Supplementary information

Folic acid targeted redox responsive poly lactic acid-based nanoparticles co-delivering Pirarubicin & Salinomycin suppress breast cancer tumor growth *in-vivo*

Priya Gupta[1], Ankushi Bansal[1*], Harshdeep kaur[1], Mohd Anees[1], Neetu Singh[1], Ritu Kulshreshtha[2] and Harpal Singh[1*]

[1]Centre for Biomedical Engineering, Indian Institute of Technology Delhi, New Delhi, India
[2]Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology, New Delhi, India

**Email:* harpal.singh@cbme.iitd.ac.in *and* harpal2000@yahoo.com
**Email:* bansalankushi21@gmail.com

1. Synthesis of [S-(PLA-b-PEG-COOH)]$_2$ block copolymer

1.1 Synthesis of (OH-PLA-S-S-PLA-OH): OH-PLA-S-S-PLA-OH was synthesized via the ring-opening polymerization of L-lactide, employing 2-HEDS as a bifunctional disulfide initiator (M/I molar ratio = 139) and stannous octoate as reaction catalyst (0.005 w/w % of L-lactide). The reaction took place at 170ºC for 3 hours under continuous N_2 flow. Upon completion, the reaction product was precipitated in diethyl ether and washed twice with methanol to eliminate the unreacted compounds. Subsequently, the precipitated polymer was dried under vacuum to obtain a powdered form, & then stored at -20ºC for future use.

1.2 End group modification of (OH-PLA-S-S-PLA-OH) with succinic anhydride: The -OH groups of the (OH-PLA-S-S-PLA-OH) was converted to -COOH groups by reacting [S-(PLA-OH)]$_2$ with succinic anhydride. Firstly, dried (OH-PLA-S-S-PLA-OH) (1 eq, 0.125 mmoles) was dissolved in dry DCM under N_2 atmosphere followed by subsequent addition of DMAP (6 eq, 0.75 mmol). Following this, succinic anhydride (8 eq, 1 mmol) and TEA (6 eq, 0.75 mmol) were introduced to the polymer solution, and the reaction mixture was stirred at r.t. for 36 hours. After the completion of reaction, the DCM solution was washed with 0.5 M HCl to remove the DMAP and TEA, followed by water washes. The polymer was then precipitated into an excess of ice-cold methanol to eliminate excess succinic anhydride and subsequently dried under high vacuum to obtain the desired modified polymer, (HOOC-PLA-S-S-PLA-COOH), The resulting modified polymer, (HOOC-PLA-S-S-PLA-COOH), was stored at a low temperature.

1.3 PEGylation of (HOOC-PLA-S-S-PLA-COOH): Conjugation of PEG-diol to (HOOC-PLA-S-S-PLA-COOH) was done using DCC/DMAP chemistry via Steglich esterification to get [S-(PLA-b-PEG-OH)]$_2$. Initially, [S-(PLA-COOH)]$_2$ (1 eq, 0.1 mmol) was dissolved in DCM and DCC (5 eq, 0.5 mmol) and DMAP (2 eq, 0.2 mmol) were added under a N_2 atmosphere. In a separate flask, PEG-diol (4 kDa, 10 eq, 1 mmol) was dissolved in dry DCM

under nitrogen atmosphere. The PLA solution was then slowly added under N_2 into the PEG solution, ensuring that there was always an excess of PEG relative to PLA. The reaction proceeded at r.t. for 3 days. After the completion of the reaction, the excess DCC, DCU byproduct, and DMAP were extracted using an acid wash (0.5 M HCl) & water wash. The resulting [S-(PLA-b-PEG-OH)]$_2$ was precipitated in an ice-cold methanol and dried under high vacuum.

1.4 Conjugation of Folic acid to [S-(PLA-b-PEG-COOH)]$_2$: Folic acid was conjugated to [S-(PLA-b-PEG-COOH)]$_2$ via -NH_2 group of FA and COOH group of the co-polymer to form amide linkage. The reaction was carried out in the presence of DCC and DMAP, using DMF and DCM dual solvent system due to solubility difference of polymer and FA. PEGylated polymer (1 eq, 0.004 mmol) was dissolved in DCM followed by addition of DCC (10eq, 0.04 mmol) and DMAP (5 eq, 0.02 mmol) to activate the -COOH group of the polymer. The reaction mixture was stirred at room temperature overnight under N_2 atmosphere. FA (5 eq, 0.02 mmol) was then reacted with activated polymer for another 24 h at r.t. FA conjugated polymer was obtained by precipitating with mixture of diethyl ether and methanol. The final product thus obtained, expressed as FA-[S-(PLA-b-PEG-COOH)]$_2$ was yellowish in colour, dried and characterized for further studies using UV−visible spectroscopy (Perkin Elmer, U.S.A.)

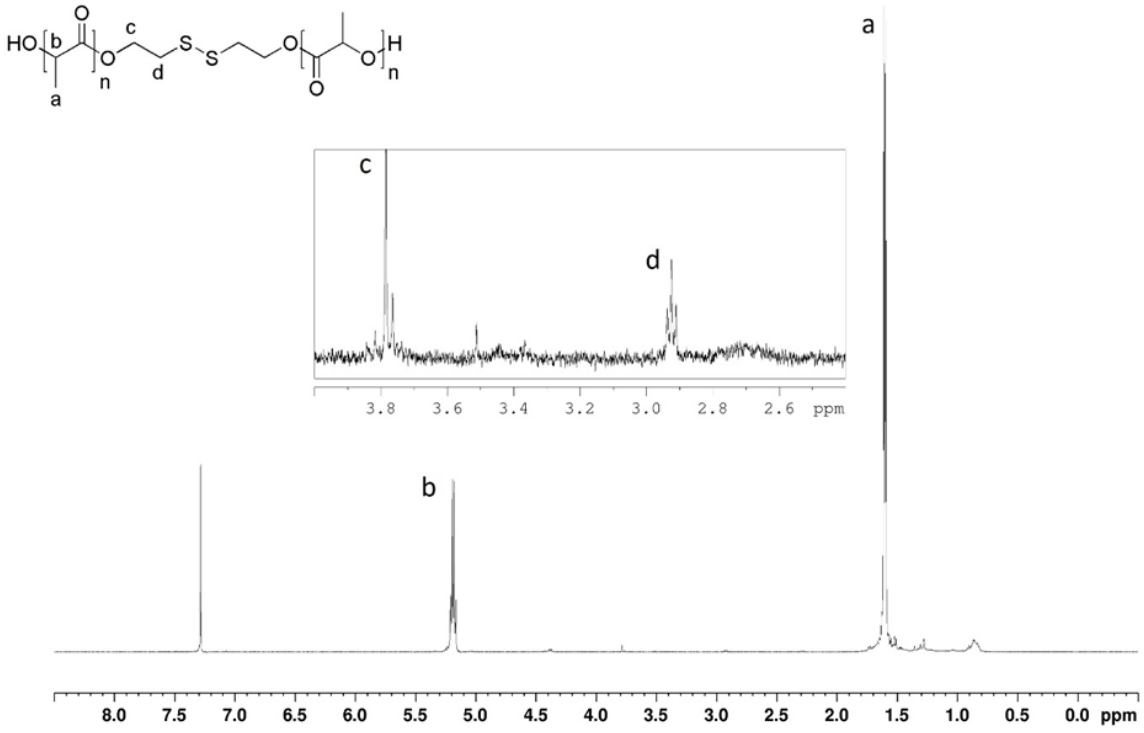

Figure S1: ^{1}H NMR spectra of [S-(PLA-OH)]$_2$

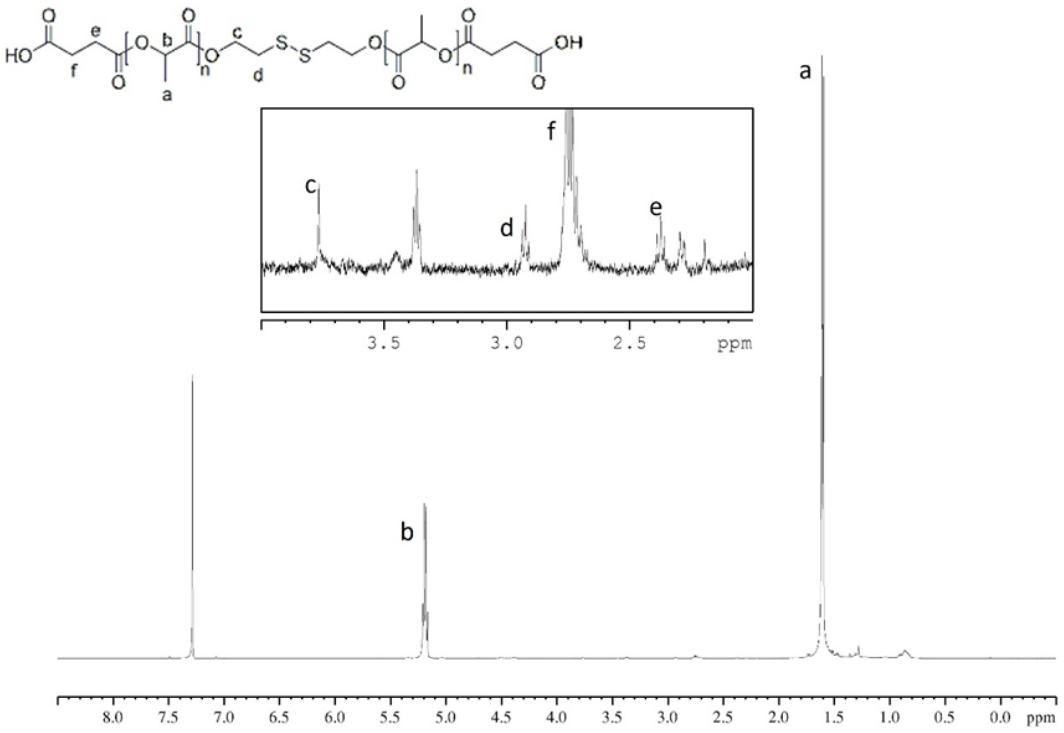

Figure S2: ^{1}H NMR spectra of [S-(PLA-COOH)]$_2$

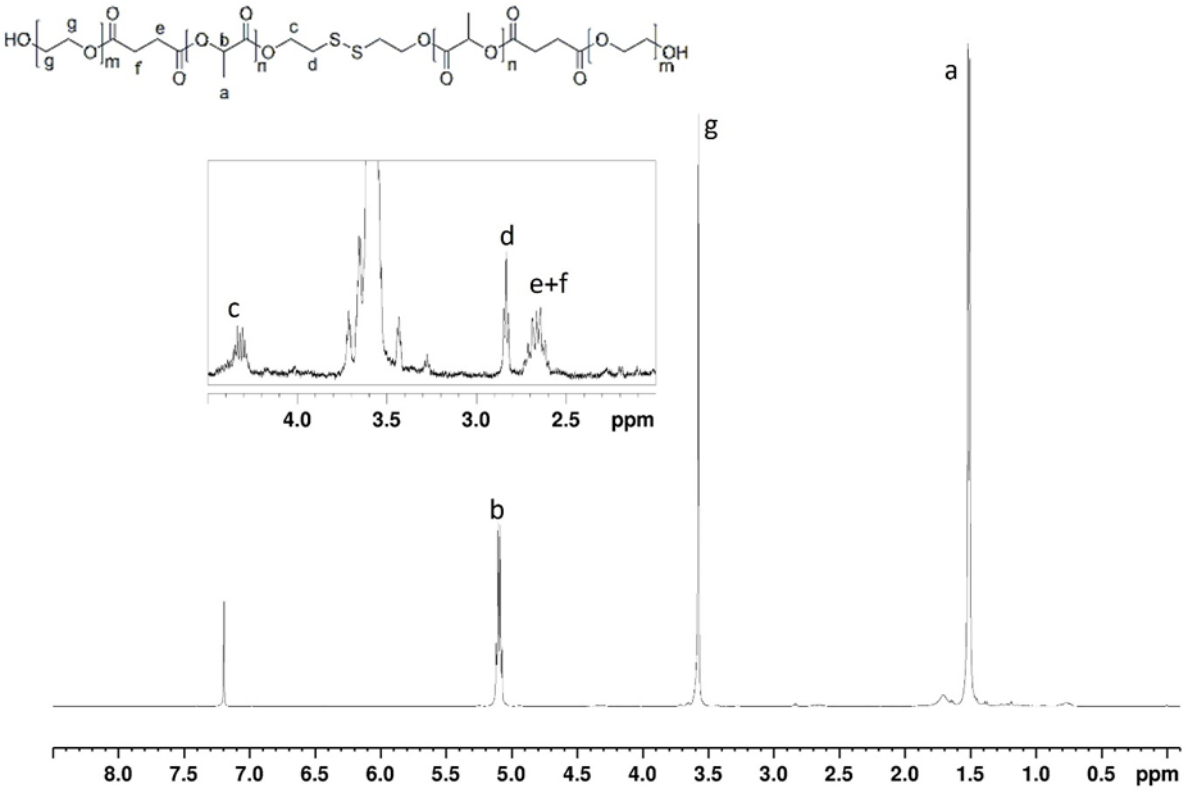

Figure S3: ^{1}H NMR spectra of [S-(PLA-b-PEG-OH)]$_2$

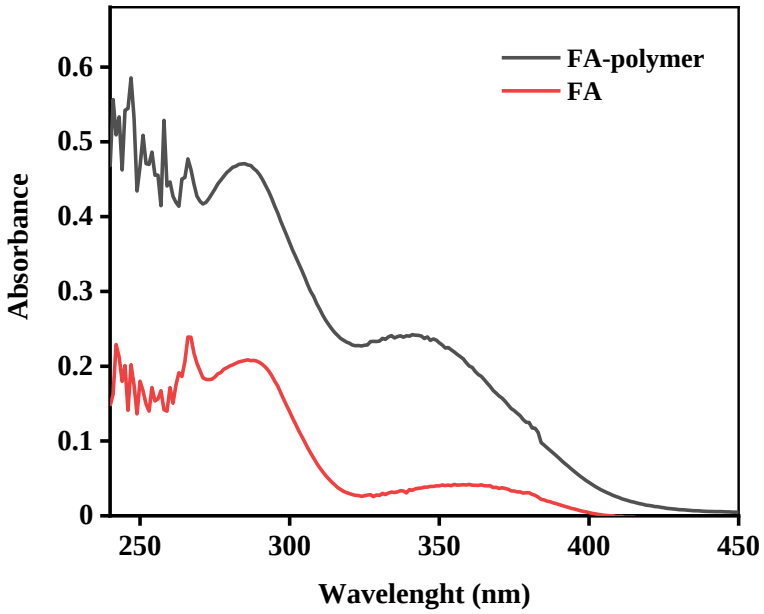

Figure S4: UV absorption spectra of FA conjugated polymer and FA in DMF

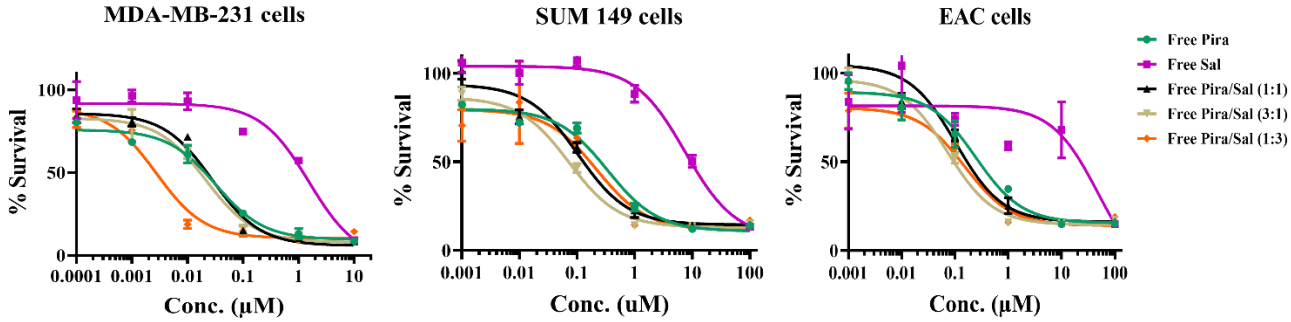

Figure S5: Non-linear regression curves of *in-vitro* cell proliferation inhibition assay on breast cancer cells MDA-BB-231, SUM-149 and EAC upon treatment with free Pira/ Sal and their combination

Table S1: IC_{50} (µM) values of free Pira/Sal and their combination on breast cancer cells

	SUM-149	MDA-MB-231	EAC
Pira	0.3360	0.0425	0.2466
Sal	7.5380	1.5330	48.510
Pira:Sal (1:1)	0.1046	0.0275	0.1046
Pira:Sal (3:1)	0.0724	0.0214	0.0724
Pira:Sal (1:3)	0.2201	0.0027	0.1530

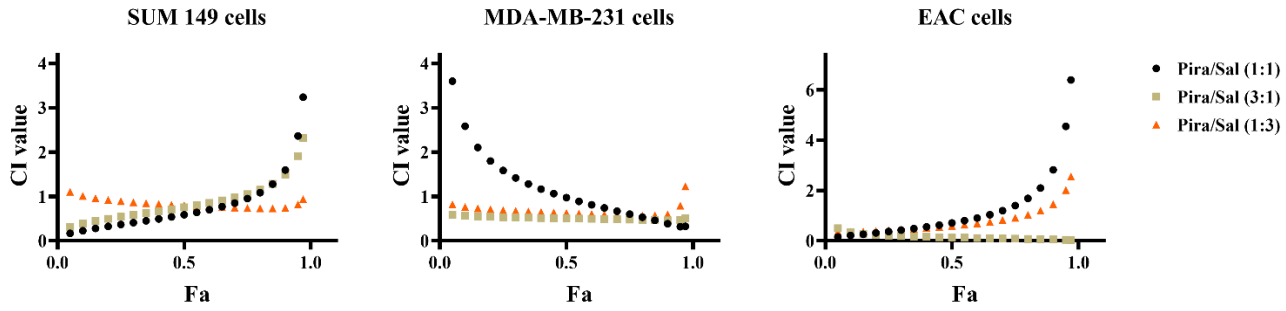

Figure S6: Combination index vs fraction affected (Fa) plots of free Pira/Sal and their combinations in different ratios (Pira:Sal - 1:1, 3:1 and 1:3) on breast cancer cells; SUM-149, MDA-MB-231 and EAC.

Table S2: Combination index (CI values) of free Pira/Sal and their combination on breast cancer cells at Fa = 0.5

	SUM-149	MDA-MB-231	EAC
Pira:Sal (1:1)	0.589	0.974	0.709
Pira:Sal (3:1)	0.757	0.507	0.136
Pira:Sal (1:3)	0.800	0.627	0.597