A Self-Assembled Albumin-Based Nanoprobe for In Vivo Ratiometric Photoacoustic pH Imaging

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The characteristics of tumor microenvironment such as high interstitial fluid pressure, hypoxia, as well as low extracellular pH, are closely related to a lot of prognostic factors controlling the tumor growth and metastasis.[1] In particular, the acidic tumor pH would not only increase the potential of tumor migration and invasion,[2] but also obstruct the activity of harbor p53 and p-glycoprotein, leading to the multidrug resistance (MDR) in chemotherapy.[3] Therefore, it is important to develop biocompatible nanoprobes to noninvasively visualize the tumor microenvironment pH for better tumor diagnosis and prognosis, so as to predict the potential of cancer metastasis and MDR, as well as to design the most appropriate therapeutic strategy for each patient to realize personalized medicine.

In order to detect the tumor pH, microelectrodes are inserted into the tumor for pH measurement. Such an invasive method, although is accurate, can only measure the pH at one location each time and is not able to provide the pH mapping of the whole tumor.[4] Recently fluorescent probes such as small fluorescent organic molecules, nanoparticles, and fluorescent proteins have been developed for in vivo pH measurements.[4] However, the qualitative measurement of pH using fluorescence imaging may also be affected by many effects such as autofluorescence background, limited light penetration depth, as well as possible quenching of fluorescence in the complicated biological issues.[5] It is thus important and urgently needed to develop an accurate and easy-to-operate method for real-time in vivo noninvasive pH imaging.

Different from fluorescence imaging, photoacoustic (PA) imaging as a newly emerged imaging modality, which relies on absorption of light and emission of ultrasound signals, shows greatly improved tissue penetration depth and better in vivo spatial resolution compared to traditional optical imaging techniques.[7] Although the use of PA imaging for pH detection has been proposed in several recent proof-of-concept studies,[8] a safe nanoprobe that can quantitatively detect the pH inside tumor microenvironments by PA imaging upon systemic administration remains to be developed to our best knowledge.

In this work, a pH-responsive albumin-based nanoprobe is fabricated for in vivo ratiometric photoacoustic pH imaging. It is found that two types of near-infrared (NIR) dyes, benzo[a]phenoxazine (BPOx) and IR825, are able induce self-assembly of human serum albumin (HSA), to form albumin–dye nanocomplexes, which after chemical cross-linking exhibit greatly enhanced stability. With IR825, whose absorbance and fluorescence are inert to pH change, serving as the internal reference, the pH-responsive dye BPOx could act as a pH indicator under both ratiometric photoacoustic and fluorescence imaging. Accurate detection of tumor pH by photoacoustic imaging was realized after intravenous injection of this nanoprobe. The gradual acidification of the tumor microenvironment during the tumor growth, as well as the instant tumor pH changes upon injection of external buffers, was vividly observed by this method. Although ex vivo fluorescence imaging by the same probe also discovered the same trend, the quantitative measurement of tissue pH by fluorescence imaging appeared to be difficult. Side-by-side comparison further illustrates that while ratiometric fluorescent pH imaging is largely affected by covering samples with even a thin layer of pork tissue, ratiometric photoacoustic pH imaging is relatively independent of the tissue depth. Our work develops a rather simple pH-responsive photoacoustic nanoprobe based on HSA, the most abundant protein in the human body. This probe and the quantitative photoacoustic pH detection method are featured with high safety, easy-to-operate, and depth-independent accuracy for real-time in vivo pH imaging of the whole tumor, promising for future cancer prognosis and therapeutic planning.

HSA, the major component of serum proteins, has been widely used to load and delivery various hydrophobic drug and dye molecules via simple hydrophobic interaction.[9] It has been found that certain types of hydrophobic drug molecules (e.g., paclitaxel) are able to induce self-assembly of HSA to form nanoparticles in the range of 80–130 nm.[9a,9e] The strategy to
synthesize the HSA–BPOx–IR825 nanoparticles is shown in Figure 1a. We firstly mixed HSA and BPOx dye (predissolved in dimethylsulfoxide (DMSO)) was added into the aforementioned mixture at the HSA:BPOx molar ratio of 1:10 under rigorous stirring overnight. Afterwards, IR825 (predissolved in methanol), which is a NIR dye previously reported by our labs,[9d,10] at the molar ratio of 1:1 following the established method.[9b,9d] Dye-loaded HSA–BPOx–IR825 nanoparticles were obtained after dialysis of the mixtures against saline to remove organic solvents and free dyes. The hydrodynamic diameter of HSA–BPOx–IR825 was measured by dynamic light scattering (DLS) to be ≈100 nm, which was similar to that of HSA–BPOx (≈90 nm), but much larger than that of HSA–IR825 (≈8 nm), indicating that the protein assembly was induced by the hydrophobic BPOx dye instead of IR825 (Figure 1b).

UV–vis–NIR absorbance spectra of different dye-loaded nanoparticles were measured (Figure 1c). Under pH 7.4, HSA–BPOx–IR825 showed characteristic peaks of both BPOx and IR828 at 580 nm and 825 nm, respectively. Although the existence of BPOx would induce self-assembly of HSA to form nanoparticles, the hydrodynamic diameters of HSA–BPOx–IR825 would decrease from 100 to 8 nm upon dilution, indicating that those HSA–BPOx–IR825 nanoparticles were not stable enough and would dissociate apart at low concentrations (Figure S1a, Supporting Information).

To improve the stability of those albumin nanoparticles, glutaraldehyde was used to induce covalent cross-linking of HSA.[11] The size and...
absorbance spectrum of such cross-linked HSA–BPOx–IR825 (C–HSA–BPOx–IR825) appeared to be similar to that of HSA–BPOx–IR825 before crosslinking (Figure 1b,c). The obtained C–HSA–BPOx–IR825 nanoparticles were monodispersed with an average size at ≈80 nm under transmission electronic microscope (TEM) (Figure 1d). The loading capacities of BPOx and IR828 in the C–HSA–BPOx–IR825 formation were determined to be 2.8% (w/w) and 1.3% (w/w), respectively, by UV–vis–NIR absorbance spectra. Importantly, the nanoparticle sizes of C–HSA–BPOx–IR825 showed no obvious change after being diluted into rather low concentrations (Figure S1b, Supporting Information), and the release of both types of dye molecules from C–HSA–BPOx–IR825 appeared to be much slower compared that from HSA–BPOx–IR825 before crosslinking (Figure S2, Supporting Information), suggesting the greatly enhanced nanoparticle stability after chemical crosslinking. Those C–HSA–BPOx–IR825 nanoparticles also showed great stability in different physiological solutions (Figure S3, Supporting Information), and appeared to be nontoxic to cells even under high concentrations (Figure 4, Supporting Information).

The protonation and intramolecular charge-transfer process in the BPOx dye as the decrease of pH, as illustrated in Figure 1e, would lead to red-shifted and enhanced absorption of BPOx, as well as increased fluorescence emission[12] (Figure S5, Supporting Information). On the other hand, it has been found that IR825 after being complexed with HSA would show a strong absorbance at 825 nm, as well as greatly enhanced fluorescence under ≈600 nm excitation, owing to the fixed intramolecular rotation of this molecule when it is inserted into the hydrophobic pocket of HSA.[9b,9d] The absorbance and fluorescence of IR825, different from that of BPOx, are inert to pH changes and may serve as the internal reference when BPOx is used for pH sensing. Therefore, the C–HSA–BPOx–IR825 showed strong pH-dependent absorbance and fluorescence spectra (Figure 1f,g). While the absorbance peak at 825 nm and fluorescence emission at 630–640 nm, both of which were contributed by IR825, showed no appreciable change as the variation of pH, the BPOx absorbance at 670–680 nm and its fluorescence emission at 700–710 nm remarkably increased as the decrease of pH, especially in the pH range of 5.0–7.0 (Figure S6, Supporting Information). Notably, the presence of various metal ions such as K⁺, Na⁺, Ca²⁺ commonly found in the physiological environment would not affect the absorbance and fluorescence properties of this nanoprobe (Figure S7, Supporting Information).

Due to the strong pH-dependent absorption of C–HSA–BPOx–IR825, photoacoustic (PA) imaging of C–HSA–BPOx–IR825 dispersed in citrate buffer solutions with different pH values was carried out (Figure 2a). The PA imaging were captured at 680 nm and 825 nm, which corresponded to the absorption peaks of BPOx and IR825, respectively. The PA intensity at 680 nm (owing to BPOx absorbance) showed an obvious increase as the decrease of pH values, while the signals at 825 nm (owing to IR825 absorbance) remained unchanged. Interestingly, 680/825 PA signal ratios showed excellent pH dependence in the range of 5.0–7.0 (Figure 2b), potentially suitable for detecting pH values in the tumor microenvironment by ratiometric photoacoustic imaging.

Based on the aforementioned pH-dependent PA signals, we carried out the in vivo PA imaging on subcutaneous 4T1 tumor model via intratumoral injection of C–HSA–BPOx–IR825. For comparison, the same amount of C–HSA–BPOx–IR825 was

![Figure 2.](image-url) PA signal of C–HSA–BPOx–IR825. a) PA imaging of C–HSA–BPOx–IR825 dispersed in buffers with different pH values. b) The I_{680}/I_{825} signal intensity ratios of C–HSA–BPOx–IR825 measured under different pH values. A nearly linear relationship was found in the pH range of 5.0–7.0. c) PA imaging of a mouse with its tumor (left side) or muscle (right side) injected with C–HSA–BPOx–IR825. d) PA signals at 680 nm and 825 nm based on PA imaging in (d). e) The quantitative analysis (680/825 ratio and pH value) in the tumor and muscle.
injected into the muscle on the other side of this mouse. In vivo PA imaging was then conducted. As shown in Figure 2c, the PA signal at 680 nm was much stronger than that at 825 nm in the tumor, while the 680/825 PA signal ratio was obviously lower in the muscle (Figure 2d). Based on the standard calibration curve (Figure 3b), the tumor and muscle pH values
were determined to be ≈6.7 and > 7.0, respectively (Figure 2e). Therefore, the in vivo PA imaging with C–HSA–BPOx–IR825 probe could detect the weak acidic pH in the tumor.

Next, we would like to use C–HSA–BPOx–IR825 for tumor pH imaging by intravenous (i.v.) injection of those nanoparticles in to mice bearing 4T1 tumors at different stages (6, 10, 14, and 18 d post-inoculation). PA imaging was conducted 24 h after i.v. injection of C–HSA–BPOx–IR825 (dose = 3.5 mg kg\(^{-1}\) BOPx, 1.6 mg kg\(^{-1}\) IR825). As tumors growing large, higher PA signals were observed in the tumor, with the signals at 680 nm became particularly stronger (Figure 3a). Interestingly, the 680/825 PA signal ratios at the tumor showed an obvious increase as the tumor growth (Figure 3b). By calibrating with the standard curve (Figure 3c), the pH values in these tumors were determined to be ≈6.8, ≈6.6, ≈6.2, and ≈6.0, at day 6, 10, 14 and 18 post tumor inoculation (Figure 3c). This is expected because as the tumor grow larger, the tumor microenvironment would indeed become more acidic due to the production of lactate by tumor cells via the anaerobic glycolytic pathway under hypoxic environment.[13]

We then wondered whether our nanoprobe could detect instant pH changes inside the tumor. 24 h after i.v. injection of C–HSA–BPOx–IR825, PA imaging was carried out before and right after challenging the tumor pH by intratumoral injection of citrate buffer (pH 5.5) or NaHCO\(_3\) solution (pH 8.4). The PA images given in Figure 3d,e revealed that, as the alternation of tumor microenvironment pH, the PA signals at 680 nm showed obvious change (Figure 3d,e). Based on the 680/825 PA signal ratios, the tumor pH values were determined to be ≈5.8 post injection of the citrate buffer, and >7.0 post injection of the NaHCO\(_3\) solution (Figure 3f,g). Hence, in vivo PA imaging as well as the quantitative analysis (680/825 ratio) using HSA–BPOx–IR825 could be used for real-time pH detection in the tumor microenvironment.

Apart from PA imaging, fluorescence imaging was also carried out by recording fluorescent signals of both BPOx and IR825 dispersed in citrate buffers with different pH values (Figure 4a). The fluorescent signals at 710 nm (emission peak of BPOx) exhibited an obvious decrease as the increased pH, especially in the range of 5.0–7.0, while the signals at 640 nm (emission peak of IR825) hardly changed. The fluorescence intensity ratios between 640 nm and 710 nm (640/710) also exhibited a sharp decrease in that range as the
increase of pH (Figure 4b,c). Note that the 710/640 fluorescence intensity ratios measured under this in vivo fluorescence imaging system (Maestro) appeared to be quite different from those measured by the fluorometer (Figure 1g, Figure S6, Supporting Information), likely owing to the reason that the CCD detector in our in vivo imaging system could be more sensitive to light at longer wavelengths.

Although C–HSA–BPOx–IR825 is fluorescent, in vivo fluorescence pH imaging of tumor-bearing mice after i.v. injection of C–HSA–BPOx–IR825 was not so successful owing to the autofluorescence background as well as the limited fluorescence ratiometric response (Figure S8, Supporting Information). Therefore, ex vivo imaging of major organs as well as tumors collected from mice at 24 h after i.v. injection of C–HSA–BPOx–IR825 was conducted (Figure 4d). The tumor showed the highest fluorescence signals at both wavelengths, suggesting the rather efficient tumor homing of nanoparticles due to the enhanced permeability and retention (EPR)
effect (Figure 4e). We compared the fluorescence signals in different organs at two different wavelengths (710 nm/640 nm). As shown in Figure 4f, the 710/640 fluorescence signal ratio in the tumor was also obviously higher than that in other organs owing to the acidic tumor microenvironment. In addition, this ratio also showed a significant increase as the tumor growth based on ex vivo fluorescence imaging of tumors at different stages (Figure 4g–i). However, the 710/640 fluorescence signal ratios measured by ex vivo imaging appeared to be much higher than those recorded in the calibration curve by imaging solution samples, making quantitative pH imaging by the fluorescence method to be not accurate. This is likely owing to the better tissue penetration of emission light at 710 nm compared to that at 640 nm, as well as possible interference from tissue autofluorescence and quenching.

At last, we side-by-side compared pH detection using our C–HSA–BPOx–IR825 nanoprobe by two imaging modalities, photoacoustic imaging and fluorescence imaging, for samples covered by pork tissues with different thicknesses. The PA signals at both 680 nm and 825 nm of C–HSA–BPOx–IR825 nanoprobe showed slight decrease as the thickness of the covered pork tissue changed from 0 to 3 mm (Figure 5a–c). Importantly, the 680/825 PA signal ratios, which were used to determine the pH values, showed no significant change, suggesting that our ratiometric photoacoustic pH sensing method remains to be accurate for targets covered by tissues with considerable depth (Figure 5d). Notably, we further found that even for a target sample covered by a pork tissue as thick as 10 mm, its photoacoustic signals were still detectable, while the ratiometric values showed little change as the increase of tissue thickness (Figure S9, Supporting Information). In marked contrast, the fluorescence signals from C–HSA–BPOx–IR825 at both wavelengths sharply decreased to the background level for samples covered by just a thin layer of pork tissue with the thickness at 1.5 mm (Figure 5e–g). In the meantime, the 710/640 fluorescence signal ratios varied significantly as the increase of pork tissue thicknesses, making ratiometric fluorescence pH imaging not accurate for targets buried even by a thin layer of tissues (Figure 5h). Therefore, photoacoustic imaging is an obviously preferred technique over fluorescence imaging for quantitative in vivo monitoring of tumor pH, without being significantly affected by the tissue depth during imaging.

In summary, a self-assembled albumin-based nanoprobe, C–HSA–BPOx–IR825 is successfully fabricated in our work for real-time pH sensing of tumors under noninvasive ratiometric photoacoustic imaging. In this system, HSA is a biocompatible nanocarrier that can simultaneously bind with two different organic dyes: BPOx as a pH-sensitive dye for real-time pH sensing, and IR825 as a pH-inert NIR dye for internal reference. Ratiometric photoacoustic imaging in combination with quantitative analysis using C–HSA–BPOx–IR825 can be used to detect the tumor microenvironment pH, which plays important roles in regulating the proliferation, metastasis, and MDR of cancer cells. Unlike the conventional fluorescence imaging method which has very limited tissue penetration and is not suitable for quantitative in vivo pH detection, ratiometric photoacoustic imaging for pH sensing appears to be rather accurate even for targets covered by tissues with considerable thicknesses. Therefore, this work provides a great tool for in vivo noninvasive, real-time, quantitative pH imaging, particularly useful for the investigation of tumor microenvironment, as well as for the prognosis of cancer. Considering the safe use of HSA as a natural carrier, as well as the existence of PA imaging instruments in clinical trials, the probe and methodology developed here may potentially become clinically adoptable in the future.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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