Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

A Hydrogel-based Implantable Multidrug Antitubercular Formulation Outperforms Oral Delivery.

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Table S1. Table showing the dosage regimens used for oral and hydrogel-mediated delivery of a combination of antituberculosis drugs.

		Oral X/2	**Oral X**	**TB-Gel X/2**	**TB-Gel X**
	Dose biweekly			X/2 (1 mL) (For 5 mice)	X (1 mL) (For 5 mice)
	Dose/week	X/4	X/2		
Isoniazid		1.875 mg x 2	3.75 mg x 2	3.75 mg	7.5 mg
Ethambutol		7.5 mg x 2	15 mg x 2	15 mg	30 mg
Pyrazinamide		7.5 mg x 2	15 mg x 2	15 mg	30 mg
Rifampicin		0.25 mg x 2	0.5 mg x 2	0.5 mg	1 mg
	Total Dose biweekly	**X/2**	**X**	**X/2**	**X**

Table S2. LC-MS/MS QTRAP 4500 parameters for quantification of antituberculosis drugs in MRM mode.

S. No.	Name	Q1 Mass (Da)[a]	Q3 Mass (Da)[b]	DP (volts)[c]	CE (volts)[d]	EP (volts)[e]
1	INH	138.1	121.1	45	15	10
2	RIF	824	792.5	100	16	10
3	EMB	205.1	116.1	55	21	10
4	PYZ	124.1	81.1	80	30	10

[a]Q1: Precursor ion mass. [b]Q3: Product ion mass. [c]DP: Declustering potential.
[d]CE: Collision energy. [e]EP: Exit potential.

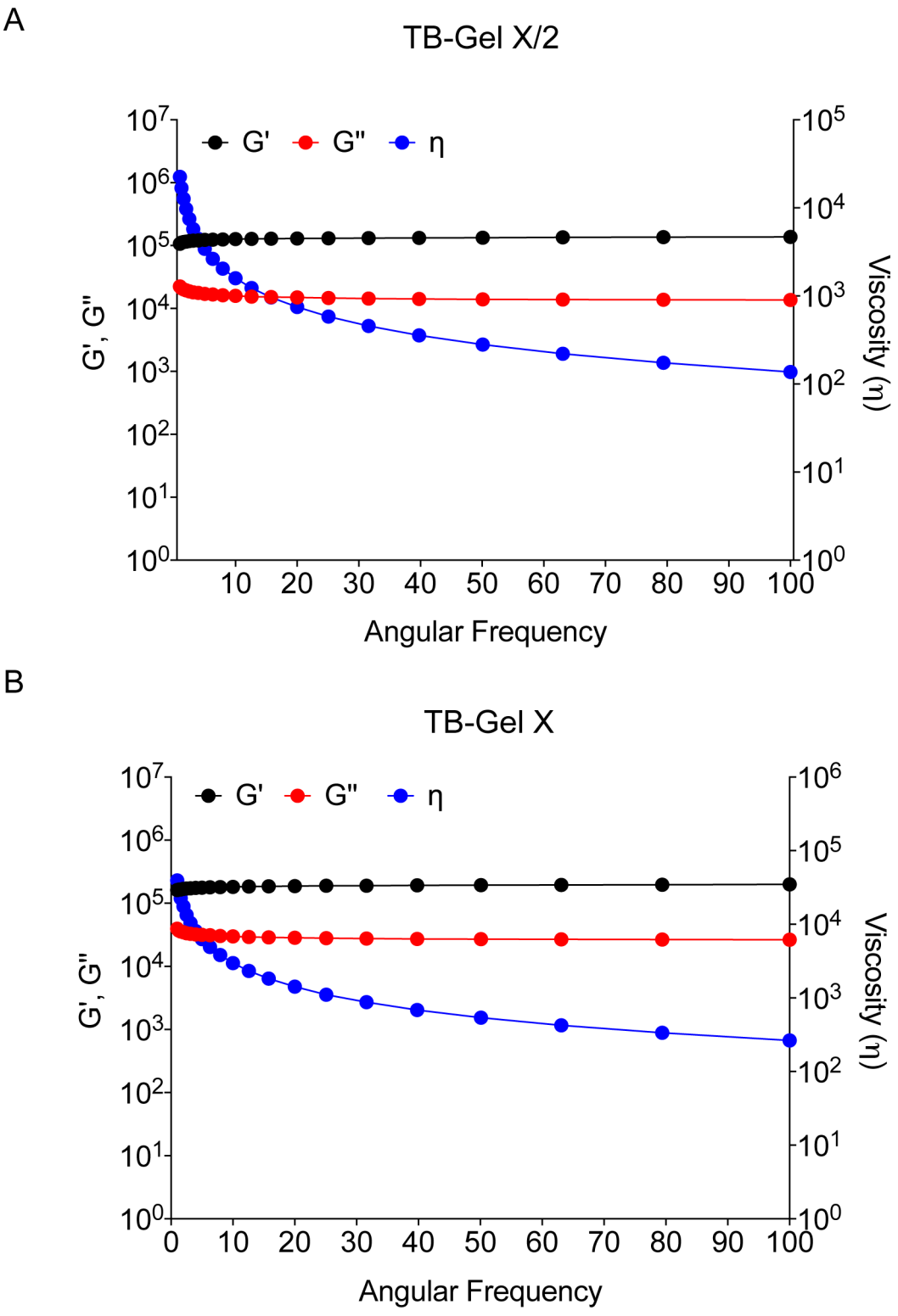

Figure S1. Frequency sweep profiles of gels understudy confirming their elastic behavior (with no cross-over points achieved where G' = G") even at a frequency as high as 100 rad s^{-1}.

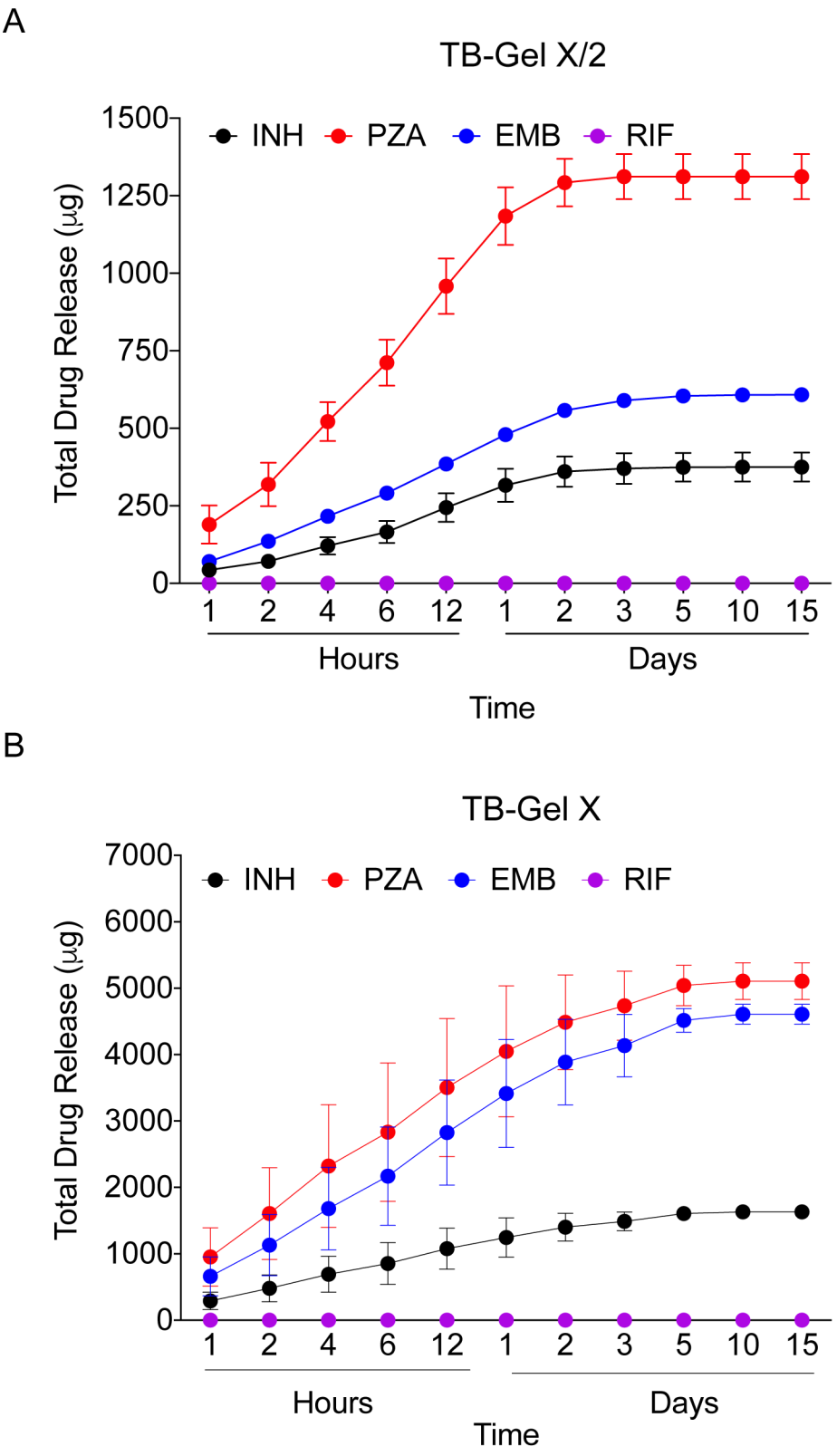

Figure S2. Total drug release (relative) at different time points for each antitubercular drug from TB-Gel X/2 (A) and TB-Gel X (B).

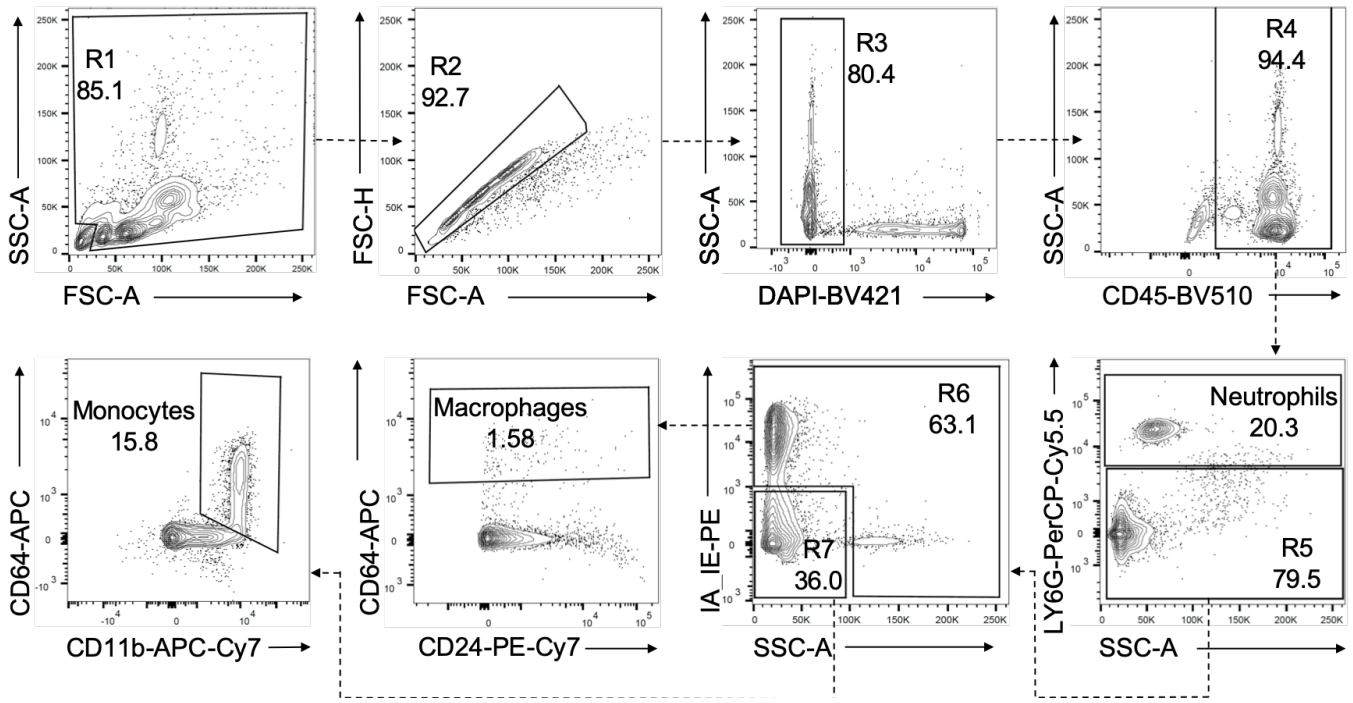

Figure S3. Gating strategy for analyzing the proportion of neutrophil, monocyte, and macrophage populations. The cell debris was excluded through FSC-A vs SSC-A (R1) and cell doublets were eliminated by using FSC-A vs FSC-H (R2). Further, the live cells were identified by excluding $DAPI^+$ cells (R3). $Ly6G^+$ neutrophils were separated from other leukocytes (R4). Leukocytes (R4) were further differentiated depending upon SSC-A vs MHC class II expression as IA/IE^- $SSC\text{-}A^{lo}$ population containing monocytes (R7) and the remaining populations (R6) having macrophages. From the R6 population, CD64+ cells were selected as macrophages. From R7 populations, monocytes were gated based on the expression of CD11b and CD64.

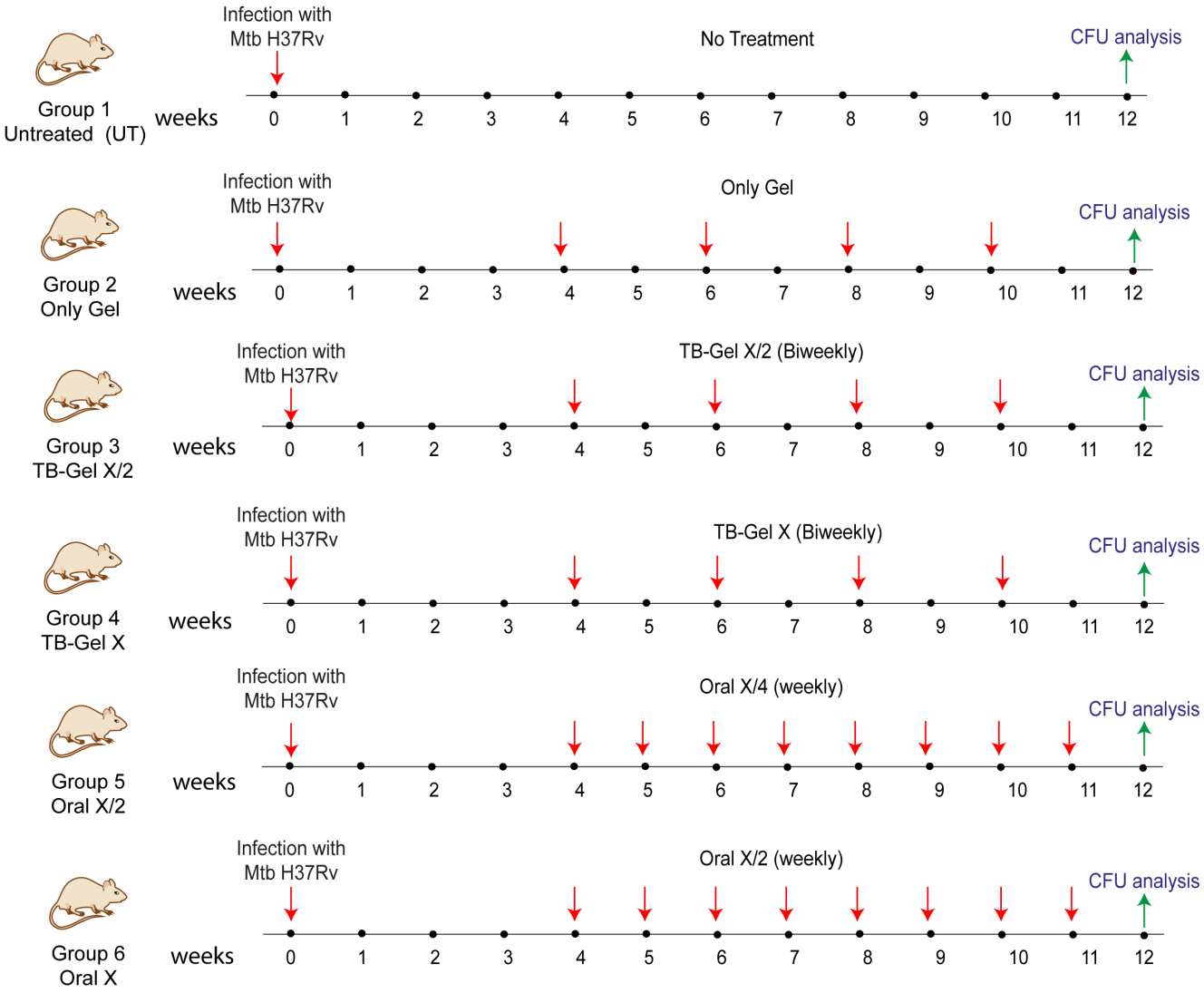

Figure S4. Schema showing the study plan where BALB/c mice were infected with *M. tuberculosis* and subjected to different treatments.

Experimental Section.

Materials. Rifampicin, isoniazid, pyrazinamide, ethambutol, hematoxylin, eosin, DPX mounting media, Tween 80, catalase, oleic acid, and IR 820 were purchased from Sigma-Aldrich. Trisodium citrate, 7H11, 7H9 broth, and dextrose were purchased from HiMedia. PANTA antibiotics were purchased from BD. Albumin was purchased from Bio Basic. Formaldehyde, lithium carbonate, xylene were purchased from Thomas Baker. Rheoplus MCR102 (Anton-Paar) was used for rheological experiments. Zeiss scanning electron microscope used for SEM imaging, and Microm HM550 (Thermo Scientific) was used for sectioning. Nikon microscope was used for brightfield imaging. LC-MS grade solvents Water (Riedel-de haen; Cat no. 39253-4L) Methanol (Honeywell; Cat No. 34966), Formic acid (Honeywell; Cat. No. 56302-50ML), and Ammonium Formate (Fluka; Cat. No. 14266-25G) were used for LC-MS/MS work.

Drug entrapment and release studies. Rifampicin (1.0 mg), Isoniazid (7.5 mg), Pyrazinamide (30.0 mg), and Ethambutol (30.0 mg) were mixed along with gelator (70 mg) in 1 mL of water, and heated till a clear solution was formed. The solution was allowed to cool at room temperture to form TB-Gel. Rheology and SEM of TB-Gel were performed as mentioned previously.[1]

We prepared the TB-Gel as mentioned above, and placed the TB-Gel (0.2 mL) in inserts of corning® HTS Transwell®-24 well plate, and incubated the inserts with 1 mL of PBS having 0.05% Tween 80 at 37 °C. We then collected the release medium at different time points and incubated the inserts with fresh PBS (1 mL). Experiment was repeated three times, and released INH, PYZ, ETM, and RIF were quantified by LC-MS/MS. Similar methodology was used for TB Gel X/2.

Sample preparation and LC-MS/MS method for quantitation. Four TB drugs Isoniazid (1mg/mL), rifampicin (1 mg/mL), ethambutol (1 mg/mL), and pyrazinamide (1mg/mL) were reconstituted in a mixture of methanol and water (1:1, v/v), and centrifuged at 12000 rpm for 10 min to remove any debris, and the supernatant was used. Quantitation was done by LC-MS/MS using Linear Ion Trap Quadrupole (QTRAP 4500, SCIEX, USA) with Turbo VTM source and

electrospray ionization (ESI) probe coupled to a high-pressure UHPLC (ExionLCTM AC, SCIEX, USA). Liquid chromatographic separation was done using an Acquity BEH C18, 2.1 × 50 mm column (Waters Ltd.) at 55°C, using a binary mobile phase with Solvent A (CH_3OH, water, and HCOOH in 58:41:1 ratio with 5 mM ammonium formate) and Solvent B (CH_3OH and HCOOH in 99:1 ratio with 5 mM ammonium formate) in gradient flow. Total run time was optimized to 8 min with the following gradient, Solvent A and B (80:20) for 0.0 to 2.4 min, Solvent A: B (10:90) from 2.50 to 4.9 min, Solvent B (100%) from 5 to 6.50 min, and then Solvent A: B (80:20) from 6.70 to 8.0 min.

For quantitation of drugs, Multiple Reaction Monitoring (MRM) in the positive ion mode was done using Q1 precursor ion (m/z) and Q3 product ion (m/z) during MRM scan. Q1/Q3 transition of 138.1/121.1 for INH, of 824.0/792.5 for RIF, of 205.1/116.1 for EMB, and 124.1/81.1 for PZA during MRM scan was used (**Table S2**). Other parameters used were Collision Gas (CAD) low, collision cell exit potential (CXP) 15, dwell time 30 msec, curtain gas 25.0 psi, Ion Source Gas 1 (GS1) 50.0 psi, GS2 50.0 psi, ion spray voltage, 5500.0 V, and temperature 500 ^oC. Standard curves were generated for absolute quantitation of four drugs using 9 different concentrations (1.95 to 500 ng/mL). Drugs were serially diluted in water: methanol (1:1 v/v) for making different concentrations. Linear regression line was plotted with correlation coefficient values of at least 0.99 using MultiQuant™ 3.0.2 (SCIEX, USA) and Microsoft excel. For sample preparation of released TB drugs, 1 mL of a mixture of water: methanol (1:1, v/v) were added to dried PBS samples with released drugs collected at different time points and vortexed for 5 min. Each vial was serially diluted and centrifuged at 12000 rpm for 5 min. Diluted samples were transferred into injection vials, and a 10 μL sample was injected on the column. Each drug was quantified using the respective standard curve.

Biocompatibility studies. Male BALB/c mice (5-6 weeks) were randomized into four groups with 6 mice in each group. Group 1 mice were left untreated, group 2 and group 3 were injected

subcutaneously with 0.2 mL of A13 hydrogel and TB gel respectively. For positive control, group 4 mice were given a topical treatment of 62.5 mg of imiquimod (5%, Imiquimod cream) on their back (after shaving) for 14 days to cause inflammation.

Flow cytometry. After 14 days, mice were euthanized and blood was collected in a tube containing anticoagulant by cardiac puncture. RBC lysis was performed using the manufacturer's protocol for the optimal erythrocyte lysis. Cells were then washed with 1 X PBS to stop lysis. The supernatant was discarded, and cells were resuspended in FACS Buffer (1X PBS supplemented with 4% Goat serum and 2mM EDTA (ethylenediaminetetraacetic acid)). Cells (~10^7) were blocked with 4% goat serum in PBS and stained with a cocktail of antibodies for 15 min at 4 ^oC. Then unbound antibodies were washed away using FACS buffer twice. Following antibodies were used Ly6C-FITC (BioLegend, cat#128006), IA/IE-PE (BioLegend, cat#107608), Ly6G-PerCP-Cy5.5 (BioLegend, cat#127616), CD45-BV510 (BioLegend, cat#103138), CD24-PE-Cy7 (BD Biosciences, cat#560536), CD64-Alexa 647 (BD Biosciences, cat#558539), and CD11b- APC-Cy7 (BD Biosciences, cat#557657). Dead cells were stained using DAPI (ThermoFisher, cat#D1306,). Cells were then analysed on BD FACSverse flow cytometer. The acquired data were further analyzed using FlowJo v10 software.

NIR fluorescence probe release studies. Near-infrared (NIR) dye (IR 820, 85 µg) entrapped A13 gel (7%) (1mL) was prepared by heating the aqueous suspension of IR 820, all four antitubercular drugs, and A13 gelator in water. NIR-TB-Gel was injected subcutaneously in BALB/c mice (n = 3, 200 µL/mouse). In another set of mice (n = 3), subcutaneous injection of an aqueous solution of NIR dye was injected. Whole-body NIR fluorescence imaging at different time points was carried out using SPECTRUM In Vivo Imaging System (IVIS, Perkin Elmer, USA) by setting an excitation filter at 710 nm and emission filter at 820 nm with an exposure time of 5 sec. Animals were anesthetized using 2.0% isoflurane in the induction chamber and transferred to IVIS under isoflurane for imaging. Image analysis was performed using Living Image software.

Murine lung infection model. Murine TB lung infection was established in 6-8 weeks old female BALB/c mice at Tubercular Aerosol Challenge Facility (TACF), ICGEB, New Delhi. A culture of 0.6 OD of H37Rv in saline was used for infection. Mice were put in autoclaved cages and were placed in the infection chamber of the aerosol challenge machine for 20 min. After 24 h of infection, four mice were randomly sacrificed and cleaned once in Lysol solution, and dissected in the laminar hood. The dissected lungs were washed in saline and arranged in Petri dishes for photographs. Lungs were homogenized in 2 mL saline, and serially diluted and plated on 7H11 plates containing OADC and PANTA. Plates were incubated at 37 ºC for 2-3 weeks. The infection load was again checked in 6 mice after four weeks post-infection before starting the treatment. Mice were randomized into six groups having six mice in each group, and subject to different treatments as mentioned in **Table S1**. Group 1 mice were left untreated. Group 2 mice were treated with free gel once in two weeks (biweekly) for eight weeks. Mice in group 3 were orally treated every week with an X/2 dose for eight weeks. X/2 dose is a combination of 5 mg/kg of rifampicin, 37.5 mg/kg of Isoniazid, 150 mg/kg of pyrazinamide, and 150 mg/kg of ethambutol. Group 4 mice were orally treated every week with X dose for 8 weeks X dose is 10 mg/kg of rifampicin, 75 mg/kg of Isoniazid, 300 mg/kg of pyrazinamide, and 300 mg/kg of ethambutol. We gave a subcutaneous injection of TB Gel with X/2 dose in group 5 mice and TB Gel with X dose in group 6 mice after every two weeks for eight weeks. The treatment was continued for eight weeks. Mice were sacrificed, cleansed by dipping in Lysol solution, dissected out in a laminar hood, and lungs were harvested out. The dissected lungs were washed in saline and arranged in Petri dishes for photographs. A small piece of the organ was cut and preserved in normal buffered formalin for histopathology. The remaining portion of the lungs was weighed and homogenized in 2 mL saline, and serially diluted and plated on 7H11 plates. Plates were incubated at 37 ºC for 2-3 weeks, and the colonies were enumerated.

Hematoxylin and eosin staining. The formalin-fixed lung tissue was sectioned on a cryotome (Microm HM550, Thermo Scientific). The sections were fixed with 4% paraformaldehyde for 5 min, followed by PBS wash. The lung tissue sections were dipped in hematoxylin solution for 10 min followed by rinsing with MiliQ water for 1-2 min to remove extra strain and then dipped in lithium carbonate solution (1.5% w/v), a bluing agent for 1 min. Slides were then stained with eosin solution for 3 min, followed by washing with MiliQ water twice. Sections were then dehydrated with a gradient of alcohol (70, 80, and 100%) followed by two washes in xylene for 2 min each. Slides were dried and mounted with DPX and kept for 24 h to solidify the DPX. Images were acquired on the Nikon microscope.

References:

1. S. Pal, N. Medatwal, S. Kumar, A. Kar, V. Komalla, P. S. Yavvari, D. Mishra, Z. A. Rizvi, S. Nandan, D. Malakar, M. Pillai, A. Awasthi, P. Das, R. D. Sharma, A. Srivastava, S. Sengupta, U. Dasgupta and A. Bajaj, *ACS Cent. Sci.* **2019**, *5*, 1648.