Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Supplementary Information

Zinc Complex of a Neutral Pyridine-based Amphiphile: A Highly Efficient and Potentially Therapeutic Bactericidal Material

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EXPERIMENTAL SECTION

Compounds and Reagents

Brain-Heart Infusion (BHI) Broth, Nutrient Broth (NB) and crystal violet (CV) were purchased from HiMedia, Mumbai, India. Dimethyl sulfoxide (DMSO), and absolute ethanol were procured from Merck, Mumbai, India. Magnesium chloride salt and N-2-Hydroxyethyl Piperazine N-2 Ethane Sulphonic acid (HEPES Buffer) was procured from Sisco Research Laboratories SRL, Mumbai, India. 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (cFDA-SE), propidium iodide (PI), 3,3'-dipropylthiadicarbocyanine iodide $DiSC_35$, 1-N-phenyl-naphthylamine (NPN), congo red (CR), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Dulbecco's Modified Eagle Medium (DMEM), trypsin–EDTA, valinomycin, polymyxin B, erythromycin and zinc perchlorate were procured from Sigma Aldrich Chemicals, USA. Fetal bovine serum (FBS) was procured from PAA Laboratories, USA.

Apparent Binding Constant for Formation of Compound 1-Zinc Complex

To determine binding stoichiometry and binding affinity of zinc towards C1 at room temperature (at 298 K), UV-visible titration spectra was pursued. A 25 μM solution of C1 prepared in buffered methanol (4:1 methanol: water) solvent was titrated with $Zn(ClO_4)_2$ solution so that the effective Zn(II) concentration was varied between 0-1250 μM. UV-visible absorption spectra was recorded in a Cary UV-100 spectrophotometer (Varian, USA) using 10 mm path length cuvette in the range of 200-500 nm wavelength. The apparent binding constant for the formation of C1-Zn was determined using modified Benesi-Hildebrand (B-H) plot [S1] and the following equation:

$$1/(A - A_0) = 1/\{K (A_{max} - A_0) C\} + 1/(A_{max} - A_0) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where A_0 is the absorbance of C1 at absorbance maximum (λ = 262 nm), A is the observed absorbance at that particular wavelength in the presence of a certain concentration of the metal ion (C), A_{max} is the maximum absorbance value that was obtained at λ = 262 nm during titration with varying Zn(II) concentrations. The apparent binding constant K (M^{-1}) was determined from the slope of the linear plot and C is the concentration of Zn(II) added during titration studies.

Bacterial Strains and Growth Conditions

The target bacterial strains used in the present study included Gram-positive *Staphylococcus aureus* MTCC 96 (*S. aureus*) and *Listeria monocytogenes* Scott A (*L. monocytogenes*) and Gram-negative *Escherichia coli* MTCC 433 (*E. coli*) and *Enterobacter aerogenes* MTCC 2822 (*E. aerogenes*). *S. aureus* MTCC 96 and *L. monocytogenes* Scott A were propagated in BHI broth at 37^{o}C and 180 rpm for 12 h. *E. coli* MTCC 433 and *E. aerogenes* MTCC 2822 were grown in NB medium at 37^{o}C and 180 rpm for 12 h. All the bacterial strains were grown from frozen stocks and sub-cultured prior to their use in experiments.

Minimum Inhibitory Concentration (MIC) of C1-Zn

MIC of C1-Zn was ascertained against Gram-positive bacteria *S. aureus* MTCC 96 and *L. monocytogenes* Scott A and Gram-negative bacteria *E. coli* MTCC 433 and *E. aerogenes* MTCC 2822. The bacterial strains were inoculated at 1% level in microtitre wells (approximately 5×10^5 CFU per well) having 100 µL of the specific growth medium and propagated overnight at 37^{o}C and 180 rpm in presence of varying concentrations of C1-Zn. The growth of the bacterial strains was verified by measuring absorbance at 600 nm in a microtitre plate reader (Infinite M200, TECAN, Switzerland). MIC of C1-Zn was defined as the minimum amphiphile concentration that resulted in growth inhibition of the target bacteria (A_{600} <0.1). The MIC values were determined from six independent experiments and expressed as average values.

Antibacterial Activity of Amphiphiles

The bactericidal activity of amphiphiles was determined by the following experiments:

Field Emission Scanning Electron Microscope (FESEM) Analysis

Overnight grown cells of *S. aureus* MTCC 96 and *E. coli* MTCC 433 were collected by centrifugation, washed twice with sterile phosphate buffered saline (PBS) and resuspended in the same. Approximately 10^6 CFU/mL cells were treated with $Zn(ClO_4)_2$, C1 and C1-Zn (6.0 µM each) for 6 h at 37^{o}C. Control samples consisted of untreated cells incubated in PBS for the same period. Treated as well as untreated cells were washed twice with PBS and fixed in 2.5% glutaraldehyde for 90 mins at 4^{o}C. Following fixation, cells were washed and resuspended in sterile MilliQ grade water. A 10 µL aliquot of each sample was spotted on separate aluminium foil covered glass stubs and air dried in a laminar hood. Subsequently,

the samples were coated with gold plasmon and examined in a field emission scanning electron microscope (Zeiss Sigma, USA) and their images were recorded.

cFDA- SE Leakage Assay

A stock solution of cFDA-SE (500 μM) was prepared in ethanol and stored at -20^{o}C. Target cells of *S. aureus* MTCC 96 and *E. coli* MTCC 433 were harvested from overnight grown cultures by centrifugation at 8000 rpm for 5 min. The cell pellet was washed twice with sterile PBS, resuspended in the same to achieve a cell concentration of 10^6 CFU/mL and labelled with cFDA-SE (final concentration of 50 μM) for 20 min at 37°C. The cells were then centrifuged, washed twice with sterile PBS to remove excess cFDA-SE molecules, resuspended in 1.0 mL of sterile PBS and treated with C1, C2 and C1-Zn (6.0 μM each) at 37^{o}C and 180 rpm for 6 h. In case of control sample, only DMSO was added to cFDA-SE labelled cells and incubated under the same conditions without amphiphile. Leakage of cFDA from cells was determined by measuring fluorescence of the cell free supernatant at an excitation wavelength of 488 nm and emission wavelength of 518 nm in a spectrofluorimeter (LS-55 Perkin-Elmer, USA). The fluorescence measurements were recorded after subtracting the fluorescence of effluxed dye from control samples. Fluorescence measurements were recorded from three independent experimental samples.

Propidium Iodide (PI) Uptake Assay

A stock solution of PI (1.5 mM) was prepared in sterile MilliQ water. Target cells of *S. aureus* MTCC 96 and *E. coli* MTCC 433 were harvested from overnight grown cultures and resuspended in sterile PBS. Cells (approximately 10^6 CFU/mL) were treated with C1, C2 and C1-Zn (6.0 μM each) at 37^{o}C and 180 rpm for 6 h. Cells incubated in DMSO under the same conditions without amphiphile was included as control. Following incubation, cells were washed with sterile PBS, resuspended in the same and incubated with PI (final concentration of 30 μM) for 30 min at 37°C in a circulating water bath incubator (Amersham, USA). Subsequently, the cells were centrifuged and washed with sterile PBS to remove excess dye, resuspended in PBS and fluorescence was measured in a spectrofluorimeter (LS-55 Perkin-Elmer, USA) at an excitation wavelength of 535 nm and emission wavelength of 617 nm. The values obtained for untreated cells were subtracted from all experimental values. Fluorescence measurements were acquired for three independent samples.

Flow Cytometry Analysis

The effect of $Zn(ClO_4)_2$, C1, C2 and C1-Zn on the viability of *S. aureus* MTCC 96 and *E. coli* MTCC 433 was studied by flow cytometry (FCM), which was performed on a FACS Calibur flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) equipped with a 15-mW, 488-nm, air-cooled argon ion laser. The target bacterial cells were pre-labelled with cFDA-SE following the protocol mentioned earlier and treated in separate sets with $Zn(ClO_4)_2$, C1, C2 and C1-Zn (6.0 μM each) for 6 h at 37°C in a circulating water bath incubator (Amersham, USA). Following treatment, the cells were analysed along with unlabelled-untreated cells as well as labelled-untreated cells (control). For FCM analysis, approximately 10^6 cells per ml were analyzed at a low flow rate setting (150-500 cells per second) and the FACS instrument was adjusted to acquire 50,000 fluorescent events. Appropriate voltage and threshold parameter were adjusted for unlabeled-untreated cells. The corresponding signal of unlabeled-untreated cells was set in the lower left quadrant in order to compensate for cellular autofluorescence. All the signals were collected in logarithmic mode. Green fluorescence of cFDA-SE stained cells was detected in the FL-1 channel (band pass filter of 530 nm). Acquisition of fluorescence data was accomplished by setting a gate in the forward-angle light scatter (FSC) vs. sideward scatter (SSC) plot, which facilitated discrimination of bacterial cells from other artefacts. Data were acquired and analyzed with the CellQuest Pro software (BD CellQuestTM Pro Version 6.0, Becton-Dickinson, USA) and the WinMDI software program WinMDI ver 2.9 (http://en.bio-soft.net/other/WinMDI). The data was analysed with the aid of statistical tables.

Fluorescence Microscope Analysis

Cells of *S. aureus* MTCC 96 and *E. coli* MTCC 433 suspended in sterile PBS (approximately 10^6 CFU/mL) were treated with C1, C2 and C1-Zn (6.0 μM each) at 37°C and 180 rpm for 6 h. In case of control sample, DMSO was added to the cells and incubated under the same conditions. For comparison, cells treated with 6.0 μM $Zn(ClO_4)_2$ at 37°C and 180 rpm for 6 h were also included. Subsequently, cells were washed twice with sterile PBS to remove unbound amphiphile or $Zn(ClO_4)_2$ and labelled with cFDA-SE and PI in separate sets as described earlier. The stained samples were fixed in 2.5% glutaraldehyde and washed twice with sterile PBS. A 10 μl aliquot of the stained sample was spotted on a clean glass slide, air dried and observed under fluorescence microscope (Eclipse Ti-U, Nikon, USA) with a filter that allowed blue light excitation at 445-495 nm for cFDA-SE and green light excitation at 495-570 nm in case of PI stained cells. The images of the cells were recorded.

Membrane Depolarization Assay

Cells of *S. aureus* MTCC 96 and *E. coli* MTCC 433 were grown till mid-logarithmic phase (A_{600} = 0.4-0.5). The cells were harvested by centrifugation, washed and resuspended in a buffer solution (5.0 mM HEPES, 5.0 mM glucose, pH 7.4) to achieve the desired cell concentration (A_{600} of 0.05) and incubated with 0.4 μM $DiSC_35$ for 1 h at 37°C followed by 15 min incubation in 100 mM KCl. Subsequently the cell suspensions (1.0 mL) in separate sets were placed in a cuvette to which $Zn(ClO_4)_2$, C1, C2 and C1-Zn (6.0 μM each) was added and the fluorescence emission intensity (λ_{Ex} = 622 nm and λ_{Em} = 670 nm) was monitored in short time intervals in a spectrofluorimeter (LS-55 Perkin-Elmer, USA) with excitation and emission slit width of 10 nm each. Valinomycin (30 μM) treated cells and DMSO treated cells were included as positive control and negative control samples, respectively. Fluorescence measurements were taken for three independent experimental samples.

Outer Membrane Permeabilization Assay

A stock solution of NPN (500 μM) was made in acetone. Initially, target cells of *E. coli* MTCC 433 were grown in nutrient broth (NB) medium at 37°C in a shaker incubator till mid-log phase (A_{600} of 0.5). The cells were centrifuged, washed twice with 5.0 mM HEPES buffer (pH 7.4) and resuspended in the same buffer. NPN was added (at a final concentration of 10 μM) to 1.0 ml aliquot of target cells, which was interacted in separate cuvettes with $Zn(ClO_4)_2$, C1, C2 and C1-Zn (6.0 μM each). Enhancement in the fluorescence intensity of NPN was measured at regular intervals in a spectrofluorimeter (LS-55 Perkin-Elmer, USA) at an excitation and emission wavelength of 350 nm and 420 nm, respectively. Polymixin B (1.0 μg/mL) treated cells and DMSO treated cells were used as positive control and negative control samples, respectively. Fluorescence measurements were taken for three independent samples.

Bactericidal Activity of C1-Zn in Simulated Body Fluid (SBF)

Varying concentrations of C1-Zn (3.0 μM, 6.0 μM and 9.0 μM each) were added separately to target cells of *S. aureus* MTCC 96 (6.0 $\log_{10}$ CFU/mL) suspended in SBF and incubated at 37°C and 180 rpm. The composition of SBF was as reported earlier. [S2] Viable cell numbers ($\log_{10}$ CFU/mL) were determined at regular intervals by serial dilution and plating. The viable cell count was also determined at the specified intervals for cells suspended in sterile phosphate buffer saline (PBS).

Antibiofilm Activity

The antibiofilm activity of the amphiphiles was evaluated by performing the following experiments:

Microtitre Well Assay for Determination of Biofilm Eradication

S. aureus MTCC 96 biofilm was grown sterile in 96 well microtitre plate by following a standard protocol described earlier. [S3] For the growth *S. aureus* biofilm, BHI media supplemented with 0.25% glucose was used. In a parallel set, *S. aureus* biofilm was also grown in BHI media having 0.25% glucose and supplemented with 50 μM Zn(II). In these experiments, $Zn(ClO_4)_2$ was used as a source of Zn(II). Following 24 h of biofilm growth in static and humid condition at 37°C, the spent media from the microtitre plate wells was aspirated and the biofilm was incubated in fresh growth media in separate sets having varying concentrations of C1, C2 and C1-Zn (8.0 μM - 256 μM each) for 24 h in a static and humid chamber at 37°C. *S. aureus* biofilm grown in BHI media with 0.25% glucose and supplemented with 50 μM Zn(II) in the absence of any amphiphile was considered as the control sample. Following incubation, the growth media was removed from all the samples and the wells were washed with sterile PBS (200 μL) to remove any non-adherent planktonic bacterial cells. The wells were then air dried for 45 min and the biofilm was stained with a 1% (v/v) crystal violet solution (150 μL) for 45 min. Subsequently, the wells were washed thrice with sterile water (200 μL) to remove excess unbound stain. The dye incorporated by biofilms was solubilized with 95% ethanol (200 μL) and the biofilm biomass was estimated by transferring the ethanol-solubilized dye solution from each well into fresh well and measuring absorbance at 595 nm in a microtiter plate reader (Infinite M200, TECAN, Switzerland). The results were expressed as percentage biofilm biomass compared to control. The minimum biofilm eradication concentration ($MBEC_{90}$) for the amphiphiles alone or in combination with 50 μM Zn(II) was determined as the concentration, which resulted in 90% reduction in biofilm biomass as compared to untreated control sample. All the experiments were performed in triplicates and a one way analysis of variance (ANOVA) was performed using Sigma Plot.

In a parallel set of experiment, estimation of biofilm extra-polymeric substance (EPS) was accomplished by congo red binding assay. Biofilm of *S. aureus* MTCC 96 was grown and treated with varying concentrations of amphiphiles with appropriate controls as described above. Subsequently, the growth media was removed and the wells were washed with sterile

PBS (200 μL) to remove non-adherent bacterial cells. The wells were air dried and the biofilm in each well was stained with 1% congo red dye for 1 min. The excess dye was aspirated and the bound dye was solubilised in 200 μL of DMSO and its absorbance was measured at 490 nm in a microtiter plate reader (Infinite M200, TECAN, Switzerland). The results were expressed as percentage biofilm biomass compared to control. All experiments were performed in triplicates and a one way analysis of variance (ANOVA) was performed using Sigma Plot.

Fluorescence Microscope Analysis

Biofilm of *S. aureus* MTCC 96 was grown in microtitre plate wells as described above and treated in separate sets with C1, C2, C1-Zn (16 μM each) or 16 μM C1 in presence of 50 μM Zn(II). An appropriate control sample as described in the previous section was also included. Subsequently, the biofilm in each well were washed twice with sterile PBS to remove unbound amphiphile and stained in separate sets with 50 μM cFDA-SE or 1% congo red using a method described previously. [S3] The stained samples were washed twice with sterile PBS to remove unbound dye, air dried and observed under fluorescence microscope (Eclipse Ti-U, Nikon, USA) with a filter that allowed blue light excitation at 445-495 nm for cFDA-SE and green light excitation at 495-570 nm in case of CR stained cells. Fluorescence images of control and amphiphile-treated biofilms were recorded.

Microtitre Well Assay for Determination of Biofilm Inhibition

Biofilm of *S. aureus* MTCC 96 was grown in sterile 96 well microtitre plate in BHI medium incorporated with 0.25% glucose and varying concentrations of C1, C2 or C1-Zn (8.0 μM and 16 μM each). In a separate set of experiment, *S. aureus* MTCC 96 biofilm was grown in sterile 96 well microtitre plate in BHI medium incorporated with 0.25% glucose and varying concentrations of C1 (8.0 μM and 16 μM) and Zn(II). In these experiments, the ratio of C1:Zn(II) was varied (1:2, 1:4 and 1:6). Following 24 h of biofilm growth, biofilm biomass was estimated by crystal violet staining as mentioned before. The results were expressed as percentage biofilm biomass compared to control. The minimum biofilm inhibition concentration ($MBIC_{90}$) for the amphiphiles alone or in combination with Zn(II) was determined as the concentration, which resulted in 90% inhibition of biofilm growth as compared to untreated control sample. All the experiments were performed in triplicates and a one way analysis of variance (ANOVA) was performed using Sigma Plot.

Reference

S1. H. A. Benesi and J. H. Hildebrand, *J. Am. Chem. Soc.*, 1949, **71**, 2703.

S2. T. Kokubo, H. Kushitani, S. Sakka, T. Kitsugi, T. Yamamuro, *J. Biomed. Mater. Res.*, 1990, **24**, 721.

S3. S. Goswami, D. Thiyagarajan, G. Das and A. Ramesh, *ACS Appl. Mater. Interfaces*, 2014, **6**, 16384.

RESULTS

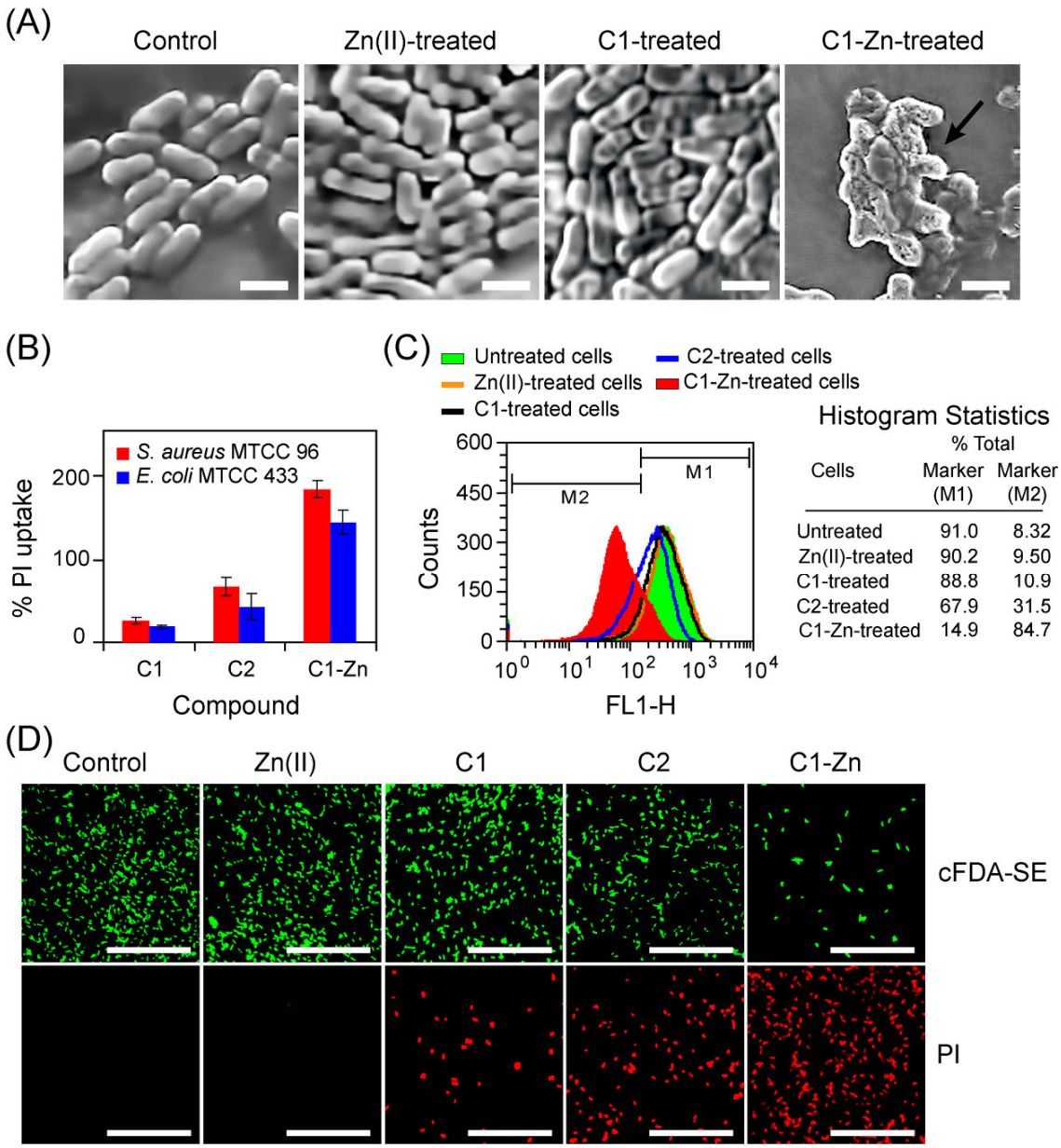

Figure S1. (A) FESEM images of *E. coli* MTCC 433 treated with Zn(II), C1 and C1-Zn (6.0 μM each). Arrow in the panel for C1-Zn-treated cells indicates damaged cell. Scale bar for the images is 1.0 μm. (B) PI uptake assay for target bacterial cells treated with amphiphiles (6.0 μM each for 6 h). (C) Flow cytometry analysis of cFDA-SE labelled cells of *E. coli* MTCC 433 treated with Zn(II) and the amphiphiles (6.0 μM each). (D) Fluorescence microscope analysis of *E. coli* MTCC 433 cells treated with Zn(II) and amphiphiles (6.0 μM each for 6 h). Scale bar for the images is 50 μm.

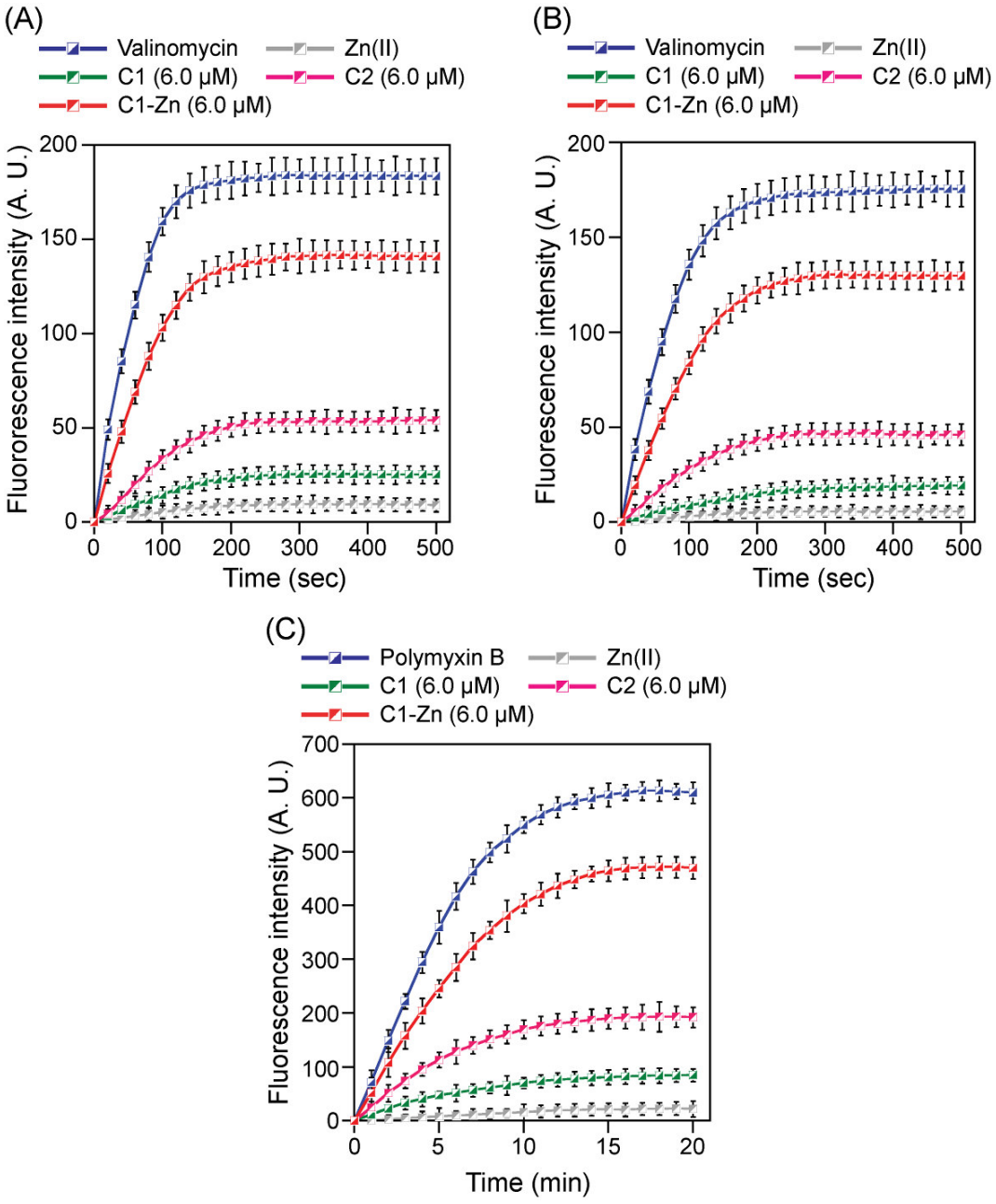

Figure S2. DiSC$_3$5-based membrane depolarization assay for (A) *S. aureus* MTCC 96 and (B) *E. coli* MTCC 433 cells treated with Zn(II) and amphiphiles. Cells treated with 30 μM valinomycin were used as positive control in the assay. (C) NPN-based outer membrane permeabilization assay for *E. coli* MTCC 433 cells treated with Zn(II) and amphiphiles. Cells treated with 1.0 μg/mL polymyxin B were used as positive control in the assay.

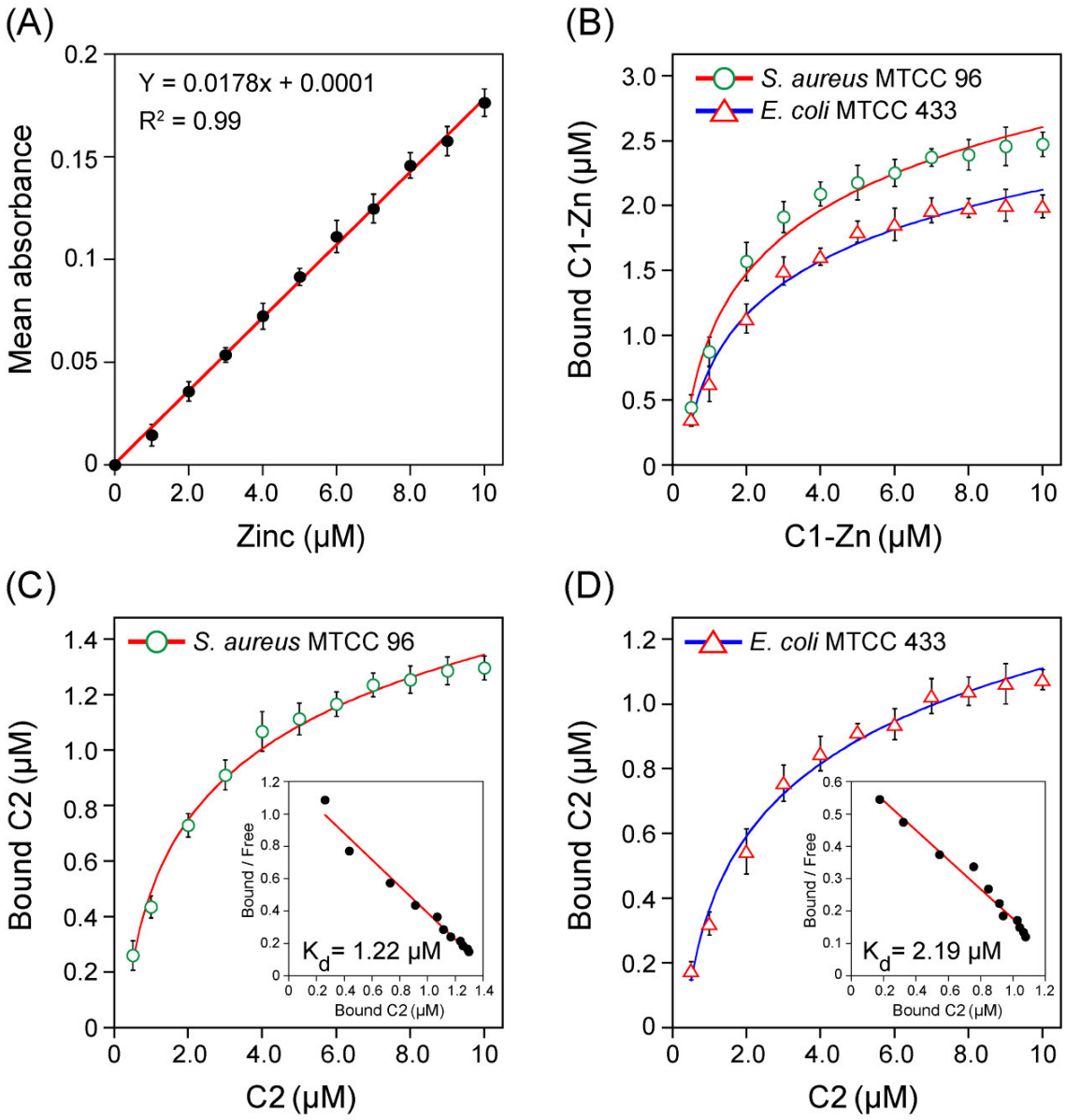

Figure S3. (A) Calibration plot of absorbance versus concentration for zinc measured by atomic absorption spectroscopy. (B) Concentration-dependent binding of C1-Zn on bacterial cells. (C-D) Concentration-dependent binding of C2 on bacterial cells. Inset in (C) and (D) indicate Scatchard plot for determination of binding affinity of C2.

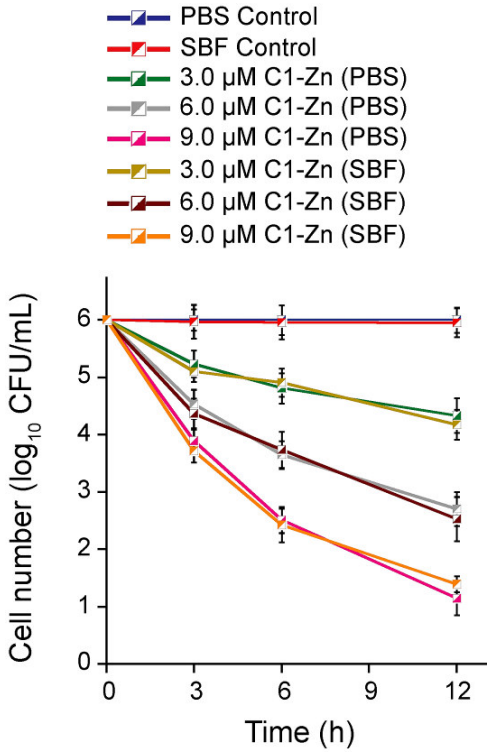

Figure S4. Time-kill curve for C1-Zn against *S. aureus* MTCC 96 cells suspended in SBF and PBS.

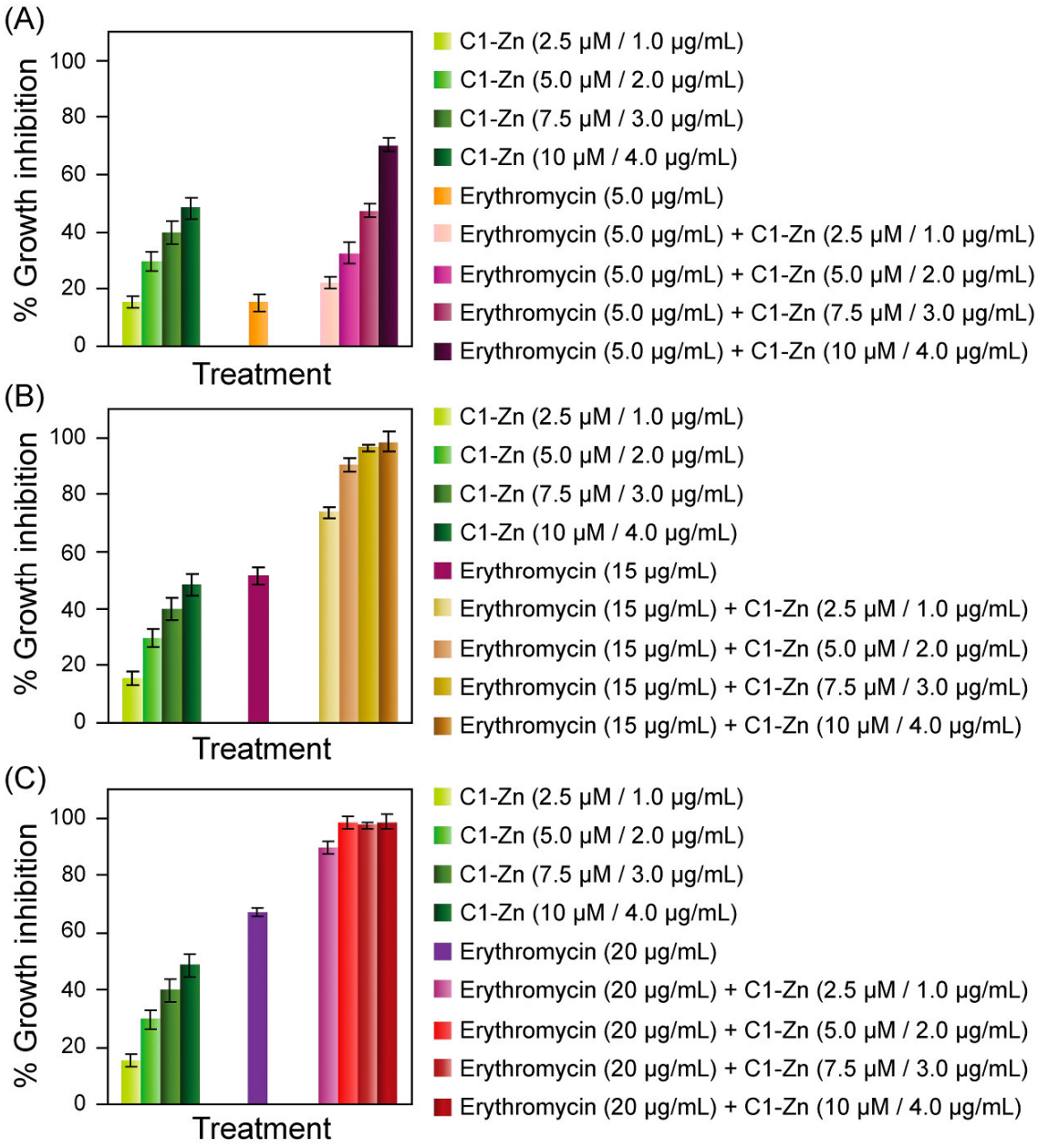

Figure S5. Effect of combined treatment of C1-Zn and erythromycin on the growth of *E. coli* MTCC 433. The concentrations of C1-Zn are indicated in both µM as well as µg/mL in parenthesis.

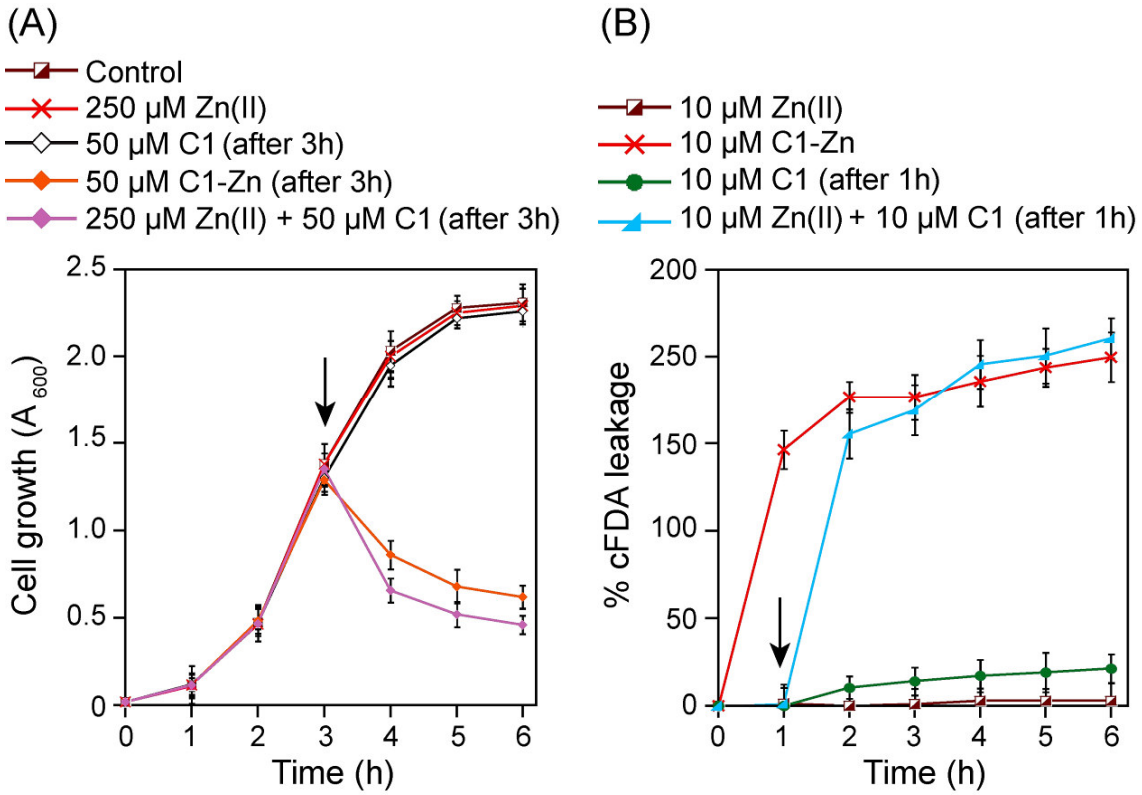

Figure S6. (A) Effect of addition of C1 on *E. coli* MTCC 433 cells grown in presence of 250 μM Zn(II). (B) cFDA-Se leakage assay in *E. coli* MTCC 433 cells incubated in 5.0 mM HEPES buffer in presence of 10 μM Zn(II) followed by addition of C1. Arrow in (C) and (D) indicate the time of addition of C1.

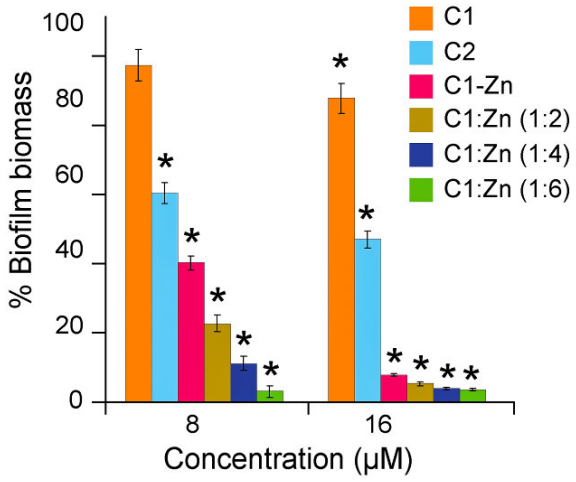

Figure S7. Effect of C1 on *S. aureus* MTCC 96 biofilm grown in a media supplemented with varying levels of zinc. The ratio of C1: Zn(II) chosen in the experiments is indicated. Amphiphile (C1) concentrations used in the experiments were 8.0 μM and 16 μM. * indicates *p* value < 0.001 in ANOVA.

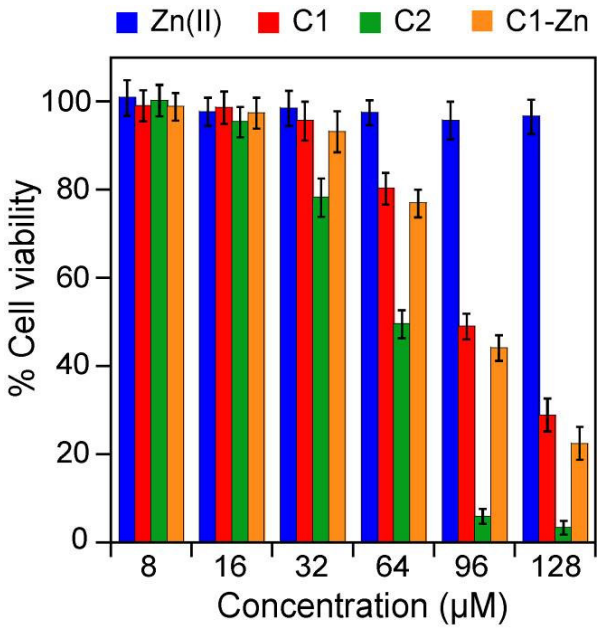

Figure S8. MTT-based assay to ascertain *in vitro* cytotoxicity of C1, C2 and C1-Zn on the viability of cultured HeLa cells. Each data point represents mean ± SD values from six independent samples.

Table S1. Minimum inhibitory concentration (MIC) of C1, C2 and C1-Zn against target bacteria.

Target Bacteria	MIC of Amphiphiles		
	C1*	C2*	C1-Zn
Staphylococcus aureus MTCC 96	25 μM / 9.2 μg/mL	16 μM / 10.4 μg/mL	8.0 μM / 3.5 μg/mL
Listeria monocytogenes Scott A	25 μM / 9.2 μg/mL	16 μM / 10.4 μg/mL	8.0 μM / 3.5 μg/mL
Escherichia coli MTCC 433	100 μM / 36.8 μg/mL	64 μM / 41.6 μg/mL	32 μM / 14 μg/mL
Enterobacter aerogenes MTCC 2822	128 μM / 47 μg/mL	80 μM / 52 μg/mL	64 μM / 28 μg/mL

* MIC of C1 and C2 represent the MIC of compound 5 and compound 6, respectively, as described in Goswami et al., *Journal of Materials Chemistry B* **2013**, *1*, 2612-2623.

Table S2. Fold reduction in MIC of erythromycin in combination with C1-Zn and determination of fractional inhibitory concentration (FIC) index.

Indicator strain	C1-Zn	Folds reduction in MIC of erythromycin	FIC index[a]	Effect[b]
E. coli MTCC 433	2.5 µM	4.0	0.32	SYN
	5.0 µM	4.0	0.40	SYN
	7.5 µM	8.0	0.35	SYN
	10 µM	16.0	0.37	SYN

[a] Fractional inhibitory concentration (FIC) index was assessed as described in the experimental section. [b] An FIC index of ≤ 0.5 is considered as synergism, FIC index of > 0.5-1.0 indicates additive effect, FIC index >1.0 - <4.0 represents indifference and FIC index of ≥ 4.0 indicates antagonism. SYN: Synergism

Table S3. Minimum biofilm eradication concentration ($MBEC_{90}$) of C1, C2, C1-Zn and C1 supplemented with Zn(II) in different ratios against *S. aureus* MTCC 96 biofilm.

Amphiphiles	$MBEC_{90}$ against *S. aureus* MTCC 96 biofilm
C1	> 256 µM
C2	64 µM
C1-Zn	32 µM
C1+ 50 µM Zn(II)	16 µM

Table S4. Minimum biofilm inhibitory concentration ($MBIC_{90}$) of C1, C2, C1-Zn and C1 supplemented with zinc in various ratios against *S. aureus* MTCC 96 biofilm.

Amphiphiles	$MBIC_{90}$ against *S. aureus* MTCC 96 biofilm
C1	256 µM
C2	32 µM
C1-Zn	16 µM
C1+ Zn(II) (1:2)	16 µM
C1+ Zn(II) (1:4)	8.0 µM
C1+ Zn(II) (1:6)	8.0 µM