Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

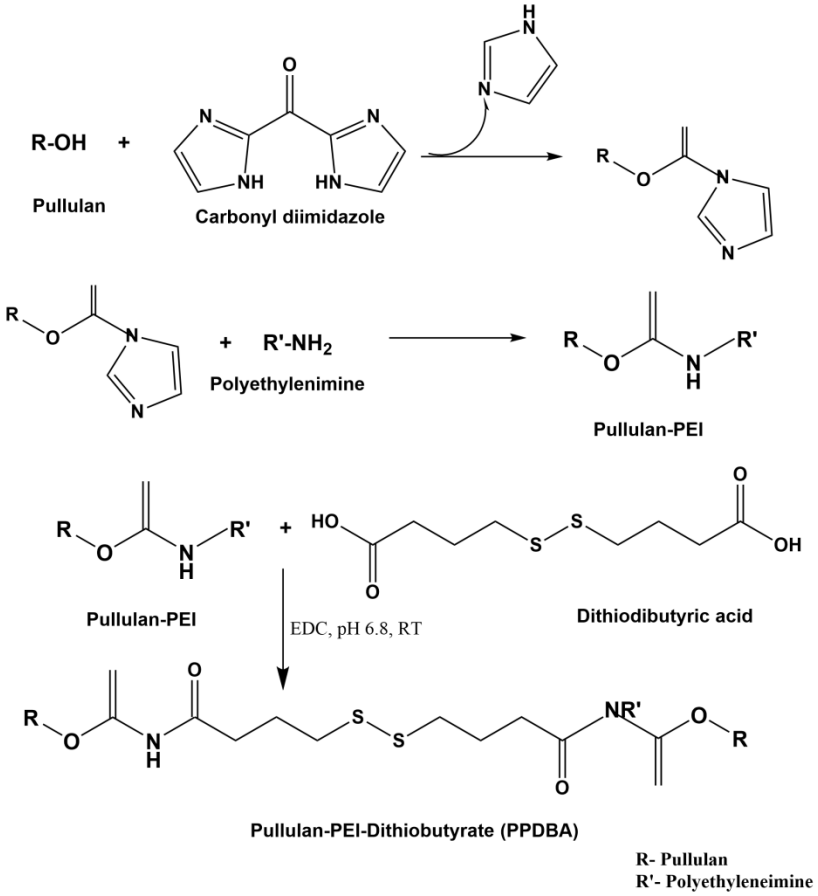
Supplementary Information

Synergistic effect on cancer cell by the intracellular delivery of p53 gene/Dox and the P-gp inhibition by pullulan thiomers: In vitro and In vivo evaluations

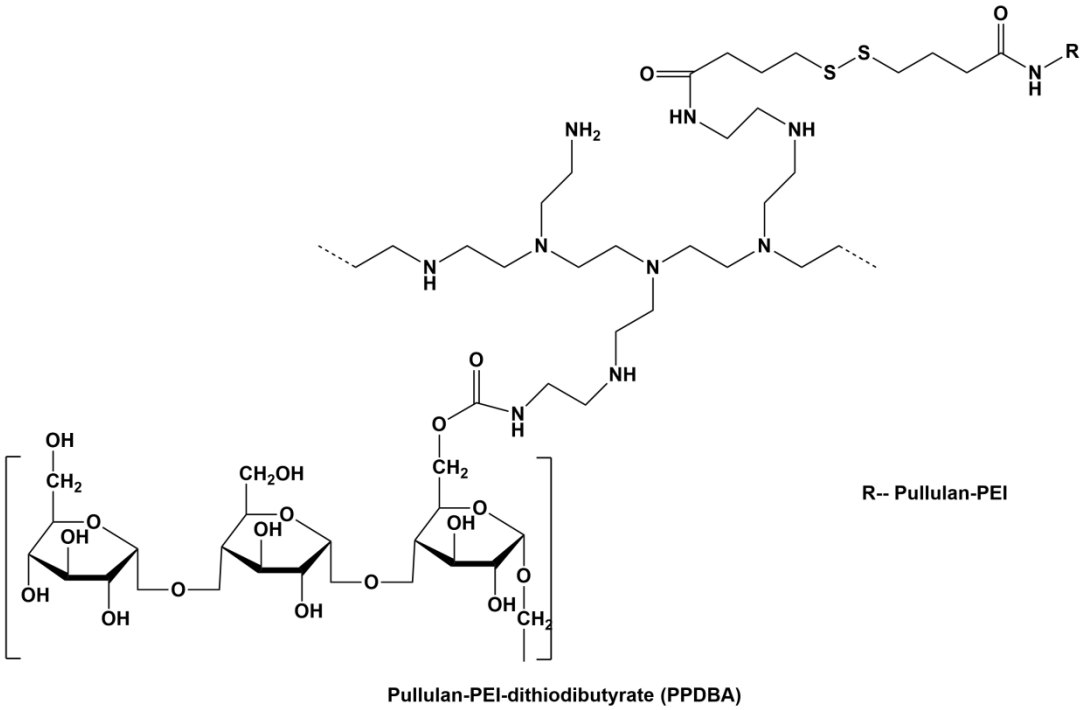
Priya S S and Rekha M R*
Biosurface Technology Division, Biomedical Technology Wing,
Sree Chitra Tirunal Institute for Medical Sciences and Technology,
Thiruvananthapuram, Kerala, India

***Corresponding author address**
Dr. Rekha MR
Scientist F
Biosurface Technology Division, Biomedical Technology Wing,
Sree Chitra Tirunal Institute for Medical Sciences and Technology,
Thiruvananthapuram-695012, Kerala, India
Phone No.: 91-471-2520214
Email: rekhamr@sctimst.ac.in

Supplementary Data



Scheme depicting the synthesis route of PPDBA

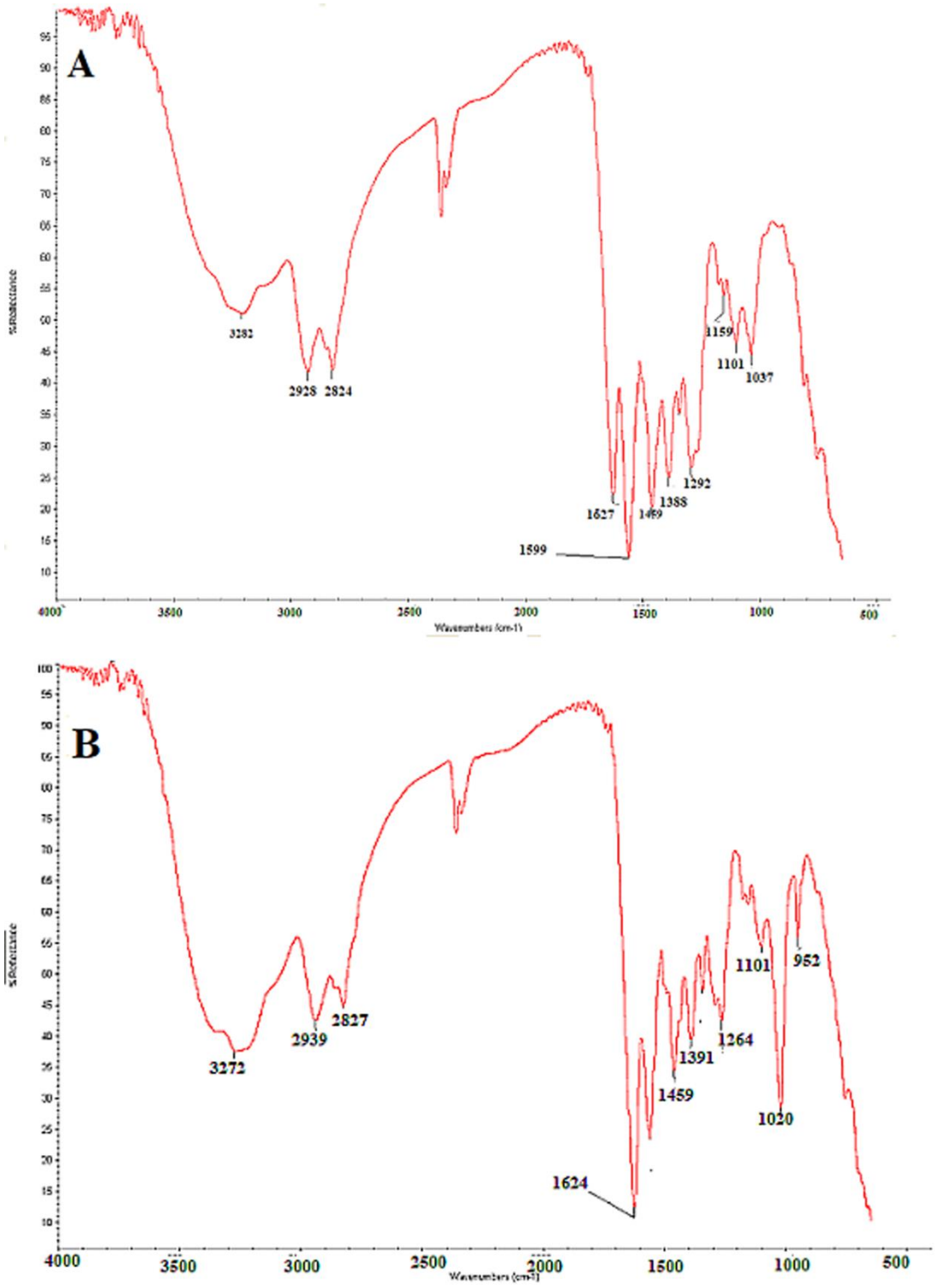


SL.NO	Sample ID	s-s linkage (mg/g of polymer)	Thiol content (mg/g of polymer)
1	PPDBA I	54.12±0.02	72.21±0.011
2	PPDBA II	71.05±0.017	75.14±0.34
3	PPDBA III	78.11±1.55	84.25±2.32
4	PDPBA I	65.31±0.98	87.08±0.42
5	PDBA II	79.27±2.02	95.05±3.01
6	PDBA III	83.22±0.013	98.16±0.31

Table 1: The thiol and disulfide content of the pullulan derivative, PPDBA I, II, III polymers and its corresponding controls PDBAI, II and III.

SL.NO	Sample ID	Amount of amino group (mg/10mg of polymer)
1	PPDBA I	3.03 ± 0.13
2	PPDBA II	2.83 ±0.12
3	PPDBA III	2.79 ±0.32
4	PDBA I	4.28 ±0.01
5	PDBA II	4.09 ±0.03
6	PDBA III	4.12 ±0.21

Table 2: The amino content of PPDBA I, II, III and PDBAI, II, III polymers determined via $CuSO_4$ assay.

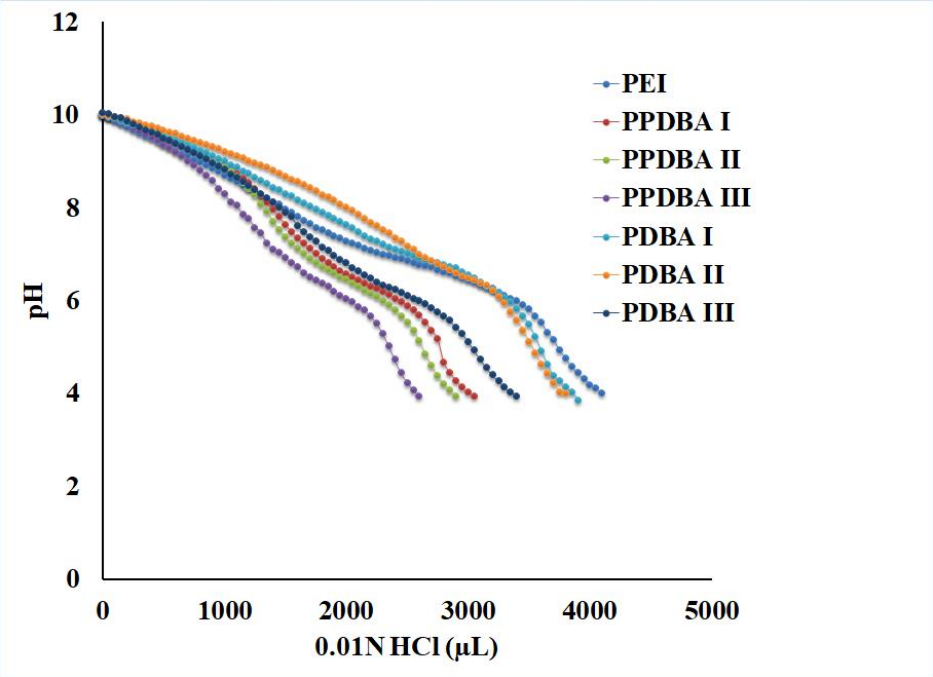


Supplementary Figure 1: FTIR spectra of A) PPDBA II and B) PDBAII.

3.2 Biophysical characterization – buffering capacity

2 Buffering capacity

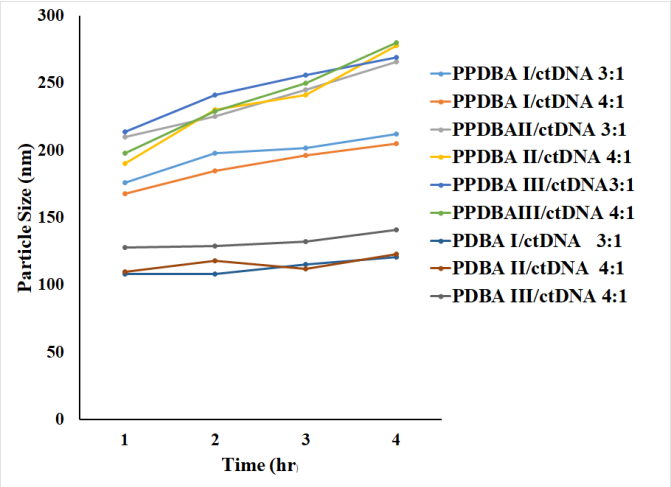
The buffering capacity of PPDBA I/II/III, PDBA I/II/III and PEI was determined by acid-base titration in the pH range 10 to 4. As shown in supplementary figure2, the titration curve confirmed that both pullulan based polymers and PEI derivatives are displaying good buffering capacity. Among the polymers, the buffering capacity was in the order PPDBA I> PPDBA II > PPDBAIII,



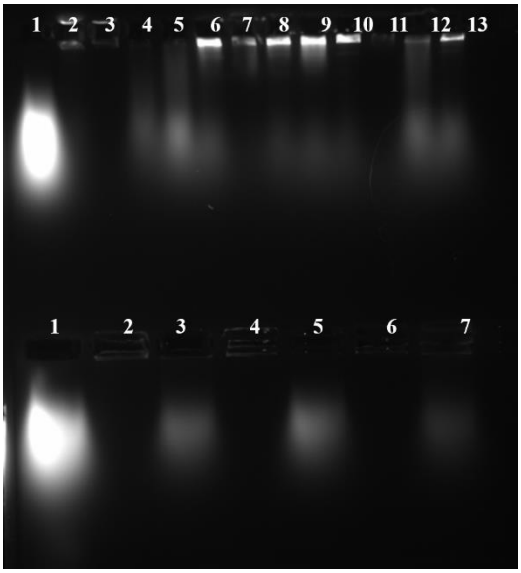
Supplementary Figure 2: Buffering capacity of PPDBA I/II/III , PDBA I/II/III and PEI.

3.2.3 Stability of nanoplexes in presence of DTT

No considerable size change was seen in the DTT treated nanoplexes of PPDBA I in comparison with untreated ones. The size increased from around 150 nm up to 270 nm in the case of PPDBA II and III, after incubation with DTT for 4hr at room temperature (Supplementary Figure 3). There was no considerable size variation in the case of control groups ie PDPA I/II/III. Agarose gel electrophoresis (Supplementary Figure 5) shows that on incubating with 10mM DTT, the DNA bands at the sample loading site become more brighter with a tail like portion observed outside the well in case of PPDBA II/III polyplexes. However, no such band of the free DNA was observed both in PPDBA I and control groups PDBA I/II/III when exposed with DTT. This can be attributed to the lower degree of conjugation in PPDBA I which result in tight binding of the polymer to DNA and as evident from the particle size data too. On the other hand, the combined treatment of heparin/DTT on the selected polyplex ratios of PPDBA I/II/III and PDBA I/II/III released the DNA bands in the gel.

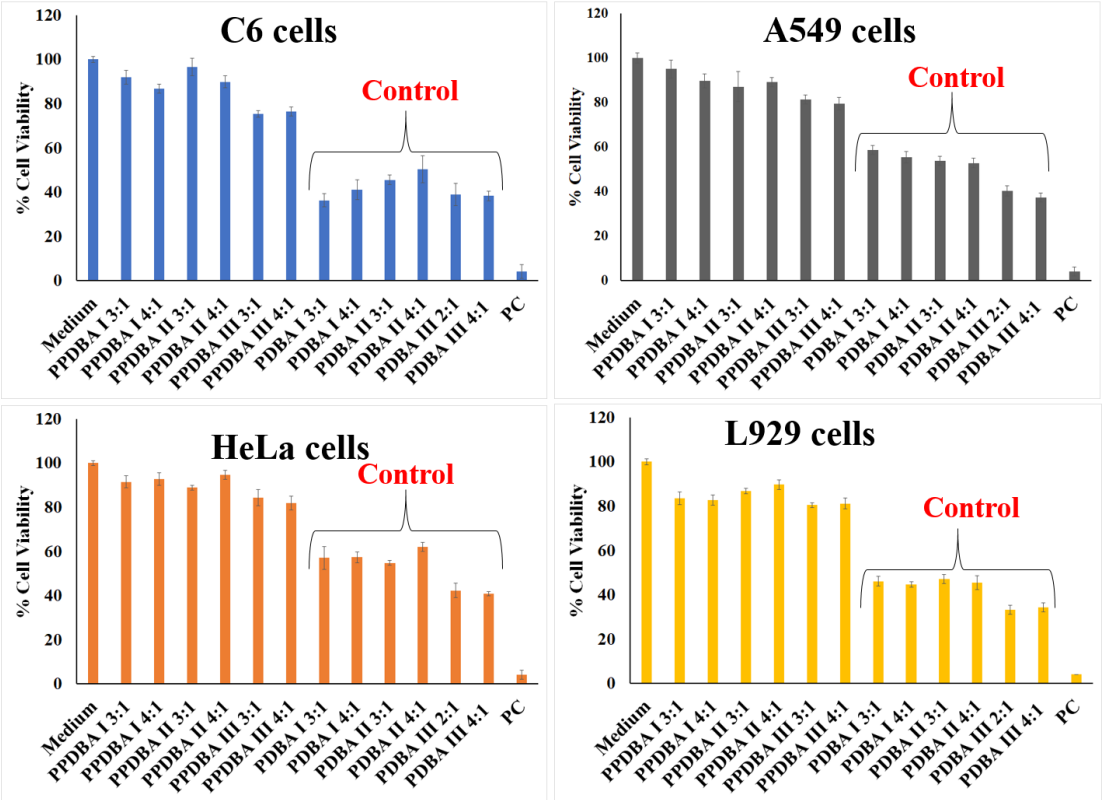


Supplementary Figure 3: Size variations of PPDBA I/II/III and PDBA I/II/III nanoplexes on exposure with 10mM DTT, measured using DLS.



Supplementary Figure 4: agarose gel electrophoresis (upper) lane 1- ctDNA alone control, 2-3 PPDBA I/ctDNA polyplexes, lane 6-7 PPDBA II/ctDNA polyplexes, lane 10-11 PPDBA III/ctDNA polyplexes, all with polymer to DNA ratios 3:1 & 4:1, treated with DTT alone. Whereas lane 4-5, PPDBA I/ctDNA polyplexes, lane 8-9 PPDBA II/ctDNA polyplexes, lane 12-13 PPDBA III/ctDNA polyplexes, all with polymer to DNA ratios 3:1 & 4:1, treated with heparin/DTT. Lane I (lower) indicates ctDNA, lane 2,4,6 (lower) indicates the control PDBAI/II/III polyplexes respectively where treated with DTT alone, whereas lane 3,5,7 (lower) shows the above polyplex with heparin/DTT treatment.

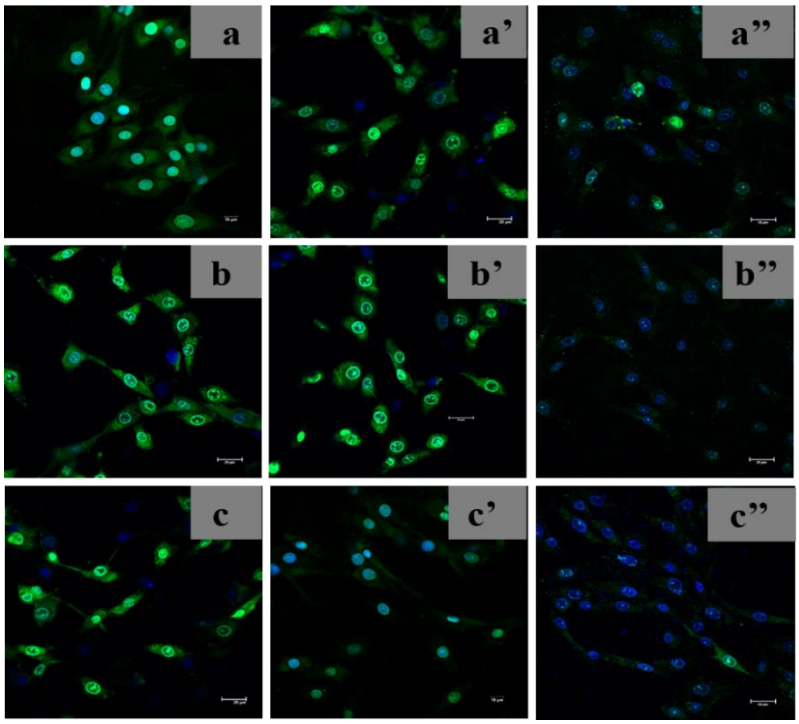
3.5.1 Cytotoxicity evaluation of the polymer



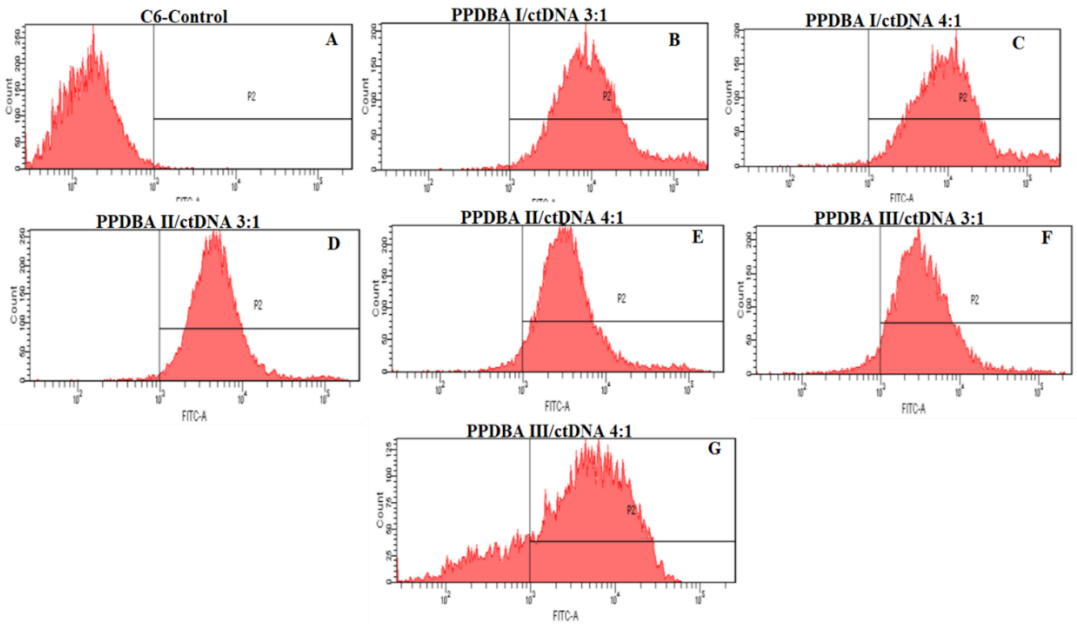
Supplementary figure 5: MTT assay of the selected PPDBA and PDBA nanoplexes at polymer to DNA ratios of 3:1 and 4:1 in different cancer cell lines such as C6, A549 and HeLa and the normal fibroblast cell, L929. Data is shown as mean ±SD, n= 4

3.5.2 Evaluation of cellular uptake

It should be noted that YOYO-I-DNA was widely confined to nuclei. Most of the green fluorescence was found to be coincided with the blue fluorescence in the nuclei at all selected ratios of PPDBA polyplexes. On the contrary, the PDBA nanoplex treated cells showed visible signs of toxicity. Though it maintained normal spindle-shaped structure in most of the cells, nuclear membrane rupture was clearly visible. The flow cytometry analysis was simultaneously carried out with all the selected ratios of PPDBA nanoplexes and the percentage uptake was as follows, PPDBAI/ctDNA 3:1 nanoplex is 96.5% and PPDBAI/ctDNA 4:1 is 95.8%. Similarly, the percentage uptake of PPDBA II/ctDNA 3:1 and 4:1 is 96.4% and 91.3% and PPDBA III/ctDNA3:1 and 4:1 is 90.2% and 74.6% respectively.

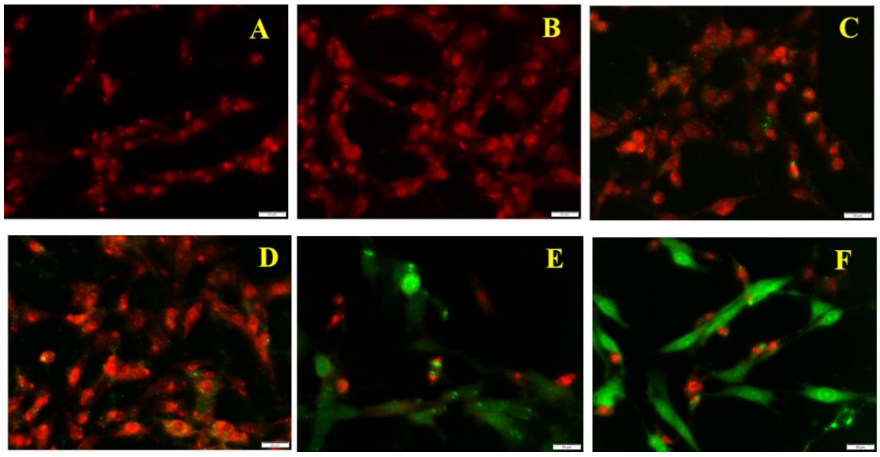


Supplementary figure 6: Cellular uptake of PPDBA polymers based nanoplexes where nucleus is stained with hoechst and DNA tagged with YOYO-I a) PPDBA I/ctDNA 3:1 a') PPDBAI/ctDNA 4:1 nanoplex a'') PDBA I/ctDNA 4:1 nanoplex b) PPDBA II/ctDNA 3:1 b') PPDBA II/ctDNA 4:1 b'') PDBA II/ctDNA 3:1 c) PPDBA III/ctDNA 3:1 c') PPDBA III/ctDNA 4:1 c'') PDBA III/ctDNA 3:1 nanoplexes.Magnification is 60x.

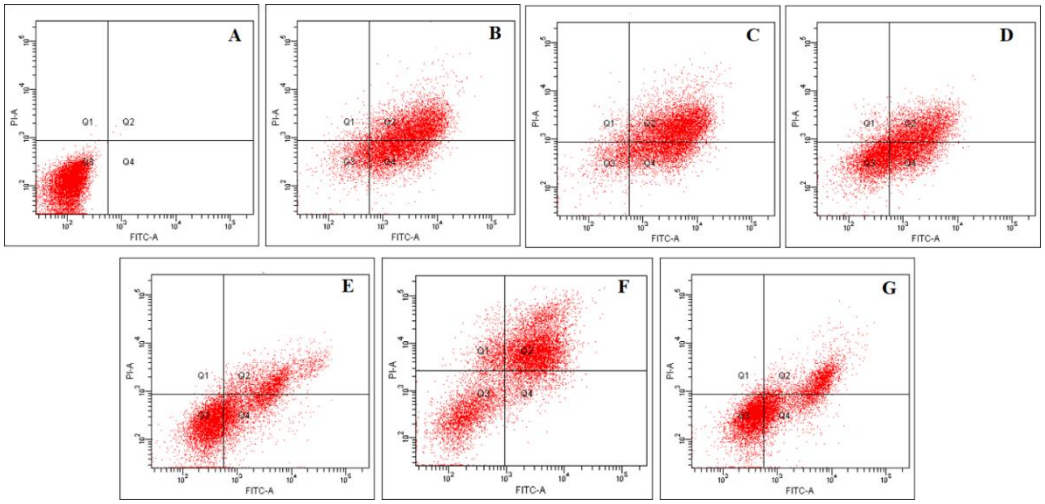


Supplementary figure 7: Flow cytometry data of A) control unstained B) PPDTB I/ctDNA nanoplex of ratio 3:1 with percentage uptake of 96.5 ± 8.12 % C) PPDTB I/ctDNA 4:1, percentage uptake is 98.8± 1.81% D) PPDTB II/ctDNA 3:1 nanoplex with uptake of 96.4±2.21% E) PPDTB II/ctDNA 4:1 is 91.3% F) PPDTB III/ctDNA 3:1 & 4:1 with percent uptake of 90.2±3.65 and 74.6 ± 5.4% respectively.

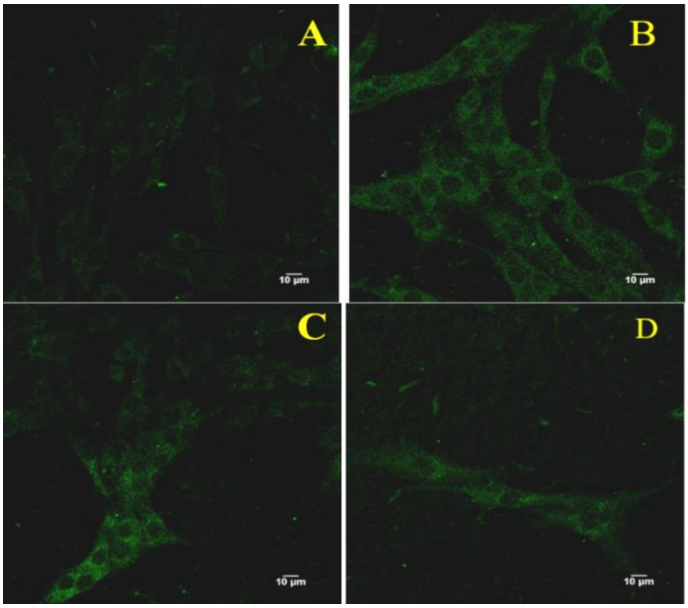
3.5.3 Transfection efficacy studies



Supplementary figure 8: Live and dead assay of A)PPDBA I/p53 3:1 transfected cells B) PPDBA I/p53 4:1 C) PPDBA II/p53 3:1 D) PPDBA II/p53 4:1 E) PPDBA III/p53 3:1 F) PPDBA III/p53 4:1 treated cells. The red colour represents dead cells and green colour indicates the live cells. The magnification is 60x.



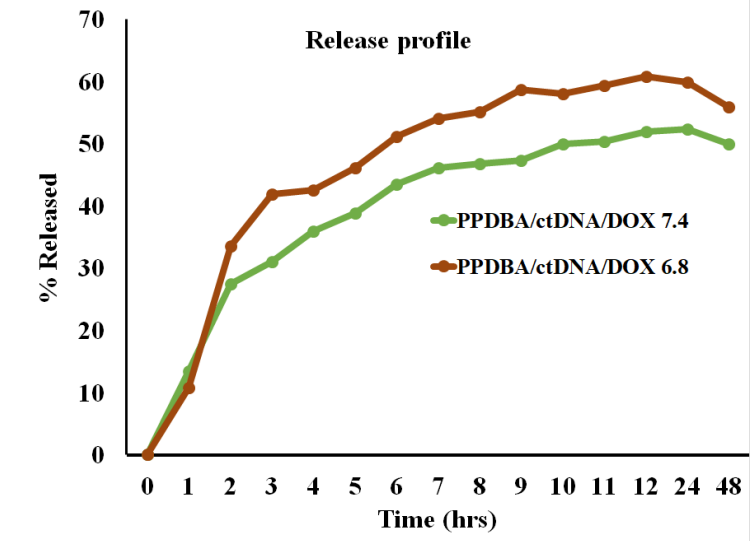
Supplementary Figure 9: Apoptosis by annexin V staining, where A) normal untreated control B) PPDBAI/p53 3:1 nanoplexes treated cells, percentage apoptosis is 85.5±5.23%, C) PPDBAI/p53 4:1 nanoplexes with percentage apoptosis is 87.8±9.62% D) PPDBAII/p53 3:1 ratio, percent apoptosis is 64.1±8.01% and E) PPDBA II/p53 4:1, percentage apoptosis is 52.9±4.22%, F) PPDBAIII/p53 3:1 and the percent apoptosis is 53.1±5.38% and G) PPDBAIII/p53 4:1, percentage apoptosis is 50.1±11.2%.



Supplementary Figure 10: Immunofluorescence analysis to establish p53 expression in C6 cells. (A) Control cell without any treatment (B) cells treated with PPDBA I/p53 nanoplex at 4:1 ratio (C) cells treated with PPDBA II/p53 nanoplex at 4:1 ratio and (D) cells treated with PPDBA III/p53 nanoplex at 4:1 ratio.

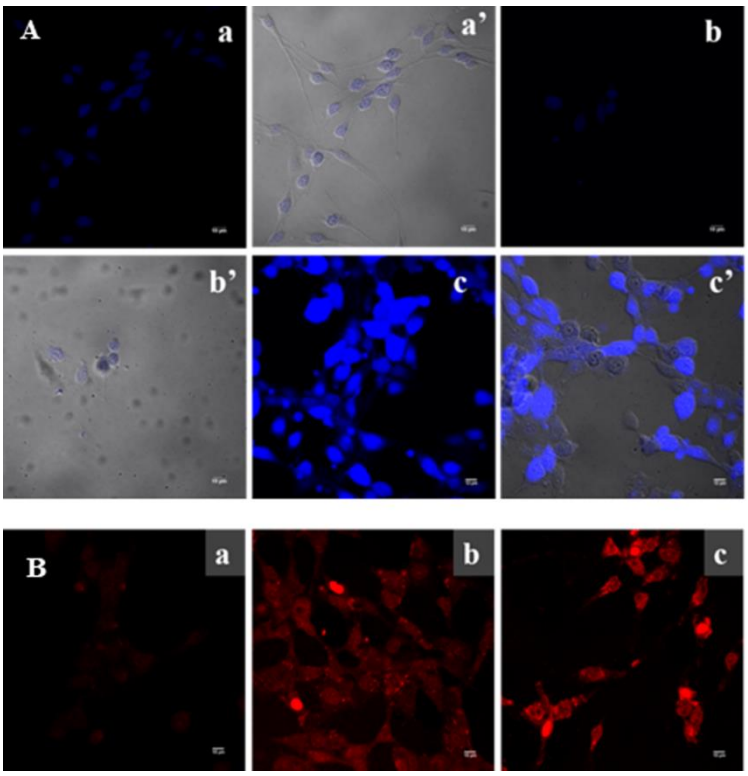
Drug Release studies

The drug DOX (5µM) was intercalated with DNA(10µg) before forming the complex with the PPDBA polymer to form the nanocomplex with the ratio 4:1. The selected nanoplex ratio of PPDBA II (4:1) along with the drug DOX (5µM) was sealed in a dialysis bag (molecular weight cutoff 3000) containing a final volume of 2mL, which was immersed in 5mL of phosphate buffer (pH 7.4, pH 6.8) in a 50cc falcon tube. At predetermined time intervals, sample was drawn periodically (200µL) and refilled with the same volume of fresh medium. Free DOX content was determined by fluorescence measurement using microplate reader (Synergy H1, USA). Percentage DOX released from the nanoplex was calculated using DOX as standard.



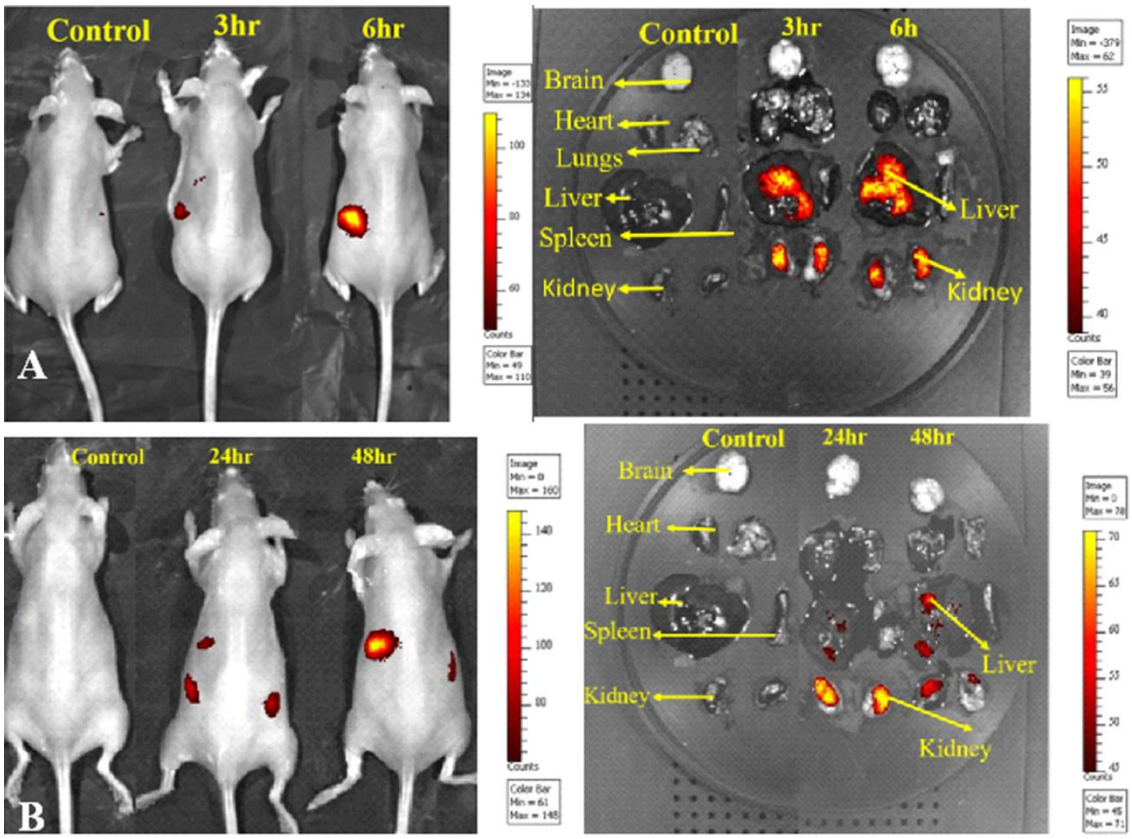
Supplementary Figure 11: Release profile of doxorubicin from the drug loaded PPDBA II nanoplex at pH 6.8 and 7.4.

3.8. Role of glutathione



Supplementary figure 12: (A) GSH level in cells, fluroscent and the corresponding bright field merged images are given (a-a') glutathione level in untreated C6 cells (b-b') cells pretreated buthionine sulfoximine followed with nanoplex treatment (c-c') cells pretreated with PPDBA II nanoplex at 4:1 ratio followed with DOX. (B) Role of GSH in DOX retention in C6 cells a) Cells pretreated with buthionine sulfoximine and PPDBA II 4:1 nanoplex followed by DOX treatment b) PPDBA nanoplex pretreated, followed by DOX treatement c) Glutathione monoester pretreated followed by PPDBA II 4:1 nanoplexes and DOX. Magnification 60x.

3.10. *In vivo* organ distribution of PPDBA nanoplexes



Supplemenatray figure 13: Organ distribution of PPDBA polyplexes A) *in vivo* imaging of BALB/c mice post injection of PPDBA nanoplexes at 3 and 6 hrs and different organs following dissection. Similarly, B) the distribution of PPDBA nanoplexes following 24 and 48hrs post injection and different organs following dissection. The normal control is the untreated mcie injected with saline only.