) Y. Zhao, C. Ye, W. Liu, R. Chen, X. Jiang, *Angew. Chem., Int. Ed. Engl.* **2014**, 53, 8127.
- [16] a) S. Liu, T. H. Zeng, M. Hofmann, E. Burcombe, J. Wei, R. Jiang, J. Kong, Y. Chen, *ACS Nano* **2011**, 5, 6971; b) Y. Tu, M. Lv, P. Xiu, T. Huynh, M. Zhang, M. Castelli, Z. Liu, Q. Huang, C. Fan, H. Fang, R. Zhou, *Nat. Nanotechnol.* **2013**, 8, 594.
- [17] a) K. Hola, Y. Zhang, Y. Wang, E. P. Giannelis, R. Zboril, A. L. Rogach, *Nano Today* **2014**, 9, 590; b) G. Hong, S. Diao, A. L. Antaris, H. Dai, *Chem. Rev.* **2015**, 115, 10816.
- [18] G. Hong, S. Diao, A. L. Antaris, H. Dai, *Chem. Rev.* **2015**, 115, 10816.
- [19] a) H. Nikaido, *Annu. Rev. Biochem.* **2009**, 78, 119; b) D. Du, Z. Wang, N. R. James, J. E. Voss, E. Klimont, T. Ohene-Agyei, H. Venter, W. Chiu, B. F. Luisi, *Nature* **2014**, 509, 512.
- [20] M. K. Higgins, E. Bokma, E. Koronakis, C. Hughes, V. Koronakis, *Proc. Natl. Acad. Sci. USA* **2004**, 101, 9994.
- [21] J. Sun, Z. Deng, A. Yan, *Biochem. Biophys. Res. Commun.* **2014**, 453, 254.
- [22] a) S. Silver, *FEMS Microbiol. Rev.* **2003**, 27, 341; b) A. F. Halbus, T. S. Horozov, V. N. Paunov, *Adv. Colloid Interface Sci.* **2017**, 249, 134; c) D. Westmeier, A. Hahlbrock, C. Reinhardt, J. Fröhlich-Nowoisky, S. Wessler, C. Vallet, U. Pöschl, S. K. Knauer, R. H. Stauber, *Chem. Soc. Rev.* **2018**, 47, 5312.
- [23] a) L. J. Piddock, *Nat. Rev. Microbiol.* **2006**, 4, 629; b) M. Stavri, L. J. Piddock, S. Gibbons, *J. Antimicrob. Chemother.* **2007**, 59, 1247.
- [24] T. H. Kim, J. P. Sirdaarta, Q. Zhang, E. Eftekhari, J. St. John, D. Kennedy, I. E. Cock, Q. Li, *Nano Res.* **2018**, 11, 2204.
- [25] S. Zhang, H. Song, P. Guo, J. Zhou, X. Chen, *Carbon* **2010**, 48, 4211.
- [26] B. Kong, A. Zhu, C. Ding, X. Zhao, B. Li, Y. Tian, *Adv. Mater.* **2012**, 24, 5844.
- [27] I. Chopra, M. Roberts, *Microbiol. Mol. Biol. Rev.* **2001**, 65, 232.
- [28] a) G. T. Hermanson, in *Bioconjugation Techniques*, 2nd ed., Academic Press, Boston **2013**, p. 259; b) M. Tsakos, E. S. Schaffert, L. L. Clement, N. L. Villadsen, T. B. Poulsen, *Nat. Prod. Rep.* **2015**, 32, 605.
- [29] L. D. Field, S. Sternhell, J. R. Kalman, *Organic Structures from Spectra*, 3rd ed., John Wiley & Sons, Inc., Hoboken, NJ **2002**.
- [30] a) Y. P. Sun, B. Zhou, Y. Lin, W. Wang, K. A. Fernando, P. Pathak, M. J. Mezziani, B. A. Harruff, X. Wang, H. Wang, P. G. Luo, H. Yang, M. E. Kose, B. Chen, L. M. Vaca, S. Y. Xie, *J. Am. Chem. Soc.* **2006**, 128, 7756; b) H. Liu, T. Ye, C. Mao, *Angew. Chem., Int. Ed. Engl.* **2007**, 46, 6473.
- [31] Y. Ni, D. Lin, S. Kokot, *Anal. Chim. Acta* **2008**, 606, 19.
- [32] C. I. Brinzila, N. Monteiro, M. J. Pacheco, L. Cirfaco, I. Siminiceanu, A. Lopes, *Environ. Sci. Pollut. Res.* **2014**, 21, 8457.
- [33] J. Tang, B. Kong, H. Wu, M. Xu, Y. Wang, Y. Wang, D. Zhao, G. Zheng, *Adv. Mater.* **2013**, 25, 6569.
- [34] X. Wang, L. Cao, S. T. Yang, F. Lu, M. J. Mezziani, L. Tian, K. W. Sun, M. A. Bloodgood, Y. P. Sun, *Angew. Chem., Int. Ed.* **2010**, 49, 5310.
- [35] M. Balouiri, M. Sadiki, S. K. Ibsouda, *J. Pharm. Anal.* **2016**, 6, 71.
- [36] G. V. Sanchez, R. N. Master, R. B. Clark, M. Fyyaz, P. Duvvuri, G. Ekta, J. Bordon, *Emerging Infect. Dis.* **2013**, 19, 133.
- [37] a) N. Safdar, J. Handelsman, D. G. Maki, *Lancet Infect. Dis.* **2004**, 4, 519; b) S. Chen, Y. Guo, S. Chen, H. Yu, Z. Ge, X. Zhang, P. Zhang, J. Tang, *J. Mater. Chem.* **2012**, 22, 9092.
- [38] T. Bergmiller, A. M. C. Andersson, K. Tomasek, E. Balleza, D. J. Kiviet, R. Hauschild, G. Tkačik, C. C. Guet, *Science* **2017**, 356, 311.
- [39] H. C. Neu, *Science* **1992**, 257, 1064.
- [40] S. Kang, M. Herzberg, D. F. Rodrigues, M. Elimelech, *Langmuir* **2008**, 24, 6409.
- [41] a) W. Bing, H. Sun, Z. Yan, J. Ren, X. Qu, *Small* **2016**, 12, 4713; b) H. J. Lin, C. F. Huang, Y. J. Li, C. H. Lin, S. G. Harroun, B. Unnikrishnan, C.-C. Huang, Y. C. Su, *Adv. Healthcare Mater.* **2016**, 5, 2545; c) R. Jijie, A. Barras, J. Bouckaert, N. Dumitrascu, S. Szunerits, R. Boukherroub, *Colloids Surf., B* **2018**, 170, 347.
- [42] a) X. Bao, Y. Yuan, J. Chen, B. Zhang, D. Li, D. Zhou, P. Jing, G. Xu, Y. Wang, K. Holá, D. Shen, C. Wu, L. Song, C. Liu, R. Zbořil, S. Qu, *Light: Sci. Appl.* **2018**, 7, 1; b) M. Qian, Y. Du, S. Wang, C. Li, H. Jiang, W. Shi, J. Chen, Y. Wang, E. Wagner, R. Huang, *ACS Appl. Mater. Interfaces* **2018**, 10, 4031.
- [43] R. Sean Norman, J. W. Stone, A. Gole, C. J. Murphy, T. L. Sabo-Attwood, *Nano Lett.* **2008**, 8, 302.
- [44] Y. Yang, P. He, Y. Wang, H. Bai, S. Wang, J. F. Xu, X. Zhang, *Angew. Chem., Int. Ed.* **2017**, 56, 16239.
- [45] M. C. Wu, A. R. Deokar, J. H. Liao, P. Y. Shih, Y. C. Ling, *ACS Nano* **2013**, 7, 1281.
- [46] J. H. Jorgensen, J. D. Turnidge, in *Manual of Clinical Microbiology*, 11th ed. (Eds: J. H. Jorgensen, M. A. Pfaller, K. C. Carroll), American Society of Microbiology, Washington, DC **2015**, pp. 1253–1273.
- [47] J. N. Eloff, *Planta Med.* **1998**, 64, 711.
- [48] A. N. Fletcher, *Photochem. Photobiol.* **1969**, 9, 439.

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