Modular Design of High-Brightness pH-Activatable Near-Infrared BODIPY Probes for Noninvasive Fluorescence Detection of Deep-Seated Early Breast Cancer Bone Metastasis: Remarkable Axial Substituent Effect on Performance

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ABSTRACT: We herein report a series of high-brightness pH-activatable near-infrared (NIR) BODIPY probes for high-contrast intravitral imaging of deep-seated early breast cancer bone metastasis by harnessing the axial substituent effect. These probes exhibit tunable $pK_a$, higher brightness, and anti-quenching capabilities in aqueous solution, which can be simultaneously adjusted by axial steric substituents. The optimized probe BODO-3 bearing axial dimethyl substituents exhibited a higher $pK_a$ value of 5.6 and a brighter NIR fluorescence under tumor acidic pH, showing 10.3-fold and 6.5-fold enhanced brightness ($\varepsilon\Phi$) at pH 5.5 and 6.5, respectively. Due to the higher brightness, BODO-3 with a brilliant NIR emission at 700 nm allows for deep optical penetrations of 5 and 8 mm at pH 6.5 and 4.5, respectively. Meanwhile, covalent functionalization with glucose (BODO-3-Glu) could further enhance breast cancer and its soft tissue metastasis imaging in vivo. Notably, covalent functionalization with bisphosphonate (BODO-3-PO$_3$H$_2$) allowed the successful targeting and visualization of deep-seated bone metastases of breast cancer with a high tumor to normal contrast of 8/1, outperforming X-rays in early detection. This strategy may provide insights for designing high-brightness activatable NIR probes for detecting deep-seated tumors and metastases.

INTRODUCTION

Breast cancer is the most common malignancy in women worldwide, and its metastases at distant sites cause 90% of breast cancer mortality.1,2 Bone is one of the most frequent sites of distant metastases, which occurs in 70% of advanced breast cancer patients.3,4 Early breast cancer is considered curable if it is treated before metastatic spread; however, tumors can rarely be cured once they have metastasized to the bones.5 Moreover, patients with metastatic bone cancer suffer from a series of severe skeletal complications, such as considerable bone pain, hypercalcemia, and pathologic fractures.6 Hence, early and accurate detection of bone metastases from breast cancer is very important for diagnosis and treatment, which can greatly improve patients’ quality of life and increase the survival rate. Clinically, bone scintigraphy is often employed for the detection of breast cancer bone metastasis due to its high sensitivity, most frequently using radioactive $^{99m}$Tc-methylene diphosphonate ($^{99m}$Tc-MDP) as an imaging agent.7 However, $^{99m}$Tc-MDP is not ideal for imaging breast cancer bone metastasis because of its poor specificity and low spatial resolution, possibly resulting in false-positive results. In addition, computed tomography, magnetic resonance imaging, and positron emission tomography are being utilized for breast cancer bone metastasis detection in vivo.8,9 Nevertheless, the highly sensitive and specific detection of early breast cancer bone metastasis with excellent spatiotemporal resolution remains challenging.

Fluorescence imaging has recently attracted great attention in disease detection due to its high sensitivity, spatiotemporal resolution, and real-time imaging capabilities, especially in the near-infrared (NIR, 650−900 nm) region, allowing high-contrast intraoperative image-guided surgery.10−13 Although a few "always-on" NIR fluorescent probes have been reported to image bone tissues,14−22 stimuli-responsive fluorescent probes that can sensitively distinguish breast cancer bone metastasis from normal bone are still elusive.23 A low microenvironment pH (6.5−6.8) is a typical cancer hallmark that can delineate tumors from normal tissues.24,25 Despite the development of a number of pH-activatable fluorescent probes for tumor
in vivo imaging of subcutaneous breast cancer and deep-seated bone metastases, respectively. Deep-tissue bioimaging was achieved using these probes, and covalent functionalization with glucose and bisphosphonate further improved intravital imaging of subcutaneous breast cancer and deep-seated bone metastases, respectively.

To date, deep-tissue bioimaging is still a huge challenge for real-time intravital imaging of acidic bone metastases in vivo. On the other hand, there remains a challenge to apply these pH-activatable probes for noninvasive fluorescence detection of breast cancer bone metastases at early stages due to their small sizes and mineralized extracellular microenvironments, which are significantly different from soft tissue tumors and make them difficult to target via the enhanced permeability and retention (EPR) effect. We previously developed a series of pH-activatable NIR BODIPY probes for tracking breast cancer metastases in different organs, showing high ex vivo fluorescence contrast. However, due to the low tissue penetration abilities, these probes were not able to directly illuminate metastases in living mice, requiring first taking out the metastasized tumors for imaging. In addition, bone metastases from breast cancer exist mainly in deep osseous tissues and it is difficult to detect them in the early stages. To the best of our knowledge, there have been no reports using pH-activatable NIR probes for noninvasive fluorescence detection of early breast cancer bone metastasis in living animals. Therefore, there remains a pressing demand to develop highly sensitive and specific pH-activatable NIR probes for real-time intravital imaging of deep-seated bone metastases, further improving treatment outcomes of metastatic breast cancer.

To date, deep-tissue bioimaging is still a huge challenge for small molecular fluorescent probes in the near-infrared-I (NIR-I, 650–900 nm) window because of compromised tissue penetration depth, which partly impairs their abilities for deep-seated bone metastasis imaging in vivo. Due to the much-decreased tissue absorption, scattering, and autofluorescence of near-infrared-II (NIR-II, 1000–1700 nm) light, one common approach for deeper body penetration is the application of NIR-II dyes in biological imaging. However, small molecular NIR-II dyes suffer from low brightness and chemical and photo stabilities. In this study, we propose a brightness enhancement strategy to greatly increase the tissue penetration depth of NIR-I probes to achieve intravital imaging of deep-seated early breast cancer bone metastasis, because they possess brilliant bright fluorescence to penetrate deeply even if part of the energy is absorbed and scattered by normal tissues and bones. We chose high-brightness pH-activatable NIR BODIPY probes to demonstrate the proof of concept, possessing the following three chemical properties: (i) high NIR brightness under tumor acidic pH, enabling deeper tissue penetration, (ii) an appropriate pH-Ka to sensitively distinguish bone metastases from normal bones, (iii) active-targeting moiety, delivering the fluorophore into a specific tissue where the breast cancer bone metastases are located. However, simultaneous control of the pH-Ka and brightness of these NIR BODIPY probes is still synthetically challenging. One generally adopted design strategy for tuning the pH-Ka of a pH-responsive BODIPY probe is altering the substituents of the amine group in the fluorophore. Nevertheless, the pH-Ka value of the optimal pH-activatable NIR BODIPY probe that we previously developed using this strategy is ~4.5, which is much lower than tumor acidic pH and is not bright enough to penetrate deeply in vivo. Thus, how to further increase the pH-Ka value and brightness of these NIR BODIPY probes would be a key issue for achieving high-contrast fluorescence detection of deep-seated bone metastases in vivo.

Endeavoring toward developing high-brightness pH-sensitive probes for noninvasive fluorescence detection of deep-seated early breast cancer bone metastasis in vivo, we herein designed and synthesized a series of pH-activatable NIR BODIPY probes with different axial substituents, which is different from previous work focused on investigation of the poly(ethylene glycol) (PEG) length effect (Figure 1). Each of the probes consists of a substituted aniline moiety, a NIR BODIPY core, and two functionalized PEG tails, which enables...
sensitive response to tumor acidic pH, produces NIR fluorescence emission, and increases the water solubility and tumor-targeting ability, respectively. With the axial substituent strategy, the $pK_a$ and brightness of these NIR BODIPY probes could be simultaneously tuned with remarkable fluorescence intensity enhancement under tumor acidic pH. In comparison to the control probe BODO-1, for example, optimized BODO-3 bearing axial dimethyl substituents exhibited a higher $pK_a$ value of 5.6 and a much brighter NIR fluorescence under tumor acidic pH, showing 10.3-fold and 6.5-fold enhanced brightness ($\Phi$) at pH 5.5 and 6.5, respectively. More importantly, by virtue of its higher brightness, the NIR fluorescence of BODO-3 could readily penetrate through 5 mm of chicken breast tissue ex vivo and in vivo, providing a maximum penetration depth of up to 8 mm. In addition, BODO-3-Glu, covalently functionalized with glucose, could effectively accumulate and activate in subcutaneous breast cancer and metastases around the bone after intravenous (i.v.) administration, displaying a high metastasis to normal contrast of up to 9/1. Notably, BODO-3-PO$_2$H$_2$ covalently functionalized with bisphosphonate, was able to selectively target and visualize deep-seated bone metastases of breast cancer much earlier in comparison to X-rays in living mice, showing a high metastasis to normal contrast of up to 8/1. We believe that this earlier in comparison to X-rays in living mice, showing a high metastasis to normal contrast of up to 8/1. We believe that this

**RESULTS AND DISCUSSION**

**Design and Synthesis of High-Brightness pH-Activatable NIR BODIPY Probes.** We envisioned that the introduction of steric substituents on the N,N-diethylaminobenzaldehyde moiety would be able to increase the brightness of NIR BODIPY probes via suppressing nonradiative decay and tuning the $pK_a$ simultaneously. With this idea in mind, we designed and synthesized 12 NIR BODIPY probes with different axial substituents by a straightforward three-step procedure (Figure 2a). As the first step, 2a–i were synthesized via acid-catalyzed condensation of 2,6-substituted N,N-diethylaminobenzaldehydes (1) with 2,4-dimethylpyrrole or 2-methylpyrrole, followed by oxidation with chloranil (TCQ) and treatment with BF$_3$·OEt$_2$ in one pot. Subsequently, each of the BODIPYS 2 was condensed with propargyl benzaldehyde derivatives to obtain NIR BODIPYs 3 under a modified Knoevenagel condition that we previously developed. Finally, to render the NIR BODIPY core water-soluble, NIR BODIPYs 3 were reacted with PEG-azide ($M_n = 2000$) by a copper(I)-catalyzed alkyne–azide cycloaddition reaction to generate a water-soluble series of BOD and BODO probes. A PEG length of 2000 g/mol was used to functionalize NIR BODIPY because of its ability to reduce liver uptake and increase tumor accumulation tropism. The photophysical data of key probes are summarized in Figure 2b and Table S1.

**Spectroscopic Properties and Axial Substituent Effects on NIR BODIPY Probes.** With these PEGylated BODIPY probes in hand, we first measured their UV–vis absorption and fluorescence emission spectra in aqueous solutions (pH 4.0 citrate buffer) (Figure 3a, Figure S1, and Table S2). All of them exhibited excellent solubility in aqueous solutions and showed NIR absorption and emission maxima ($\lambda_{abs}/\lambda_{em}$) at about 650/670 and 680/705 nm for the series of BOD and BODO probes, respectively. To further confirm the pH-dependent fluorescence properties, we measured the pH titration profiles of the NIR BODIPY probes from pH 3.1 to 7.9 (Figure 3b,c and Figure S2). The series of BOD and BODO (BODO-1 to BODO-6) are highly sensitive to acidic pH and displayed a significant fluorescence increase at the lowered pH, showing $pK_a$ values of 4.6, 5.6, 5.0, 4.6, 5.1, 5.6, 5.3, 4.9, and 4.0, respectively. Importantly, no NIR fluorescence was observed at physiological pH. In addition, fluorescence images of these probes in different buffers
demonstrate exquisite control of pH transitions (Figure 3d). These NIR BODIPY probes are “OFF” at neutral pH but turn “ON” at acidic pH by harnessing the photoinduced electron transfer (PeT) mechanism that occurs from the p-anilino moiety to the NIR BODIPY core. Nevertheless, BODO-7, BODO-8, and BODO-9 containing axial halogen substituents (F, Cl, Br) are “always-on” probes and their fluorescence intensities slightly changed regardless of the pH value. Interestingly, BOD-2 and BOD-3 bearing two methyl groups as axial substituents are most sensitive to the tumor acidic pH and both show an optimal pK_a value of 5.6, probably because the methyl group does not significantly alter the highest occupied molecular orbital (HOMO) energy level of the p-anilino moiety. Moreover, BOD-2 containing only one methyl group as an axial substituent shows a lower pK_a value of 5.1. These results demonstrated that the pK_a of these NIR BODIPY probes are mainly controlled by the p-anilino moiety and could be well tuned via axial substituents.

To further investigate the pH-dependent brightness of these NIR BODIPY probes, we next compared their fluorescence intensities at the same pH value (Figure 3e,f). The relative fluorescence intensities of series of BOD and BODO probes were normalized to the fluorescence intensities of BOD-1 and BODO-1, respectively. In comparison with BOD-1, BOD-2 and BOD-3 containing axial steric substituents exhibited higher relative fluorescence intensities, especially for BOD-2, showing 6.3-fold, 5.8-fold, and 4.6-fold enhanced fluorescence intensity at pH 5.6, 6.0, and 6.6, respectively (Figure 3e). In addition, BOD-2 showed 6.3-fold and 3.6-fold higher brightness (εΦ) in comparison to BOD-1 at pH 5.5 and 6.5, respectively (Table S1). Similarly, BODO-2, BODO-3, and BODO-4 bearing axial steric substituents produced a brilliant increase in fluorescent brightness in comparison to BODO-1,
especially for BODO-3, showing 8-fold, 7.3-fold, and 5-fold enhanced fluorescence intensity at pH 5.6, 6.0, and 6.6, respectively (Figure 3f). In addition, BODO-3 showed 10.3-fold and 6.5-fold higher brightness ($\varepsilon\Phi$) in comparison to BODO-1 at pH 5.5 and 6.5, respectively (Table S1). In addition, in comparison to BODO-2, BODO-3 displayed even brighter NIR fluorescence, indicating that increasing the number of axial methyl substituents of the $p$-anilino moiety would further enhance the fluorescence intensity by inhibition of its intramolecular rotation. In contrast, BODO-5 and BODO-6, both containing a freely rotating $p$-anilino moiety, showed lower relative fluorescence intensities. These results indicated that the fluorescence intensities of these NIR BODIPY probes could be significantly increased via the introduction of axial steric substituents in the $p$-anilino moiety.42

To verify the fluorescence enhancement mechanism, we next mixed these NIR BODIPY probes with 10% fetal bovine serum (FBS) in different pH buffers (Figure 3g,h and Figure S3). Typically, organic dyes consisting of hydrophobic $\pi$-systems have strong interactions with plasma proteins, which have been used to increase the brightness of flexible NIR dyes.43−45 The mechanism could be ascribed to the protein binding that restricts the free intramolecular rotation and changes the rigidity of the fluorophore. Thus, the rigid conformation of a NIR probe can be evaluated after binding to the protein, showing fewer fluorescence intensity variations for a rigid dye. The results suggested that those pH-activatable NIR BODIPY probes bearing axial steric substituents are more rigid with fewer fluorescence intensity variations. The fluorescence intensities of BODO-1, BODO-2, BODO-3, BODO-4, and BODO-5, for example, increased by 65%, 40%, 26%, 24%, and 53% at pH 5.0 after incubation with 10% FBS, respectively.
Similar results were also observed for a series of BOD probes (Figure 3h). Moreover, these NIR BODIPY probes were still pH-responsive after binding to proteins and showed higher fluorescence intensity at a lower pH value. To further confirm this phenomenon, we examined the relationship between viscosity and fluorescence intensity of a series of BOD probes. Similarly, BODO-2 and BODO-3 exhibited fewer fluorescence intensity variations as the ratio of glycerol was increased (Figure S4). These results indicated that BODO-3 possessing higher brightness, for example, could be attributed to the relatively rigid conformation adjusted by axial dimethyl substituents, which enables inhibition of the free rotation of the p-anilino moiety and consequently minimization of nonradiative decay. In addition, these pH-activatable NIR BODIPY probes were still pH-responsive after binding to proteins and showed higher fluorescence intensity at a lower pH value. To further confirm this phenomenon, we examined the relationship between viscosity and fluorescence intensity of a series of BOD probes. Similarly, BODO-2 and BODO-3 exhibited fewer fluorescence intensity variations as the ratio of glycerol was increased (Figure S4). These results indicated that BODO-3 possessing higher brightness, for example, could be attributed to the relatively rigid conformation adjusted by axial dimethyl substituents, which enables inhibition of the free rotation of the p-anilino moiety and consequently minimization of nonradiative decay. In addition, these pH-activatable NIR BODIPY probes were still pH-responsive after binding to proteins and showed higher fluorescence intensity at a lower pH value.

To further evaluate the cell imaging abilities of these pH-activatable NIR BODIPY probes, we first incubated them with 4T1 breast cancer cells at the same concentration (5 μM). All pH-responsive probes would turn on in the lysosomes of cancer cells due to the low pH (Figure S6). Especially for BOD-2 and BODO-3, they showed the highest fluorescence intensities, which were consistent with the results in the two other cancer cell lines HeLa and PC3 (Figure S7). Interestingly, BOD-3 and BODO-4 both bearing axial dimethoxy substituents displayed weak NIR fluorescence signals, probably because of the slow accumulation and activation in cancer cells (Figure S8). Furthermore, all of these NIR BODIPY probes showed negligible cytotoxicity in HeLa cells (Figure S9). Therefore, given the pKᵣ brightness,
antiquenching capability, and cell imaging properties, BOD-2 and BODO-3 were selected for further studies.

**Optical Penetration Study of BOD-2 and BODO-3 Ex Vivo and In Vivo.** Encouraged by the excellent performances of BOD-2 and BODO-3 in aqueous solutions and cells, we then assessed their optical penetration capabilities ex vivo and in vivo. In comparison to BOD-1 and BOD-3, BOD-2 exhibited better tissue penetration capability regardless of the pH value and better penetration depth due to the higher brightness, which could readily penetrate through 5 and 1 mm of chicken breast tissue at pH 4.5 and 6.5, respectively (Figure S10). To next investigate the optical penetration capability of BODO-3, we examined fluorescence images of BOD-1, BOD-2, and BODO-3 covered with different thicknesses of chicken breast slices using a Cy5.5 filter (Figure 4a). All of these probes displayed excellent deep-tissue penetration abilities at a lowered pH value of 4.5, while significant attenuation of the image intensities of BOD-1 and BOD-2 were observed with an increase in pH value. Notably, only BODO-3, which outperformed BOD-2 (1 mm), was able to penetrate through 5 mm of chicken breast tissue at pH 6.5 (Figure 4a), showing high ratios of fluorescence intensities of up to 5.5/1 (pH 5.5) and 3.4/1 (pH 6.5) at a depth of 5 mm (Figure 4b). More importantly, BODO-3 enabled deep penetration at a maximum depth of up to 8 mm, outperforming indocyanine green (ICG) in the NIR-I field (Figure S11). Overall, these results demonstrated that BODO-3 holds great promise for deep-penetration imaging, which can be attributed to the higher brightness and longer NIR emission wavelength that reduced scattering. To further assess its optical penetration capability in vivo, BODO-1 and BODO-3 in pH 5.6 citrate buffer were injected intramuscularly into the left and right thighs of the same mice, respectively. In comparison to BOD-1, BODO-3 exhibited much stronger NIR fluorescence and showed a 3.2-fold enhanced fluorescence intensity at a depth of 5 mm, which was consistent with the ex vivo results (Figure 4d,e and Figure S12). Having confirmed the capability of BODO-3 to image deeply in vivo, we applied BODO-3 to detect deep-seated bone metastasis of 4T1 breast cancer after i.v. injection of 2.0 nmol (10 μM, 200 μL) of BOD-3-PO₃H₂ and BOD-3-Glu, respectively. Arrowheads: bone metastasis (yellow); spread of tumor (purple). (b) Bioluminescence and fluorescence images of harvested femurs and organs from 4T1 bone metastasis bearing mice sacrificed at 24 h postinjection. Abbreviations: He, heart; Li, liver; Lu, lung; Ki, kidneys; Sp, spleen; Pa, pancreas; Mu, muscle. (c) Bioluminescence and fluorescence images of harvested femurs and organs from 4T1 bone metastasis bearing mice at the late stage sacrificed at 24 h postinjection. The left hindlimb (i) was separated into two parts of left femur and resected metastasis in soft tissues (ii). Abbreviations: RF, right femur; LF, left femur. (d) Femur to organ ratios of BOD-3-PO₃H₂ and BOD-3-Glu fluorescence intensity by ROI analysis at 24 h postinjection. (e) Tumor to organ ratios of BOD-3-Glu fluorescence intensity by ROI analysis at 24 h postinjection. (f) White light images of normal femur and a metastatic femur: histology and fluorescence microscopy analysis of BOD-3-PO₃H₂ accumulation and activation in normal femur and femur metastases. BOD-3-PO₃H₂ is shown in red. Scale bar: 100 μm.
metastases in the breast cancer metastasis bearing mice. The metastatic tumors can be confirmed via bioluminescence imaging because of the engineered luciferase expression of cancer cells. BODO-3, indeed, was able to noninvasively illuminate bone metastases in the living mice without taking out the metastatic bones first, which is different from the case in our previous work.23 In addition, breast cancer metastases in other organs, including heart and pancreas, were also detected simultaneously (Figure 4f,g). However, due to the lack of active-targeting ability, a false-positive NIR fluorescence signal was also observed in the left tibia (Figure 4g).

**In Vivo NIR Fluorescence Imaging of Subcutaneous Breast Cancer Tumors.** To further improve the metastasis-targeting ability, BODO-3-PO3H2 containing two carboxylate groups as modification was first synthesized via a copper(I)-catalyzed click reaction (Figure 5a). Next, BODO-3-Glu, BODO-3-Gal, and BODO-3-PO3H2 were obtained by engineering glucosamine, galactosamine, and bisphosphonate groups into BODO-3-PO3H2 showing pKa values of 5.4, 5.4, and 5.7, respectively (Figure S13). Glucosamine was selected to attach onto BODO-3-PO3H2 because of the higher rates of glucose uptake of cancer cells, and galactose was used as a control.47 Meanwhile, bisphosphonate that can bind to hydroxyapatite (HA) was employed to covalently functionalize BODO-3-PO3H2 due to its high binding affinity to the abundant Ca2+ in the microenvironment of bone metastases. With these probes in hand, we first examined their potential binding capabilities to HA and found that only BODO-3-PO3H2 exhibited high binding to HA and remained pH responsive (Figure 5b and Figure S14). To investigate their capabilities to preferentially accumulate and activate in the breast tumors in vivo, mice bearing subcutaneous 4T1 breast cancer xenografts were injected i.v. with five selected probes and time-dependent NIR fluorescence images were collected using Cy5.5 filters (Figure 5c and Figure S15). Intense and sustained NIR fluorescence in the tumors was observed for all five probes up to 24 h, facilitating real-time fluorescence imaging. Notably, the breast tumors could be clearly distinguished from surrounding normal tissues 2 h after i.v. administration and displayed the highest fluorescence intensities at 6 h postinjection (Figure 5d). To further examine the biodistribution, the tumors and main organs were harvested and analyzed at 24 h postinjection (Figure 5c). BODO-1, BODO-3, BODO-3-Glu, and BODO-3-PO3H2 exhibited strong NIR fluorescence in the tumors and low fluorescence signals in all other organs, indicating that these four probes could effectively accumulate in the tumors. Notably, BODO-3-Glu exhibited higher tumor tropism, probably due to the overexpressed glucose transporter-1 (GLUT1) in breast cancer cells,27 and produced high tumor to normal contrasts of up to 3/1 (tumor to liver), 10/1 (tumor to kidneys), and 7/1 (tumor to muscle), respectively (Figure 5d,e). In contrast, BODO-3-Gal showed lower tumor tropism but higher liver uptake because of a high expression of galactose receptors in hepatic cells.28 In addition, BODO-3-PO3H2 also displayed rapid accumulation and high retention in the tumors even at 24 h postinjection, showing a high tumor to muscle ratio of 7.4. Therefore, BODO-3-Glu and BODO-3-PO3H2 are promising for further applications in the high-contrast fluorescence detection of deep-seated bone metastases.

**Intravital Imaging of Deep-Seated Early Breast Cancer Bone Metastasis.** To investigate if BODO-3-PO3H2 and BODO-3-Glu could sensitively detect deep-seated bone metastases of breast cancer, a challenging bone metastasis bearing mice model was established by intramedullary injection of 4T1-luc cells into the femurs. The 4T1 breast cancer cells were engineered to consistently express luciferase; thus, three modalities of X-ray, bioluminescence, and fluorescence imaging were used to monitor the tumor growth. The bioluminescence signals were clearly observed in the right femurs of mice at day 10 after inoculation, while no morphological lesions occurred via X-ray analysis (Figure 6a and Figure S16). Moreover, both the left and right legs of mice could move normally, indicating that the mice bearing 4T1 bone metastases were still in the early stages at day 10. BODO-3-PO3H2 and BODO-3-Glu, respectively, were then intravenously injected into 4T1 bone metastasis bearing mice at the same dose of 2.0 nmol (10 μM, 200 μL). The early bone metastases in the right femurs were clearly distinguished from the surrounding background tissues from 2 to 24 h, showing maximum fluorescence intensity signals at 6 h postinjection (Figure 6a and Figure S17). In contrast, no fluorescence signals were observed in the normal left legs. By day 15, the bone metastases were detected by X-ray imaging. In addition, the bone metastases in the right femurs slightly spread to the surrounding soft tissues, showing dark shadows around the right leg bones. Accordingly, the right legs gradually became swollen and immobile, especially at day 20. Notably, these two probes were able to sensitively detect deep-seated bone metastases of breast cancer in vivo regardless of the early or late stages (Figure 6a).

To further examine biodistribution, harvested femurs and organs were analyzed by bioluminescence and fluorescence imaging at 24 h postinjection (Figure 6b). Due to the bone tropism of bisphosphonate, pH-activatable BODO-3-PO3H2 selectively targeted and clearly illuminated bone metastases in the right femur with minimal background signals in all other organs, displaying a high metastasis to normal contrast of up to 8/1 (Figure 6d). Hematoxylin and eosin (H&E) staining and confocal images of tissue cryosections further confirmed that BODO-3-PO3H2 was able to efficiently accumulate and activate in the metastatic femur (Figure 6f). Interestingly, BODO-3-Glu, which exhibited excellent imaging capability in vivo, did not show high ex vivo NIR fluorescence in the metastatic femur. To determine the reason, another mouse bearing 4T1 bone metastases in the left leg was injected i.v. with BODO-3-Glu and organs were collected for imaging and histological analysis (Figure 6c and Figure S18). Intense NIR fluorescence was observed in the left hindlimb in vivo and ex vivo, showing a high metastasis to normal contrast of up to 9/1 (Figure 6e). However, when the left hindlimb was separated into two parts of resected soft tissues and left femur that both had bioluminescence signals, only the resected soft tissues displayed strong NIR fluorescence. In addition, the resected soft tissues consisting of breast cancer metastasis were further confirmed by H&E staining and exhibited a 7/1 tumor to muscle contrast (Figure 6e and Figure S18). These results suggested that BODO-3-Glu could effectively accumulate and light up in the soft tissue metastases of breast cancer.

**CONCLUSION**

In this study, a series of pH-activatable NIR BODIPY probes with different axial substituents have been rationally designed and synthesized. These NIR BODIPY probes exhibit tunable pKa, high fluorescence intensity, and antiquenching capabilities...
in polar solvents, which significantly enhanced NIR brightness up to 10-fold at acidic pH and further increased deep-tissue penetration depth up to 8 mm. Meanwhile, these pH-responsive probes could localize to breast cancer xenografts in vivo and be activated by acidic pH to produce a high tumor to normal contrast after i.v. administration. Notably, by engineering glucose groups into BODO-3, BODO-3-Glu effectively accumulated and lit up in the soft tissue metastases around the bone. More importantly, by engineering bisphosphonate groups into BODO-3, BODO-3-PO3H2 successfully targeted and clearly illuminated deep-seated early bone metastases of breast cancer in vivo with a high signal to noise ratio of up to 8, which is more sensitive than X-ray in the early detection of bone metastasis. Therefore, these high-brightness pH-activatable NIR BODIPY probes may provide a robust platform for tracking cancer metastasis, deep-tissue imaging, and fluorescence-guided surgery.

■ ASSOCIATED CONTENT

1 Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscentsci.1c01066.

Experimental section including synthesis, supplemental figures and tables, cell culture and confocal imaging, cell cytotoxicity, animal model, in vivo fluorescence imaging of subcutaneous breast cancer tumors and bone metastases, and 1H NMR and 13C NMR spectra of the synthesized compounds (PDF)

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Notes
The authors declare no competing financial interest.

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■ REFERENCES

(1) Siegel, R. L.; Miller, K. D.; Jemal, A. Cancer statistics, 2020. Ca-Cancer J. Clin. 2020, 70, 7–30.
(2) Chaffer, C. L.; Weinberg, R. A. A perspective on cancer cell metastasis. Science 2011, 331, 1559–1564.
(3) Weigelt, B.; Peterse, J. L.; van’t Veer, L. J. Breast cancer metastasis: markers and models. Nat. Rev. Cancer 2005, 5, 591–602.
(4) Weilbaecher, K. N.; Guise, T. A.; McCauley, L. K. Cancer to bone: a fatal attraction. Nat. Rev. Cancer 2011, 11, 411–425.
(5) Harbeck, N.; Penault-Llorca, F.; Cortes, J.; Gnant, M.; Houssami, N.; Poortmans, P.; Rudy, K.; Tsang, J.; Cardoso, F. Breast cancer. Nat. Rev. Dis. Primers 2019, 5, 66.
(6) Mamedova, F.; Ladeira, K.; Pinho, F.; Saraiva, N.; Bonito, N.; Pinto, L.; Goncalves, F. Bone metastases: an overview. Oncol. Rev. 2017, 11, 321.
(7) Wu, P.; Siegwart, D. J.; Xiong, H. Recent advances in the targeted fluorescent probes for the detection of metastatic breast cancer. Sci. China: Chem. 2021, 64, 1283–1296.
(8) Fernandes, R. S.; dos Santos Ferreira, D.; de Aguiar Ferreira, C.; Giammarile, F.; Rubello, D.; de Barros, A. L. B. Development of imaging probes for bone cancer in animal models. A systematic review. Biomed. Pharmacother. 2016, 83, 1253–1264.
(9) Meng, Q.; Li, Z. Molecular imaging probes for diagnosis and therapy evaluation of breast cancer. Int. J. Biomed. Imaging 2013, 2013, 1.
(10) Vahmeijer, A. L.; Hutteman, M.; van der Vorst, J. R.; van de Velde, C. J. H.; Frangioni, J. V. Image-guided cancer surgery using near-infrared fluorescence. Nat. Rev. Clin. Oncol. 2013, 10, 507–518.
(11) Zhang, R. R.; Schroeder, A. B.; Grudzinski, J. J.; Rosenthal, E. L.; Warram, J. M.; Pinchuk, A. N.; Elicieri, K. W.; Kuo, J. S.; Weichert, J. P. Beyond the margins: real-time detection of cancer using targeted fluorophores. Nat. Rev. Clin. Oncol. 2017, 14, 347–364.
(12) Zhao, T.; Huang, G.; Li, Y.; Yang, S.; Ramezani, S.; Lin, Z.; Wang, Y.; Ma, X.; Zeng, Z.; Luo, M.; de Boer, E.; Xie, J. X.; Thibodeaux, J.; Brekken, R. A.; Sun, X.; Sumer, B. D.; Gao, J. A transistor-like pH nanoprobe for tumour detection and image-guided Surgery. Nat. Biomed. Eng. 2017, 1, 0006.
(13) Hu, Z.; Fang, C.; Li, B.; Zhang, Z.; Cao, C.; Cai, M.; Su, S.; Sun, X.; Shi, X.; Li, C.; Zhou, T.; Zhang, Y.; Chi, C.; He, P.; Xia, X.; Chen, Y.; Gambhir, S. S.; Cheng, Z.; Tian, J. First-in-human liver-tumour surgery guided by multispectral fluorescence imaging in the visible and near-infrared-I/II windows. Nat. Biomed. Eng. 2020, 4, 259–271.
(14) Zaheer, A.; Lenkinski, R. E.; Mahmood, A.; Jones, A. G.; Cantley, L. C.; Frangioni, J. V. In vivo near-infrared fluorescence imaging of osteoblastic activity. Nat. Biotechnol. 2001, 19, 1148–1154.
(15) Hyun, H.; Wada, H.; Bao, K.; Gravier, J.; Yadav, Y.; Laramie, M.; Henary, M.; Frangioni, J. V.; Choi, H. S. Phosphonated near-infrared fluorophores for biomedical imaging of bone. Angew. Chem., Int. Ed. 2014, 53, 10668–10672.
(16) Harmatys, K. M.; Cole, E. L.; Smith, B. D. In vivo imaging of bone using a deep-red fluorescent molecular probe bearing multiple iminodiacetate groups. Mol. Pharmaceutics 2013, 10, 4263–4271.
(17) Peck, E. M.; Battles, P. M.; Rice, D. R.; Roland, F. M.; Norquest, K. A.; Smith, B. D. Pre-assembly of near-infrared fluorescent multivalent molecular probes for biological imaging. Bioconj.ugate Chem. 2016, 27, 1400–1410.
(18) Hu, X.; Wang, Q.; Liu, Y.; Liu, H. G.; Qin, C. X.; Cheng, K.; Robinson, W.; Gray, B. D.; Pak, K. Y.; Yu, A. X.; Cheng, Z. Optical imaging of articular cartilage degeneration using near-infrared dipicolyamine probes. Biomaterials 2014, 35, 7511–7521.
(19) Feng, Y.; Zhu, S. J.; Antaris, A. L.; Chen, H.; Xiao, Y. L.; Lu, X. W.; Jiang, L. L.; Diao, S.; Yu, K.; Wang, Y.; Herranz, S.; Yue, Y. J.; Hong, X. C.; Hong, G. S.; Cheng, Z.; Dai, H. J.; Hsueh, A. J. Live imaging of follicle stimulating hormone receptors in gonads and bones using near infrared II fluorophore. Chem. Sci. 2017, 8, 3703–3711.
earth doped nanoparticles for near-infrared II imaging. *Nano Lett.* 2019, 19, 2985−2992.

(21) Zhai, C.; Schreiber, C. L.; Padilla-Coley, S.; Oliver, A. G.; Smith, B. D. Fluorescent self-threaded peptide probes for biological imaging. *Angew. Chem., Int. Ed.* 2020, 59, 23740−23747.

(22) Li, D.; Liu, Q.; Qi, Q.; Shi, H.; Hsu, E. C.; Chen, W.; Yuan, W.; Wu, Y.; Lin, S.; Zeng, Y.; Xiao, Z.; Xu, L.; Zhang, Y.; Stoyanova, T.; Jia, W.; Cheng, Z. Gold nanodusters for NIR-II fluorescence imaging of bones. *Small* 2020, 16, 2003851.

(23) Xiong, H.; Zuo, H.; Yan, Y.; Occhialini, G.; Zhou, K.; Wan, Y.; Siegwart, D. J. High-contrast fluorescence detection of metastatic breast cancer including bone and liver micrometastases via size-controlled pH-activatable water-soluble probes. *Adv. Mater.* 2017, 29, 1700131.

(24) Hanahan, D.; Weinberg, R. A. Hallmarks of cancer: The next generation. *Cell* 2011, 144, 646−674.

(25) Webb, B. A.; Chimenti, M.; Jacobson, M. P.; Barber, D. L. Dysregulated pH: a perfect storm for cancer progression. *Nat. Rev. Cancer* 2011, 11, 671−677.

(26) Urano, Y.; Asanuma, D.; Hama, Y.; Koyama, Y.; Barrett, T.; Kamiya, M.; Nagano, T.; Watanabe, T.; Hasegawa, A.; Choyke, P. L.; Kobayashi, H. Selective molecular imaging of viable cancer cells with pH-activatable fluorescence probes. *Nat. Med.* 2009, 15, 104−109.

(27) Wang, Y.; Zhou, K.; Huang, G.; Hensley, C.; Huang, X.; Ma, X.; Zhao, T.; Sumer, B. D.; DeBerardinis, R. J.; Gao, J. A nanoparticle-based strategy for the imaging of a broad range of tumours by nonlinear amplification of microenvironment signals. *Nat. Mater.* 2014, 13, 204−212.

(28) Wang, L.; Fan, Z.; Zhang, J.; Changyi, Y.; Huang, C.; Gu, Y.; Xu, Z.; Tang, Z.; Lu, W.; Wei, X.; Li, C. Evaluating tumor metastatic potential by imaging intratumoral acidosis via pH-activatable near-infrared fluorescent probe. *Int. J. Cancer* 2015, 136, E107−116.

(29) Xiong, H.; Kos, P.; Yan, Y.; Zhou, K.; Miller, J. B.; Elkassih, S.; Siegwart, D. J. Activatable water-soluble probes enhance tumor imaging by responding to dysregulated pH and exhibiting high tumor-to-liver fluorescence emission contrast. *Biocatalytic Chem.* 2016, 27, 1737−1744.

(30) Grossi, M.; Morgunova, M.; Cheung, S.; Scholz, D.; Conroy, E.; Terrile, M.; Panarella, A.; Simpson, J. C.; Gallagher, W. M.; O’Shea, D. F. Lysozyme triggered near-infrared fluorescence imaging of cellular trafficking processes in real time. *Nat. Commun.* 2016, 7, 10855.

(31) Gui, L.; Yuan, Z.; Kassaye, H.; Zheng, J.; Yao, Y.; Wang, F.; He, Q.; Shen, Y.; Liang, L.; Chen, H. A tumor-targeting probe based on a mitophagy process for live imaging. *Chem. Commun.* 2018, 54, 9675−9678.

(32) Takahashi, S.; Kagami, Y.; Hanaoka, K.; Terai, T.; Komatsu, T.; Ueno, T.; Uchiyama, M.; Koyama-Honda, I.; Mizushima, N.; Taguchi, T.; Arai, H.; Nagano, T.; Urano, Y. Development of a series of practical fluorescent chemical tools to measure pH values in living samples. *J. Am. Chem. Soc.* 2018, 140, 5925−5933.

(33) Wang, Z.; Xia, H.; Chen, B.; Wang, Y.; Yin, Q.; Yan, Y.; Yang, Y.; Liu, J.; Liu, W.; Zhang, Q.; Wang, Y. pH-Amplified CRET nanoparticles for in vivo imaging of tumor metastatic lymph nodes. *Angew. Chem., Int. Ed.* 2021, 60, 14512−14520.

(34) Schroeder, A.; Heller, D. A.; Winslow, M. M.; Dahlman, J. E.; Pratt, G. W.; Langer, R.; Jacks, T.; Anderson, D. G. Treating metastatic cancer with nanotechnology. *Nat. Rev. Cancer* 2012, 12, 39−50.

(35) Zhu, S.; Tian, R.; Antaris, A. L.; Chen, X.; Dai, H. Near-infrared-II molecular dyes for cancer imaging and surgery. *Adv. Mater.* 2019, 31, 1900251.

(36) Li, C.; Chen, G.; Zhang, Y.; Wu, F.; Wang, Q. Advanced fluorescence imaging technology in the near-infrared-II window for biomedical applications. *J. Am. Chem. Soc.* 2020, 142, 14789−14804.

(37) Lei, Z.; Zhang, F. Molecular engineering of NIR-II fluorophores for improved biomedical detection. *Angew. Chem., Int. Ed.* 2021, 60, 16294−16308.