SUPPORTING INFORMATION

Integration of Multitargeted Polymer-Based Contrast Agents with Photoacoustic Computed Tomography: An Imaging Technique to Visualize Breast Cancer Intratumor Heterogeneity

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1. Experimental Calculations

1.1. *In vitro* cytotoxicity assessment of MTMPPPCAs

The *in vitro* cell toxicity of MTMPPPCAs was assessed using the CytoTox-Fluor™ assay. The standard equation (1) for determining the % cell viability was based on the fluorescence values of the respective samples

$$\% \text{ cell viability} = \left(\frac{F_{sample} - F_{cm}}{F_{cell} - F_{cm}}\right) x\ 100 \quad \text{(Eq. 1)}$$

where, F_{sample} = Fluorescence of sample

F_{cm} = Fluorescence of complete medium

F_{cell} = Fluorescence of control cells

1.2. Encapsulation efficiency (EE) of Squaraine (SQ650) and ICG

The EE for both SQ650 and ICG was calculated using the following equation (2).

$$EE_{SQ650} = \left(\frac{\text{Mass of ICG in MTMPPPCAs}}{\text{Mass of ICG used in the formulation}}\right) \times 100$$

$$EE_{ICG} = \left(\frac{\text{Mass of SQ650 in MTMPPPCAs}}{\text{Mass of SQ650 used in the formulation}}\right) \times 100$$

2. Results

2.1 Zeta Potential measurements

The overall surface charge of the synthesized MTMPPPCAs was determined using Malvern Zetasizer ZS (Nano-ZS90) instrument by dispersing the materials (1mg/mL) in 1×PBS (0.15 M, pH 7.4) at 25°C. The average zeta potentials of both targeted and untargeted NPs were -21.4 ± 1.7 mV and -23.03 ± 1.33 mV, respectively (Figure S1).

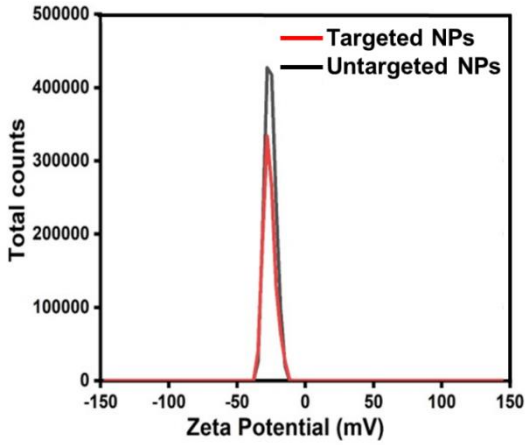

Figure S1: Zeta Potential for targeted and untargeted NPs suspended in 1×PBS at 25°C.

2.2 NP size stability studies

In order to evaluate the size stability of the synthesized MTMPPPCAs at physiological conditions, the particle stability study was performed by suspending the particles in 1×PBS (0.15 M, pH 7.4) at 25 °C for a period of 21 days (Figure S2). The results revealed the nanoparticles were stable in the suspended solution over the total duration of 21 days with no signs of aggregation or precipitation. These results were encouraging for future use in animal studies.

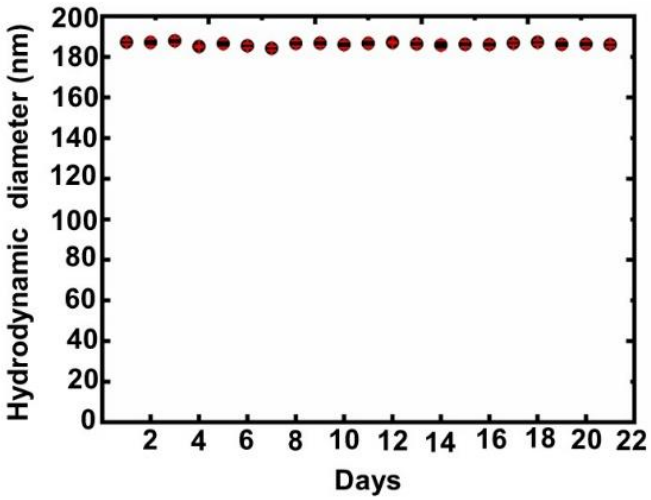

Figure S2: Hydrodynamic diameter of lyophilized MTMPPPCAs measured during 21 consecutive days. The NPs were suspended in 1×PBS at the concentration of 1 mg/mL at 25 °C. The error bars were calculated based on the average of three independent measurements.

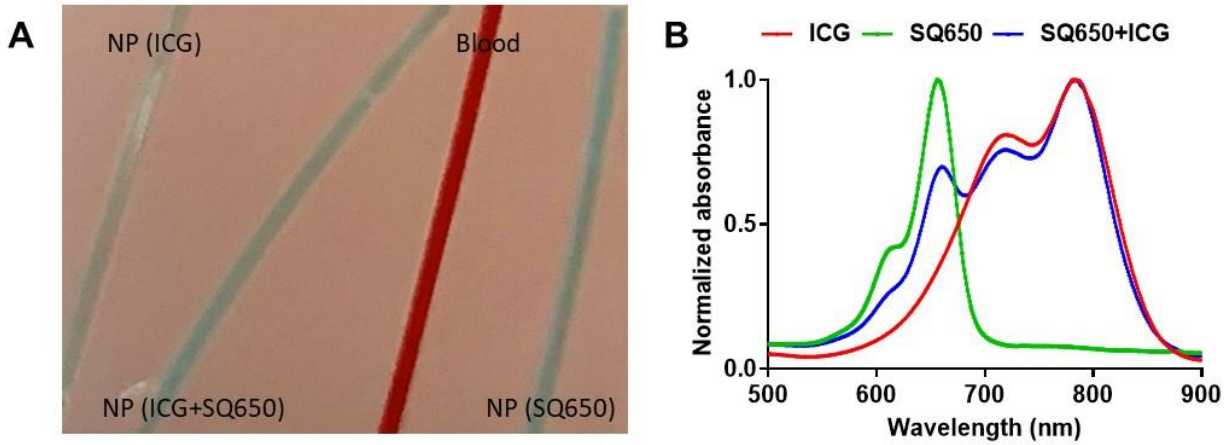

Figure S3 A. Photo of the tubes containing nanoparticles with ICG, nanoparticles SQ650, MTMPPPCAs and blood. B. Optical absorption spectra of ICG, SQ650 and MTMPPPCAs.

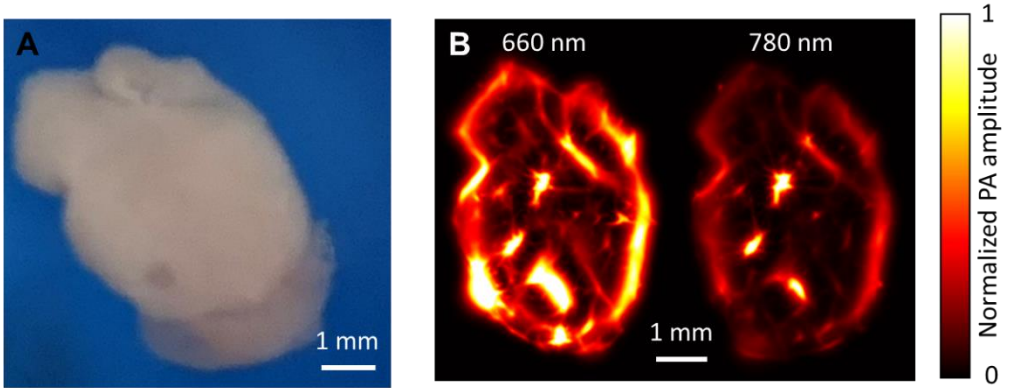

Figure S4. A. Excise breast tumor. B. Photoacoustic studies performed at 660 and 780 nm wavelengths.

2.3 Pathology studies

Pathology studies were conducted on *ex vivo* tumors excised from targeted and untargeted T-47D bearing mice to corroborate the location of ER+ and PR+ identified and visualized by fluorescence imaging. IHC and H&E studies were performed by Reveal Bioscience, a leading company in tissue pathology located in San Diego, California.

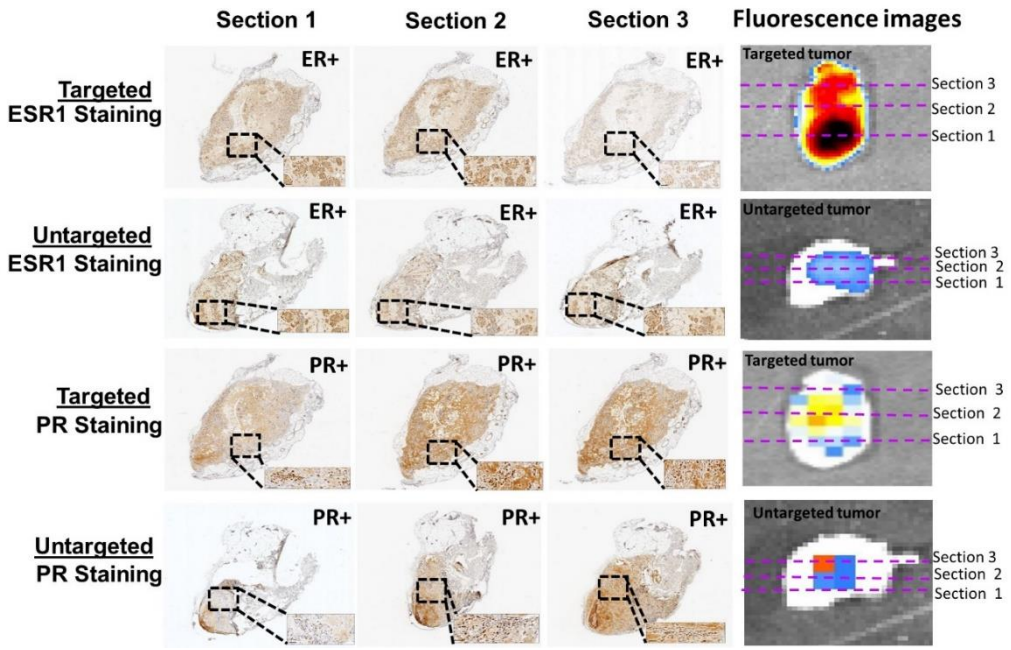

Figure S5. Immunohistochemistry (IHC) analysis of the entire excised tumors during the necropsy studies. The entire untargeted or untargeted tumors were sliced into ten different sections. Only three sections were processed for IHC and H&E staining. The sections that were analyzed are indicated in Figure S3. Magnified ER+ or PR+ immunostaining images are placed as inserts on the right side, bottom position of each picture of the tumor section. The fluorescence images depict the tumor excised from T-47D-bearing mice of the targeted and untargeted groups.

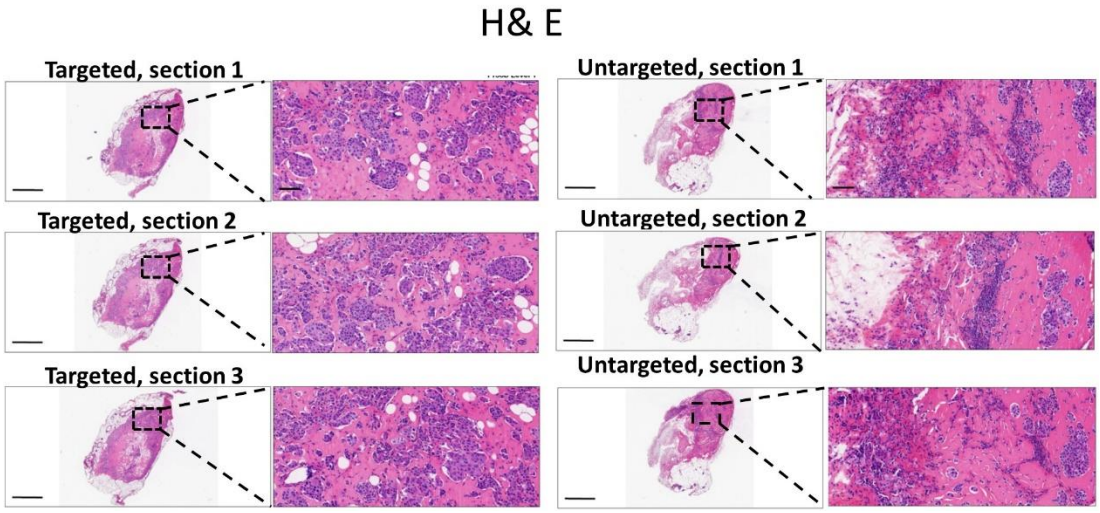

Figure S6. Representative images of Hematoxylin and eosin (H&E) staining on FFPE targeted and untargeted tumors (entire sections). The scale bar represents 900 µm.

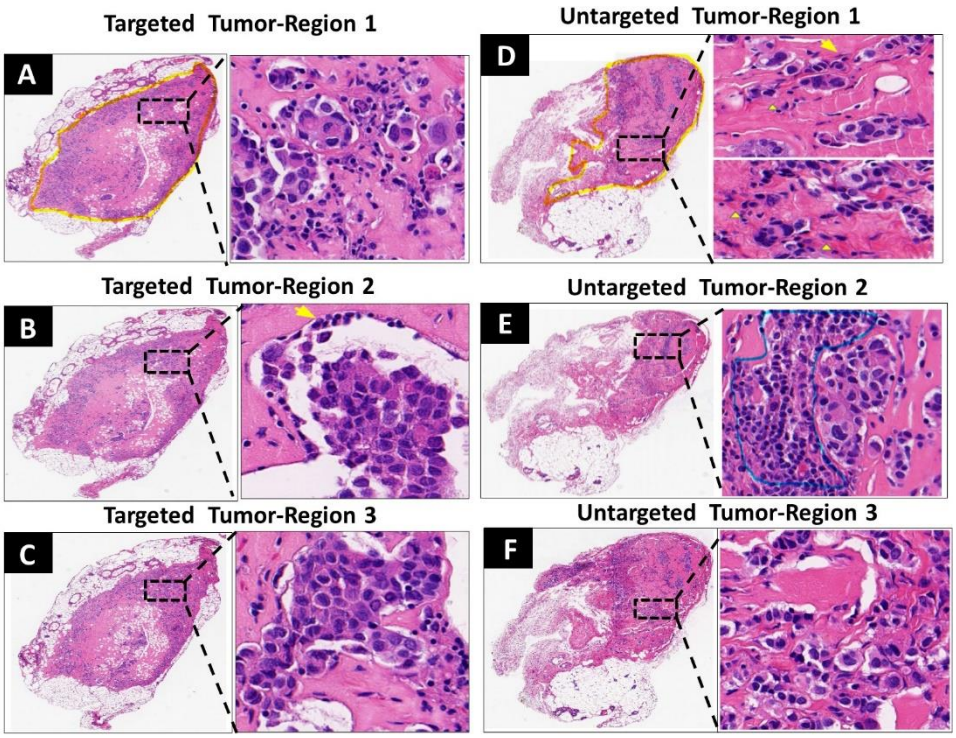

Figure S7. Pathology findings. A. Targeted Tumor-region 1 has stroma and tumor cells. Many of the tumor cell clusters have associated leukocytes (lymphocytes, some neutrophils), infiltrating the clusters with possible individual cell necrosis. The arrow **B-C. Targeted tumor sections Regions 2-3.** Both sections have more areas of tumor cell necrosis and leukocytes. Leukocytes are prominent around tumor cells. The yellow arrow points out the presence of leukocytes around tumor cells. **D. Untargeted tumor section 1.** In this region, tumor cells are in rows and clusters. Tumor is outlined in yellow. Arrow points to a row of tumor cells. Arrowheads are leukocytes. **E. Untargeted tumor section 2.** Tumor cells with large numbers of adjacent lymphocytes. This section has large areas of leukocytes, mostly lymphocytes (blue outlined). **F. Untargeted tumor section 3.** This section has more tumor cells and more lymphocytes and other leukocytes in the area of tumor cells. The tumor cells are arranged in rows and clusters.

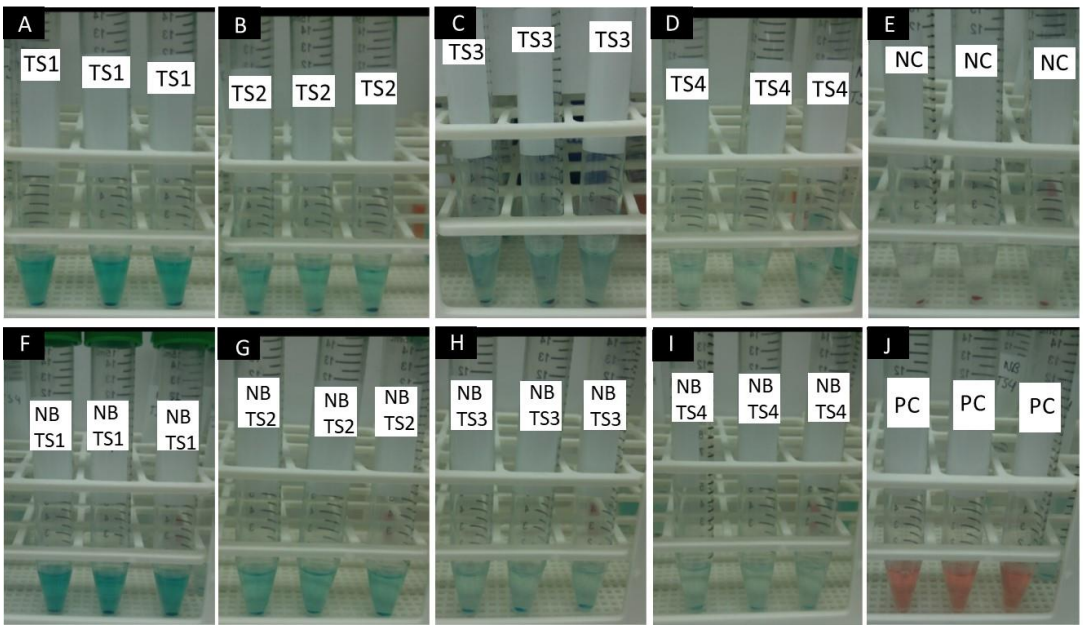

Figure S8. Hemolytic assay. Testing, negative and control samples after incubation for 3 ¼ hrs at 37 °C followed by centrifugation at 800xg for 15 min. One hundred microliters of the sample's supernatant was used to measure the level of hemoglobin in the human plasma sample and determine the hemolysis percent. The experiment was conducted in triplicate and test samples were incubated in the absence and presence of diluted total blood hemoglobin The samples incubated with blood were labeled as follows: Test Sample 1 (TS1)(MTMPPPPCAs 12 mg/ml), Test Sample 2 (TS2)(MTMPPPPCAs 6 mg/ml), Test Sample 3 (TS3)(MTMPPPPCAs 3 mg/ml), Test Sample 4 (TS4) (MTMPPPPCAs 1.5 mg/ml), Negative Control (NC) (Total blood hemoglobin diluted), Positive Control (PC) (Triton X-100 (10 mg/ml). The samples incubated in the absent of blood were labelled as: NB TS1, NB TS2, NB TS3 and NB TS4. Total blood hemoglobin diluted and TrixtonX-100 served as the negative and positive control in this experiment. No hemolysis was observed in the negative control. However, 95 % of hemolysis was observed in samples incubated with Triton X-100. A dark a pellet was observed in the bottom of the test tubes where the test samples were placed. The pellet formed by red blood cells indicates that the test samples are slightly hemolytic.