SUPPORTING INFORMATION

Converting oligo(ethyleneglycol)s into non-toxic highly selective biocompatible antimicrobial poly(ethyleneglycol)s: synthesis, antimicrobial and antibiofilm activity

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Experimental Section-

Materials- Poly(ethylene glycol) diacrylate (M_n= 575 and 700), tryptamine, diphenyl carbonate, ethyl carbazate, butyl amine, hexyl amine, octyl amine, dodecyl amine, deuterated dimethyl sulfoxide (DMSO-d_6, 99.9 atom % D), N-phenyl-1-naphthylamine (NPN), and Crystal violet solution were purchased from Sigma Aldrich. Dimethylformamide (DMF) HPLC grade, lithium bromide, Acetone, and Dextrose extrapure AR, ACS grade were purchased from Sisco Research Laboratories Pvt. Ltd. (SRL). Luria Bertani Broth, Luria Bertani Agar, 10X PBS, and EZcount MTT Cell Assay Kit were purchased from Himedia. Glacial acetic acid was purchased from Merck. Defibrinated sheep blood was purchased from Labline Trading Co. PEG standards for column calibration in size exclusion chromatography were purchased from Agilent Pvt. Ltd.

Material Characterization-

FT-IR Spectroscopy- The measurements were done using Perkin Elmer Spectrum 400 FTIR in ATR mode at room temperature in range of 600-4000 cm^{-1}.

Nuclear Magnetic Resonance (NMR)- ^{1}H and ^{13}C-NMR spectra, HSQC were recorded in JEOL JNM-ECZ500R spectrometer using TMS as internal standard and DMSO-d_6 as NMR solvent.

Size Exclusion Chromatography (SEC)- SEC measurements were performed on a i-seies plus integrated HPLC attached with a Refractive index detector (Wyatt Optilab T-Rex) system. PEG standards were used for column calibration and DMF (0.01% LiBr) was used as mobile phase at a flow rate of 0.75 mL/min at 30^{o}C. ATRA 7.3.0 software (Wyatt Technology Corporation) was used for data collection and processing.

Thermogravimetric Analysis (TGA)- TGA thermograms of all the polymers were recorded in SDT Q600 V20.9 Build 20 instrument under nitrogen atmosphere. The samples were heated in the temperature range of 30-500^{o}C with heating rate of 10^{o}C/min.

Differential Scanning Calorimetry (DSC)- DSC analysis was performed in PerkinElmer DSC 6000 under nitrogen atmosphere with the temperature range of -50 to 80^{o}C with heating rate of 10^{o}C/min. Note: All the samples were freeze dried prior to analysis to remove moisture.

Optical density (OD)- The OD for the minimum inhibitory concentration (MIC) analysis, biofilm inhibition assay, haemolysis assay, and cytotoxicity assay (MTT assay) was measured using a BioTek Epoch 2 Microplate Spectrophotometer (Agilent) to ensure accurate quantification of bacterial growth, biofilm formation, haemolytic activity, and cell viability in the respective assays.

Fluorescent Intensity- The fluorescence intensity for the NPN assay was measured using a Cary Eclipse Fluorescence Spectrophotometer (Agilent) to accurately quantify the fluorescence emitted, providing insights into membrane permeability in the bacterial cells.

Zeta potential & Dynamic Light Scattering (DLS) - The zeta potentials and particle size studies were performed using a zeta sizer ultra particle analyzer from Malvern (Model no: ZSU3305).

Synthesis-

Synthesis of indole-functionalized Poly(ethylene glycol) polymer (InPEG$_x$)- Poly(ethylene glycol) diacrylate (2 g, 3.47 mmol, M_n= 575) was stirred in a 1:2 ratio of DMF:water mixture (20 mL) for few minutes. To this solution, tryptamine (0.557 g, 3.47 mmol) was added with constant stirring and the reaction was continued for 72 hr at room temperature. After this, the

solvent was freezed using liquid nitrogen and subjected to lyophilization. The product was then washed with ethyl acetate and dried under vacuum. The obtained indole-functionalized PEG polymer ($InPEG_{575}$) appeared as light-yellow viscous polymer as final product. Similar reaction procedure was used by taking poly(ethylene glycol) diacrylate (M_n=700) as starting material to prepare $InPEG_{700}$ (**Table S1**).

Table S1: Starting material used for the synthesis of indole-functionalized PEG polymer ($InPEG_x$).

Polymer synthesized	**PEG Diacrylate (M_n)**	**PEG Diacrylate (g, mmol)**	**Tryptamine (g, mmol)**	**DMF:water (1:2)**
$InPEG_{575}$	575	2 g, 3.47 mmol	0.577 g, 3.47 mmol	20 mL
$InPEG_{700}$	700	2 g, 2.85 mmol	0.457 g, 2.85 mmol	20 mL

Synthesis of Triazolinedione derivatives (C_n-TAD, n= 4,6,8,12)- TAD derivatives are synthesized using the previously reported procedure. Briefly, diphenyl carbonate (1 g, 4.668 mmol) was reacted with 1 eq. of a primary amine (butyl, hexyl, octyl, or dodecyl amine) at 85 °C under inert conditions for 40 min. Ethyl carbazate (0.485 g, 4.668 mmol) was then added, and the mixture was heated to 140 °C for 2.5 hr and subsequently to 240 °C for 1.5 hr. The resulting mixture was cooled down and washed thoroughly with diethylether. The obtained white solid compound (urazole derivatives) was dried. The synthesized urazole (0.6 g) was oxidized to corresponding TAD derivatives using 0.2 equivalents of DABCO-Br in 10 mL of dry DCM under inert conditions at room temperature for 5 hours. The reaction mixture was filtered, and the pink filtrate was concentrated to obtain the pink-coloured TAD derivatives.

Synthesis of hydrophobically modified $InPEG_x$ with Triazolinedione derivatives (C_n-TAD, n=4,6,8,12)- To the DMF solution of $InPEG_x$ (0.1g, 1 mL), solution of C_n-TAD (1 eq. with respect to indole group) in DMF (0.5 mL) was added at room temperature in inert atmosphere and stirred for 20 min. After the completion of reaction, which is observed by the disappearance of characteristic red colour of TAD, the resultant mixture was freeze dried to remove solvent. The hydrophobically modified $InPEG_x$ was obtained as yellow viscous polymer ($InPEG_x$-C_n-TAD).

Table S2: Starting material used for the synthesis of hydrophobically modified indole-functionalized PEG polymer ($InPEG_x$-C_n-TAD).

S.No.	Post-modified Polymer synthesized	$InPEG_x$ (g, mmol)	C_n-TAD (g, mmol)
1.	$InPEG_{575}$-C_4-TAD	$InPEG_{575}$ (0.1 g, 0.136 mmol)	C_4-TAD (0.021 g, 0.136 mmol)
2.	$InPEG_{575}$-C_6-TAD	$InPEG_{575}$ (0.1 g, 0.136 mmol)	C_6-TAD (0.025 g, 0.136 mmol)
3.	$InPEG_{575}$-C_8-TAD	$InPEG_{575}$ (0.1 g, 0.136 mmol)	C_8-TAD (0.029 g, 0.136 mmol)
4.	$InPEG_{575}$-C_{12}-TAD	$InPEG_{575}$ (0.1 g, 0.136 mmol)	C_{12}-TAD (0.0236 g, 0.136 mmol)
5.	$InPEG_{700}$-C_4-TAD	$InPEG_{700}$ (0.1 g, 0.116 mmol)	C_4-TAD (0.018 g, 0.116 mmol)
6.	$InPEG_{700}$-C_6-TAD	$InPEG_{700}$ (0.1 g, 0.116 mmol)	C_6-TAD (0.021 g, 0.116 mmol)
7.	$InPEG_{700}$-C_8-TAD	$InPEG_{700}$ (0.1 g, 0.116 mmol)	C_8-TAD (0.024 g, 0.116 mmol)
8.	$InPEG_{700}$-C_{12}-TAD	$InPEG_{700}$ (0.1 g, 0.116 mmol)	C_{12}-TAD (0.031 g, 0.116 mmol)

Estimation of MIC of the polymers against Gram-positive and Gram-negative bacteria

E. coli (MTCC1302), *P. aeruginosa* (MTCC741), and *S. aureus* (MTCC96) were cultured overnight in LB broth at 37°C with shaking (120 rpm). A 1% inoculum was transferred to fresh LB media, and bacterial growth was monitored at OD_{600} until reaching 0.5 ± 0.02. Then, 10^8 CFU/mL of bacteria were added to a 96-well plate. The minimum inhibitory concentration (MIC) of polymers was determined using the standard broth microdilution method (Gupta et al., 2015; He et al., 2007; Katiyar et al., 2023; Mohamed et al., 2016; Sedaghati et al., 2016). In brief, the bacteria were incubated overnight at 37°C with different polymer concentrations OD_{600} was measured to assess bacterial growth. The OD_{600} values were converted to $\log_{10}$(CFU/mL) and a $\log_{10}$(CFU/mL) vs. concentration curve was plotted. The MIC_{90} of each polymer was determined from the graph as the concentration at which $\log_{10}$(CFU/mL) was at 10% of the control. Three biological repeats were conducted for each experiment. The conversion value used to convert OD to CFU/ml are as follows: *E. coli* (MTCC1302), OD_{600} of 1=8 x 10^8 (Cho et al., 2017); *P. aeruginosa* (MTCC741), OD_{600} of 1=2 x 10^8 (Dong-ju Kim, 2012); *S. aureus* (MTCC96), OD_{600} of 1=1.5 x 10^9

Biofilm inhibition assay (Crystal violet assay)

E. coli (MTCC1302), *P. aeruginosa* (MTCC741), and *S. aureus* (MTCC96) were cultured overnight in LB broth at 37°C with shaking (120 rpm). A 1% inoculum was transferred to fresh LB media containing 5% dextrose, and bacterial growth was monitored at OD_{600} until reaching 0.5 ± 0.02 using a BioTek Epoch 2 Microplate Spectrophotometer. From the culture, 10^8 CFU/mL of bacteria were added to a 96-well plate. Different polymer concentrations were

introduced to the bacterial suspension and incubated at 37°C to assess their effects on bacterial growth. The time required for biofilm formation *E. coli* (MTCC1302), *P. aeruginosa* (MTCC741), and *S. aureus* (MTCC96) are 96hrs (Corsini et al., 2022), 9hrs (Sathe et al., 2023), and 72hrs (Tankersley et al., 2014) respectively. So the incubation time for estimating the anti-fouling activity of the polymers were set to 96hrs for *E. coli* (MTCC1302), 9hrs for *P. aeruginosa* (MTCC741), and 72hrs for *S. aureus* (MTCC96) respectively. After incubation, the medium was removed, and the wells were gently washed three times with 1X PBS to eliminate planktonic cells. The wells were then stained with 1% crystal violet and incubated at room temperature for 20 minutes. Excess crystal violet was removed by rinsing the wells three times with 1X PBS, followed by air-drying. The bound dye was eluted with 30% acetic acid, and the optical density at 550 nm (OD_{550}) was measured using a BioTek Epoch 2 Microplate Spectrophotometer. Three biological replicates (N=3) were performed for the experiment (Arul et al., 2020; Keshvardoust et al., 2019; Panlilio et al., 2022).

.*In-vitro* toxicity assay of the polymers (Hemolysis assay)

Sheep blood was collected into a centrifuge tube and centrifuged at 1000g for 5 minutes at 4°C. After removing the supernatant, the pellet was washed twice with 1X PBS and then resuspended in 1X PBS. Fresh blood was placed in microcentrifuge tubes and treated with varying concentrations of polymers. For control purposes, 1% Triton X-100 served as the positive control, while PBS was used as the negative control. The tubes were incubated at 37°C for two hours. Following incubation, the tubes were centrifuged at 1000g for 5 minutes at 4°C. The supernatant was transferred to a fresh tube, and hemolytic activity was determined by measuring the optical density (OD_{405}) of the hemoglobin released from red blood cells using a BioTek Epoch 2 Microplate Spectrophotometer. Each condition was tested in three biological replicates (N=3). The mean OD_{405} values were converted into percentages of hemolytic activity and plotted on a graph to determine the HC_{50} value of the polymers, which represents the concentration causing 50% hemolysis (Humpola et al., 2023; Jha et al., 2017; Sæbø et al., 2023).

Membrane permeabilization of the polymers (NPN assay)

The 1-N-phenylnaphthylamine (NPN) uptake assay was conducted to assess the effect of polymers on bacterial membrane permeabilization. Bacterial cultures were collected when they reached an OD600 of 0.5 ± 0.02. The cultures were centrifuged at 3500g for 5 minutes at 4°C, and the pellets were resuspended in 1X PBS. The bacterial suspensions were then transferred

to a 96-well plate for further analysis. 50μM Polymyxin B (for P. aeruginosa) and 0.1% Triton X-100 (for S. aureus) were used as positive controls, while PBS served as negative control. Polymers were added at concentrations corresponding to their MIC, followed by a 2-hour incubation at 37°C. Subsequently, 8 μL of 0.5 mM NPN (dissolved in 1:1 acetone:PBS) was added, and fluorescence intensity was recorded using a Cary Eclipse fluorescence spectrophotometer. Each experiment was tested in three biological replicates (N=3).

Cytotoxicity assay of the polymers (MTT assay)

For the cell cytotoxicity assay, HEK293 (Human Embryonic Kidney) cells were seeded at a density of 0.2 × 106 cells per well in a 12-well tissue culture plate and incubated overnight at 37°C in a CO_2 incubator. Once the cells reached 70–80% confluency, they were treated with 1500 μg/mL of polymers at a concentration significantly higher than the MIC and incubated overnight. The MTT assay was performed using the EZ Count MTT Cell Assay Kit as per the manufacturer's instruction. The absorbance was measured at 570 nm using a BioTek Epoch Microplate Spectrophotometer. Triton X-100 (2%) was used as the positive control, resulting in maximum cell death, while untreated cells served as the cell control, showing maximum viability. Each experiment was conducted in three biological replicates (N=3) (Arul et al., 2021, 2020; Sivagnanam et al., 2022).

Figures:

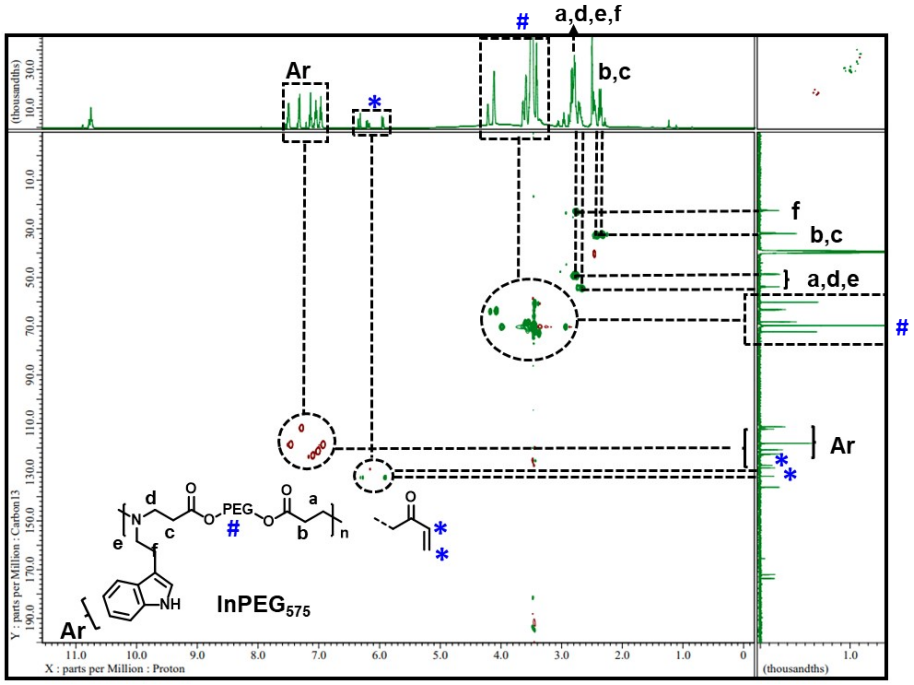


Figure S1: HSQC of $InPEG_{575}$.

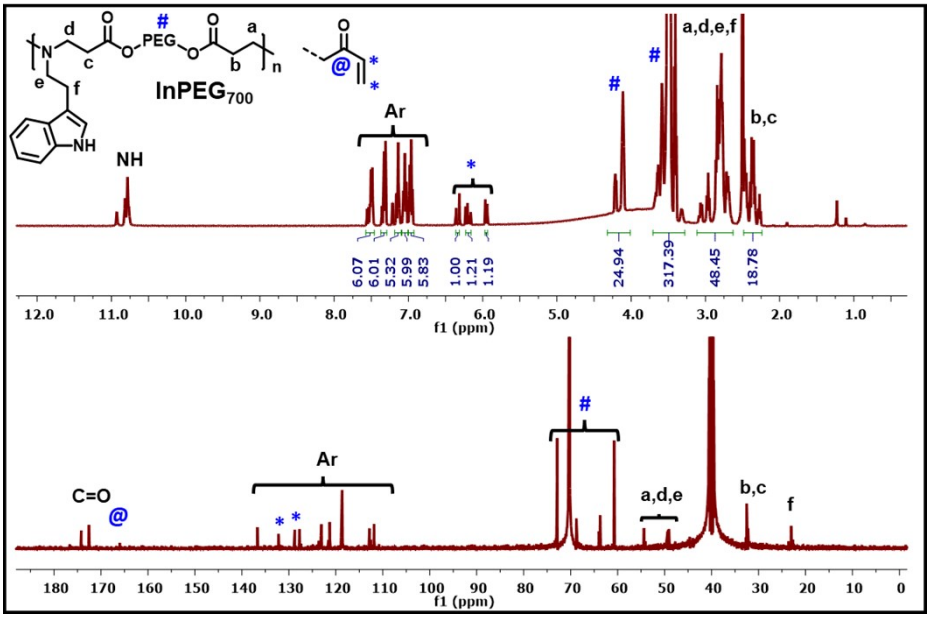


Figure S2: ^{1}H & ^{13}C-NMR spectra of $InPEG_{700}$.

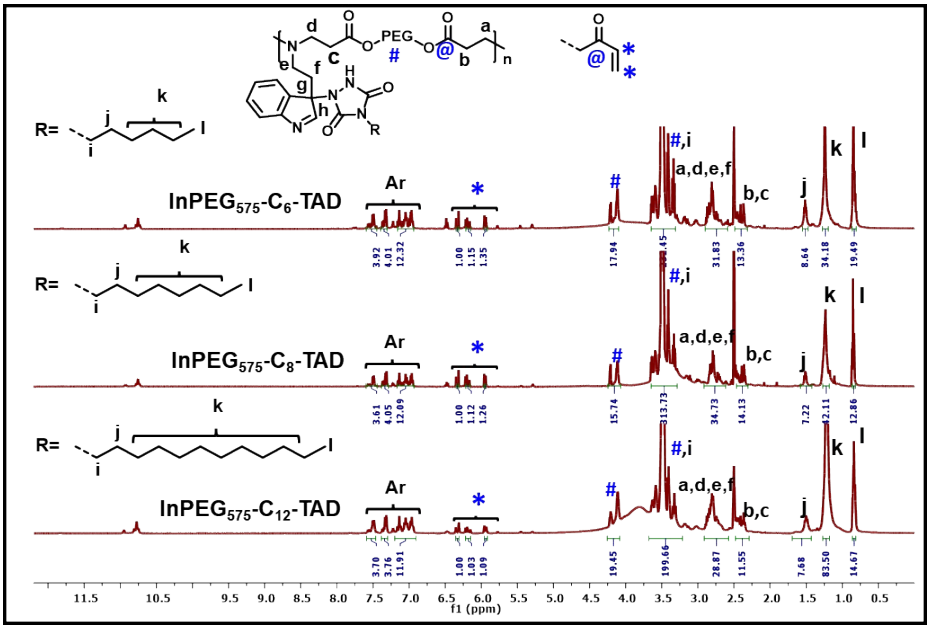


Figure S3: ^{1}H-NMR spectra of InPEG$_{575}$-C$_n$-TAD (n=6,8,12).

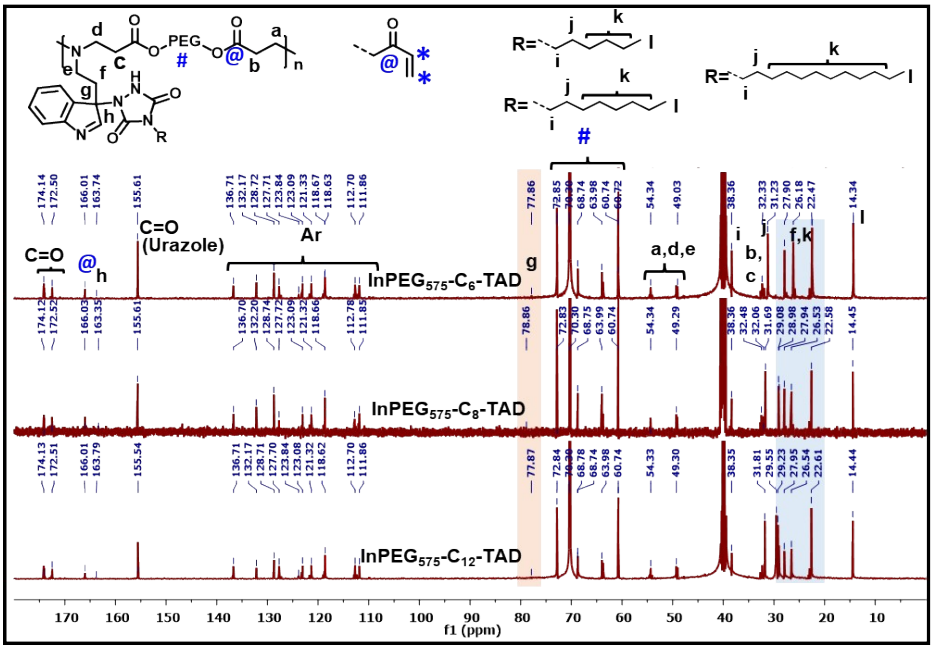


Figure S4: ^{13}C-NMR spectra of InPEG$_{575}$-C$_n$-TAD (n=6,8,12).

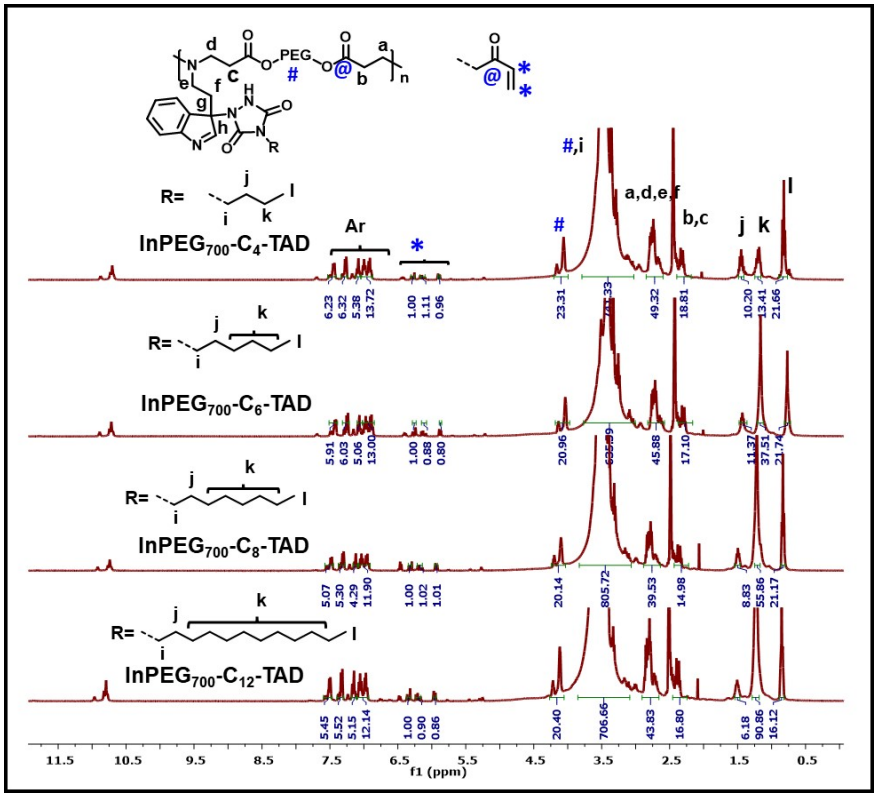


Figure S5: ^{1}H-NMR spectra of InPEG$_{700}$-C$_n$-TAD (n=4,6,8,12).

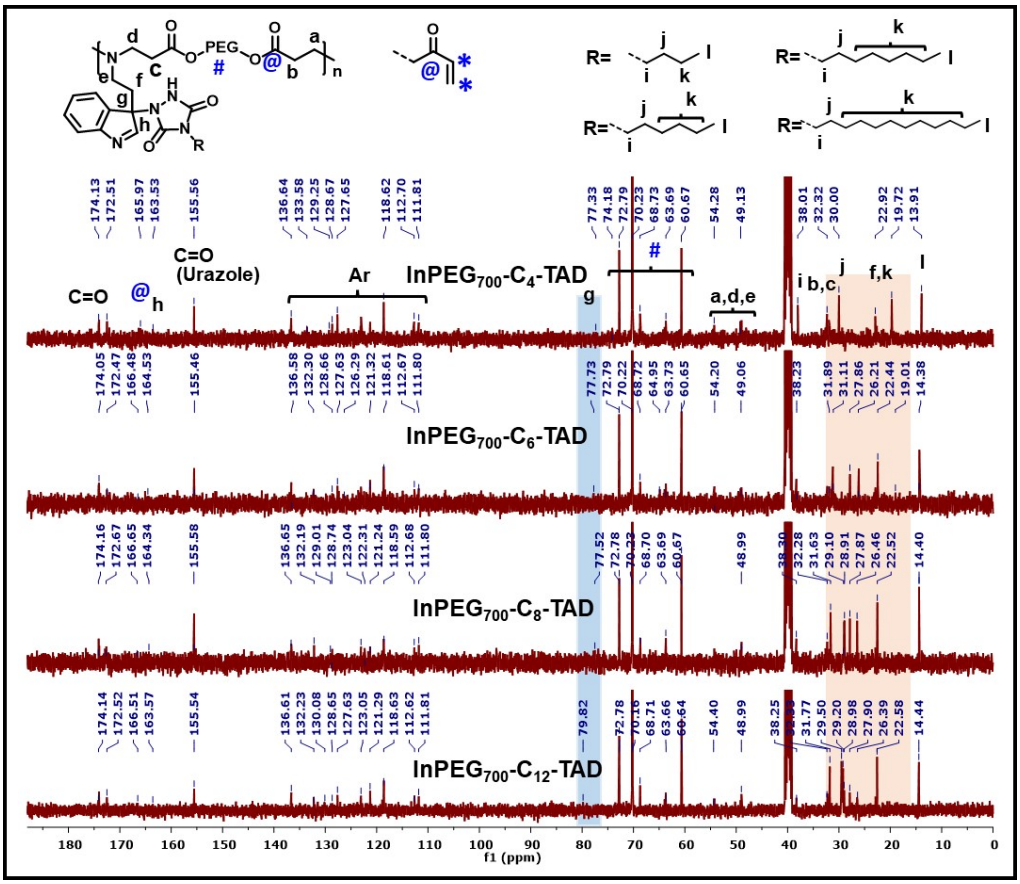


Figure S6: ^{13}C-NMR spectra of InPEG$_{700}$-C$_n$-TAD (n=4,6,8,12).

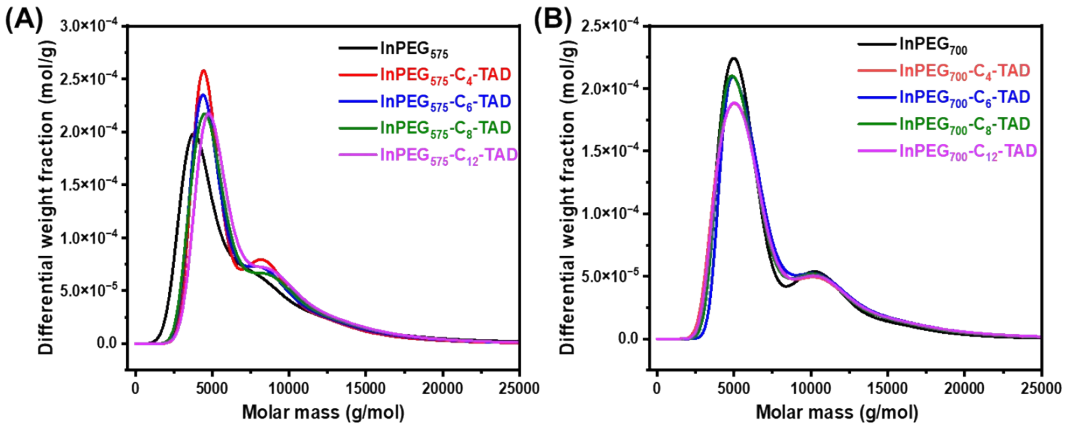


Figure S7: SEC curve for modified and unmodified polymer.

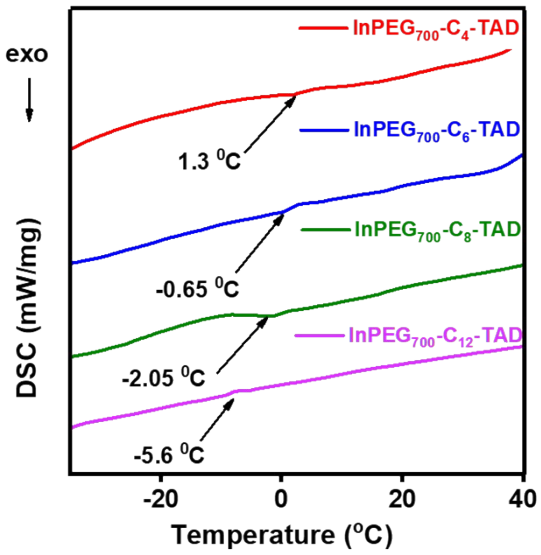
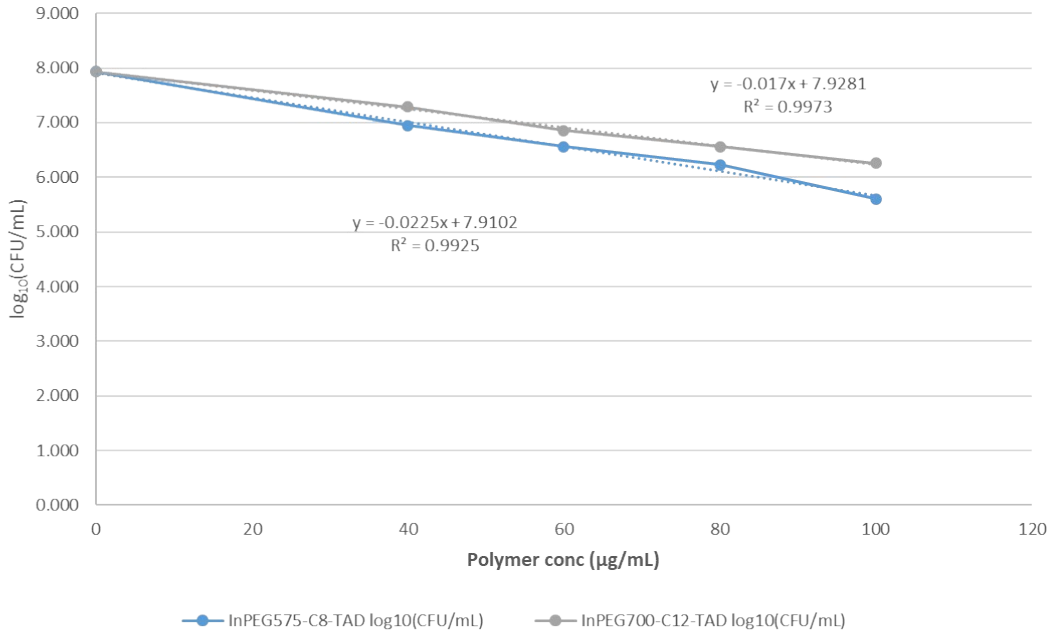


Figure S8: DSC thermogram of post-polymerization derivatives of $InPEG_{700}$-C_n-TAD (n=4,6,8,12).

Representation Raw Data for Biological studies

(1) Representative data showcasing the MIC calculation of two best polymers

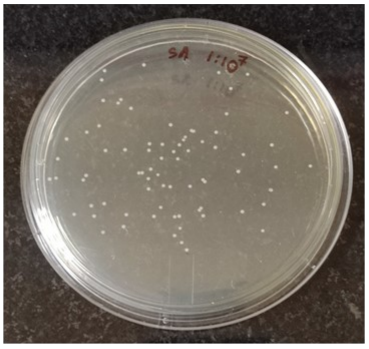
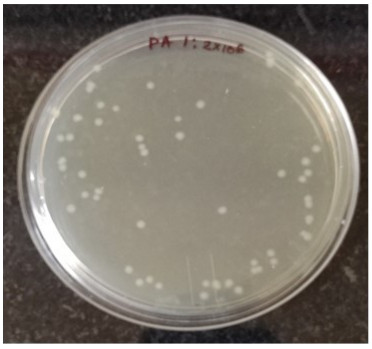


Polymer conc (µg/mL)	$InPEG_{575}$-C_8-TAD		$InPEG_{575}$-C_{12}-TAD	
	log_{10}(CFU/mL)	OD values	log_{10}(CFU/mL)	OD values
0	7.930	0.426	7.930	0.426
40	6.944	0.044	7.111	0.065
60	6.556	0.018	6.869	0.037
80	6.230	0.009	6.146	0.007
100	5.602	0.002	5.845	0.004

Calculated MIC from graph of log10(CFU/mL) vs polymer concentration	Calculated MIC from graph of OD vs polymer concentration	Reported MIC
43.6	43.4	44
49.0	48.8	49

(Bacteria = P. aeruginosa)

(2) Representative data shows the correlation between OD and CFU

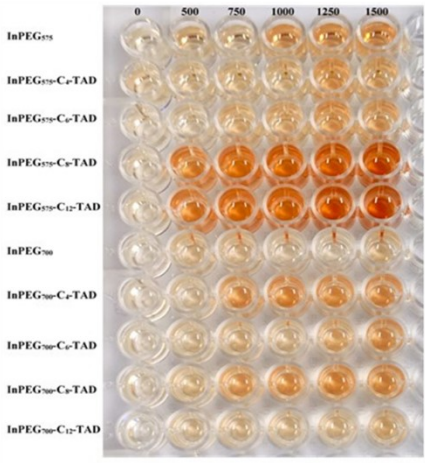
a)  b) 

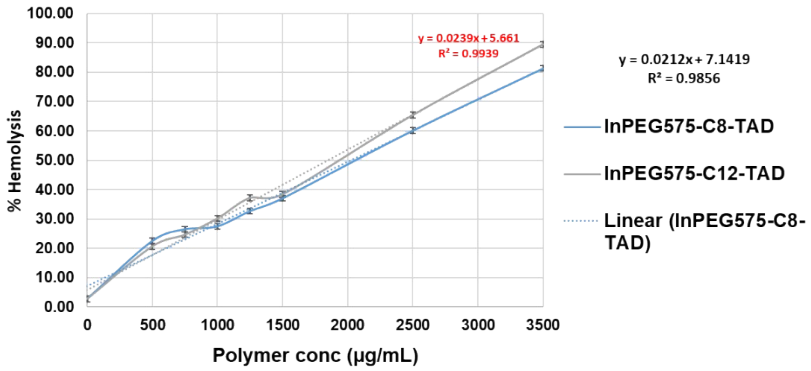
Representative figure spread plate images for (a*) S. aureus* and (b) *P. aeruginosa*

	Number of colonies in plate (100 ul of OD =0.5; df=10^7)	Calculated CFU/mL	Used CFU/mL (reported by Chang et al.)
S. aureus	76	$1.52*10^9$	$1.5*10^9$
	Number of colonies in plate (50 ul of OD =0.5; df=10^6)	Calculated CFU/mL	Used CFU/mL (reported by Dong-ju Kim et al.)
P. aeruginosa	52	$2.08*10^8$	$2*10^8$

(3) Hemolysis data:

Representative image of hemolysis assay at range of different concentrations of the polymers.





	m	C	% Hemolysis	HC50
$InPEG_{575}$-C_8-TAD	0.0212	7.1419	50	2022
$InPEG_{575}$-C_{12}-TAD	0.0239	5.661	50	1855

% Hemolysis data points at different concentration

Polymer conc (µg/mL)	Hemolysis for $InPEG_{575}$-C_8-TAD	SD	Hemolysis for $InPEG_{575}$-C_{12}-TAD	SD
0	2.71	0.3820	2.71	0.3820
500	22.46	0.2043	20.62	0.1883
750	26.48	0.1717	24.64	0.2498
1000	27.49	0.2971	30.17	0.2920
1250	32.68	0.3730	37.20	0.0247
1500	37.03	0.4386	38.37	0.4423
2500	60.13	0.4589	65.41	0.3698
3500	81.33	0.3431	89.51	0.3667

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