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Supporting Information

Simultaneous and Controlled Release of Two Different Bioactive Small Molecules from Nature Inspired Single Material

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Experimental

Materials

Branched poly (ethyleneimine) (BPEI, MW ~25000), dipentaerythritol penta-acrylate (5-Acl, Mw ~524.21), were purchased from Sigma Aldrich (Bangalore, India). Propylamine, pentylamine, hexylamine, octylamine and decylamine were purchased from Sigma Aldrich (Bangalore, India). Doxorubicin hydrochloride and Tetracycline hydrochloride, Methylene blue were obtained from Sigma Aldrich (Bangalore, India). Absolute Ethyl alcohol was purchased from TEDIA Company (United States of America). Reagent grade THF, acetone, methanol, NaCl were purchased from RANKEM (Maharashtra, India). Rhodamine-6G, fluorescein were purchased from Labo Chemie (Laboratory Reagents and Fine Chemicals Mumbai India). Dichloromethane, chloroform were obtained from Merck Specialties Pvt Ltd (Mumbai, India). Phosphate Buffer Saline (PBS) capsules of pH 7.4 was obtained from Sigma Aldrich (Bangalore India) and were dissolved in Milli-Q grade water according to the procedure as prescribed by the company (i, e. one tablet in 200 mL of water). Aluminum wire used for submerging the material under PBS buffer was purchased from local electrical shop in Guwahati city (Assam, India). All materials were used as obtained without any further purification.

General considerations

Glass vials (Borosil) were washed with acetone and ethanol prior to use for preparing solutions of polymer (BPEI) and small molecules (5-Acl). Field Emission Scanning electron microscope (FESEM) images were obtained using Carl Zeiss field emission scanning electron microscope and the samples were coated with a thin layer of gold prior of imaging. Contact angle measurements were performed using KRUSS Drop Shape Analyser-DSA25 instrument with an automatic liquid dispenser at ambient temperature. Advancing and receding contact angles were measured with deionized water droplet in four to five different locations of each sample. Release of drug molecules were monitored by Perkin-Elmer Lambda 25 UV/VIS Spectrophotometer. Bright-field and fluorescence microscopic images of polymeric material before and after drug loading were obtained using optical microscope Carl Zeiss AG, Jena Site Germany. Milli-Q grade water was used for all experiments. Digital pictures were captured using a canon power shot SX420 IS digital camera.

Preparation of Chemically Reactive Polymeric Material and Post Covalent Modifications:

The polymeric material was prepared according to the procedure reported earlier.[1] Briefly, NaCl salt (600μg/mL) was dissolved in ethanol and this salt doped ethanol was used in preparing solution of BPEI and 5-Acl, where 0.5 g BPEI and 1.325 g of 5Acl were separately dissolved in 10 mL of salt doped ethanol. Then, 1 mL of 5-Acl was mixed with 0.3 mL of BPEI and the reaction mixture was kept in shaking to form the semisolid and chemically reactive gel. Next, the polymeric gel was transferred in the glass tube to prepare the cylindrical shaped polymeric material. Afterwards the polymeric gel material was taken out, rinsed with THF for one hour and was subsequently transferred in decylamine solution (0.5 mL of decylamine in 10 mL of THF) for overnight. Next, the material was further washed thoroughly with THF for one hour to remove the weakly adhered decylamine molecules and was dried at ambient conditions. Then, the anti-wetting property was examined with digital images and contact angle measurements. Other water wetting properties were modulated in the chemically reactive material by treating the polymeric material with different amine containing small molecule solutions including propylamine (30 mg/mL), pentylamine (30 mg/mL), hexylamine (30 mg/mL), octylamine (30 mg/mL), decylamine (30 mg/mL) solutions for overnight. Then, the polymeric materials were further washed with THF for one hour to remove the loosely held small molecules and were dried at ambient conditions. Later, the antiwetting property was examined with digital images and contact angle measurements.

Loading and release of drug from polymeric material:

The drug molecules (Doxorubicin (DOX) and Tetracycline (TC)) were dissolved in ethanol solvent (0.5 mg/mL). Next, the polymeric material was placed in solution of selected drug molecules. A solution (1200 μL) of selected small molecules in ethanol is sufficient to get fully wet polymeric material (0.160g). Then, the wet polymeric material was kept in dark

for ethanol evaporation. Next, air-dried polymeric material was finally incubated in phosphate buffer saline (PBS) buffer (pH 7.4) at 37°C. The aliquot was replaced with fresh buffer at regular time intervals and the collected aliquot was analysed under UV/Visible spectrometer for examining the rate of release of the selected UV/Vis active small molecules. The polymeric material was kept completely submerged in PBS buffer (pH 7.4) during the entire course of release study.

Loading and release of dual drug molecules:

Here, the doxorubicin and tetracycline were mixed in equal proportions (0.2 mg/mL of each) in ethanol solvent. Next, the polymeric material was placed in solution of this mixture. Thereafter, the polymeric material was kept in air for evaporation of ethanol in dark. Next, drug loaded polymeric material was subsequently incubated in PBS at physiological conditions (pH 7.4, 37°C) for examining the extent of release of loaded small molecules. The PBS buffer was collected at regular intervals and was analysed under UV/Visible spectrometer. The two distinct peaks were observed in UV/visible spectra at 385 nm and 485 nm wavelengths for tetracycline and doxorubicin respectively, and this UV/Vis peaks allowed to quantify the rate of simultaneous release of two distinct bioactive small molecules.

Culture and maintenance of cells:

Primary HDF cells (human dermal fibroblast; Himedia, India) for cell proliferation study, MG63 (Human osteosarcoma; NCCS Pune, India) and MDA-MB-231 cells (human breast adenocarcinoma; NCCS Pune, India) cells for anticancer study were cultured in high glucose DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (fetal bovine serum; Gibco, Life Technologies, U.S.A.) and 1% antibiotic-antimycotic (Gibco, Life Technologies, U.S.A.) in tissue culture flasks and maintained at 37°C in a humidified incubator with 5% CO_2. Fresh medium was replenished every 3rd day.

Alamar blue cell proliferation assay:

Cell proliferation rate on polymeric matrix (DA treated) was evaluated following the alamar blue dye reduction method (Invitrogen, Life Technology, U.S.A.).[2] In brief, samples (6 x 2 mm; diameter x height) were conditioned with complete DMEM (supplemented with 10% FBS) for 24 h prior cell seeding. Thereafter, equal number of HDF cells ($3x10^4$ cells/ matrix) were seeded on polymeric matrix and incubated at 37 °C in a humidified incubator with 5% CO_2. At specific time points (day1, 3 and 5), cells with polymeric matrix was incubated with the dye (10%, v/v) for 3 h in dark. Afterwards, 100 µl of reduced media was spectrophotometrically recorded at 570 nm and 600 nm using a microplate reader (Tecan Infinite Pro, Switzerland). The cell proliferation was represented as the arbitrary unit (A.U.) normalized with day 1 value of reduced dye, which was directly related to the number of viable cells existing on the matrix.

To determine the cytotoxicity of aqueous release of matrix (if any), 20 µl of releasate in a ratio of 1:10 (releasate to media) was added to the cells (MG-63 and MDA-MB-231) and incubated for 48 h in the aforementioned conditions followed by MTT assay.

Live cell imaging:

Live cells on matrices were visualized using live/dead assay kit (Invitrogen, Life Technologies, U.S.A.) following the manufacture's protocol. In brief, $3x10^4$ cells were seeded on polymeric matrix and maintained for 5 days at 37°C in a humidified CO_2 incubator with periodical media changes. Thereafter, cells seeded polymeric matrix were stained with live/dead assay kit for 15 min at dark. Live/dead assay kit consists of two main components; calcein-AM and ethidium homodimer-1. Calcein-AM defuses through the intact membrane of live cells enzymatically cleaved and flourishes as green, whereas ethidium homodimer-1 flourishes as red upon binding to DNA after passing through the damaged cell membrane of dead cells.

Anticancer assessment of released DOX:

Cytotoxicity of MG-63 and MDA-MB-231 cells against released DOX from polymeric matrix was performed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay protocol.[3] In brief, the equal number of cells (10^4 cells/well) were seeded in each well of a 96-well plate and incubated at 37°C in a humidified CO_2 incubator for 24 h. Prior to treatment, the released DOX concentration of all groups (day 1, 3 and 7) was adjusted to 2 µg/ml. Thereafter, seeded cells were treated with released DOX (2 µg/ml) and incubated for next 48 h. Free DOX of the same concentration and sterile PBS (pH 7.4) were used as positive (+ Ve) and negative (-Ve) control respectively. Post 48 h, MTT (5 mg/ml of media) was added in each well and incubated for 4 h. After incubation, media was removed and DMSO (Dimethyl sulfoxide) was added to dissolve the formazan crystal. Absorbance was measured at 570 nm using a microplate reader (Tecan Infinite Pro, Switzerland).

Estimation of IC_{50} value:
To determine IC_{50} value, cells (MG-63 and MDA-MB-231) were treated with various concentrations (0-2µg/ml) of free and released DOX in triplicate and incubated for 48 h at 5% CO_2 at 37°C followed by MTT assay as described.

Analysis of cell morphology:
For morphological analysis, released DOX treated cells (MG-63 and MDA-MB-231) were investigated under phase-contrast and fluorescent (RFP mode, red filter) mode using a microscopy (Evos FL, Life Technologies, U.S.A) and photographed. Untreated cells were used as control reference.

Disc-diffusion assay:
Antibacterial activity of released tetracycline (TC) was carried out by disc-diffusion method against *Staphylococcus aureus* MTCC 3160 and *Escherichia coli* MTCC 40 (MTCC, IMTECH, India).[4] 100 µl of bacterial suspension (10^8 CFU/ml) was used to lawn the solidified nutrient agar plate (Himedia, India) evenly using a sterile glass L-spreader. The plates were allowed to dry for 10 mins and then the sensitivity test was performed. The released tetracycline concentration of all groups (i.e., day 1, 3 and 7) was adjusted to 2.5 mg/ml prior to disc impregnation. The sterile discs (6 mm in diameter) were impregnated with 10 µl of released tetracycline (25 µg/disc) at a concentration of 2.5 mg/ml and properly positioned on the inoculated agar plates. Free tetracycline of same concentration and sterile distilled water were used as positive (+Ve) and negative (-Ve) references, respectively. Inoculated agar plates were then incubated at 37°C for 24 h. The antibacterial activity of released tetracycline against the test bacteria was evaluated by measuring the zone of inhibition using calipers. The experiments were repeated thrice to ensure reliability

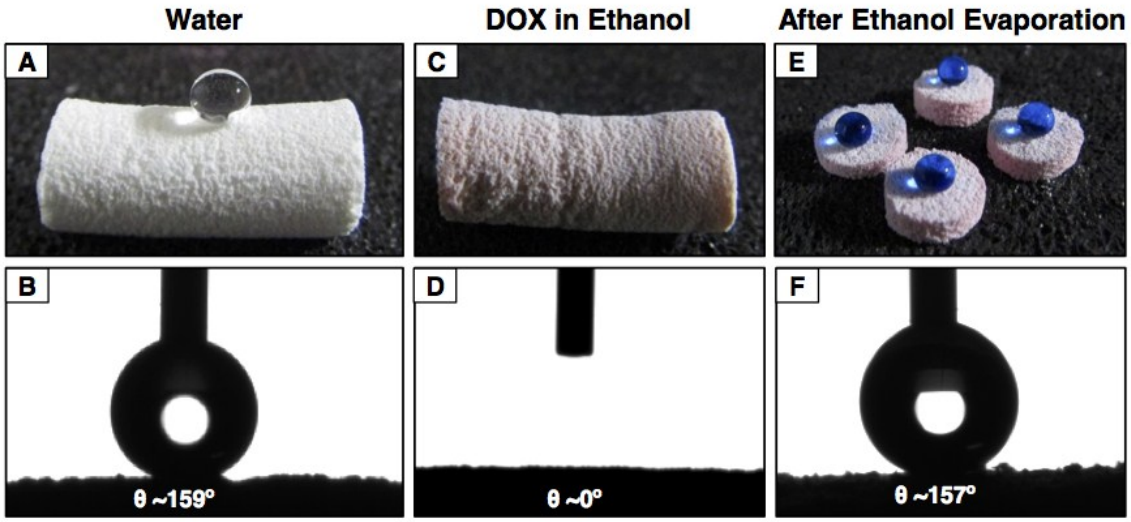

Figure S1. (A-F) Digital images (A, E) and contact angle images (B, F) of beaded water droplets on polymeric material before (A, B) and after (E-F) post loading of doxorubicin, where ethanol was evaporated out from the polymeric matrix. (C) Digital images of polymeric material that is readily soaked in solution of DOX in ethanol. (D) Contact angle image of ethanol on polymeric material.

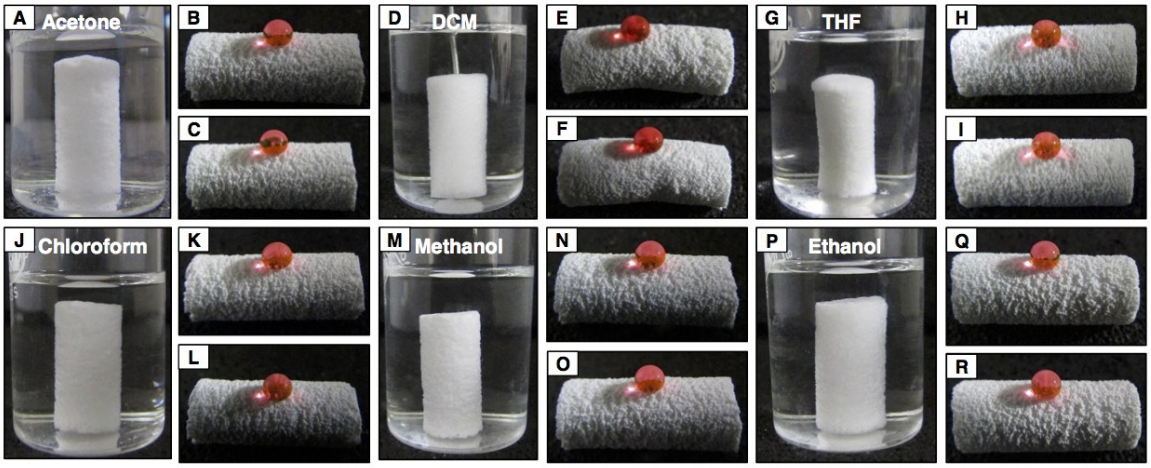

Figure S2. (B-C, E-F, H-I, K-L, N-O, Q-R) Digital images of beaded water droplet on the superhydropbic polymeric material (post modified with decylamine) before (B,E,H,K,N,Q) and after (C,F,I,L,O,R) exposing to various organic solvents—including acetone (A), dichloromethane (D), tetrahydrofuran (G), chloroform (J), methanol (M), ethanol (P) for 1 h.

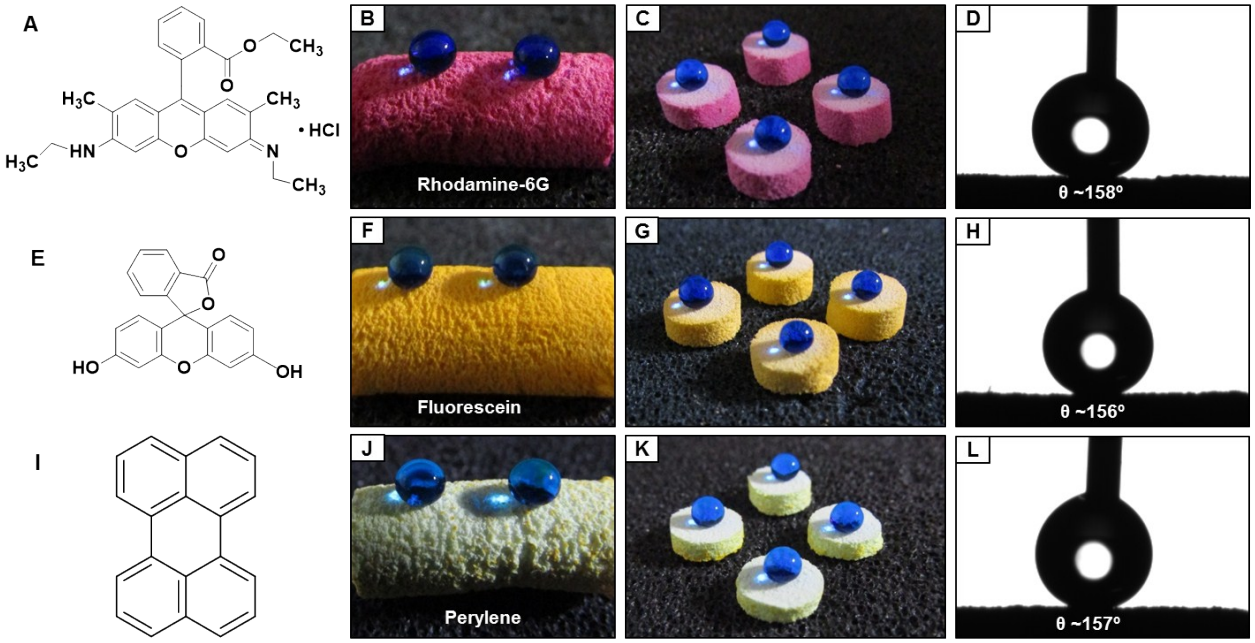

Figure S3. (A, E, I) Chemical structures of rhodamine 6G (A), fluorescein (E), perylene (I). B-L) Digital images (B, C, F, G, J, K) and contact angle images (D, H, L) of beaded water droplets (colored with methylene blue dyed) on superhydrophobic material (post modified with decylamine) that are post loaded with Rhodamine-6G (B-D), Fluorescein (F-H) and Perylene (J-L) respectively.

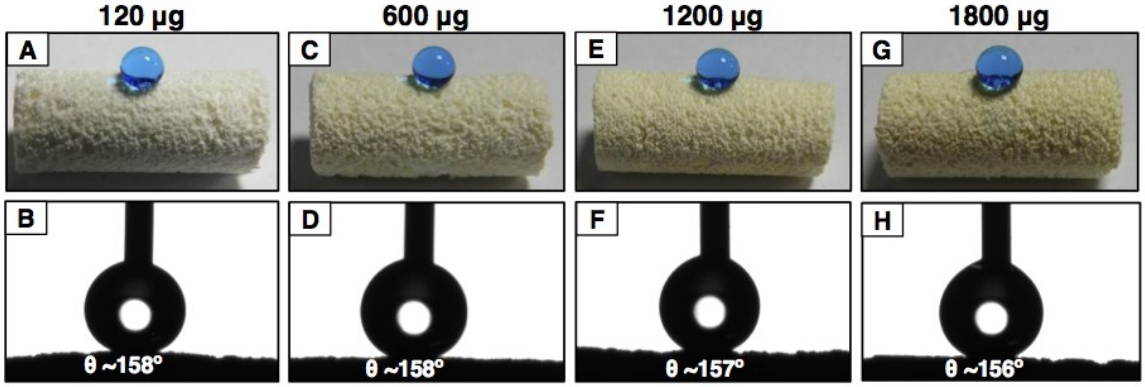

Figure S4. (A-H) Digital images (A, C, E, G) and contact angle images (B, D, F, H) of beaded water droplet (methylene blue dye is added) on the polymeric material (160 mg)—after post loading different amounts of tetracycline—including 120 µg (A, B), 600 µg (C, D), 1200 µg (E, F) and 1800 µg (G, H) respectively.

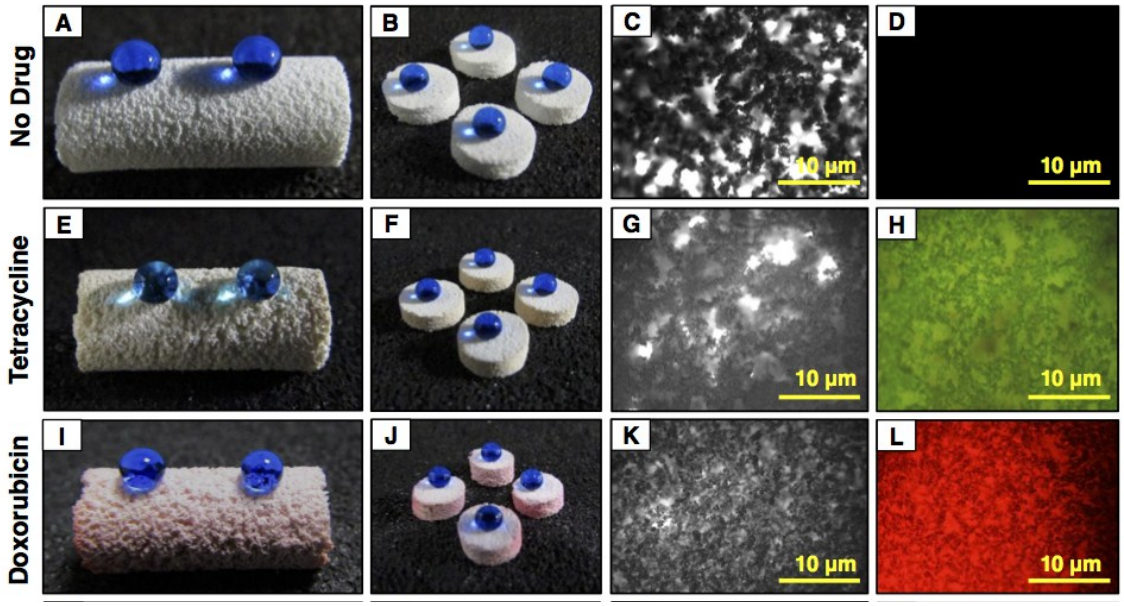

Figure S5. (A-J) Digital images (A-B, E-F, I-J), and optical microscopic images (C-D, G-H, K-L) of superhydrophobic polymeric material before (A-D) and after loading with different drug molecules, including tetracycline (E-H), doxorubicin (I-L), respectively

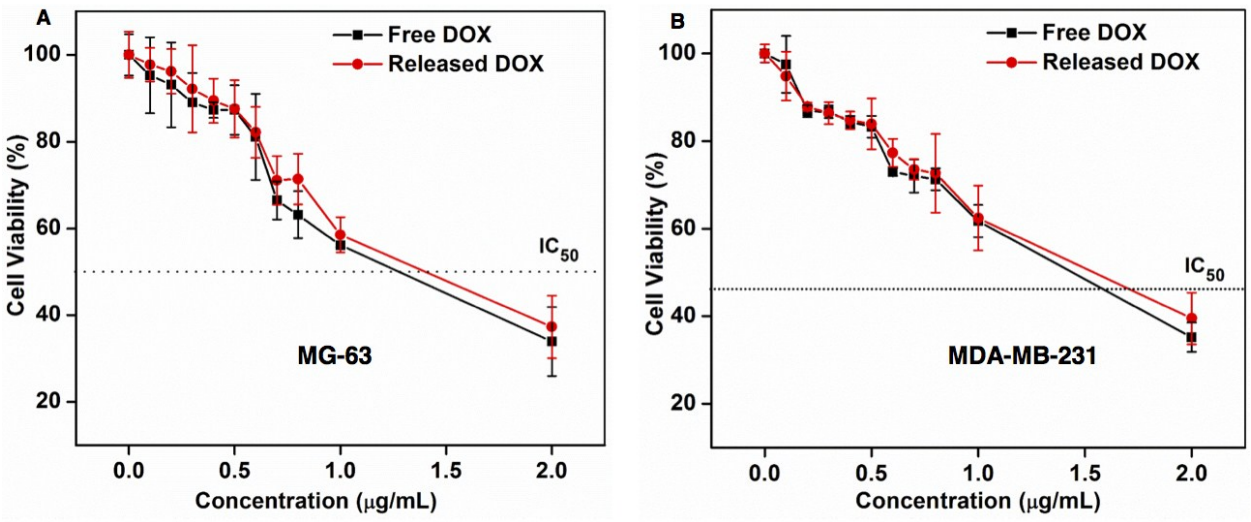

Figure S6. (A-B) Plots illustrating the anticancer activity of freshly added doxorubicin (black) and released doxorubicin from the polymeric material (red) against two different cell lines—that are MG-63 (A) and MDA-MB-231 (B)

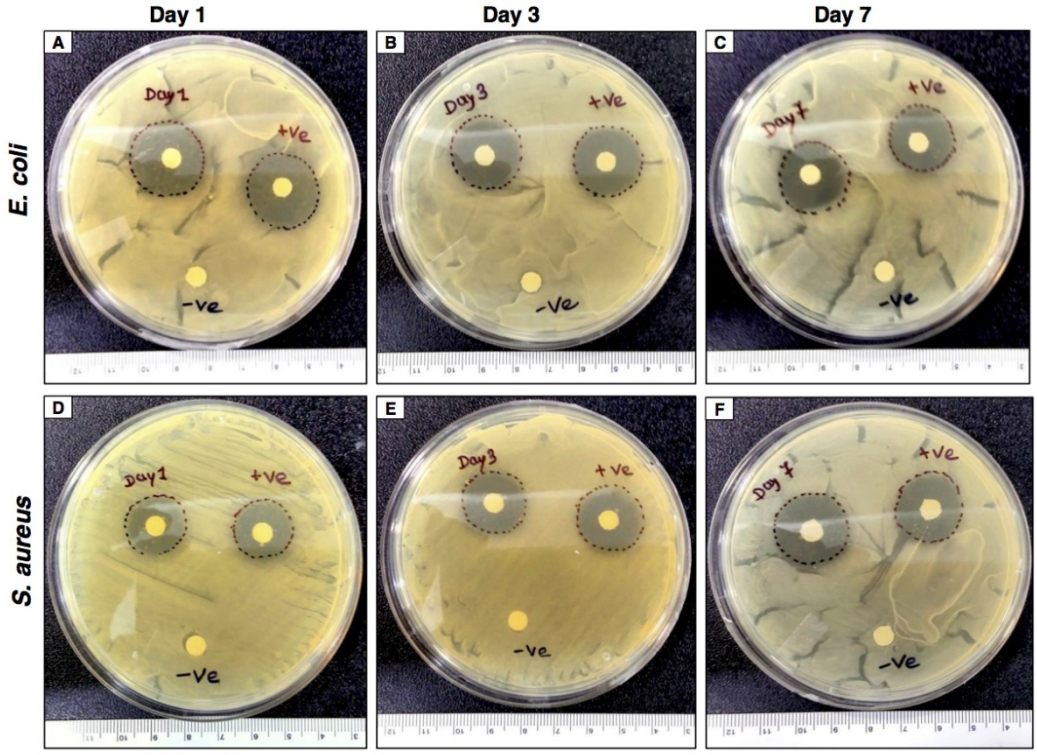

Figure S7. (A-F) Digital images of agar plate showing the average diameter of zone of inhibition (mm) of freshly added (denoted as +ve) and released tetracycline (TC) from polymeric material at various time intervals including one day (A, D), three day (B, E) and seven days (C, F) against *E. coli* (gram –ve) (A-C) and *S. aureus* (gram +ve) (D-F) bacteria's. The –ve control is PBS buffer without any TC.