Supplementary Information

Sodium bicarbonate nanoparticles modulate the tumor pH and enhance the cellular uptake of doxorubicin

Hanan Abumanhal[1,2], Lilach Koren[1], Assaf Zinger[1], Zvi Yaari[1], Nitzan Krinsky[1,3], Galoz Kaneti[1], Nitsan Dahan[4], Yael Lupu-Haber[4], Edith Suss-Toby[5], Esther Weiss-Messer[5], Michal Schlesinger-Laufer[6], Janna Shainsky-Roitman[1] and Avi Schroeder[1,*]

[1]Laboratory for Targeted Drug Delivery and Personalized Medicine Technologies, Department of Chemical Engineering, Technion – Israel Institute of Technology, Haifa 32000, Israel
[2]Russell Berrie Nanotechnology Institute, The Norman Seiden Multidisciplinary Graduate program, Technion – Israel Institute of Technology, Haifa 3200, Israel
[3]The Interdisciplinary Program for Biotechnology, Technion – Israel Institute of Technology, Haifa 32000, Israel
[4]Life Sciences and Engineering Infrastructure Center, Lorry I. Lokey Interdisciplinary Center, Technion – Israel Institute of Technology, Haifa 32000, Israel
[5]Bioimging Center, Biomedical Core Facility, Ruth and Bruce Rappaport Faculty of Medicine, Technion – Israel Institute of Technology, Haifa 32000, Israel
[6]The Pre-Clinical Research Authority Unit, Technion – Israel Institute of Technology, Haifa 32000, Israel

[*]E-mail: avids@technion.ac.il

1. pH determination of the internal aqueous phase in liposomes by pyranine

300µM pyranine solutions were prepared in 10 mM Hepes (Spectrum Chemicals Crop, Tel Aviv, Israel) buffer using DDW. The pH of the solutions is adjusted to the desired value with HCl (1M) or NaOH (1M). Determination of pH by pyranine is based on the ratio of fluorescence intensity at emission wavelength of 507 nm measured at two excitation wavelengths; 460 nm, indicating the level of the un-protonated ionized pyranine 8-hydroxy group (which is pH dependent), and 415 nm, indicating the level of total pyranine in the system irrespective of whether its 8-hydroxy group is ionized or not (the pH-independent isosbestic point). The 460/415-nm excitation ratio (emission at 510) is a direct measure of level of pyranine ionization. The liposomes internal phase pH is calculated from the 460/415-nm excitation ratio of the fluorescence intensities using the equation (1):

$$\mathrm{pH} = \mathrm{p}K_\mathrm{a} + \log\left(\frac{R - R_\mathrm{a}}{R_\mathrm{b} - R}\right)$$

Where R is the F460/F415 ratio, pKa is the apparent pKa of the fluorescent probe. R_a and R_b are the fluorescence intensity ratios of the protonated and un-protonated forms of the probe, respectively. A calibration curve was made using pyranine solutions in various pH values which was fitted to the values obtained by Avnir at el[32] using solver,XLaM in the Microsoft excel program.

The values of pK_a, R_a, and R_b are obtained using the following equation (2):

$$R = R_\mathrm{a} + \frac{R_\mathrm{b} - R_\mathrm{a}}{1 + \exp[(\mathrm{p}K_\mathrm{a} - \mathrm{pH}) \cdot \ln 10]}$$

2. lung and lymph metastasis in mice

At the end of the *in vivo* efficacy experiment, mice were scanned using In-Vivo-Imaging-System (IVIS) for the detection of lung and lymph node metastasis in all mice groups. Metastasis were observed in control and free dox treated groups only (Figure 1). Tumors were covered and mice were scanned for the detection of metastasis by luminesce signal in the lungs 10 minutes after luciferin (Perkin Elmer) intra peritoneal (IP) injection. After sacrificing the mice lymph nodes were extracted and scanned to ensure that the signal observed is related to the lymph nodes.

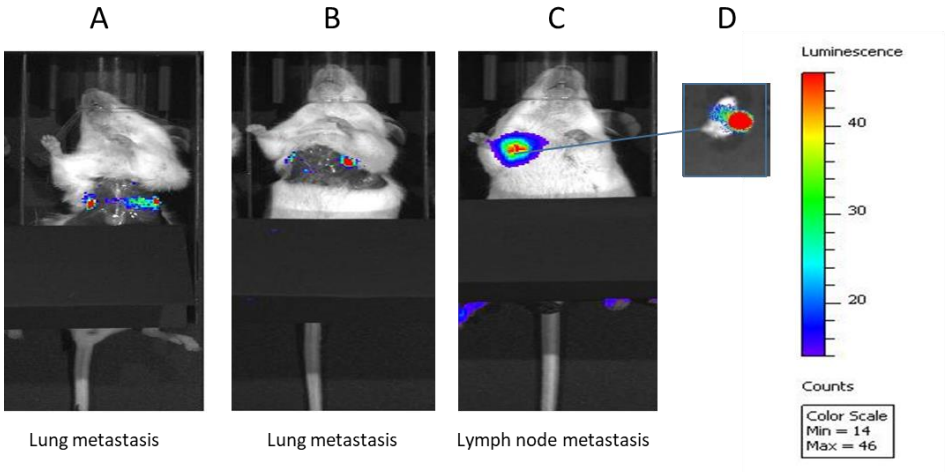

Figure S1: mice were scanned for lung and lymph node metastasis at the end of the experiment, (A-B) lung metastasis in control and free dox treated mice, (C) lymph node metastasis in control mouse, (D) lymph node metastasis after extraction.

3. *In vitro*- doxorubicin activity in different pH values.

The pH difference obtained in mice between untreated (7.13) and treated (7.38) groups was 0.25. Doxorubicin activity was examined in-vitro in these two conditions. By this modification in pH values, 25% increase in doxorubicin activity was obtained **(P value<0.001)** Figure S2(B). compared to in-vivo data that was obtained, in groups treated with combined therapy, tumors' size and weight was 35% and 30% smaller than in groups treated with drug only.

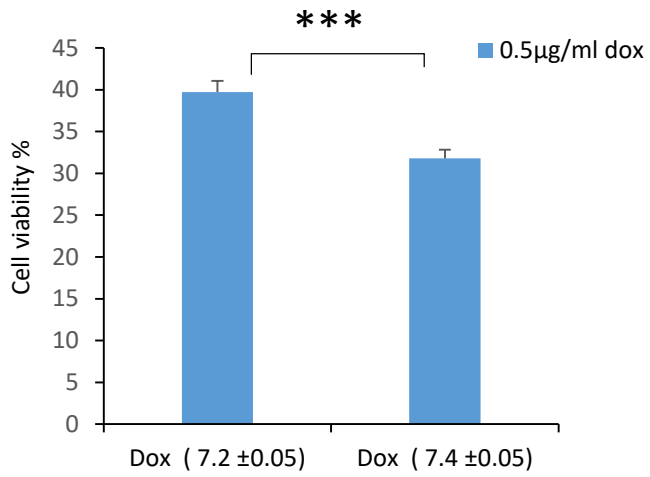

Figure S2: Cell viability 24 hours after doxorubicin treatment in two different media pH conditions (7.2±0.05 and 7.4±0.05)

4. Sodium calibration curve by ICP

Concentration of each sample (liposomes, Free sodium bicarbonate solution) was measured using a standard calibration curve at 0.01, 0.05, 0.1, 0.5, 1.0, 5.0,10,20 and 50 ppm (Figure S3. Sodium intensity was measured at 588.995nm and quantified using Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES, 5100-Agilent).

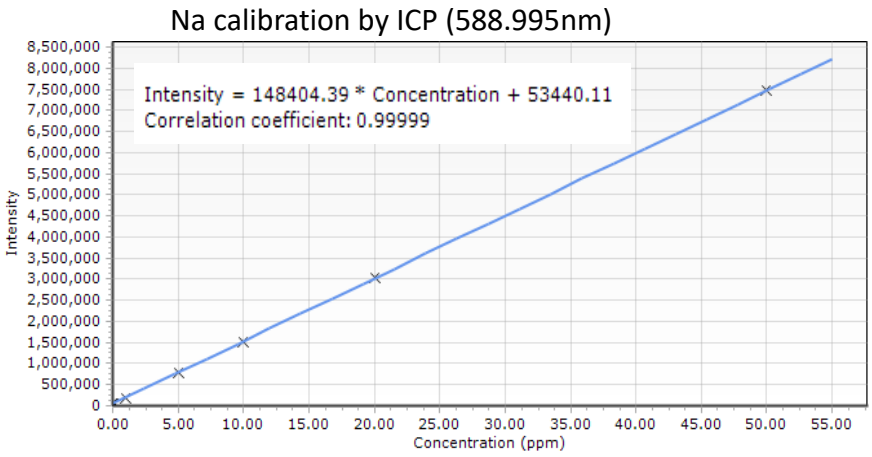

Figure S3: Sodium(Na) calibration curve used to quantify sodium bicarbonate in liposomes.

5. Combination between BES buffer (pKa 7.1) and doxorubicin

To validate the results obtained after combination with HEPES buffer, additional ionic buffer –BES with pka around 7.1(Sigma Aldrich, Rehovot, Israel) was examined on cells after combination with doxorubicin. Similar results of HEPES buffer was also obtained in this case Figure S4, no effect on doxorubicin activity compared to the drug alone. pH of the media was also measured and no significant differences were observed to media without buffer addition.

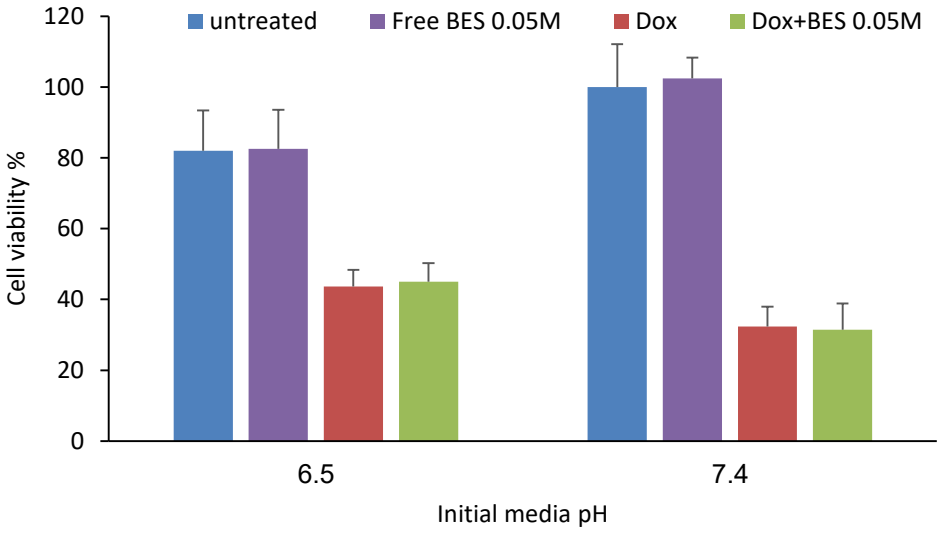

Figure S4: Doxorubicin activity after combination with BES buffer (50mM).

6. Breast cancer cells viability in different media pH

4T1 breast cancer cells viability was measured 24h after cells incubation in media with different pH points (pH 6- pH 8), it was noticed that decreasing the pH affects cancer cells viability in cell culture. Significant decrease was observed at media pH 6. Figure S5.

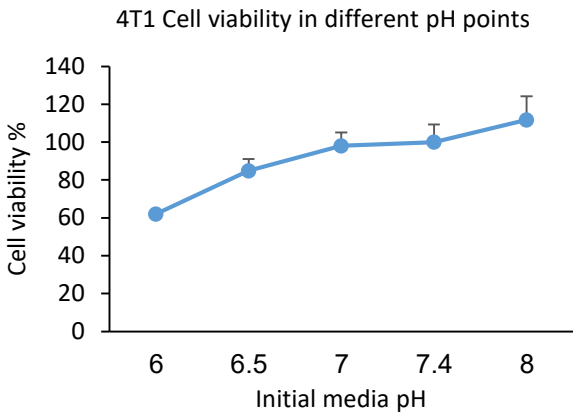

Figure S5: 4T1 breast cancer cells viability in different media pH points.

7. Doxorubicin cellular uptake and distribution

Using GE InCell Analyzer. Doxorubicin fluorescent intensity was quantified in the cells cytoplasm and nuclei. The mean fluorescent intensity in the cytoplasm was similar for each treated group to the values obtained in the nuclei as shown in FigureS6.

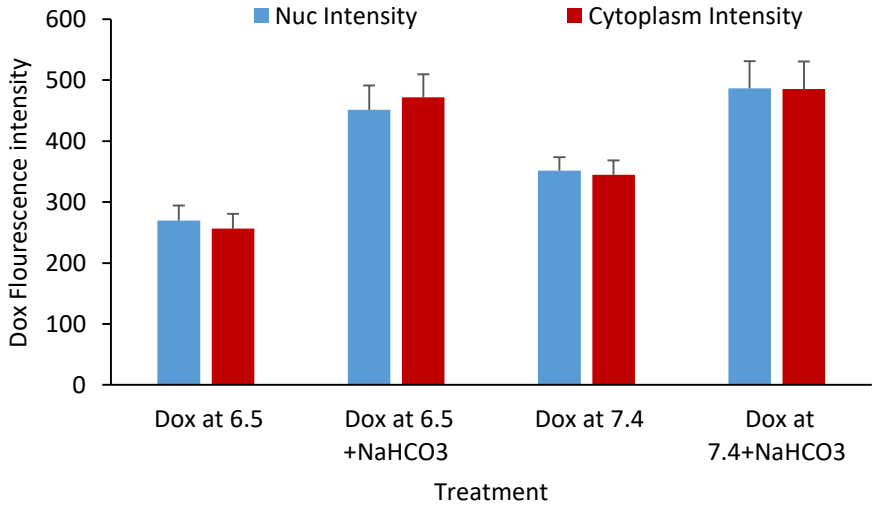

Figure S6: doxorubicin mean fluorescence intensity as distributed in the cytoplasm and nuclei for the different treated groups (media pH, with or without bicarbonate)