pH-sensitive and biodegradable charge-transfer nanocomplex for second near-infrared photoacoustic tumor imaging

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pH-sensitive and biodegradable organic charge-transfer nanocomplex acts as new class of photoacoustic probe for tumor acidic microenvironment imaging in the second near-infrared window.

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ABSTRACT

The emerging photoacoustic imaging (PAI), especially in the NIR window, offers reliable sensing for deep-penetration and high-resolution closing to clinical requirements. However, the contrast agents that are capable of specifically responding to the intricate biological environments, more importantly, indicate promising biodegradability and biocompatibility are still limited. Herein, we introduce a new class of pH-sensitive organic PA contrast agent working in the second near-infrared window (NIR-II, 960 - 1700 nm), which is derived from the self-assembled charge-transfer nanocomplex (CTN) by 3, 3', 5, 5'-tetramethylbenzidine (TMB) and its dication structure (TMB⁺⁺). Such unique NIR-II-responsive CTN can specifically respond to pH change in the physiological range, allows noninvasive and sensitive visualization of the tumor acidic microenvironment (e.g. at pH 5) in mice with higher signal-to-noise ratio (SNR). Moreover, this organic CTN demonstrates the biodegradable feature in physiological condition (e.g. at pH 7.4), which provides the great biosafety without the concern of nanoparticles accumulation *in vivo*. These results clearly show the potential of TMB/TMB⁺⁺-based CTN as a promising pH-activatable and biodegradable molecular probe for specific tumor photoacoustic imaging in the NIR-II region.

KEYWORDS

Charge-transfer nanocomplex, Second near-infrared window, Photoacoustic imaging, pH-sensitive, Biodegradable, Tumor acidic microenvironment

1 Introduction

As a hybrid technique emerging in biomedical analysis, photoacoustic imaging (PAI) has been well recognized to provide reliable sensing at penetration depth and spatial resolution close to practical

requirements, which may overcome the limitations occurred in conventional optical imaging (e.g. low penetration, light diffusion etc) and ultrasonic imaging (e.g. low contrast), therefore enable its great

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possibility for clinical translation.[1-5] Despite such great potential, the conventional PAI for numerous biomedical applications based on intrinsic or extrinsic contrasts are still flawed due to the overwhelming light scattering of biological tissues and serious nonspecific PA signal readout.[1, 6] Remarkable efforts to technically improve the quality of PAI have been made in the engineering of more sensitive photoacoustic setups as well as the development of more efficient contrast agents (CAs).[7-9] Of which the development of the commercially available Q-switched Nd:YAG pulse-laser (1064 nm) and new second NIR window (NIR-II, 960 - 1700 nm) light-absorbing PA contrast materials such as silver nanoplates, copper sulfide nanoparticles, phosphorus phthalocyanine and semiconducting polymer nanoplateforms, have allowed for the photoacoustic sensing in the NIR-II window. So far, NIR-II PA imaging has received worthy attention in deep tissue, brain vasculature and tumor detection due to its centimeter penetration and higher signal-to-noise ratio (SNR).[10-17] However, these NIR-II PA contrast agents for *in vivo* applications still faced challenges that the endogenous NIR-II absorption and non-targeted contrast extravasation would dramatically affect the imaging sensitivity and specificity. In addition, the biological incompatibility and long-term accumulation of these applied PA contrast agents further raised considerable concerns for their biosafety in practice.[8, 10] Although some NIR-II contrast agents were designed for targeted PA imaging by conjugation of targeting-moieties (e.g. antibodies), intelligent and environment-responsive NIR-II PA sensing still cannot be fully achieved yet.[11, 18-20] Therefore, the development of sensitive and biodegradable NIR-II PA imaging probes that can specifically respond to external stimulation for more reliable molecular sensing of various biological and pathological implications, and more importantly, can potentially realize successful clinical translations will be highly desired.[21, 22]

Charge-transfer complex (CTC) materials are assemblies of charge-donating (D) and charge-accepting (A) molecules that have been intensely studied for several decades due to their wide range of

properties, such as the anisotropic conductivity, photoconductivity, paramagnetism, ferroelectricity and ambipolar transport.[23-29] Extensive investigations toward the intramolecular charge-transfer (ICT) have shown that the excitation energy of the transitional state could contribute to the formation of typical charge-transfer (CT) bands in the visible and NIR regions in the electromagnetic spectrum, which therefore results in an intense light harvesting, and such kind of CTC materials are expected to have multi-field applications in solar cells, ferromagnets, superconductors, sensors and devices.[30-32] In particular, the colorimetric assay based on the formation of charge-transfer nanocomplex (CTN) by TMB (3, 3', 5, 5'-tetramethylbenzidine) and cationic TMB (TMB⁺⁺) has been well applied for free radicals detection, blood test and the enzyme-linked immunosorbent assay (ELISA) in preclinical research and clinical practice.[33-35] Preliminary studies also disclosed that the TMB oxidation product (diiminium cation, TMB⁺⁺) can accept the electron (or charge) of TMB and produce charge-transfer nanocomplex, which is responsible for the blue colored CT bands with maximum absorbance at 370 and 652 nm, respectively. Further oxidation of the cationic TMB⁺⁺ or moderate changes in pH yields a yellow diimine product (maximum absorbance at 450 nm), resulting in the degradation of CTN (Figure 1a).[33, 36, 37]

Interestingly, we recently observed that this CTN shows one more broadband NIR absorption peak ranging from 750 nm to 1200 nm on the optical spectrum (Figure 1b).[37] Such strong absorption band could be greatly enhanced in the acidic environment (pH 5) compared to the neutral or slight basic condition (pH $\geq$ 7), which provides the feasibility to enable CTN as a promising pH-sensitive NIR-II absorber for biological applications. Currently, extensive studies have demonstrated the acidic microenvironment within growing tumor, mostly attributed to the glycolytic metabolism, deficient blood perfusion and hypoxia, which plays significant rules in tumor cells proliferation, metastasis and chemoresistance.[38-40] Accordingly, focusing on low tumor pH has been widely regarded as an effective

strategy for tumor diagnostic and therapeutic purposes.[41-43] In terms of the unique pH-responsive feature of CTN, in this study, the broadband NIR-absorbing and environment-sensitive CTN could be a promising pH-activatable PA probe for specific and sensitive sensing of tumor acidic microenvironment. Considering the biomedical applicability of this TMB/TMB⁺ based CTN, the potential cytotoxicity and clearance will be evaluated in combination with the biosafety studies of TMB.[44-46]

2 Experimental section

2.1 Preparation, diameter optimization and characterization of CTN

3, 3', 5, 5'-tetramethylbenzidine (TMB) and silver nitrate were purchased from Sigma-Aldrich. Unless otherwise stated, all commercial available reagents for synthesis and assay were used without further purification. The CTN nanofibers were prepared and characterized according to a previously reported method with slight modifications.[47] Typically, 1 mL of AgNO₃ aqueous solutions with different concentrations (1 - 20 mM) were added into 2 mL of TMB (10 mM) ethanol solution respectively under vigorous stirring at room temperature. The color of the reaction mixture started to change from green to dark purple after the reaction had proceeded for 2 hours. The top purple CTN nanofibers were collected by centrifugation and then washed two times with DI water for the following experiments, the bottom gray precipitated Ag nanoplates were removed. UV-Vis-NIR absorption spectra were measured using a Perkin Elmer Lambda 950 spectrometer. The material morphology of CTN was investigated by a JEOL JSM-6700F field emission SEM. A Bruker D8 X-ray diffraction (XRD) system, a Perkin-Elmer model PHI 5600 X-ray Photoelectron Spectroscopy (XPS) system and a Bruker Vertex 80v vacuum FTIR spectrometer were used to study the structure.

2.2 Cell culture and cytotoxicity test

Mouse embryo fibroblast cells NIH/3T3 and murine macrophage cells RAW264.7 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% calf

bovine serum (CBS) containing penicillin (100 I. U. mL⁻¹) and streptomycin (100 µg mL⁻¹) and maintained in an atmosphere containing 5% CO₂ and 95% humidified air at 37 °C. For the cytotoxicity test of CTN, both of two cell lines were seeded in 96-well plates (8000 cells/well in 100 µL) and incubated for 24 h. Different concentrations of CTN were added to the cell culture medium, respectively, then the cells were incubated for 24 h. The medium was then removed and washed with PBS. Fresh cell culture medium with TOX8 was then added to wells and plates were incubated for 3 h. The fluorescence at 590 nm was measured by a Tecan's Infinite M200 microplate reader with 560 nm excitation. Cell viability was expressed by the ratio of the fluorescence of cells incubated with CTN to that of control cells.

2.3 In vitro PA signal test of CTN

The laser source used is from OPO tunable (700 - 1200 nm) wavelength and fiber coupling. The output light from the fiber is then collimated and focused by condenser lens on the sample with a spot size of ~ 500 µm diameter. Meanwhile, a beam splitter and photodiode are utilized to monitor the laser intensity variation. During the experiment, the MPE were well kept within the ANSI standard. An ultrasound transducer (V303SU, Olympus) with 2 MHz central frequency is placed close to the sample to detect the PA resonance signals. The transducer is immersed in water tank for optimum optical and acoustic coupling. The PA resonance signals are firstly amplified by a low-noise amplifier (5662, Olympus) with 54 dB gain, and then recorded by an oscilloscope (LeCroy) with 500 MS/s sampling rate. Peak-to-peak voltage of the PA signals were then normalized with the laser energy at each wavelength and plotted against the wavelength to generate the PA spectrum of CTN.

For the concentration-dependent 1064 nm PA testing of CTN, a Q-switched Nd:YAG pump laser was used as the laser source of PA instrumentation. CTN solutions or fresh mouse blood were placed inside a low-density polyethylene (LDPE) tube with an inner diameter (i.d.) of 0.59 mm and outer diameter (o.d.) of 0.78 mm. LDPE tube containing samples and a single-element ultrasound transducer (UST, V323-SU/2.25 MHz, 13 mm active area, and 70% nominal

bandwidth, Panametrics) were both immersed in water for coupling of PA signals to UST. The LDPE tube was irradiated with laser pulses at wavelength 1064 nm. PA signals were collected by UST, amplified with a gain of 50 dB and subsequently band pass filtered (1-10 MHz) by a pulse/receiver unit (Olympus-NDT, 5072PR). Finally, the output signals from the pulser/receiver unit were digitized with a data acquisition card (DAQ, GaGe, compuscope 4227) operated at 25 MHz and the acquired signals were stored in the computer. PA signals of with different concentrations of CTN at 1064 nm were measured and plotted.

2.4 Deep tissue NIR-II PA imaging of CTN

CTN spots of three different concentrations were embedded in an agar gel phantom (0.1, 0.5 and 1 mg/mL, respectively). Chicken breast tissues were stacked layer by layer above agar gel phantom to obtain various imaging depths. A home-made photoacoustic tomography system was used for deep tissue and tumor imaging. The system uses a high energy Q-switched 1064 nm Nd:YAG laser (Continuum, Surelite Ex) as a PA excitation source. The laser can deliver 5 ns pulses at 10 Hz repetition rate. The near-infrared 1064 nm beam was delivered to the ultrasound scanner through right angle prisms. The narrow laser beam was expanded and homogenized using an optical diffused (OD). The ultrasound transducer (UST) were immersed in water for coupling the PA signal from the sample to the transducer. The PA signals generated by the tumor were received by a single-element transducer (V323-SU/2.25 MHz, Olympus NDT). The UST has 13 mm active area and 70% nominal bandwidth. The UST was continuously rotated in a circular geometry by a stepper motor (SM) to acquire PA signals from all angles of the tissue and tumor. The signals collected were subsequently amplified, band-pass filtered (1-10MHz) by an ultrasound amplifier and filter unit (A/F), (Olympus NDT, 5072PR) and then digitized. The signals were recorded by the PC with a 25 Ms/s data acquisition (DAQ) card from GaGe (compuscope 4227). Finally, the acquired PA signals were used to reconstruct the tumor image using a delay-and-sum back projection algorithm.

2.5 *In vivo* NIR-II PA imaging of tumor

In vivo PA imaging of tumor-bearing mouse was acquired by the homemade 1064 nm PA imaging system mentioned above. Animal experiments were performed in accordance with the approved guidelines and regulations, and were approved by the institutional Animal Care and Use committee of Nanyang Technological University, Singapore (Animal Protocol Number ARF-SBS/NIE-A0263). Subcutaneous injection of two million 4T1 xenograft tumor cells suspended in 0.2 mL of DMEM (10% FBS, 1% antibiotics) into one side of the backs of female NCr nude mouse was performed. After two weeks, the tumor-bearing mice were used for 1064 nm PA imaging. A layer of ultrasonic coupling gel was applied on the mouse's skin at tumor area. The mouse was subsequently anesthetized and placed into PA setup with its tumor protruding above to the water tank through a hole that had been sealed by transparent polyethylene film. The tumor-bearing mouse and the control mouse without tumor were monitored and imaged before and after intratumoral or subcutaneous injection of CTN (1 mg/mL, 200 μ L per mouse, $n = 3$) under 1064 nm laser with the power intensity of 5.5 mJ/cm². At different post injection times, PA images were acquired and reconstructed for the tumor and the normal tissue.

3 Results and discussion

3.1 Preparation and characterization of CTN

Here, the charge-transfer nanocomplex material used was prepared as shown in Figure 1a.[47] The diameter optimization was achieved through alteration of the relative concentration of the substrates (TMB and AgNO₃), wherein the intermolecular charge-transfer degree of the π -conjugated push-pull system was controllable. As shown in Figure 1c and Figure S-1 in the Electronic Supplementary Material (ESM), the diameters of the prepared CTN were varied from 418.4 $\pm$ 15.4 to 79.6 $\pm$ 2.2 nm and the optimal sized-CTN (< 100 nm) was chosen as the candidate for further investigation. The XRD analysis (Figure S-2a in the ESM) indicated the peaks at 10.5, 17.4 and the broad peak can be ascribed to the formation of organic CTN by charge-transfer process between TMB and TMB⁺. The XPS studies in Figure S-2b show the

occurrence of carbon, oxygen and nitrogen signals. The peaks at 401.9 and 399.8 eV in the N1s high-

computational studies using Gaussian 09 under HF/6-31+G(d,p) scheme.[48] The simulated molecular

resolution XPS spectrum (Figure 1d) can be assigned to the protonated imine TMB⁺⁺ and the neutral amine TMB of CTN. Differences in the FTIR spectra (Figure S-2c in the ESM) between TMB and CTN could also be direct evidence for the formation of CTN containing the typical phenyl ring of TMB and C=N bond of quinonediimine like cation (TMB⁺⁺). To further validate the charge-transfer of CTN, we carried out

orbitals in Figure 1e showed that the energy level of LUMO of TMB⁺⁺ (-7.0341 eV) was lower than that of TMB with an energy-gap of 0.0468 eV, which demonstrated the possibility of the CT process from TMB to TMB⁺⁺. All these results could be deduced the structure and charge-transfer mechanism of as-prepared CTN.

Figure 1 Preparation and characterization of CTN. (a) Schematic illustration of CTN formation, degradation and in vivo NIR-II PA imaging. (b) UV-Vis-NIR absorption spectrum of CTN in different pH conditions. (c) SEM images and diameter distribution of CTN. (d) High resolution XPS spectrum of CTN (N1s). (e) Frontier molecular orbitals and relative energy level of TMB and TMB⁺⁺.

3.2 In vitro photoacoustic performance of CTN

As shown in Figure 2a, the optical spectrum of the CTN aqueous solution exhibited the broadband absorption ranging from NIR-I to NIR-II window, which can be attributed to the CT bands of

TMB/TMB⁺⁺-based CTN. Most importantly, this pH-sensitive CTN was highly stable under an acidic environment (PBS, pH 5.0) for 72 h, while it was gradually degraded in normal physiological conditions (pH 7.4) (Figure 1b and 2b). Such observed

pH-responsive phenomenon of CTN could be likely attributed to the different charge-transfer efficiency between TMB and TMB⁺⁺ when upon their exposure to varying pH conditions.[49, 50] In addition, the potential changes of CTN structure under different pH environment were monitored by SEM after incubation with different buffers after 24 hours (Figure 2b), the images clearly showed the CTN nanofibers were almost degraded in pH 7.4 PBS buffer whereas no morphological changes were observed in the acidic condition. Therefore, the pH-sensitivity and biodegradability of such CTN could not only act as valuable feature for specifically responding to the critical parameter, pH, in physiological and pathological processes (e.g. tumor acidity), but also as a property that will alleviate the health risk caused by nanoparticles enrichment *in vivo*. [51, 52] Moreover, CTN was incubated with different cell lines, HEK293 (human embryonic kidney cells) and RAW264.7 (mouse macrophage cells) for 24 h. No statistical difference in the cell viability was found with either of the two cell lines even at concentrations of up to 500 $\mu\text{g/mL}$ (Figure 2c), which indicated the good biosafety

for potential biological application. We further evaluated the feasibility of CTN as a NIR-II PA contrast agent. Considering the lower native absorption from the tissue components in NIR-II region, as a proof of concept, we mainly monitored the PA signal of CTN with a 1064 nm laser excitation. The photoacoustic spectra in an aqueous solution was collected and normalized by a tunable pulse-laser illumination from 700 to 1200 nm (Figure 2d), which suggested that CTN is suitable for NIR-II PA imaging due to the excellent photon-caustic converting efficacy.[10] In addition, to reasonably optimize the CTN usage amount for efficient PA imaging contrast at 1064 nm, we determined through comparison with the PA signal of fresh mouse blood and found that the critical concentration of CTN was 0.1 mg/mL (Figure 2e). Importantly, the linear correlation between the concentration of CTN and PA intensity at 1064 nm was observed in Figure 2f with $R^2 = 0.993$, indicating the applicability for the potential of PAI quantification. These results suggest that CTN could act as an attractive contrast candidate for NIR-II PA bioimaging and sensing purpose.

Figure 2 *In vitro* studies of CTN. (a) UV-Vis-NIR absorption spectrum of CTN (0.05 mg/mL) in different PBS buffers (pH 5 and pH 7.4). (b) Sensitivity evaluation of CTN in different PBS buffers within 72 hours, scale bar, 1 μm . (c) Cell viability of NIH/3T3 and RAW264.7 cells after 24 h incubation with CTN at different concentrations. (d) Normalized PA spectra of CTN and PBS. (e) PA signal of CTN (0.1

mg/mL) and fresh mouse blood at 1064 nm. (f) Plot of the PA amplitude at 1064 nm as a function of CTN concentration.

3.3 Deep tissue photoacoustic imaging

To validate the availability of CTN for *in vivo* application, we firstly tested the penetrating ability of 1064 nm PAI with a tissue mimicking phantom made of agar gel embedding CTN (Figure 3a), and the chicken breast muscles were covered to achieve

noise ratio (SNR) of the reconstructed images gradually decreased along with the increased depths of the chicken breast tissues (Figure 3c and Figure S-4 in the ESM). It is noteworthy that the PA penetration of 0.1 mg/mL CTN was able to reach ~3 cm tissue depth while the signal of agar gel objects with higher

different thicknesses (Figure S-3 in the ESM). The PA images were obtained by increasing tissue depths from 0 to 5 cm. As shown in Figure 3b, all the agar gel cylinders with different concentrations (0.1, 0.5 and 1 mg/mL) of CTN were clearly visualized, indicating the promising PA contrast of CTN and the superior feature of NIR-II imaging. However, the signal-to-

concentrations (CTN, 0.5, 1 mg/mL) remained detectable even up to 5 cm tissue depth, which could recognize the advantages of NIR-II PAI for deep penetration and high-contrast in the particle bioimaging trials.

Figure 3 Deep tissue NIR-II PA imaging. (a) Agar gel phantom with different concentrations of CTN (0.1, 0.5 and 1 mg/mL) and schematic illustration for deep tissue penetration of NIR-II PAI. (b) Two-dimensional PA images at different depths (0 to 5 cm) with stacked chicken breast muscle blocks, laser energy density is 20 mJ/cm². (c) Plot of SNR with different CTN concentrations at 1064 nm as a function of the tissue depth.

3.4 *In vivo* photoacoustic imaging of tumor

To further examine the imaging capability of CTN for tumor sensing *in vivo*, PA scanning of 4T1 tumor-bearing mouse was conducted on a homemade PA setup under 1064 nm pulsed laser excitation (Figure S-5 in the ESM).[53] Then 200 μ L of CTN (1 mg/mL) was intratumorally injected into the tumor-bearing mice, and the control mice were treated with the same amount of CTN by subcutaneous injection. As shown in Figure 4, prior to the injection of CTN, only PA signals of the background noise were observed in both tumor-bearing and control mice, which should be derived from the tissues' absorption in NIR-II region. 5 min after CTN injection, obvious PA signals

appeared in the tumor area and normal tissue were observed, and no significant difference was found at this stage. Then the PA amplitudes in the tumor were markedly enhanced over time, which should be attributed to the CTN response under the acidic tumor microenvironment. In contrast, the mean PA intensity at the focused region of the control mice was gradually weakened mainly due to the degradation of CTN under normal physiological condition. At 4 hours post injection, the extremely significant difference ($p < 0.001$) of PA intensity was observed between tumor and normal tissue. After 24 hours, there were no detectable PA signals in control mice whereas the tumor-bearing mice still showed

significant PA signals, which was corresponded with the pH-sensitivity investigations of CTN *in vitro* (Figure 2b).

To confirm whether such significant PA signal enhancement was specifically attributed to the acidic tumor microenvironment, we have incubated CTN (50 $\mu\text{g/mL}$) with both 4T1 tumor cells and NIH-3T3 fibroblast cells for 4 hours, then we tested the PA signals of the cell lysates. As shown in Figure S-6 in the ESM, there was no obvious difference between co-incubation groups and the group with CTN alone. This result indicated that the lysosomes and endosomes could not significantly enhance the CTN PA signals within both tumor and normal cells. Moreover, we have performed the PA sensing in tumor models after 4T1 cells seeding for different stages (Figure S-7 in the ESM). From the results, PA signals were gradually increased in both tumor models after CTN injection. However, there were significant differences of the PA intensity between 7 days tumor (small size) and 14 days tumor (large size) after 2 hours. These results demonstrated the capability of CTN being potential NIR-II PA probe for both specific tumor acidic microenvironment sensing and different stages tumor detection.

4 Conclusions

In conclusion, we have successfully developed a novel,

organic self-assembled, pH-sensitive and biodegradable photoacoustic probe, CTN, for NIR-II PA tumor imaging in mice. The results of these studies demonstrated that CTN is suitable for *in vivo* NIR-II PA imaging with good contrast and deep penetration up to 5 cm due to the strong CT band in the NIR-II region where the tissue background is minimal; Such self-assembled CTN is pH-responsive and the PA signal can be significantly enhanced in acidic conditions compared to in neutral conditions, which allows specific sensing of the tumor acidic microenvironment. More important, the biodegradable feature of CTN in physiological condition (pH 7.4) and the preliminary cell viability evaluation indicated negligible biosafety concern. This is particularly vital for *in vivo* imaging applications. Furthermore, refinements of this CTN to improve the sensitivity or specific targeting, as well as functional diversification will allow for better biocompatibility and utility of *in vivo*. Overall, this TMB/TMB⁺⁺-based organic CTN can serve not only a new class of NIR-II PA contrast agent but also as an pH-activatable probe for tumor imaging in mice. Given the many merits of CTN, we believe that such kind of NIR-II light absorbing charge-transfer nanocomplexes hold great potentials for further advancing PA imaging in biology and medicine.

Figure 4 *In vivo* NIR-II PA imaging of tumor. (a) Representative PA images of the 4T1 tumor bearing mice and normal mice before and postinjection of CTN. (b) Normalized mean PA amplitudes as a function of time at 1064 nm. Energy density, 5.5 mJ/cm². Statistically significant difference in PA amplitude (a.u.) between tumor and normal tissue (*p < 0.05, **p < 0.01, ***p < 0.001, n = 3).

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