Light-Triggered Release of Ciprofloxacin from *in situ* Forming "Click" Hydrogels for Antibacterial Wound Dressings

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SUPPLEMENTARY INFORMATION

EXPERIMENTAL SECTION

General Considerations

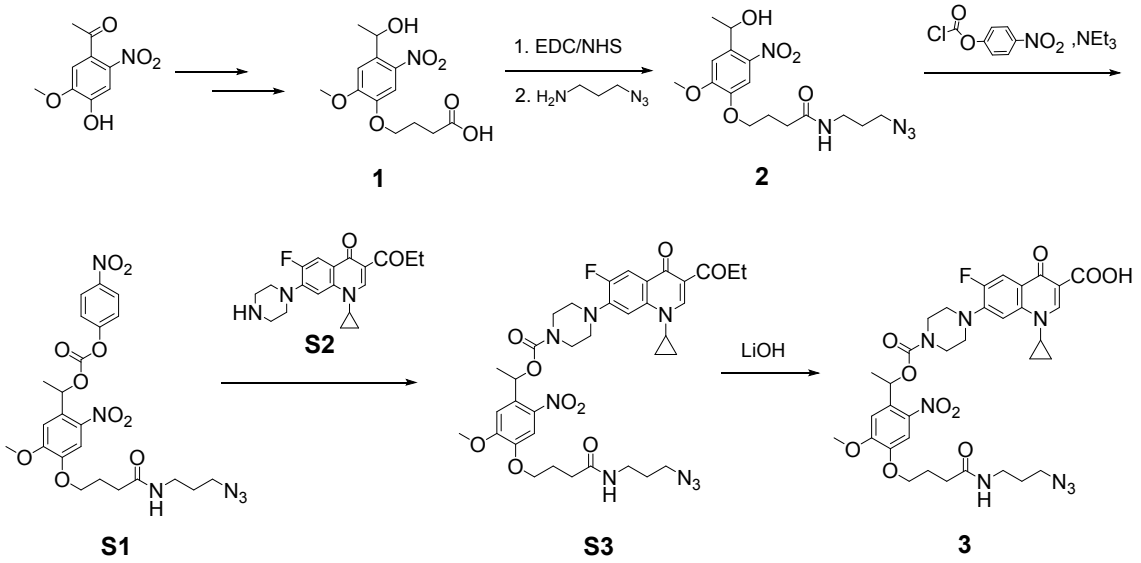
Reagents. 4-arm PEG-NH_2 (molar mass 20 000 g mol^{-1}) was purchased from Jenkem Technology, USA; 4-nitrophenyl chloroformate was purchased from VWR, Australia; DBCO-acid was purchased from Click Chemistry Tools, USA; N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC.HCl) was purchased from Carbosynth, UK; all other chemicals were purchased from Sigma-Aldrich, Australia and used as received. 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoic acid (compound **1**)[1] and ciprofloxacin ethyl ester[2] were synthesized following previously reported procedures. 4-arm PEG-DBCO and 4-arm PEG-N_3 were synthesized according to our previously developed methods.[3]

NMR Spectroscopy. ^1H and ^{13}C NMR spectra were recorded in either $CDCl_3$ or DMSO-d_6 on a Bruker Advance III 400 spectrometer at 298 K. Chemical shifts are reported as δ in parts per million (ppm) and referenced to the chemical shift of the residual solvent resonances ($CDCl_3$ ^1H: δ = 7.26 ppm, ^{13}C: δ = 77.16 ppm; DMSO-d_6 ^1H: δ = 2.52 ppm, ^{13}C: δ = 39.52 ppm). The resonance multiplicities are described as s (singlet), d (doublet), t (triplet), q (quartet) or m (multiplet).

Rheology Measurements. Rheology studies were carried out using an Anton Paar Physica rheometer with a plate-plate configuration. The lower plate is made of quartz and the upper plate from stainless steel with a diameter of 8 mm. Tests were carried out by applying a 1% strain with a frequency of 1 Hz to ensure measurements were performed in the viscoelastic region. In a typical experiment, a solution (15 µL) containing a mixture of polymer precursors in phosphate buffered saline (PBS) (10 wt%) was transferred to the lower plate and the upper plate was lowered to a measurement gap of 0.3 mm and the storage (G') and loss (G") moduli were immediately recorded.

High Performance Liquid Chromatography (HPLC). HPLC analysis was performed on a Hewlett-Packard 1100 series HPLC system (Agilent Technologies, CA) with a reverse-phase VydacTM analytical (C18, 300 Å, 5 µm, 4.6 mm x 150 mm) column. The eluent gradient was 0% to 20% solvent B (solvent A: 0.1% trifluoroacetic acid (TFA)/H_2O; solvent B: 0.1% TFA/CH_3CN) for the first 20 min followed by a elution gradient of 20% to 70% of solvent B over a 30 min period ((solvent A: 0.1% TFA/H_2O; solvent B: 0.1% TFA/CH_3CN)). The flow rate was 1 mL min^{-1}.

Synthetic Procedures



Scheme S1. Synthetic procedures for PC-CIP.

Synthesis of N-(3-azidopropyl)-4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanamide (**2**). Compound **1** (0.586 g, 2 mmol) was dissolved in DMF (20 mL) at 0 °C followed by addition of EDC.HCl (0.472 g, 3 mmol) and N-hydroxysuccinimide (0.357 g, 3 mmol). The solution was stirred at 0 °C for 2.5 h and at ambient temperature for 2 h. DMF was removed *in vacuo* and the residue was dissolved in ethyl acetate (EtOAc, 50 mL) and washed with water (50 mL x 2). The organic phase was dried over $MgSO_4$, concentrated *in vacuo* and used in the next step.

The above product was dissolved in DMF (10 mL) followed by addition of 3-azido-1-propanamine (0.426 g, 4.25 mmol) and NEt_3 (0.442 g, 4.3 mmol). Subsequently, the solution was stirred overnight at ambient temperature. DMF was removed *in vacuo* and the residue was dissolved in EtOAc (50 mL) and washed with water (50 mL). The organic phase was dried over $MgSO_4$, concentrated *in vacuo* and purified by silica gel flash chromatography, eluting with EtOAc to give the product as slightly yellow powder (yield: 70%). ^{1}H NMR (400 MHz, , DMSO-d_6): 7.90 (1H, t, *J* = 5.5 Hz), 7.57 (1H, s), 7.19 (1H, s), 6.28 (1H, d, *J* = 6.5 Hz), 4.08 (2H, t, *J* = 6.5 Hz), 3.97 (3H, s), 3.11 (2H, d, *J* = 5.8 Hz), 2.25 (2H, t, *J* = 7.4 Hz), 1.98 (2H, d, *J* = 10.9 Hz), 1.73 (3H, d, *J* = 6.5 Hz), 1.65 (2H, t, *J* = 6.8 Hz).

Synthesis of 1-(4-(5-((3-azidopropyl)amino)-5-oxopentyl)-5-methoxy-2-nitrophenyl)ethyl (4-nitrophenyl) carbonate compound (**S1**). Compound **2** (0.38 g, 1 mmol) was dissolved into EtOAc (20 mL) followed by addition of 4-nitrophenyl chloroformate (0.4 g, 2 mmol), NEt_3 (0.2 g, 2 mmol) and 4-dimethylaminopyridine (5 mg, 41 mmol) at 0 °C. The solution was stirred at 0 °C for 1 hour. The reaction solution was then washed with 1 M HCl (50 mL). The organic phase was dried over $MgSO_4$, concentrated *in vacuo* and purified by silica gel flash chromatography, eluting with EtOAc/Methanol (v/v = 9/1) to give the product as slightly yellow powder (yield: 73%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.34 – 8.26 (2H, m), 7.91 (1H, t, *J* = 5.5 Hz), 7.59 (1H, s), 7.57 – 7.49 (2H, m), 7.20 (1H, s), 6.29 (1H, d, *J* = 6.5 Hz), 4.08 (2H, t, *J* = 6.5 Hz), 3.97 (3H, s), 3.11 (2H, d, *J* = 5.8 Hz), 2.25 (2H, t, *J* = 7.4 Hz), 1.98 (2H, d, *J* = 10.9 Hz), 1.73 (3H, d, *J* = 6.5 Hz), 1.65 (2H, t, *J* = 6.8 Hz).

Synthesis of ethyl 1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylate (**S2**). Ciprofloxacin (0.5 g, 1.5 mmol) was suspended in ethanol (50 mL) followed by addition of concentrated H_2SO_4 (1 mL). The mixture was refluxed for 5 hours at 105 °C. Ethanol was removed *in vacuo* and the residue was redissolved in DCM (30 mL) and wash with saturated aqueous sodium bicarbonate solution (30 mL). The organic phase was dried over $MgSO_4$ and concentrated *in vacuo* to give the product as white powder (yield: 67%). ^{1}H NMR (400MHz, $CDCl_3$): δ 8.46 (1H, s), 7.98 (1H, d, *J* = 13.4 Hz), 7.23 – 7.16 (3H, m), 4.32 (2H, q, *J* = 7.1 Hz), 3.50 – 3.30 (1H, m), 3.17 (4H, dd, *J* = 6.1, 3.7 Hz), 3.03 (4H, dd, *J* = 6.0, 3.7 Hz), 1.34 (3H, t, *J* = 7.1 Hz), 1.25 (2H, q, *J* = 7.2 Hz), 1.13 – 1.01 (2H, m).

Synthesis of ethyl 7-(4-((1-(4-(5((3-azidopropyl)amino)-5-oxopentyl)-5-methoxy-2-nitrophenyl)ethoxy)carbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (**S3**). Compound **S1** (216 mg, 0.4 mmol) and compound **S2** (329 mg, 1 mmol) were dissolved in DMF (15 mL) followed by addition of NEt_3 (1 g, 10 mmol). The solution was stirred at ambient temperature for 1 hour and then diluted by addition of EtOAc (45 mL). The reaction solution was washed with 1 M HCl solution (30 mL) and brine (30 mL). The organic phase was dried $MgSO_4$, concentrated *in vacuo* and purified by silica gel flash chromatography, eluting with EtOAc/Methanol (v/v = 9/1) to give the product as yellow oil (yield: 94%) which solidified on standing. ^{1}H NMP (400 MHz, $CDCl_3$): δ 8.42 (1H, s), 7.90 (1H, d, *J* = 13.1 Hz), 7.47 (1H, s), 7.19 (1H, d, *J* = 7.1 Hz), 6.90 (1H, s), 6.32 (1H, q, *J* = 6.4 Hz), 6.16 (1H, t, *J* = 5.6 Hz), 4.29 (2H, q, *J* = 7.1 Hz), 4.08 – 3.99 (2H, m), 3.88 (3H, σ), 3.80 – 3.48 (4H, m), 3.41 – 3.32 (1H, m), 3.27 (4H, q, *J* = 6.3 Hz), 3.16 (4H, s), 2.34 (2H, t, *J* = 7.2 Hz), 2.19 – 2.03 (2H, m), 1.71 (1H, m), 1.59 (3H, d, *J* = 6.5 Hz), 1.32 (2H, m), 1.25 (2H, q, *J* = 7.1 Hζ), 1.06 (2H, q, *J* = 6.6 Hz).

Synthesis of 7-(4-((1-(4-(5((3-azidopropyl)amino)-5-oxopentyl)-5-methoxy-2-nitrophenyl)ethoxy)carbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**3**). Compound **S3** (0.29 g, 0.38 mmol) and LiOH (1.5 g, 62.5 mmol) were dissolved in water (12 mL) and methanol (38 mL). The solution was stirred at ambient temperature for 2 hr and acidified by adding 1M aqueous HCl. Methanol was removed *in vacuo* and the residue was extracted with CH_2Cl_2 (20 mL x 3). The organic phase was dried over $MgSO_4$, concentrated *in vacuo* and purified by silica gel flash column chromatography, eluting with EtOAc/Methanol/Acetic acid (v/v/v = 9/1/0.1) to give the product as yellow powder (yield: 86%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 14.83 (1H, s), 8.71 (1H, s), 7.98 (1H, d, *J* = 12.8 Hz), 7.49 (1H, s), 7.30 (1H, d, *J* = 7.0 Hz), 6.89 (1H, s), 6.33 (1H, q, *J* = 6.5 Hz), 5.79 (1H, m), 4.09-3,99 (2H, m), 3.89 (3H, s), 3.85 – 3.51 (1H, m), 3.42-3.50 (1H, m), 3.35 – 3.13 (8H, m), 2.34 (2H, t, *J* = 7.1 Hz), 2.12 (2H, t, *J* = 6.6 Hz), 1.67-1.77 (2H, m), 1.61 (3H, d, *J* = 6.5 H), 1.33 (2H, q, *J* = 6.8 Hz), 1.10-1.16 (2H, m). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.07, 172.22, 166.85, 154.88, 154.11, 153.71, 152.38, 147.58, 147.26, 145.60, 145.50, 140.21, 139.02, 133.18, 120.34, 120.26, 112.69, 112.45, 109.26, 108.32, 108.20, 105.19, 77.35, 77.23, 77.03, 76.71, 69.98, 68.59, 56.35, 49.41, 37.25, 35.34, 32.76, 28.80, 24.82, 22.04, 20.37, 8.27. ESI+/MS: Calculated: 737.3 Found: 737.2.

Fabrication of PC-CIP conjugated hydrogel. PEG-DBCO (1.4 mg, *ca.* 0.07 μmol) was dissolved in CH_2Cl_2 (1 mL) followed by addition of PC-CIP solution (1.4 μM) in CH_2Cl_2 (40 μL). The mixture was vortexed and allowed to react for 30 min at ambient temperature. CH_2Cl_2 was removed by placing

the solution under a gentle flow of N_2 gas for 1 h. The obtained product was redissolved in PBS solution (20 μL) and was mixed with 4-arm PEG-N_3 in PBS solution (2.8 mM, 20 μL) and immediately transferred onto a 24 x 60 mm coverslip and covered with another round coverslip (9 mm in diameter) on top of the gel and allowed to gel for 1 hour. The gel was peeled off from the coverslip and soaked in Milli-Q water for 12 hours before further study. A control hydrogel was prepared by mixing equal volume of 4-arm PEG-DBCO (2.8 mM in PBS) with 4-arm PEG-N_3 (2.8 mM in PBS).

UV-Triggered Release of CIP from PC-CIP Conjugated Hydrogel.

Hydrogels were soaked in 20% aqueous CH_3CN for 24 hours with a solution change every 6 hours to remove any unreacted polymer precursors and PC-CIP. Subsequently, the hydrogels were placed under a UV lamp and exposed to UV light (360 nm wavelength) at an intensity of 10 mW cm^{-2} for 2 or 5 min. The gels were then submerged in 20 v/v% aqueous CH_3CN (120 μL). At a predetermined time point, an aliquot was withdrawn for HPLC analysis and replaced with fresh solution with similar volume.

Bacterial Assay on Hydrogels

S. aureus ATCC 25923 was grown overnight in nutrient broth. Bacterial density was adjusted to 10^8 colony forming unit per mL, corresponding to optical density (600 nm) of 0.1. 100 μL of this bacterial bacterial suspension was placed on Muller-Hinton Plates and a cotton swab was used to spread the cells evenly. Hydrogels (PC-CIP conjugated hydrogel and control hydrogel) were then placed on the middle surface of the culture plates. Subsequently, the gels were exposed to UV irradiation (365 nm wavelength) at the intensity of 10 mW cm^{-2} for 2 min or 5 min and incubated at 37 °C overnight. Aluminum foil was used to screen UV light surrounding the hydrogel on the plate.

A control study using unmodified CIP was also undertaken to examine the antibacterial effect. In this study, PBS solution (40 μL) containing 2.6 μg and 4.3 μg CIP respectively were deposited onto the middle of the plates and incubated at 37 °C overnight. Bacterial growth and zone of inhibition were subsequently imaged using a digital camera.

Fibroblast Cytotoxicity Tests following UV exposure

Cell preparation. NIH/3T3 cells were thawed from cryo-stage at 37 °C and cultured in DMEM with 10% FBS, 2% penicillin/streptomycin at 37 °C. Cells were then subcultured in 24 well plates at a density of 5 x 10^4 cell per well and incubated at 37 °C for 24 hours.

Gel preparation. The gels were prepared as described above and swelled in culture medium for 24 hours before use.

Biocompatibility tests. For the UV exposure tests, culture media (1 mL) was replaced with fresh media and the gels were placed into the wells. After the gel sank to the bottom, the plate was

irradiated with UV light under the same intensity used for bacterial assay studies (10 mW/cm^2) for 5 min.

To test the possibility of cytoxcity from possible small molecules diffusing from the hydrogel following UV irradiation, pre-swelled gels (without attached CIP) were placed on empty wells and irradiated with the same UV light for 5 min. Then 1 mL of culture media was added and incubated for 2 hours at 37 °C with shaking to allow any possible small molecules generated by the photo-cleavage to diffuse into culture media. Afterwards, the culture media in the cell culture plate was removed and replaced by this solution. For the control study, culture media (1 mL) was changed with fresh media. All cell plates were incubated for another 24 hours at 37 °C before imaging. Tests were performed in triplicate and three randomly selected images were taken from each well.

Live/dead staining: Live/dead assay was performed using Live/Dead Viability/Cytotoxicity Assay Kit (Molecular Probes, Invitrogen) on NIH/3T3 cells treated as described above. Briefly, cells were gently agitated in PBS prior to treatment with 1 µM Calcein AM/8 µM ethidium homodimer. After 20 min of calcein/ethidium incubation, cells were imaged under a fluorescence microscope. Live cells were stained with green fluorescence while dead cells were stained red.

Statistical analysis: Fibroblast survival percentage was calculated based on the number of live and dead cells per imaging field. Results were expressed in mean $\pm$ standard deviation. The condition of equal variance was achieved by logarithmic transformation of the data. A one-way ANOVA was used to test for statistical significance ($P<0.05$).

References

1. B. Yan, J.-C. Boyer, D. Habault, N. R. Branda and Y. Zhao, *Journal of the American Chemical Society*, 2012, **134**, 16558-16561.
2. X. Li, Y.-K. Zhang, J. J. Plattner, W. Mao, M. R. K. Alley, Y. Xia, V. Hernandez, Y. Zhou, C. Z. Ding, J. Li, Z. Shao, H. Zhang and M. Xu, *Bioorganic & Medicinal Chemistry Letters*, 2013, **23**, 963-966.
3. V. X. Truong, K. M. Tsang, G. P. Simon, R. L. Boyd, R. A. Evans, H. Thissen and J. S. Forsythe, *Biomacromolecules*, 2015, **16**, 2246-2253.

SUPPLEMENTARY FIGURES

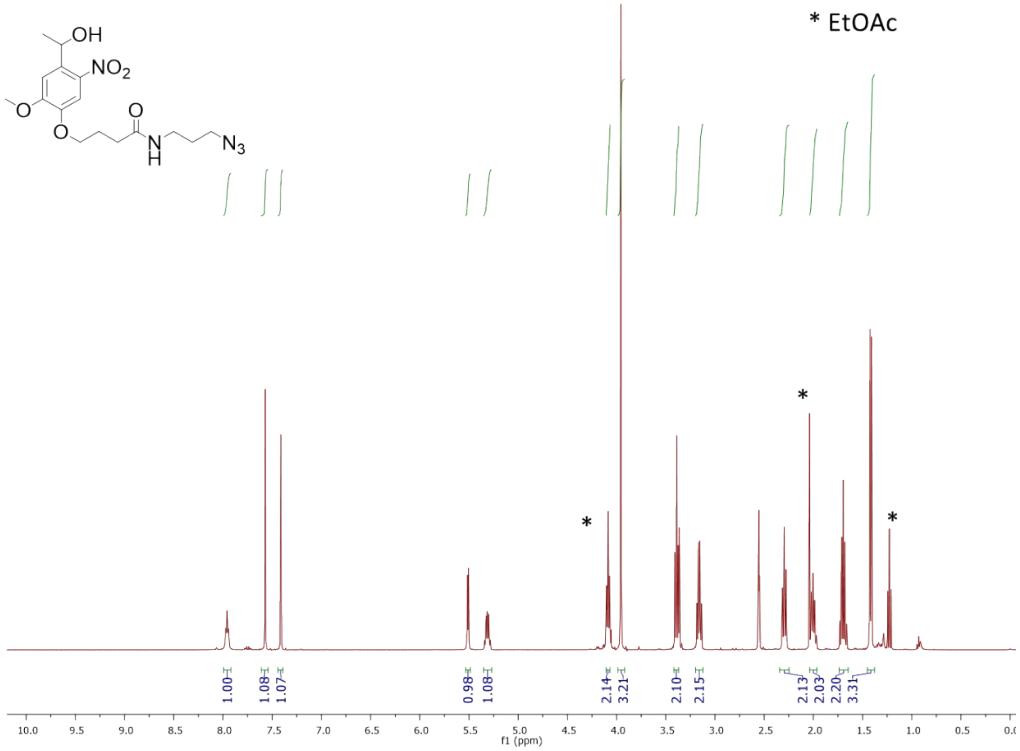


Fig. S1 ^{1}H NMR spectrum of compound **2** (DMSO-d_6, 400 MHz).

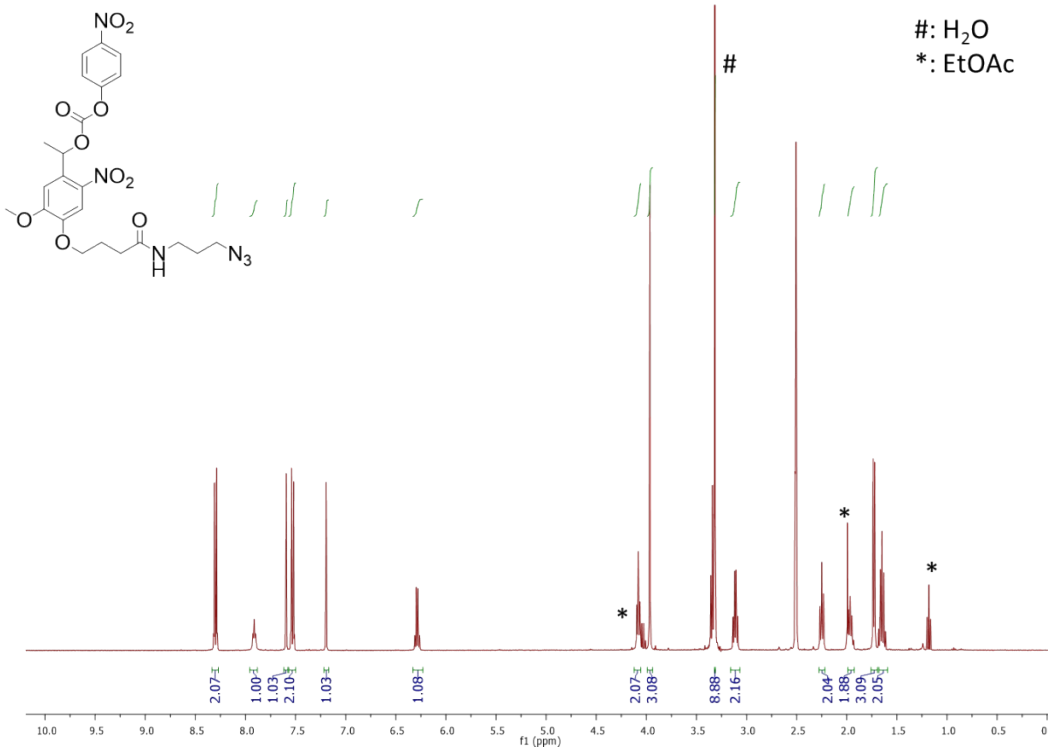


Fig. S2 ^{1}H NMR spectrum of compound **S1** (DMSO-d_6, 400 MHz).

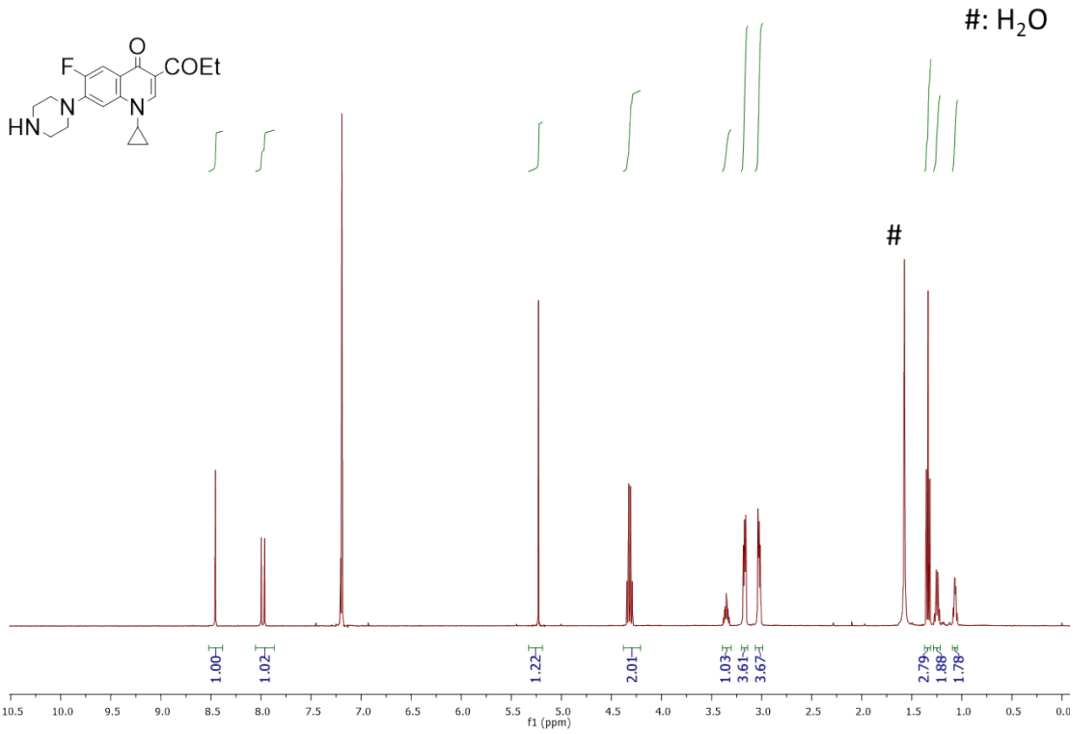


Fig. S3 ^{1}H NMR spectrum of compound **S2** ($CDCl_3$, 400 MHz).

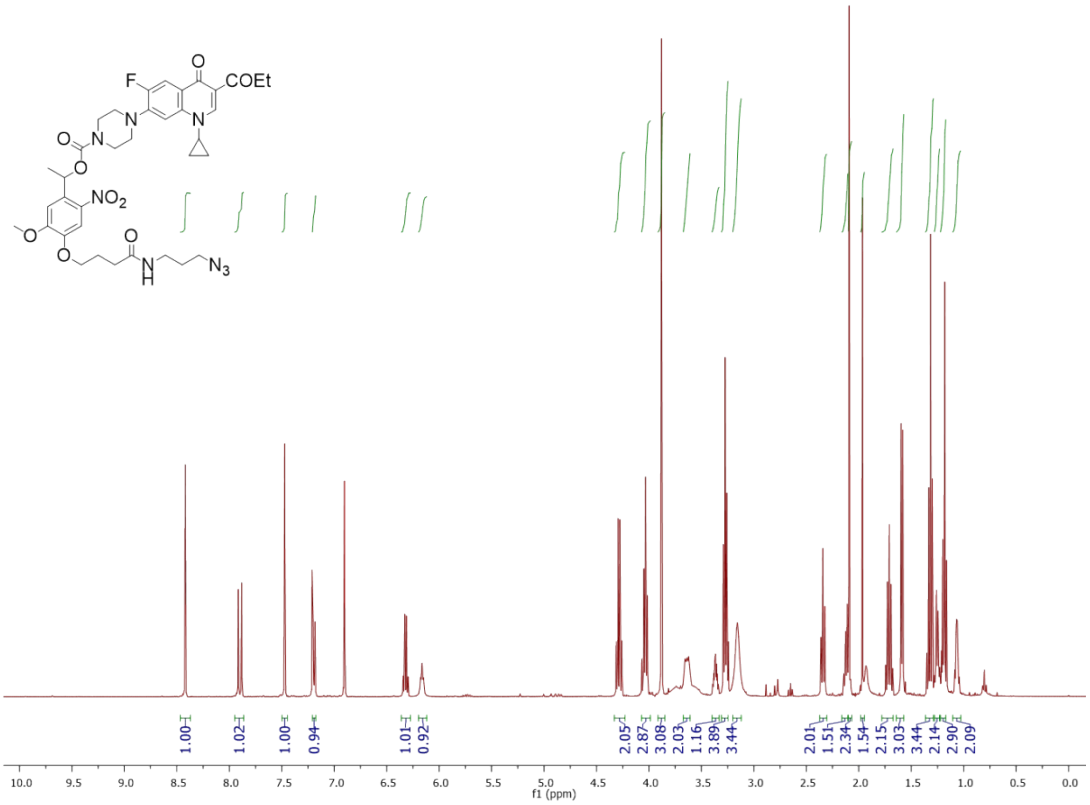


Fig. S4 ^{1}H NMR spectrum of compound **S3** ($CDCl_3$, 400 MHz).

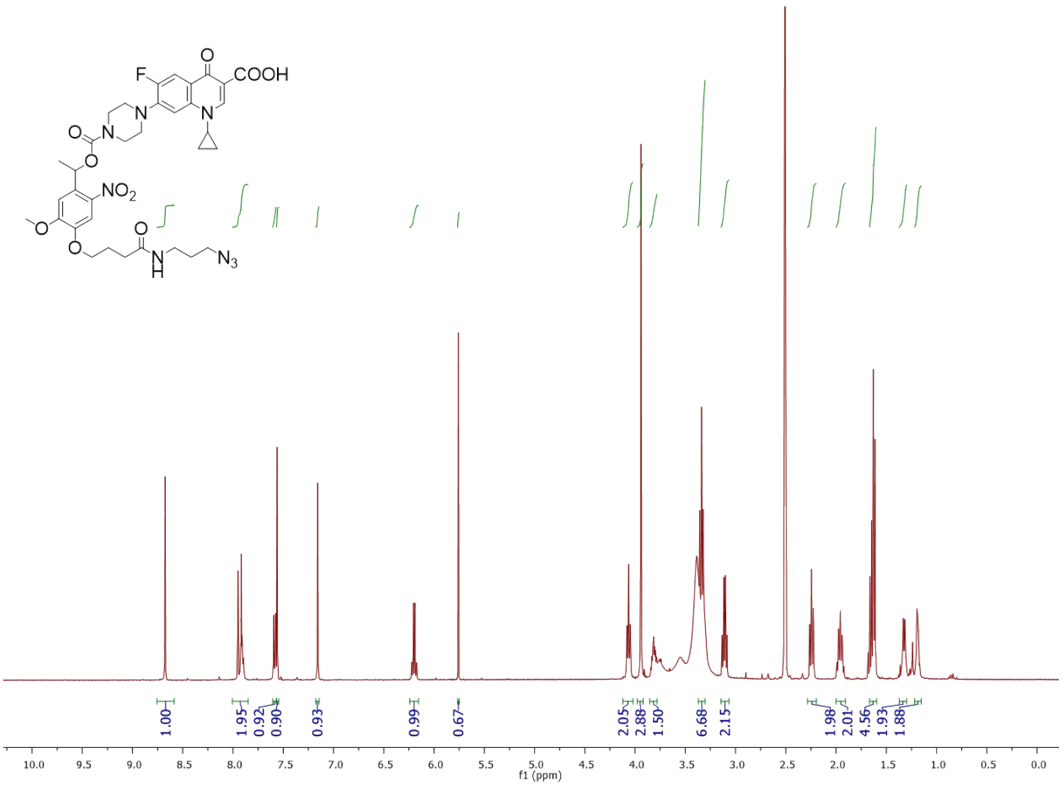


Fig. S5 ^{1}H NMR spectrum of compound **3** (DMSO-d_6, 400 MHz).

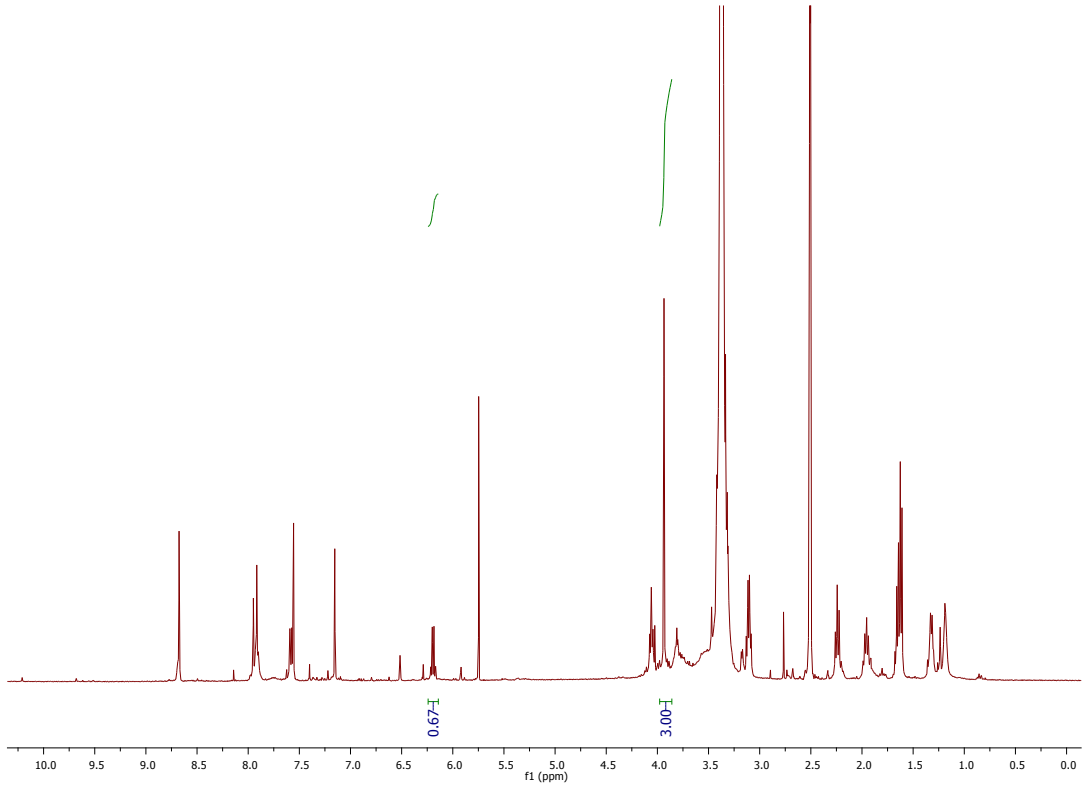


Fig. S6 ^{1}H NMR spectrum of compound **3** (DMSO-d_6, 400 MHz) after irradiation with UV for 8 min showing the decrease of the chemical shift corresponding to the proton adjacent to the carbamate group.

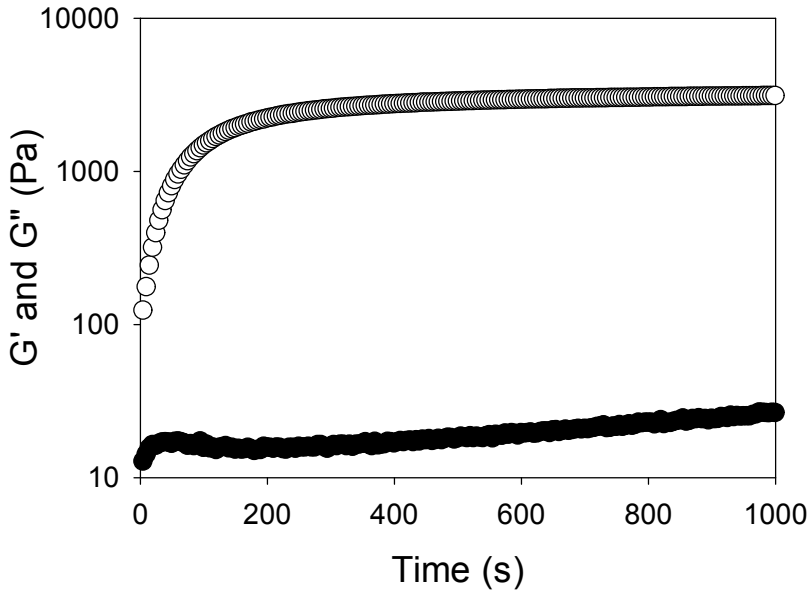


Fig. S7 Rheological analysis of gelation of 4-arm PEG-DBCO and 4-arm PEG-N_3 with a 1:1 molar ratio of DBCO to azide showing evolution of G' (empty circle) and G" (filled circle) with time.

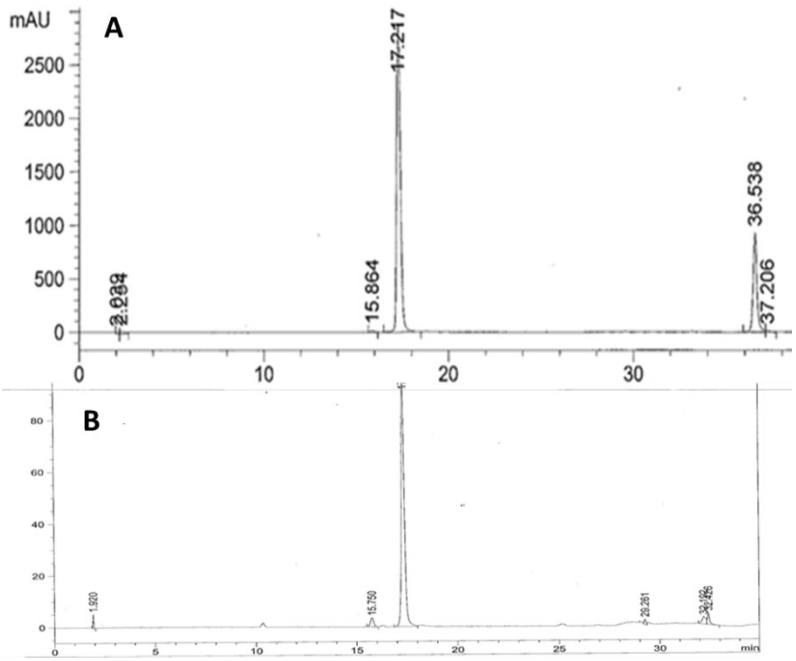


Fig. S8 HPLC profile of (**A**) mixture of PC-CIP and CIP; and (**B**) solution from PC-CIP conjugated hydrogel after 2 min of UV irradiation.

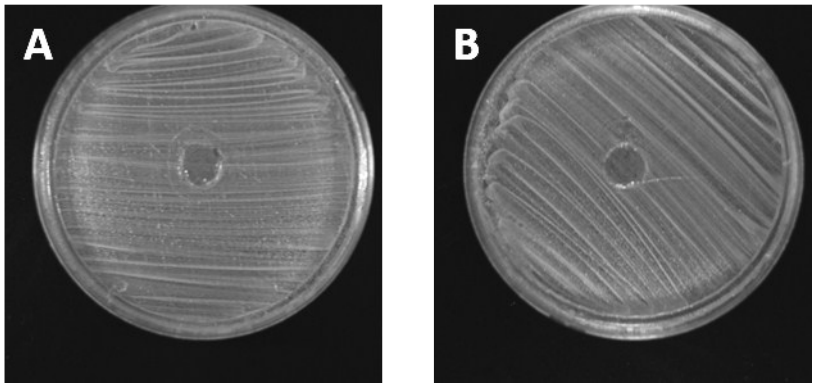


Fig. S9 Bacterial assay on control hydrogel without conjugated PC-CIP before (**A**) and after (**B**) 5 min of UV irradiation and overnight incubation. The position where the hydrogel was placed, in the middle of the culture plate, is evident. There is no inhibition of bacterial growth in or around this position.

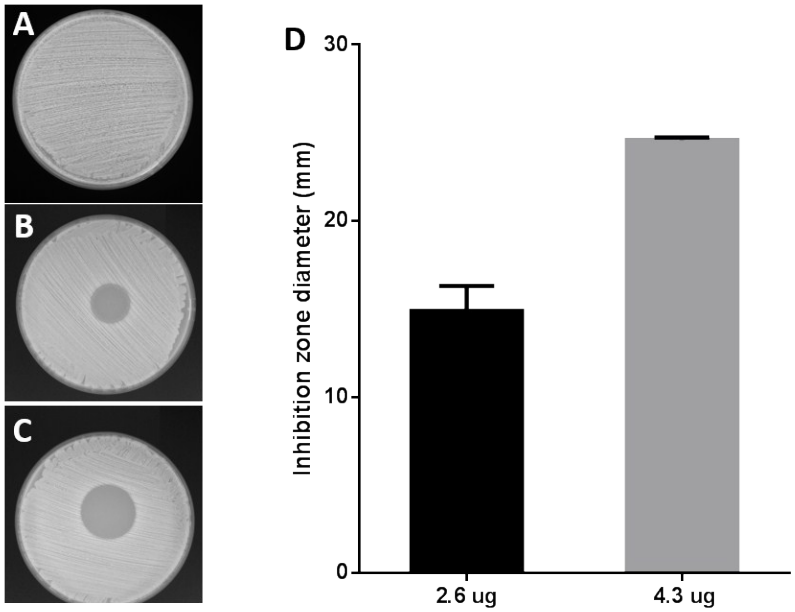


Fig. S10 Antimicrobial activity from native CIP. Inhibition of *S. aureus.* growth on Muller – Hinton agar plates of with (**A**) no CIP; (**B**) drop of solution containing 2.6 µg of CIP; (**C**) drop of solution containing 4.3 µg CIP; and (**D**) corresponding measurements of inhibition zone. The amounts of CIP used were similar to those released from hydrogels after UV treatment for 3 min and 5 min respectively.

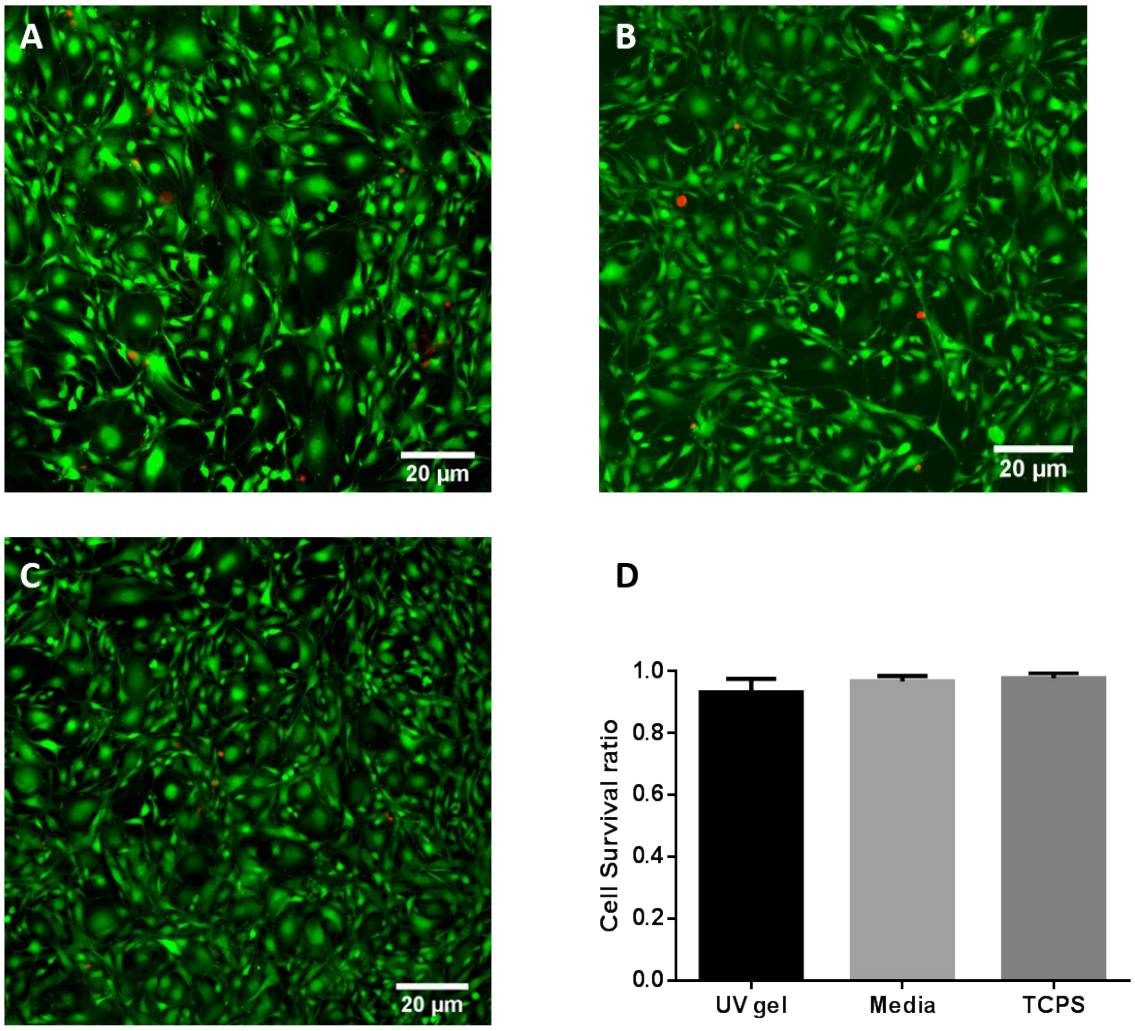


Fig. S11 Representative live-dead images of NIH/3T3 fibroblast cells after 24hr incubation. (**A**) Cells and PC-CIP hydrogel directly exposed to UV. (**B**) Cells exposed to media collected following UV exposure of the PC-CIP hydrogel from a separate culture well. (**C**) Cells cultured without PC-CIP hydrogel/UV exposure. **(D)** Corresponding measurements of cell survival ratio. Cell survival ratio were 93.2±4.3%, 96.8±1.8% and 97.8±1.8% for **(A)**, **(B)** and **(C)** respectively. UV exposure was 5 min, 10 $mWcm^{-2}$. There was no statistical difference between the test samples and the TCPS control (one-way ANOVA).