Supporting Information

Impact of Ionizable Groups in Star Polymer Nanoparticles on NLRP3 Inflammasome Activation

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Synthesis of Macro chain transfer agent (CTA): 250 mg (0.1 mmol) of 4-arm-PEG_{2000}-NH_2 was dissolved in 5 mL anhydrous tetrahydrofuran (THF) in a round bottom flask. In a separate vial, 189 mg (0.5 mmol) of 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid N-succinimidyl ester was dissolved in 2 ml of dichloromethane. This solution was then added dropwise to the round bottom flask and allowed to stir for 24 hrs at room temperature under inert atmosphere. The unreacted impurities were removed via dialysis against a 1:1 mixture of dichloromethane and methanol using a 3.5 kDa membrane for 48 hours. The solution was then rotovapped and lyophilized overnight to yield the final product. Yield - 173.23 mg (69%). ^{1}H NMR (400 MHz, CDCl3) δ ppm: 7.90 (t, 8.00 H), 7.57 (m, 4.00 H), 7.38 (m, 8.00 H), 3.76-3.42 (m, 208.00 H), 2.73 (s, 15.00 H), 1.97 (d, 12.00 H), 1.85 (m, 14.00 H).

Synthesis of 4-arm-PEG_{2000}-DM_{100}-X_{100} block copolymers: Polymerization was done using both hydrophobic and ionic monomers, where the hydrophobic monomer was kept constant while the ionic monomer was varied. The hydrophobic monomer chosen was dodecyl methacrylate (DM), as prior work had shown a positive correlation between hydrophobicity and activation of NLRP3 inflammasome. The ionic monomers tested were 2-(dimethylamino)-ethyl methacrylate (DMA), 2-(diisopropylamino)-ethyl methacrylate (DiPA), 2-(tert-butylamino)-ethyl methacrylate (TBM) and 2-N-morpholinoethyl methacrylate (MEM). The number of units of both hydrophobic and ionic monomers was fixed at 100, giving rise to four different combinations - DM_{100}-DMA_{100}, DM_{100}-$DiPA_{100}$, DM_{100}-TBM_{100} and DM_{100}-MEM_{100}. The typical synthesis protocol for the block copolymers has been outlined for 4-arm-PEG_{2000}-DM_{100}-DMA_{100}. Similar procedure was followed for the other polymers by varying the ionic monomer.

Synthesis of block copolymer 4-arm-PEG_{2000}-DM_{100}-DMA_{100}: 4 arm-PEG-CTA (16 mg, 0.0045 mmol), dodecyl methacrylate (116 mg, 0.45 mmol), 2-(dimethylamino)-ethyl methacrylate (72 mg, 0.45 mmol) and recrystallized azodiisobutyronitrile (AIBN) (0.30 mg, 0.0018 mmol) were dispersed in 0.5 mL anhydrous tetrahydrofuran in a Schlenk flask. The flask was then sealed, and the solution subjected to five freeze-pump-thaw cycles followed by nitrogen purge to remove all the dissolved oxygen. This was then kept in a preheated oil bath at 70 °C and allowed to stir for 24 hrs. To quench the polymerization reaction, the flask was kept on ice. The polymer was then precipitated in ice-cold methanol, centrifuged at 4000 g for 20 min, and the supernatant decanted. The precipitate was then lyophilized overnight to obtain the final block copolymer. Yield = 93 mg

(47 %). ^{1}H NMR (400 MHz, CDCl3) δ ppm: 4.10 (br s, 194.00 H), 3.94 (br s, 173.00 H), 3.76-3.42 (s, 208.00 H), 2.32 (s, 590.00 H), 1.63 (br s, 199.00 H), 1.29 (s, 1745.00 H), 0.93 (s, 632.00 H). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm: 76.3, 69.6, 55.9, 44.5, 30.9, 28.6, 27.2, 25.0, 21.7, 13.1.

4-arm-PEG$_{2000}$-DM$_{100}$-DiPA$_{100}$: Reactants: 4 arm-PEG-CTA (15 mg, 0.0041 mmol), dodecyl methacrylate (105 mg, 0.41 mmol), 2-(diisopropylamino)-ethyl methacrylate (88 mg, 0.41 mmol), recrystallized azodiisobutyronitrile (AIBN) (0.27 mg, 0.0016 mmol), and anhydrous tetrahydrofuran (0.5 mL). ^{1}H NMR (400 MHz, CDCl3) δ ppm: 4.02-3.80 (br d,189.00 H), 3.76-3.42 (s, 208.00 H), 2.65 (br s, 90.00 H), 1.64 (br s, 183.00 H), 1.29 (s, 1204.00 H), 1.05 (s, 625.00 H), 0.91 (s, 356.00 H). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm: 75.9, 69.4, 30.9, 28.5, 25.1, 21.7, 19.8, 13.1.

4-arm-PEG$_{2000}$-DM$_{100}$-TBM$_{100}$: Reactants: 4 arm-PEG-CTA (20 mg, 0.0055 mmol), dodecyl methacrylate (140 mg, 0.55 mmol), 2-(tert-butylamino)-ethyl methacrylate (78 mg, 0.55 mmol), recrystallized azodiisobutyronitrile (AIBN) (0.36 mg, 0.0022 mmol), and anhydrous tetrahydrofuran (0.5 mL). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.06 (br s, 93.00 H), 3.92 (br s, 135.00 H), 3.76-3.42 (s, 208.00 H), 2.82 (br s, 117.00 H), 2.05-1.74 (br s, 255.00 H), 1.61 (br s, 183.00 H), 1.28 (s, 1399.00 H), 1.13 (s, 501.00 H), 0.88 (t, 407.00 H). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm: 76.3, 69.8, 39.9, 30.8, 28.3, 24.6, 21.7, 12.9.

4-arm-PEG$_{2000}$-DM$_{100}$-MEM$_{100}$: Reactants: 4 arm-PEG-CTA (18 mg, 0.005 mmol), dodecyl methacrylate (126 mg, 0.5 mmol), 2-N-morpholinoethyl methacrylate (72 mg, 0.5 mmol), recrystallized azodiisobutyronitrile (AIBN) (0.33 mg, 0.002 mmol), and anhydrous tetrahydrofuran (0.5 mL). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.09 (br s, 114.00 H), 3.93 (br s, 113.00 H), 3.76-3.42 (t, 208.00 H), 2.63 (br s, 407.00 H), 2.52 (br s, 201.00 H), 1.63 (br s, 122.00 H), 1.29 (br s, 1111.00 H), 0.92 (t, 342.00 H). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm: 75.7, 69.4, 65.9, 53.0, 40.3, 33.7, 31.2, 28.6, 21.7, 12.9.

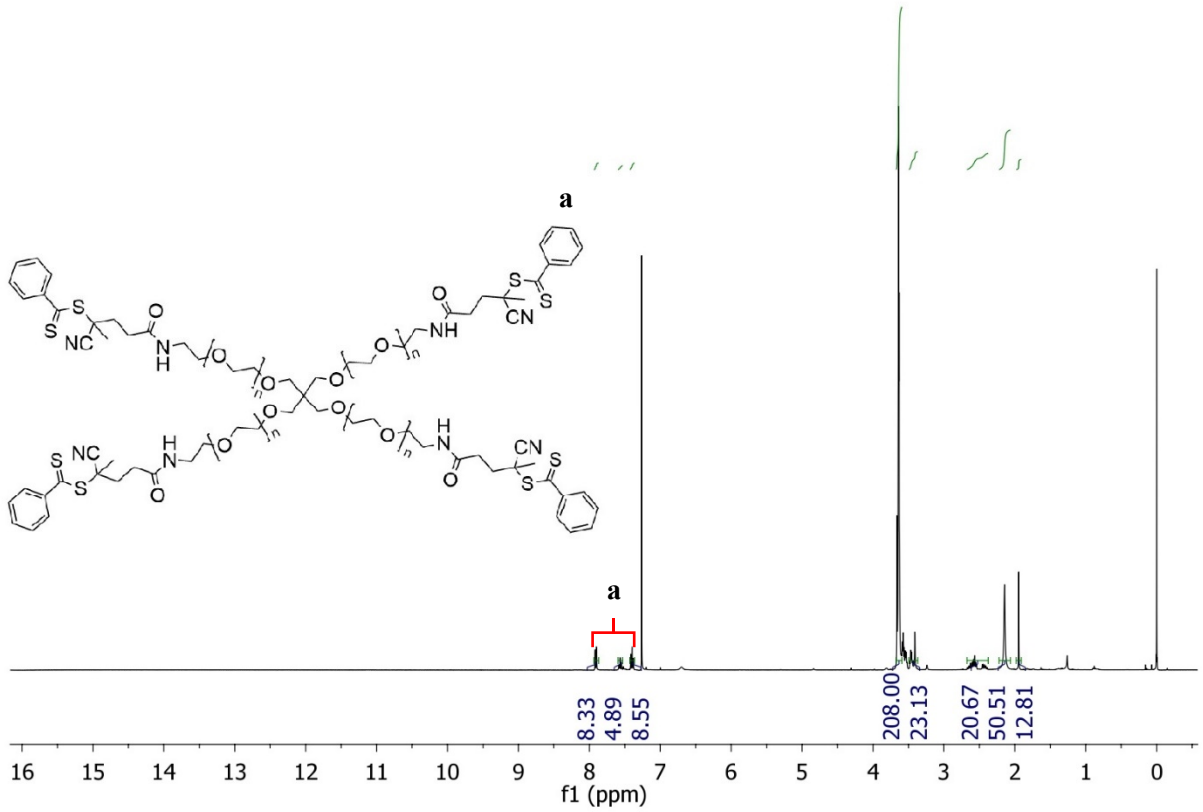

Figure S1. Structure and NMR characterization of PEG-CTA. ^{1}H NMR plot of 4-arm-PEG$_{2000}$-CTA in $CDCl_3$. The presence of the aromatic peaks highlighted "a" and the absence of any peak at around 5.00 ppm confirms that the PEG-CTA reaction went 100 %.

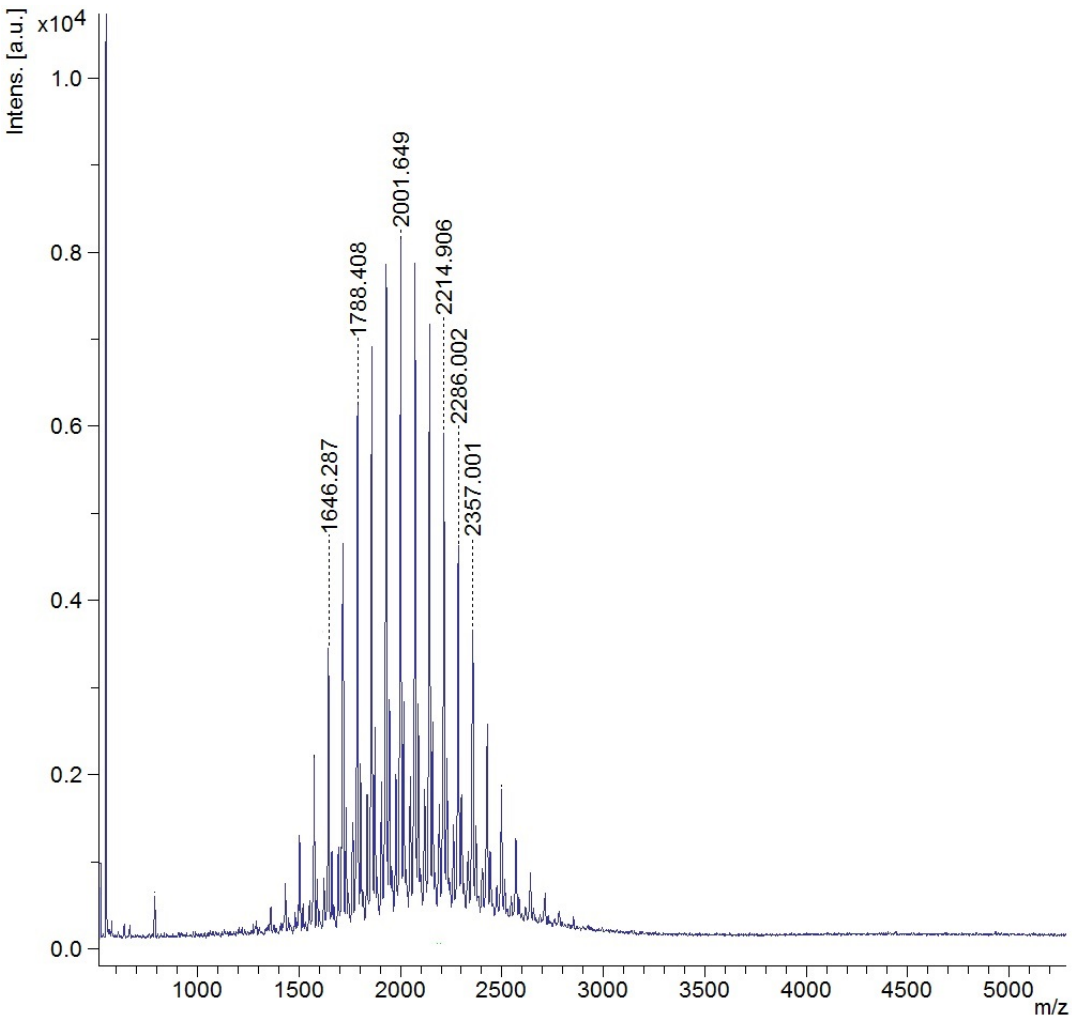

Figure S2. MALDI characterization of PEG-CTA. MALDI-TOF mass spectra for the 4-arm-PEG-CTA with the mass breakdown of the peaks, corroborating the formation of the desired product.

S.No.	Polymer	Feed Ratio		Obtained Ratio		Molecular weight (g/mol)	Size (d nm) ± SD	Zeta Potential (mV)
		x	y	X	y			
1	DM_x-DMA_y	100	100	94	95	42,360	191.7 ± 4	33.8 ± 4.2
2	DM_x-$DiPA_y$	100	100	58	46	28,100	210.1 ± 7.4	20.8 ± 2.3
3	DM_x-TBM_y	100	100	69	53	28,600	109.5 ± 23.1	35.5 ± 3.6
4	DM_x-MEM_y	100	100	65	62	32,400	163.5 ± 20.4	20.4 ± 2.0

Table ST1. Star Polymer and their Nanoparticle physicochemical properties

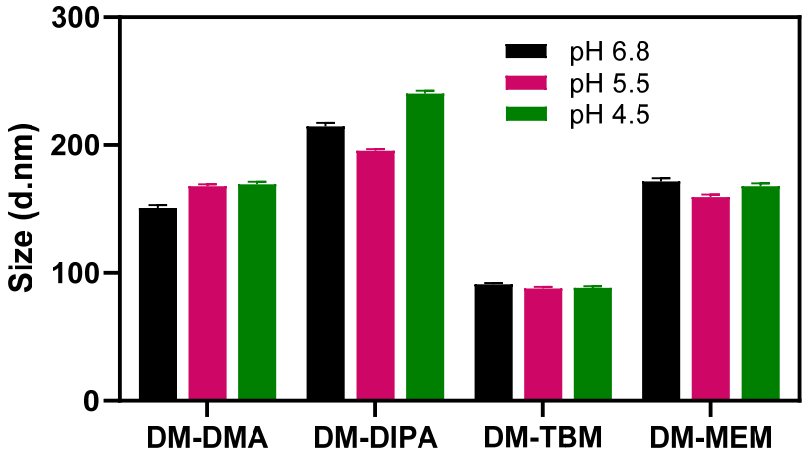

Figure S3: pH-dependent size analysis of Star polymer Nanoparticles Representative plot for the hydrodynamic diameter of the various star polymer nanoparticles at varying pH determined by dynamic light scattering. The concentration of the particles is 0.1 mg/ml.

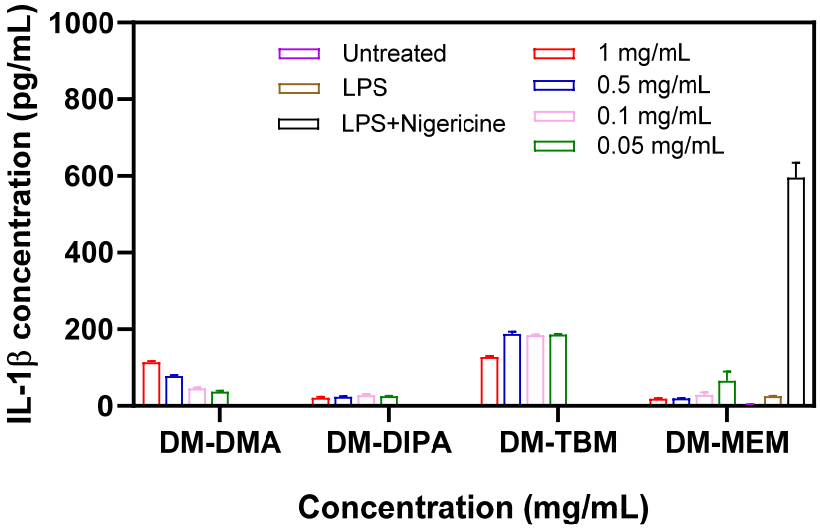

Figure S4: Effect of star polymer nanoparticles on IL-1β release and inflammasome complex formation in caspase-1 KO iBMDMs. IL-1β levels in the supernatant of LPS primed iBMDMs knocked out for caspase-1 incubated with four concentrations (ranging from 1 mg/ml to 0.05 mg/ml) of the NPs, for 24 hrs quantified by ELISA.

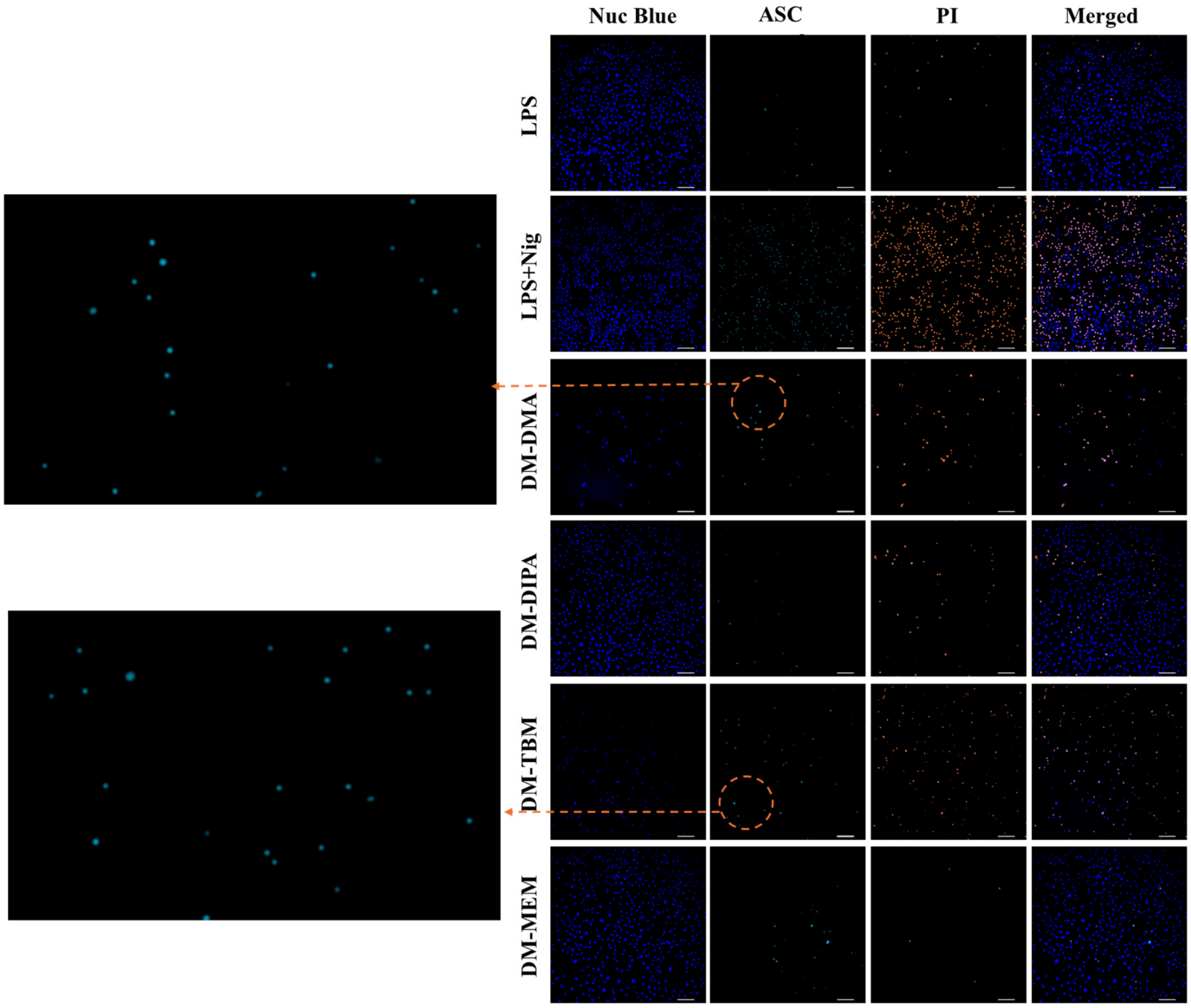

Figure S5: Investigating the effect of star polymer nanoparticles on NLRP3 inflammasome complex formation. Representative confocal microscopic images of iBMDMs primed with LPS and treated with 0.5 mg/ml concentration of the NPs for 16 hrs. Blue fluorescence correlates with nuclei stained with NucBlue, red fluorescence represents PI-positive dead cells and cyan fluorescent dots depict the ASC specks formed in the inflammasome complex. Magnification = 20x; Scale bar = 50 μm.

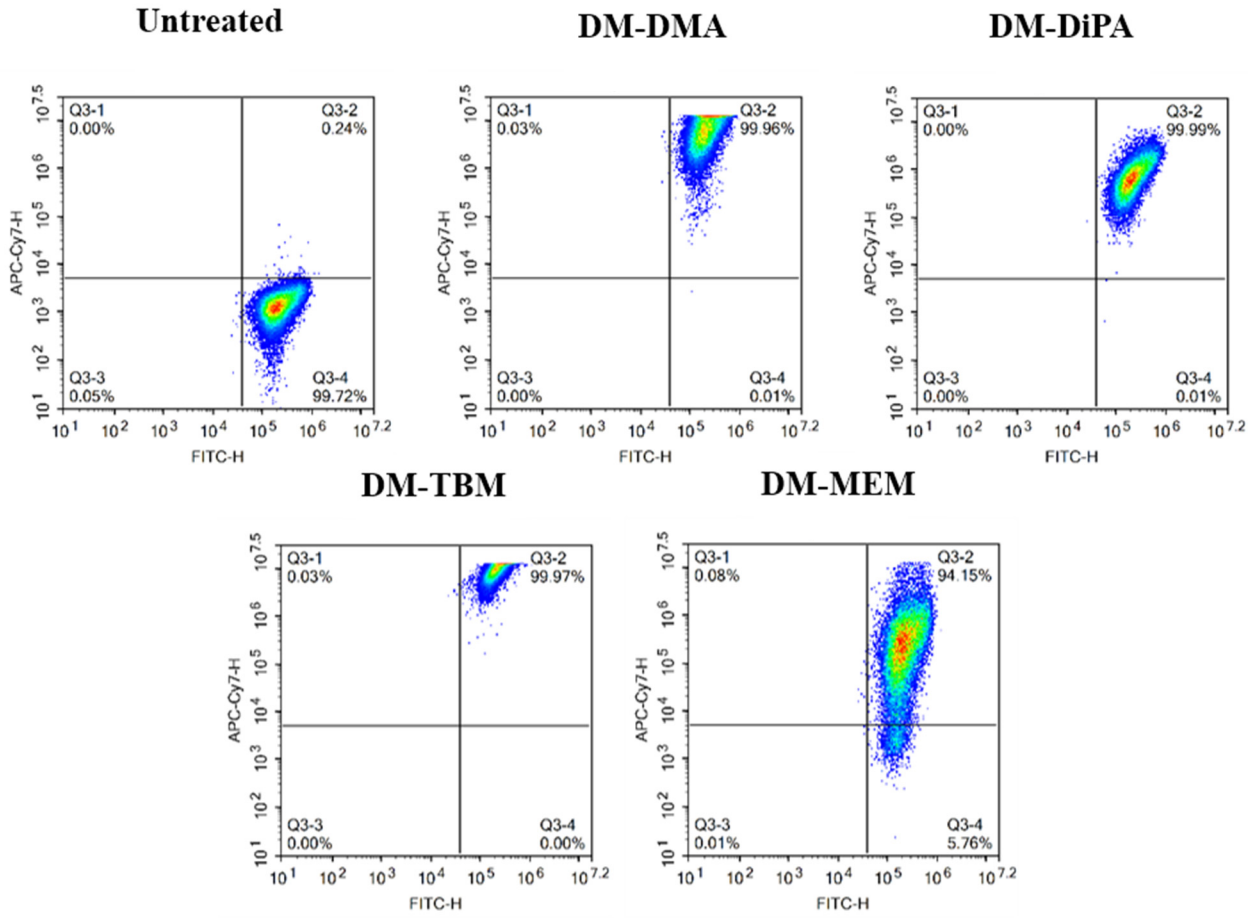

Figure S6: Cellular internalization of Star polymer Nanoparticles by iBMDMs analyzed via flow cytometry Representative density plots for the cellular internalization of 0.5 mg/ml of DiR dye-loaded polymer nanoparticles by the CFSE-stained iBMDMs. The cells in the lower right quadrant represent only CFSE-positive cells while the cells in the upper right quadrant are depictive of CFSE and DiR double positive cells, highlighting the cell population with internalized NPs.

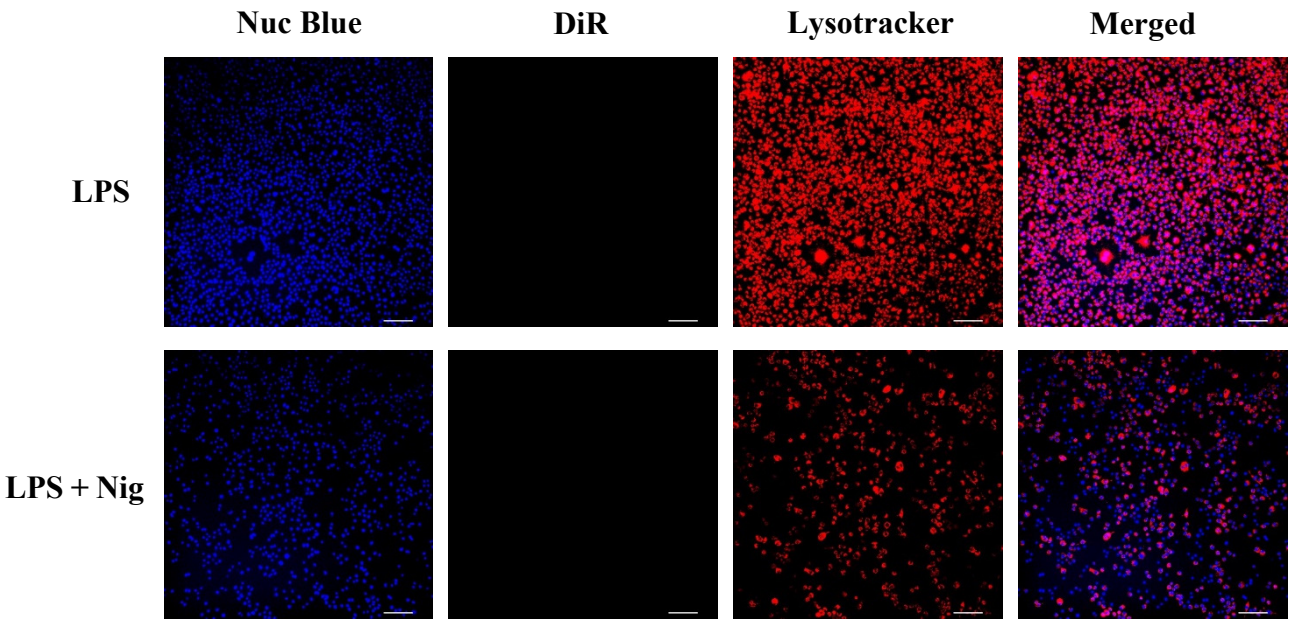

Figure S7: Representative confocal images of controls used for Lysosomal Rupture. iBMDMs were treated with LPS for 4 hrs and LPS followed by Nigericin for 2 hrs as the negative and positive controls respectively. Blue fluorescence represents nuclei stained by NucBlue, DiR channel signifies internalized DiR-loaded NPs (which in this case is negative as no particles were added), and red fluorescence represents intact lysosomes. Scale bar = 50 μM

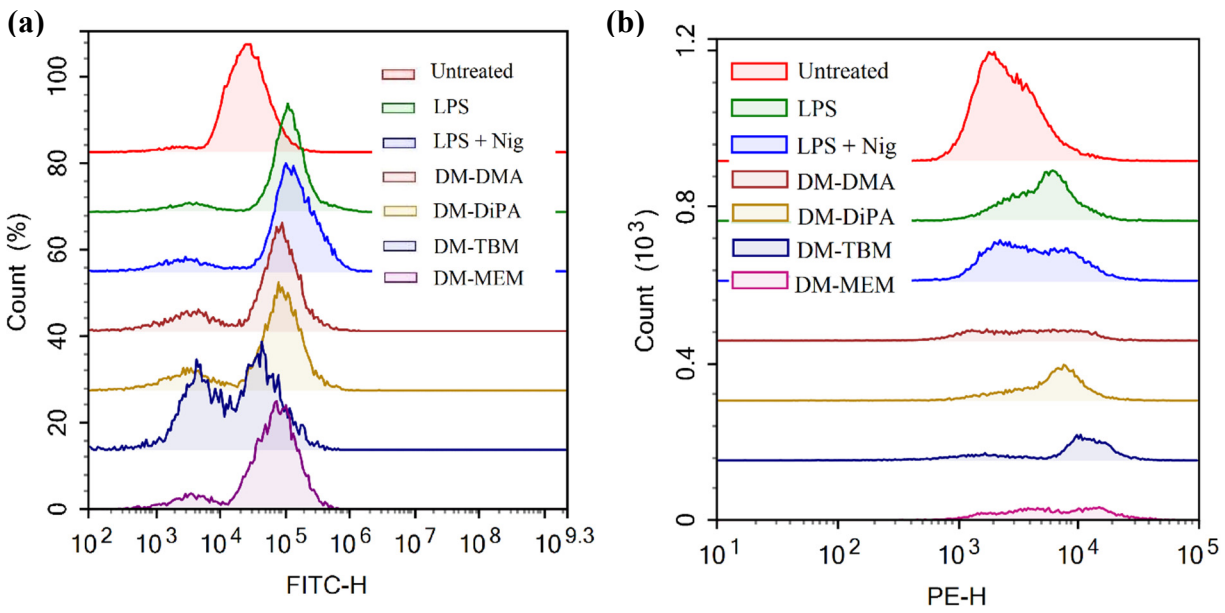

Figure S8: Mechanistic evaluation of calcium influx and mitochondrial ROS production effectuated by Star Polymer nanoparticles. (a) Representative flow cytometry overlay plots depicting the shift in Fluo-4-AM fluorescence intensity for LPS-primed iBMDMs treated with 0.5 mg/mL of the NPs for 24 hours. The plot represents the percentage of Fluo-4-AM positive cells, or the degree of calcium influx caused by NPs in 24 hrs. **(b)** Representative flow cytometry overlay plots depicting the MitoSOX fluorescence intensity shift for LPS-primed iBMDMs treated with 0.5 mg/mL of the NPs for 24 hours. The plot represents the percentage of MitoSOX-positive cells i.e the mitochondrial ROS production caused by NPs in 24 hrs**.**