## Supplementary file to the manuscript

# CD-340 functionalized doxorubicin-loaded nanoparticle induces apoptosis and reduces tumor volume along with drug-related cardiotoxicity in mice

Laboni Mondal[1], Biswajit Mukherjee[1]*, Kaushik Das[2], Sanchari Bhattacharya[1], Debasmita Dutta[1], Shreyasi Chakraborty[1], Murari Mohan Pal[1], Raghuvir H Gaonkar[3], and Mita Chatterjee Debnath[3]

[1]*Department of Pharmaceutical Technology, Jadavpur University, Kolkata- 700032, India.*

[2]*Department of Biological Chemistry, Indian Association for the Cultivation of Science, Kolkata-700032, India.*

[3]*Infectious Diseases and Immunology Division, CSIR-Indian Institute of Chemical Biology, Kolkata-700032, India.*

***** Corresponding author
Dr. Biswajit Mukherjee,
Department of Pharmaceutical Technology,
Jadavpur University, Kolkata- 700032, India.
Telephone:+ 91-33-2457 2588
Fax: + 91-33-2414 6677
Email: biswajit.mukherjee@jadavpuruniversity.in, biswajit55@yahoo.com

## *Preparation of PLGA nanoparticles*

Multiple emulsion solvent evaporation method with appropriate modifications in the pH of the aqueous phase was adopted for doxorubicin nanoparticles preparation for efficient drug loading.

In this method, 2.5% (w/v) and 1.5% (w/v) of polyvinyl alcohol (PVA, MW 125,000) solutions were suspended in phosphate buffer saline and pH was adjusted to 8. They were used as aqueous phase. In 2.5 % PVA solution DOX (10mg) was dissolved. PLGA (85:15) (~100 mg) was dissolved in 1ml of dichloromethane (DCM). Variations in organic phage by using different co-surfactants such as Pluronic F-68 (0.5%w/v), Pluronic F127 (0.5%w/v), Tween 80 (14% v/v), and TPGS (0.03 % w/v) along with PVA (2.5%) were studied and the effect of these formulation variations were observed to optimize the nanoparticle formation along with higher loading of DOX. All these formulation variations have been shown in Table 1.

The organic polymeric solution was emulsified with a dropwise gradual addition of 1ml of DOX in 2.5% PVA solution to the organic phase and homogenized for 5 mins using a homogenizer (at 16000 rpm, IKA-R-104, Germany). The primary w/o emulsion was quickly added to 75 ml of 1.5% PVA solution and homogenized (16000 rpm) for 8 mins to get water/oil/water (w/o/w) type multiple emulsion. When the nanoparticles were formed, the particle suspension was kept on a bath type sonicator (TRANS-O-SONIC, Mumbai, India, 30 ± 3 kHz, in cold water) for 30 mins to break the agglomerate formed (if any) and after sonication this final emulsion was kept on a magnetic stirrer overnight for complete removal of the organic solvent. DCM was allowed to diffuse out and evaporate to get homogenous nanoparticles. Then the emulsion was centrifuged at 5000 rpm and 15000 rpm in a cold centrifuge (Hermle refrigerated centrifuge, Wehingen, Deutschland) to separate the larger and smaller particles. Particles were resuspended in Milli-Q water (Millipore Corp., Billerica, MA, USA) and allowed for three consecutive washing in cold centrifuge (16000 rpm for 10

min each) to remove the excess of PVA, if any, attached to the nanoparticle surface. After the final washing, the particles were frozen at −20°C and lyophilized (without any cryoprotectant) in a Freeze dryer (Laboratory Freeze Dryer, Instrumentation India Ltd., Kolkata, India) for 10 h to obtain a solid dry product. Blank nanoparticles (BL-NP) were prepared by following the same procedure but the drug was not added.

Antibody conjugation to the nanoparticles has been described in the main publication.

## *Characterization of DOX-NP*

**Determination of drug loading:**

The percentage of drug loading and drug loading efficiency were calculated using the following equations.

Actual drug loading (weight %) = (Amount of drug present in nanoparticles/Total weight of nanoparticles analyzed) X 100 (%)

Drug loading efficiency (%) = (Actual drug loading/Theoretical drug loading) X 100 (%)

**Particle size and zeta potential**

Lyophilized DOX-NP and DOX-Ab-NP were resuspended in a small amount (in 2 ml) of Milli-Q water which was further sonicated (for 15 min) and vortexed (for few mins) before analysis, to break any lump present in the suspension. Samples were subjected to analysis for average particle size, polydispersity indices (PDI) and zeta potential by Zetasizer Nano ZS 90 (Malvern Instruments, Malvern, UK) using Data Transfer Assistance (DTA) software (Malvern Zetasizer Limited, Malvern, UK).

**Scanning electron microscopy (SEM)**

For assessment of surface morphology, DOX-NP was fixed onto metallic stubs with a double-sided adhesive carbon tape. Samples were spread as a thin layer. Excess samples were removed and the stubs were then coated with platinum in vacuum using JEOL JFC 1600 auto fine coater (JEOL, Tokyo, Japan) at an acceleration voltage of 5 kV. All the samples were visualized under the scanning electron microscope (SEM, JEOL JSM 6700 F, JEOL, Tokyo, Japan).

**Transmission electron microscopy (TEM)**

TEM was performed with the nanoparticles to analyze the internal morphology and drug distribution. Freeze-dried nanoparticles were resuspended in Milli-Q water and dropped carefully on the carbon-coated copper grid (300#; Ted Pella Inc., CA, USA) that was further air-dried for 10 h and subjected to imaging through the TEM instrument (JEM 2100; JEOL, Tokyo, Japan).

**Atomic force microscopy (AFM)**

To analyze particle surface/three dimensional morphology and particle size of nanoparticles, AFM analysis was performed by AFM (AFM; Dimension Icon, Bruker, Karlsruhe, Germany) under ambient conditions by mode Peak Force QNM (Quantitative Nano Mechanical mapping) using silicon nitride probe having a resonance frequency 150–350 kHz and a force constant 0.4 N/m. Lyophilized nanoparticles were dispersed in Milli-Q water. After sonication and vortexing for few mins, a drop of the sample was carefully placed on a pre-cleaned glass slide that was further vacuum dried. This prepared glass slide was examined for AFM imaging.

## *Stability study*

### Accelerated stability analysis

Effect of temperature and relative humidity (RH) on the formulations (DOX-NP and DOX-Ab-NP) was examined by performing the stability study as per the International Conference on Harmonization (ICH) guidelines (ICH, 2003). The optimized nanoparticles were analyzed for stability testing for three-months with a frequency of one-month sampling. Definite amounts of DOX-NP and DOX-Ab-NP were weighed and kept in zone III at 4-8°C (in the refrigerator), 30°C, 75% RH and 40°C, 75% RH for the period of 30, 60 and 90 days. Samples were withdrawn from the stability chamber at the specified time intervals (after 30, 60 and 90 days). SEM and drug content studies were performed to examine the stability criteria for those samples.

### Hydrolytic stability study

Hydrolytic stability of DOX-Ab-NP was performed at different pH buffers (citrate buffer, pH-3, acetate buffer, pH 5, phosphate buffer, pH 7.4 and bicarbonate buffer pH 10) for 4 weeks as described by Bhattacharya *et al*, 2018. Samples were collected at a predetermined time points and weight variations were calculated according to the formula.

Weight change (%) = $[(W_0-W_t)/W_0] \times 100$

Where, $W_0$ and $W_t$ represent as the initial weight and weight at time t, respectively

## *In-vitro cellular studies*

### Assessment of cytotoxicity by MTT assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay was conducted to assess the cytotoxicity. Cytotoxicity of DOX-NP and DOX-Ab-NP were determined by using three breast cancer-specific cells (i.e. SKBR-3, MCF-7, and MDA-MB-231) that differ in their expression level of HER2 antigen on their surface. SKBR-3 expresses the protein more than MCF-7 and MDA-MB 231 does not express the same. All the three cell types were seeded at 5,000 per well density in 96-well plates. After 24 h of seeding, drug either as a solution (free-drug) or encapsulated in nanoparticles (DOX-NP and DOX-Ab-NP) at different concentrations (0.1–25 μM/ml) was added to the wells. After 24h of incubation time, 10 μl MTT (Sigma-Aldrich Co, St Louis, MO, USA) was added, and the plates were incubated at 37°C for 4h in a cell culture incubator (ESCO, US), DMSO was added in which the intracellular formazan crystals were solubilized and the color intensity was measured at 540 nm using a microplate reader (Spectromax, Japan). The antiproliferative effect of DOX, DOX-NP, and DOX-Ab-NP was calculated as a percentage of cell growth inhibition with respect to dose and compared to the respective controls.

### Cellular internalization of nanoparticles by confocal microscopy

Binding and internalization of DOX-NP and DOX-Ab-NP in HER2 overexpressing cells and HER2 negative cells were examined by confocal microscopy (TCS-SP8 confocal microscope, Leica, Germany). SKBR-3, MCF-7, and MDAMB 231 cells were seeded in pre-cleaned coverslips in the six-well plate at $5x10^4$ cells per well with 1 ml growth medium. After 24 h, the cells were treated with the pure drug, DOX-NP and DOX-Ab-NP and incubated at 37°C for 1 h and 6 h. The media was removed and cells were washed twice at respective time points with sterile PBS (pH 7.4) to remove any uninternalized fraction. The cells were then fixed using 4% *p*- formaldehyde (PFA) for 10 min. PFA was then removed and the cells were

washed with sterile PBS twice. Then the counterstaining was done for the nuclei of the cells. Finally, the coverslips were taken out of the wells and placed on pre-cleaned glass slides which were further fixed and viewed under a confocal microscope (TCS-SP8 confocal microscope, Leica, Germany).

**Caspase activation**

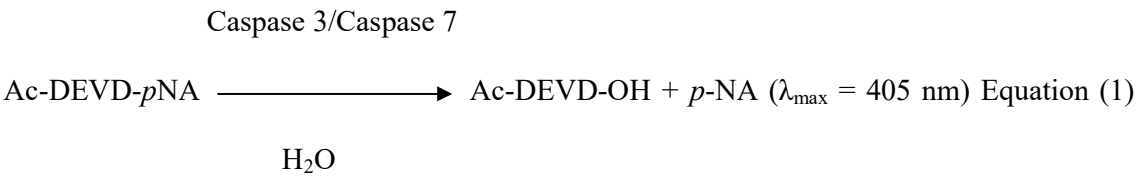
***Determination of the relative level of HER2 antigen in breast cancer cells***

The relative level of HER2 in SKBR-3, MCF-7, and MDA-MB-231 has been analyzed in a comparative approach. All the three types of cells were seeded into 35 mm culture plates and allowed to grow till confluency. The cells were lysed with Laemmli Buffer. Samples were heated at 94°C for 10 min and loaded (equal amount of sample, 20 µl each) onto SDS-PAGE gels. After completion of the run, the protein was transferred onto the PVDF membrane (run time 1 hr with 15 V). Membrane was then blocked with 5% bovine serum albumin (BSA) in Tris buffer saline (TBS). Primary antibody incubation was done overnight against HER2/β-Actin (Sigma; 1:5000) antibody. On the following day, the unbound antibody was removed by washing with TBST (TBS + Tween 20 [0.001%]; pH 7.4) followed by incubation with HRP-tagged secondary antibody (1:5000; anti-mouse; Sigma) for an hour. The unbound antibody was removed again by TBST washing and the membrane was subjected to development by the ECL method. Band intensity was measured by Image J.

***Caspase 3/7 activity assay***

Caspase 3/7 activity was measured in SKBR-3, MCF-7, and MDA-MB-231 cells after challenging the cells with DOX/ DOX-NP/DOX-Ab-NP for 24 h in a fluorimetric approach using caspase 3/7 assay kit (Abcam, Cambridge, UK).[21] Briefly, 1-5 X $10^6$ cells were suspended in 50 µl of chilled cell lysis buffer (Abcam, Cambridge, UK) and incubated for 30 min and centrifuged at 10,000g for 5 min. The supernatant was taken in a 96-well plate and incubated with 50 µl of 2X Reaction Buffer (containing 10 mM DTT, Abcam). Ac-DEVD-*p*-

NA substrate was added to the wells (200 µM final concentration, Abcam) (Equation-1) and incubated at 37°C for 90 min. Absorbance was measured at 405 nm on a microplate reader. Accordingly, the comparative graphical analysis was performed by GraphPad Prism 5.




## *In vivo studies*

**Pharmacokinetic study**

100 µl of plasma sample was precipitated with 300 µl acetonitrile containing 500 ng/ml tolbutamide (internal standard). Samples were vortex-mixed for 10 mins, centrifuged at 4000 rpm for 10 mins at 10ºC and 100 µl of supernatant was taken out from each sample and diluted with 100 µl water, mixed and loaded into LC-MS/MS (LC: Shimadzu Model 20AC, MS: AB-SCIEX, Model: API4000, Software: Analyst 1.6). Analytes were eluted using YMC Triat C18 column (30 x 2.1 mm, YMC Corp-Japan) and gradient elution of two solvents, solvent A (0.1 % formic acid in water) and solvent B (0.1% formic acid in 80:20 acetonitrile and water). Flow rate was 0.8 ml/min and total run time was 3.0 min.

**Supplementary Figures**

**Figure S1** Fourier transform infrared spectroscopy (FTIR) spectra of individual components, physical mixture, and nanoparticle formulations. PLGA: Poly lactic-*co*-glycolic acid; PVA: Polyvinyl alcohol; DOX: doxorubicin; DOX-NP: doxorubicin-loaded nanoparticles; BL-NP: Blank nanoparticles; BS3: Suberic acid bis (3-sulfo-N-hydroxy succinimide ester; DOX-BS-NP: doxorubicin-loaded BS3 linked nanoparticles.

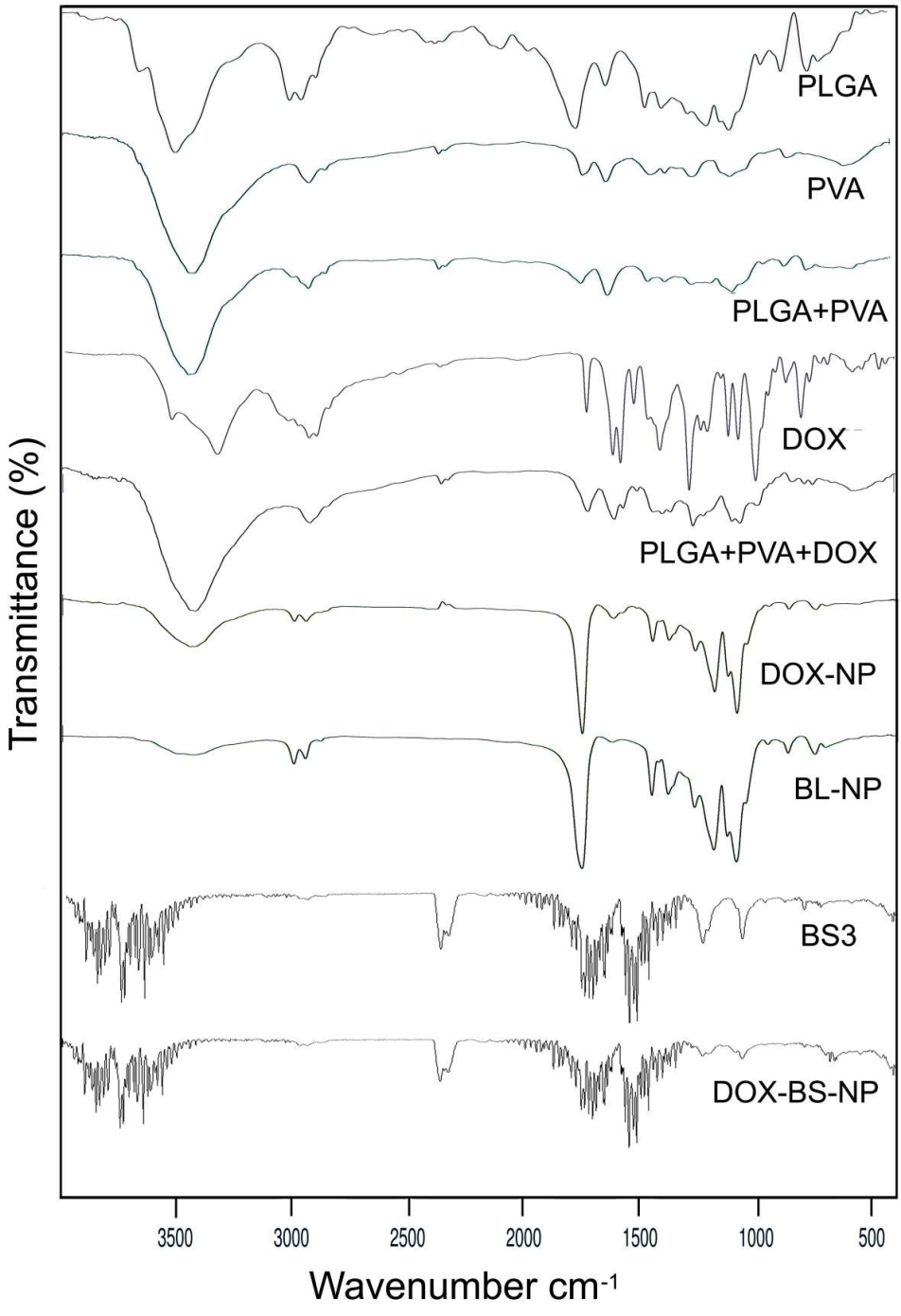


**Figure S2** Particle size distribution, Zeta potential and SEM images of formulations prepared by using surfactant PVA (polyvinyl alcohol) (A, B, C respectively), TWEEN (tween 80) (D, E, F respectively), PVA and PLU-68 (Pluronic F-68) (G, H, I respectively), PVA and PLU-127 (Pluronic F-127) (J, K, L respectively), PVA and TPGS (M, N, O). Particle size distribution, Zeta potential and SEM images of antibody-conjugated DOX nanoparticle; DOX-Ab-NP (P, Q, R respectively)

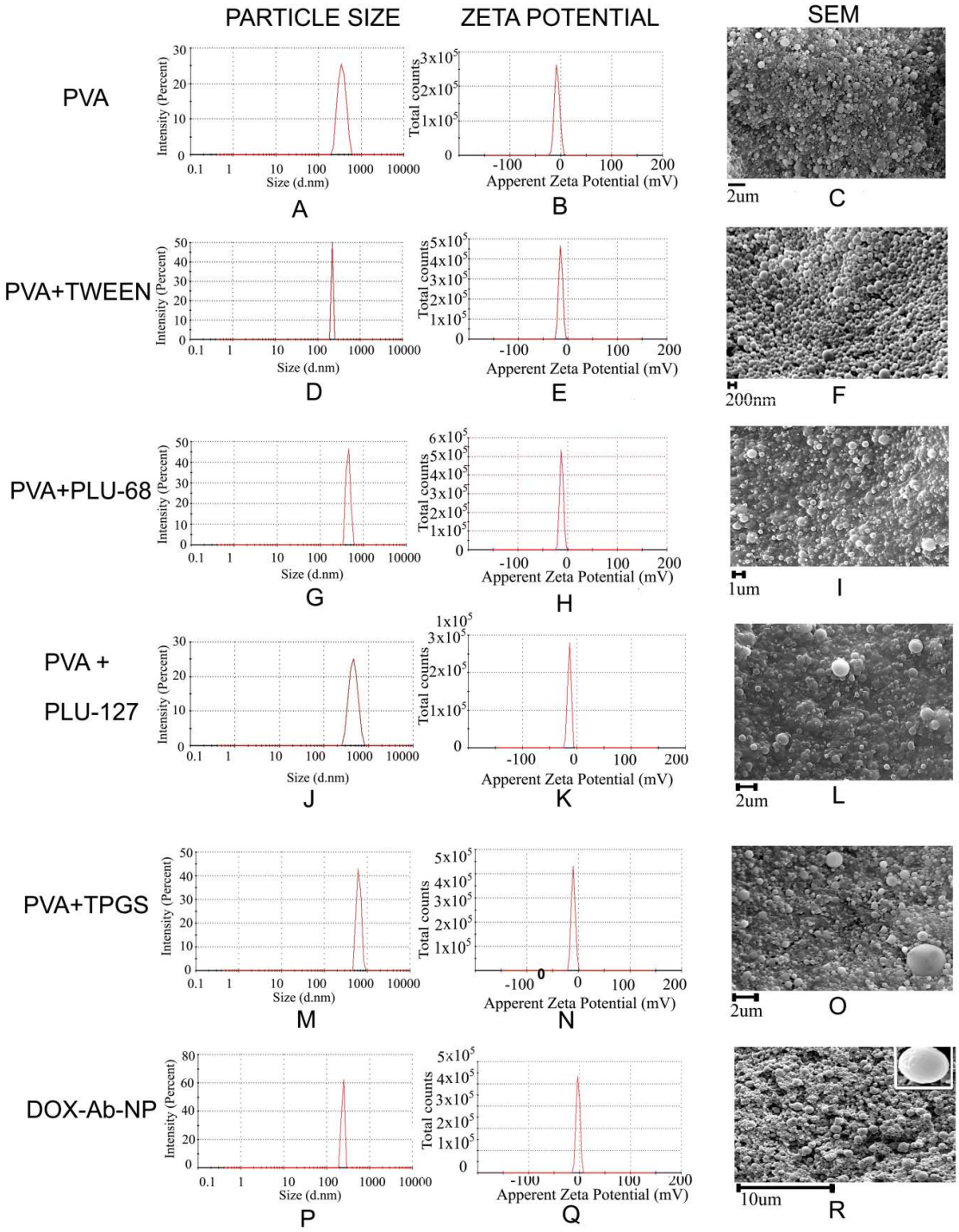


**Figure S3** SEM images of DOX-NP and DOX-Ab-NP stored A; at 30°C, 75% relative humidity, B; at 40°C, 75% relative humidity after 30 days, 60 days and 90 days and C; in refrigerator. D; Hydrolytic stability study of DOX-NP at different buffers (citrate buffer (pH 3), acetate buffer (pH 5), phosphate buffer (pH 7.4) and bicarbonate buffer (pH 10)

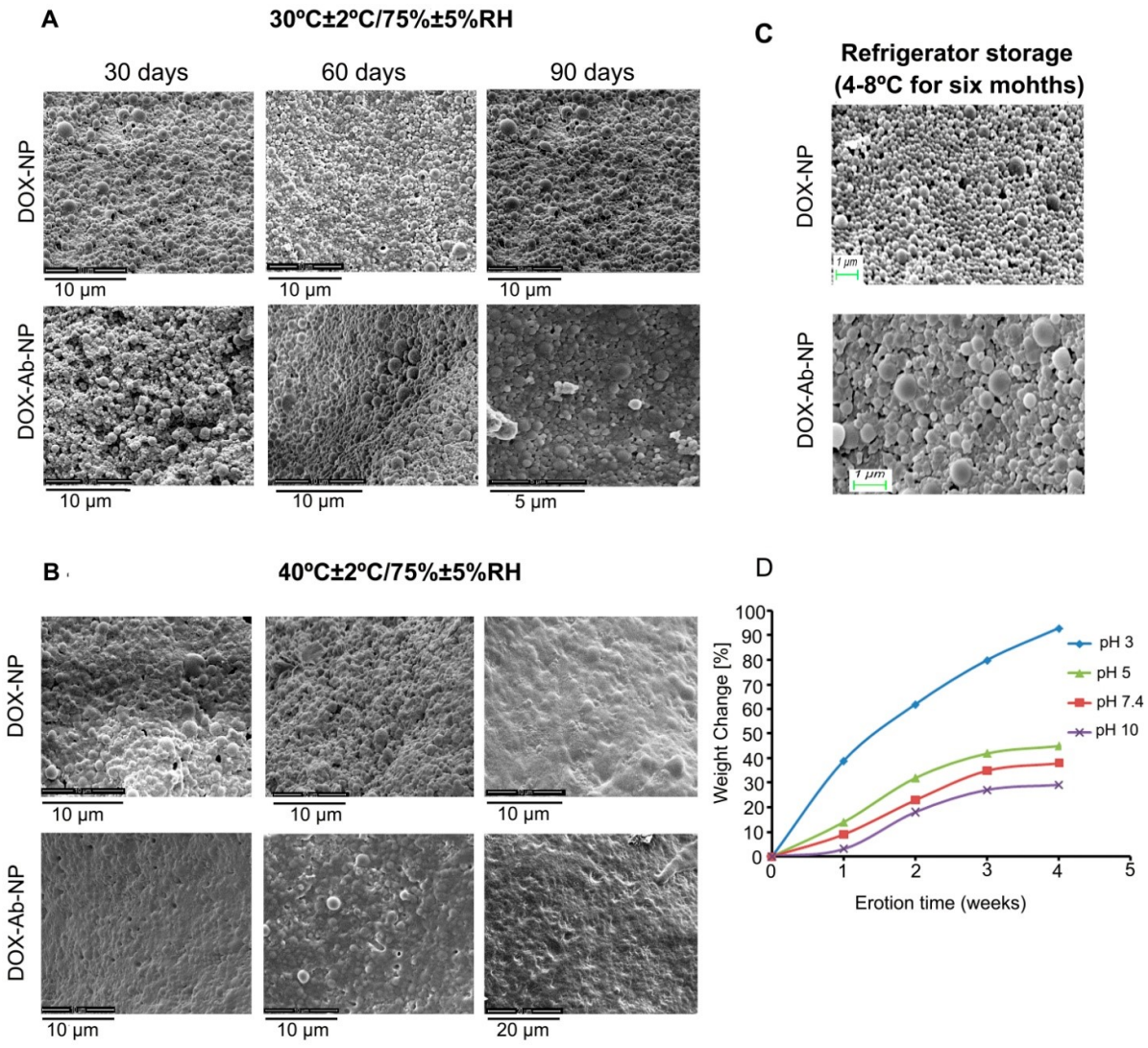


**Figure S4** Individual tumor growth curve of each mouse bearing tumor of all groups (saline treated (mouse 1, 2, 3) DOX treated (mouse 1, 2, 3) DOX-NP treated (mouse 1, 2, 3), DOX-Ab-NP treated (mouse 1, 2, 3)