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ARTICLE

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

Lei Li, Deepanjali Patil, Greg Petruncio, Kathleen K. Harnden, Jisha V. Somasekharan, Mikell Paige, Lihong V. Wang, and Carolina Salvador-Morales*

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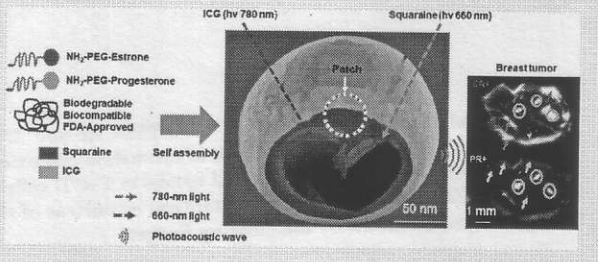
ABSTRACT: **One of the primary challenges in breast cancer diagnosis and treatment is intratumor heterogeneity (ITH), *i.e.*, the coexistence of different genetically and epigenetically distinct malignant cells within the same tumor. Thus, the identification of ITH is critical for designing better treatments and hence to increase patient survival rates. Herein, we report a noninvasive hybrid imaging technology that integrates multitargeted and multiplexed patchy polymeric photoacoustic contrast agents (MTMPPPCAs) with single-impulse panoramic photoacoustic computed tomography (SIP-PACT). The target specificity ability of MTMPPPCAs to distinguish estrogen and progesterone receptor-positive breast tumors was demonstrated through both fluorescence and photoacoustic measurements and validated by tissue pathology analysis. This work provides the proof-of-concept of the MTMPPPCAs/SIP-PACT system to identify ITH in nonmetastatic tumors, with both high molecular specificity and real-time detection capability.**



KEYWORDS: *breast cancer, intratumor heterogeneity, multitargeted, single-impulse panoramic photoacoustic computed tomography, patchy particles*

Breast cancer accounts for the second leading cause of cancer death globally (11.6%) with an alarming mortality rate of 6.6% as per 2018 global cancer statistics.[1,2] One of the primary challenges in breast cancer diagnosis and treatment is intratumor heterogeneity (ITH). ITH is the coexistence of different genetically and epigenetically distinct malignant cells within the same tumor.[3−5] It is responsible for 30% of cancer-related deaths worldwide in women, as it is closely associated with cancer progression, resistance to therapy, and recurrences.[6] The current treatment options for breast cancer are guided by critical factors, including molecular subtypes,[7,8] locations, metastatic stages, previous treatments, and other parameters.[3,4,6] Luminal A (LA), luminal B (LB), human epidermal growth factor receptor 2 (HER2)-enriched cells, and triple-negative breast cancer (TNBC) are the main subtypes treated in clinical settings.[9] LA and LB are present in 70% of breast cancer tumors,[10,11] whereas HER2 and TNBC are found in 15−30% and 15% of breast cancers, respectively.[12−14] TNBC is the most aggressive subtype and has a worse prognosis than the other three.[15]

Currently, positron emission tomography/computed tomography (PET/CT) is the only imaging modality that is capable of rendering ITH information based on metabolic activity,[16−19] cell proliferation, and estrogen receptor (ER) status in breast cancer tumors.[20−22] However, the main challenges of using PET/CT include the high cost, long-term exposure to radiation, and the side effects of radiotracers. Furthermore,

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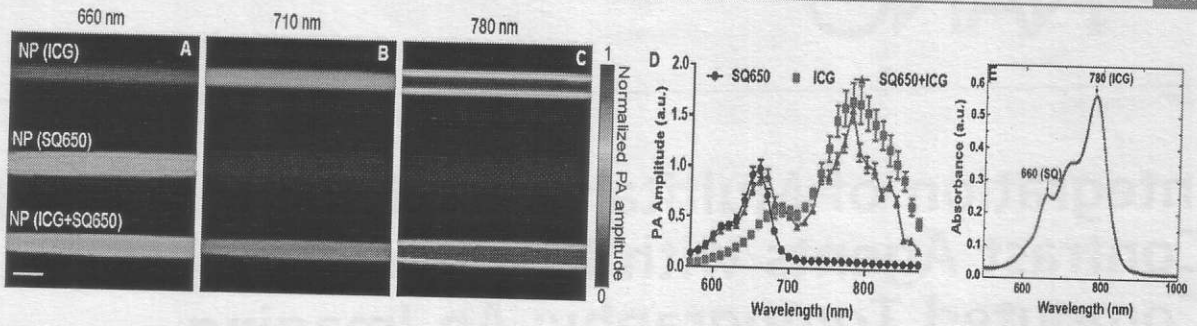

Figure 6. PA spectra of MTMPPPCAs. (A–C) PACT images of nanoparticles with ICG, nanoparticles with SQ650, and MTMPPPCAs in silicone tubes at wavelengths of 660, 710, and 780 nm, respectively. Scale bar, 1 mm. (D) PA spectra of MTMPPPCAs with ICG (green line), nanoparticles with SQ650 (blue line), and the MTMPPPCAs (red line). (E) Absorption spectrum of MTMPPPCAs.

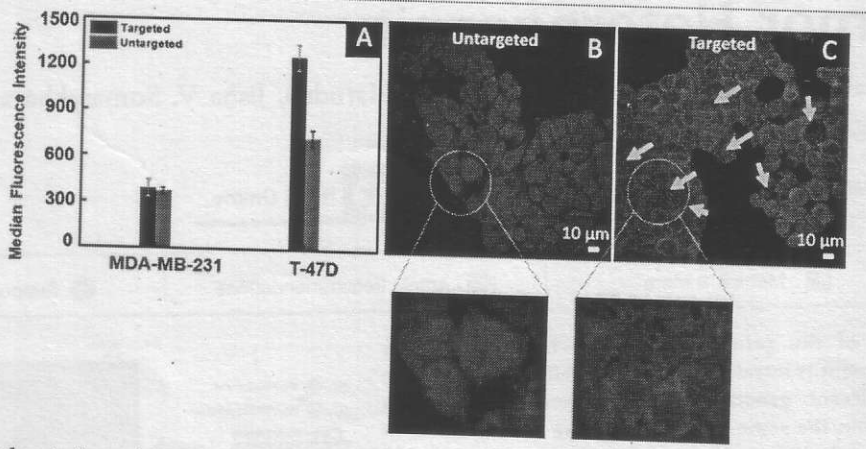

Figure 7. Cellular uptake studies of MTMPPPCAs and UTMPPPCAs in T-47D and MDA-MB-231 breast cancer cells. (A) The MTMPPPCAs uptake doubled after 24 h of incubation compared with UTMPPPCAs. Error bar, standard deviation (n = 3). (B, C) Confocal microscopy images of MTMPPPCAs cell uptake in T-47D cells after 24 h of incubation with the section insets. The yellow arrows point out the presence of MTMPPPCAs in the cytoplasm or nucleus of the cell. There is a higher cell uptake in the targeted sample compared to the untargeted one, as shown by the close-up images.

technique to assess the molecular specificity of NH_2-PEG-estrone and NH_2-PEG-progesterone (Figure 4). The SPR responses of the analyte (*i.e.*, NH_2-PEG-estrone) were plotted at different concentrations (Figure 4A–C). The SPR results show that the sharp shape of the sensorgrams in the association stage of the interaction indicates the binding of NH_2-PEG-estrone to full-length recombinant human estrogen alpha receptor (FLER-α) at different micromolar concentrations (Figure 4A). Fitting the sensorgram data to an appropriate kinetic binding model allowed the calculation of the kinetic disassociation (K_D) rate constant. Assuming 1:1 binding affinities and fitting the dose response plot revealed that the K_D value of FLER-α was 1.5 μM. This value indicates an excellent binding affinity between the analyte (*i.e.*, NH_2-PEG-estrone) and the ligand (*i.e.*, FLER-α). The fact that NH_2-PEG-estrone shows little binding or negligible binding to estrogen-related receptor beta (ERR-β) (Figure 4B) or HER2 (negative controls) (Figure 4C), respectively, demonstrates the preferential binding of NH_2-PEG-estrone to FLER-α. With respect to the molecular interaction between NH_2-PEG-progesterone and the PR, we observed specific binding at different concentrations, as shown in Figure 4 D–F. Following the same rationale described above, we found that the K_D for progesterone was 10 μM (Figure 4D–F). The binding affinity response was higher for NH_2-PEG-progesterone compared to ERR-β (Figure 4E) and FLER-α (Figure 4F) at different concentrations, which suggests its molecular specificity with the progesterone receptor.

Physicochemical Characterization of MTMPPPCAs. A self-assembled construct with the targeting moieties on the PLGA shell surface and the PA reporter dyes forming the inner lining of the MTMPPPCAs' core was successfully achieved. The mean diameters of MTMPPPCAs and untargeted multiplexed patchy polymeric PA contrast agents (UTMPPPCAs) were 169.3 ± 5.6 nm and 180.1 ± 2.4 nm respectively, showing a narrow size distribution (Figure 5A). The average zeta potentials of both targeted and untargeted nanoparticles were −21.4 ± 1.7 mV and −23.03 ± 1.33 mV, correspondingly (Figure S1). Furthermore, the mean particle size of MTMPPPCAs suspended in 1× PBS did not change within 21 days, exhibiting excellent long-term size stability (Figure S2). Two-dimensional stochastic optical reconstruction microscopy (STORM) images clearly showed the patchy nature of the MTMPPPCAs' surface (Figure 5B–D). The donut-like shape confirmed the MTMPPPCAs' hollow core structure (Figure 5B–D). The presence of NH_2-PEG-estrone-Alexa 647 and NH_2-PEG-progesterone-Cy3B is observed in both the nanoparticle's surface and patch, but it is more prominent in the latter, as shown in Figures 5B and C, respectively. The single-labeled experiments served as control samples for the two-color experiment. The dual STORM imaging experiment is presented in Figure 5D. In this picture, the presence of NH_2-PEG-estrone-Alexa-647/NH_2-PEG-progesterone is indicated

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