Near-infrared-labeled tetracycline derivative is an effective marker of bone deposition in mice

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ABSTRACT

Bone-specific compounds have been used effectively for the detection of bone mineralization, growth, and morphological changes. These agents typically contain iminodiacetic acid groups that can form complexes with apatite and fluoresce in the visible spectrum. We exploited a subset of these chemical chelators to produce a near infrared (NIR) optical bone marker for preclinical animal imaging. By conjugating target compounds to IRDye® 800CW, we extended the effective fluorescence signal detection to the NIR region without affecting the compound’s ability to function as a marker of the mineralization process. Calcein and a tetracycline-derivative (BoneTag™ agent; BT), bound specifically to differentiated mineralized osteoblast cultures, with the latter exhibiting 6-fold higher signal intensities. Subsequent in vivo testing demonstrated effective skeletal labeling with IRDye 800CW BT. We were able to identify a changing mineralization front in bone sections from 1) normal growing mice injected with IRDye 800CW BT six weeks prior to the administration of IRDye 680 BT and 2) an osteoporosis mouse model comparing cortical bone in sham-treated and ovariectomized mice. These results provide evidence that the NIR-labeled BT is effective as a general marker of skeletal features and an indicator of the bone mineralization and remodeling processes.

Keywords: IRDye® 800CW
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Near-infrared
Molecular imaging
Whole animal imaging
Fluorescence microscopy
Immunofluorescent assay
Mineralization
Bone is a dynamic tissue that undergoes continuous remodeling in response to diverse stimuli such as circulating hormone fluctuations or mechanical weight-bearing exercise. The process of bone remodeling is initiated by osteoblasts, which are bone-forming cells that also stimulate the differentiation of osteoclasts for resorption of bone prior to its new deposition and mineralization [1,2]. Numbers and activities of osteoblasts and osteoclasts are tightly controlled by extracellular cytokine levels, to which these cells also contribute, and by matrix receptor-ligand interactions, such that a balance of the two cell types is normally maintained. Perturbations to the balance can occur in response to genetic, mechanical or biological events. The consequences may include microscopic bone damage, or severely compromised bone architectural remodeling, leading to osteoporosis, atherosclerosis, and extreme bone pain [3-6].

To investigate mechanisms of aberrant bone remodeling, a number of different fluorescent stains that are able to detect and quantify bone mineralization have been developed. Among the most successful of these are divalent cation chelating agents such as calcine, DCAF (2,4-bis[N,N′-di(carboxymethyl)–aminomethyl]fluorescein) [7-9], tetracycline and its derivatives [8,10,11], Alizarin red derivatives [8,10,11], Xylenol orange [8,13], or bone-targeting drugs such as pamidronate [14]. Incorporation of these probes involves direct binding to areas undergoing calcification at the bone/osteonectin (unmineralized bone) interface. These fluorescent agents all have the spectral limitation of excitation/ emission spectra that reside in the visible region, which is not ideal for noninvasive animal imaging. Aminobisphosphonates, which are used to treat osteoporosis and cancer bone metastasis, also have been labeled and used for fluorescent imaging, because these readily bind hydroxyapatite, inhibiting osteoclast resorption of bone matrix. However, because this chemical class interferes with bone biology, its utility as a probe for dynamic bone properties is limited.

Near-infrared (NIR) optical imaging has been applied with considerable success to the noninvasive and longitudinal visualization of dynamic processes in vivo. We have previously exploited the superior sensitivity and extremely low background [15-19] of the NIR dye, IRDye® 800CW (ex 774 nm; em 805 nm) to investigate disease progression mechanisms and assess relative molecular changes that reflect efficacy of a particular drug or compound. Applications for a NIR bone-targeting agent include detection of microcalcification [20], interrogation of remodeling and deposition of mineralized bone during normal development or in the context of cancer and disease progression, and the evaluation of bone tissue changes in disease models such as osteoporosis. Osteoporosis is the direct result of an imbalance between osteoblast and osteoclast activity and is a relevant disease state in which to examine an optical imaging agent associated with bone mineralization. As an animal model for preclinical testing of agents to treat osteoporosis, the U.S. Food and Drug Administration recommends the use of ovariectomy-related osteopenia in mice [21], so we adopted this model for the studies described here.

In this study, our goal was to develop and validate an NIR bone labeling agent that could be used to visualize and monitor rates of change in mineralized bone. We tested several compounds for optimal labeling efficiency and stability after conjugation to IRDye 800CW. Fibroblasts and differentiated osteoblasts were used in cell-based screening assays to confirm that the labeled IRDye 800CW conjugates bound specifically to bone-derived cells that synthesized mineralized matrix in culture, and to identify the compound with greatest affinity. We optimized dose and validated the selected compound in vivo using ovariectomized (OVX) nu/nu mice imaged noninvasively to provide evidence that the labeled agent was specifically deposited in bone tissues. Subsequently, excised skeletal tissue was sectioned for NIR fluorescence microscopic imaging. We obtained high resolution images of the label location in femur, tibia, and vertebrae, highlighting differences in bone mineralization between normal and OVX mice. The resulting probe allows anatomic visualization of bone structure for localization, and is a useful biomarker for monitoring bone dynamics and changes in bone morphology.

**MATERIALS AND METHODS**

**Cell Culture, Reagents, and Instrumentation**

NCCLS (National Committee for Clinical Laboratory Standards)-recommended quality control organisms, *Escherichia coli* (gram negative; ATCC 25922), *Staphylococcus aureus* (gram positive; ATCC 25923), and *Pseudomonas aeruginosa* (gram negative; ATCC 27853) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). MC3T3-E1
(subclone 4) preosteoblast and A431 epidermoid carcinoma cell lines were also purchased from ATCC and maintained in alpha minimum essential medium, supplemented with ribonucleosides, deoxyribonucleosides, 2 mM L-glutamine, and 1 mM sodium pyruvate (GIBCO, Carlsbad, CA), and 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), but lacking ascorbic acid. MC3T3-E1 cells were differentiated from preosteoblasts using L-ascorbic acid and sodium monobasic phosphate [22]. Tetracycline (T-4062), 10% neutral buffered formalin (HT50-1-2), silver nitrate (209139), and Alizarin red S (A5533) were purchased from Sigma-Aldrich (Dallas, TX, USA). IRDye® 800CW NHS ester, Odyssey® Blocking Buffer, an Odyssey Infrared Imaging System, an Aerius Automated Infrared Imaging System, and a Pearl® Imager were provided by LI-COR Biosciences (Lincoln, NE, USA). A Synergy 4 Microplate Reader was purchased from Biotek Instruments (Winooski, VT, USA).

**Synthesis of bone-specific agents**

IRDye 800CW NHS ester was conjugated via a primary aliphatic amine to calcium-chelating agents calcein and tetracycline or a tetracycline derivative (BoneTag agent [BT]). The BT was designed to have a suitable amine group remote from the chelating portion of the molecule. For calcein, the amine linker for the labeling was introduced by an initial reaction of an activated carboxylate with hexamethylene diamine. The incubation period for IRDye 800CW NHS ester binding to the amine of each moiety was approximately 45 min at ambient temperature in 1 M potassium phosphate buffer at pH 8.5. The labeled conjugate was purified by high-performance liquid chromatography (HPLC) using an Omnifit column, and monitored at 260 nm and either 680 or 780 nm. The system was equilibrated with ultrapure water and 500 mM sodium phosphate buffer (PBS, pH 7.0) and was eluted at 2.5 mL/min (~22 bar pressure) using a gradient of 100% ultrapure water and then 0 to 50% 500 mM PBS (pH 7.0) for 25 min. The sample was desalted and passed through a 0.2 µm PES (polyethersulfone) syringe filter into a sterile container. The IRDye 800CW BT and calcein conjugates were aliquoted and lyophilized.

The minimum inhibitory concentration was used to compare antibiotic activity before and after conjugation with the fluorophore, using a range of 0 - 128 µg/mL for tetracycline, tetracycline derivative, and IRDye 800CW BT. An inoculum was prepared from single colonies amplified in liquid culture until visual comparison was confirmed to match the 0.5 McFarland Standard (10^6 cfu/mL) and the turbidity standard visual comparison card. Once concentrations were established, 10^6 cfu/mL of bacterial suspension was added to 2 mL of media containing antibiotic series. Cultures were incubated for 18 hours at 37°C with shaking. Tubes were screened for effective inhibition of bacterial growth using the 0.5 McFarland standard and the turbidity standard visual comparison card [23].

**Cell-based assays**

IRDye 800CW BT and IRDye 800CW calcein binding was evaluated by immunofluorescent cell-based assay. MC3T3-E1 preosteoblasts were differentiated by culture in 50 µM ascorbic acid (AA), followed by addition of 3.0 mM inorganic phosphate. Initial characterizations of MC3T3-E1 cells and their subclones showed that the concentration of phosphate added during differentiation in culture is critical to achieve mineralization of matrix in the culture that resembles woven bone rather than non-collagenous mineral deposition that does not pertain to bone formation [22]. Therefore, we selected a phosphate concentration appropriate to organized collagen mineralization. Osteoblasts were incubated (24 h) with increasing concentrations of IRDye 800CW conjugated to either calcein or BT (0 - 100 nM), and compared for binding with their undifferentiated counterparts. The assays were stopped by fixation with 4% formaldehyde solution for 20 min, followed by four washes in 1X PBS + 0.02% Triton X-100 to remove unbound dye and permeabilize the cells. The plates were blocked in Odyssey Blocking buffer for 1.5 h and incubated for an additional hour with TO-PRO-3 DNA stain (ex/em: 642/669 nm) diluted 1:5000 for normalization of cell number. Washing steps were repeated with Odyssey Blocking buffer + 0.02% Tween® 20 and the plate was scanned with an Aerius Automated Infrared Imaging System. To confirm specificity of binding, we performed a competitive challenge analysis by preincubation with 0.02 - 10 µM Tetracycline or Tetracycline derivative for 30 min, followed by IRDye 800CW BT incubation overnight. Samples were run in triplicate. Fluorescence intensity of IRDye 800CW BT (800 nm), indicating mineralization, was normalized by ratio-metric analysis to the signal from TO-PRO-3 DNA stain (700 nm), indicating cell number.

Mineralization in cell culture was confirmed by Alizarin red S staining procedures [24]. Briefly, Alizarin red S (2%) was dissolved in distilled water, and pH adjusted to 4.1 - 4.3 using 0.5% ammonium hydroxide. Cultures were fixed with 10% neutral
Figure 1. (A) Confluent cultures of undifferentiated and differentiated MC3T3-E1 cells (subclone 4) were subjected to Alizarin red S staining that confirmed the presence of mineralized deposits. (B) Representative fluorescent microscopic images for undifferentiated and differentiated MC3T3-E1 (subclone 4) cultures presented at the same scale (20X). (C) IRDye® 800CW BT (visible at 800 nm) binds preferentially to osteoblasts following differentiation in culture. Confluent cultures of undifferentiated and differentiated MC3T3-E1 cells (subclone 4) were incubated in serum-free media (alpha minimum essential medium) containing increasing concentrations of IRDye 800CW BT (0 - 100 nM). After 24 h, cells were fixed, permeabilized, blocked, counterstained with TO-PRO-3 (visible at 700 nm), and imaged. Relative Fluorescent Units (RFU) were determined by applying a ratiometric analysis of fluorescence (800 nm normalized to 700 nm signal) in triplicate wells. Data are plotted as means ± standard deviations.

Figure 2. Osteoblasts have significantly higher affinity for IRDye 800CW conjugates than tumor cells, and exhibit preferential binding to the conjugated BT. A431 cells (A) or differentiated MC3T3-E1 cells (B) were incubated with 100 nM IRDye 800CW BT or IRDye 800CW calcein for 1 h, then fixed, permeabilized, blocked, counterstained with TO-PRO-3 (visible at 700 nm), and imaged. Relative Fluorescent Units (RFU) were determined by applying a ratiometric analysis of fluorescence (800 nm normalized to 700 nm signal) in triplicate wells. Data are plotted as mean ± STDEV.
buffered formalin (30 min), washed, and stained with Alizarin red S solution for 10 - 15 min. Unincorporated dye was removed by washing with distilled water. The absorbance signal from calcium deposits was measured at 540 nm. Data were normalized to DNA/RNA 260/280 nm readings.

**Ovariectomy (OVX)**

All animals used in the imaging experiments were cared for and maintained under the supervision and guidelines of the University of Nebraska-Lincoln Institutional Animal Care and Use Committee. Mice were maintained on a purified maintenance diet (AIN-93M) obtained from Harlan Teklad (Madison, WI, USA). All mice were anesthetized with 2% isoflurane throughout all procedures. Female athymic nude (nu/nu) mice were obtained from Charles River Laboratories, Inc. (Cambridge, MA, USA) at 8 wks of age. Animals were split into two treatment groups, sham-treated and OVX. Isoflurane-anesthetized animals were aseptically prepared. Ovaries were exposed by dorsal incision and retraction of the paraovarian fat pad. The ovary and associated oviduct were severed and removed. The incision was closed with 5-0 nonabsorbable suture in an interrupted pattern or wound-clip (stainless steel) [25]. Sham treatments followed the same surgical procedure without removal of the ovaries. Animals were maintained for an additional 8 weeks postsurgical procedure. For all imaging experiments, the fluorescent contrast agent was injected intraperitoneally. Animals were imaged and sacrificed 48 h after injection of IRDye® 800CW BT. Femora, tibiae, and vertebrae were removed for tissue sections. Mouse uterine weights were removed to evaluate the effects of ovariectomy.

An additional 2 athymic nude mice received IRDye 800CW BT, followed 42 d later with an injection of IRDye 680 BT to assess the feasibility of identifying active mineralization fronts over time. Five days after the second dye injection, mice were sacrificed and tibia and femur bones were excised and prepared for paraffin embedding as described previously.

**Fluorescent microscopy of IRDye 800CW BT deposition**

Bones were fixed in Z-fix (Anatech, Battle Creek, MI, USA) for 48 hours followed by paraffin embedding. Sections (8 µm) were imaged by fluorescent microscopy using an Olympus IX81 inverted microscope equipped with a halogen bulb and NIR filters (EX:710/75, DC:760LP, EM:810/90; Chroma Technology Corp., Rockingham, VT, USA). Cortical thickness was visualized at the mid-diaphyseal region of the femur for control and OVX mice.

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**Figure 3.** Bone labeling by IRDye 800CW BT yields higher intensity bone-specific fluorescence and more rapid systemic clearance than the calcein conjugate. Male athymic nude mice were injected intraperitoneally with 2 nmol of either IRDye 800CW BT (A) or IRDye 800CW calcein (B). Dorsal, ventral, and lateral view images were captured approximately 24 h post injection. White arrows in the dorsal and lateral views indicate kidney location.
RESULTS

Cell-based assays
To identify a suitable bone-targeting agent for development into an effective noninvasive imaging probe, we selected a subset of strong calcium chelators and chemically coupled them to the high-quantum-yield fluorophore IRDye® 800CW. The conjugates were first screened in vitro for their ability to distinguish between undifferentiated MC3T3-E1 (subclone 4) pre-osteoblasts and the same cell line following differentiation with ascorbic acid and inorganic phosphate treatment, which induces mineralization of a collagenous extracellular matrix [22]. The presence of mineralized calcium deposits after differentiation was first confirmed by Alizarin red S staining normalized to nucleic acid content (260/280 nm) (Figure 1A). Approximately 2.5-fold increased signal reflected the enrichment of mineralized deposits in the differentiated cultures (Fig 1B). Cells were then seeded in a micro-well format and incubated with increasing concentrations of IRDye 800CW BT (Figure 1C) or IRDye 800CW calcein (not shown). Binding of the BT conjugate to MC3T3-E1 cells was dose-responsive regardless of differentiation. However, the signal intensity for the BT conjugate binding to fully differentiated osteoblasts was 5- to 10-fold higher at all doses relative to the undifferentiated cells, supporting a specific function of the targeting chelator in adherence of the conjugate to mineralized bone-like matrix. Similar results were obtained using IRDye 800CW calcein. These results also validate use of undifferentiated-versus-differentiated MC3T3-E1 subclone 4 cells as a screening system for specific binding of labeled test agents to a mineralized matrix.

Targeting specificity of the labeled chelating agents was next evaluated by comparing binding affinity to cultured A431 cells (epidermoid cell line, negative control) relative to fully differentiated MC3T3-E1 cells. Neither calcein nor BT, conjugated to IRDye 800CW fluorophore, conferred significant binding or signal intensity to the A431 cells at a concentration of 100 nM (Figure 2A). Although a higher level of non-specific binding to A431 cells was noted for the BT conjugate than for the calcein conjugate, this signal intensity was only ~4% of the signal from differentiated MC3T3-E1 cells at the same concentration (Figure 2B). Importantly, the labeled BT exhibited at least 20-fold higher signal intensity compared to labeled calcein upon incubation with differentiated MC3T3-E1 cells, confirming the specificity of this targeting agent for bone-derived cells in a mineralized matrix context.

The BT compound, as a tetracycline derivative, is a broad-spectrum antibiotic and an inducer of gene expression in engineered systems. Since residual biological function could complicate in vivo studies that make use of these inducible systems, we compared...
the antibiotic efficacy of the BT before and after conjugation, using the minimum inhibitory concentration as a quantifier. The lowest concentrations of tetracycline and unlabeled BT required to inhibit visible growth of P. aeruginosa (gram negative), S. aureus (gram positive), and E. coli (gram negative) were <16 µg/mL, 1 µg/mL, and <2-3 µg/mL, respectively. In contrast, the lowest concentration of IRDye® 800CW BT required to inhibit visible growth of P. aeruginosa, S. aureus, and E. coli was >128 µg/mL in all test organisms, confirming significant loss of activity despite its strongly retained function as a mineral chelator.

**In vivo Comparison of bone-targeting agents**

To identify the most effective bone-targeting agent among those we tested, we performed a direct comparison in mice. Athymic nude mice (~0.03 kg) were injected intraperitoneally with 2 nmol (10 µM; 0.1 mg/kg) of either IRDye 800CW BT (Figure 3A) or IRDye 800CW Calcein (Figure 3B) and imaged after a 24 h clearance period. Strong fluorescence signal is visible in all parts of the skeleton in both of the representative animals shown in dorsal, ventral and lateral views. However, the signal-to-background ratio was higher for the animals receiving IRDye 800CW BT, both as a result of lower irrelevant tissue background and of higher skeletal signal. In addition, whereas the BT conjugate did not accumulate significantly in non-bone tissues, the IRDye 800CW calcein probe gave rise to intense fluorescent kidney signals (indicated by arrows in dorsal and lateral views, Figure 3). The higher background signal and clearance pattern of the calcein agent are consistent with instability of the chemical linkage between the calcein and IRDye 800CW components, leading to a higher rate of premature dissociation. Because the characteristics of IRDye 800CW BT were more favorable in vivo, we selected this agent for further evaluation and optimization.

**Dose Determination**

To determine an optimal targeting dose of IRDye 800CW BT for high signal to background in noninvasive imaging of skeletal features, we admin-

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**Figure 5.** Microscopic comparison of IRDye 800CW BT agent incorporation into femora of sham-treated and OVX mice indicates measurable differences in cortical bone labeling and thickness. (A-D) Formalin-fixed paraffin-embedded femora from sham-treated (A and B) and OVX (C and D) female athymic nude mice sacrificed 48 h following intravenous administration of IRDye 800CW BT were sectioned for examination by near infrared fluorescence microscopy. Representative images of mid-shaft femur sections (8 µm) are shown at 20X (A and C) or 60X (B and D) magnification. (E) A dual-wavelength Odyssey-scanned image (21 µm) of a sham femur cross-section illustrates both cortical and trabecular labeling. IRDye 800CW BT is depicted in green, and tissue autofluorescence is shown in red. Yellow represents regions of overlap between red and green. (F) Dual color labeling reflects the temporal span between bone mineralization fronts. A femur was excised, formalin-fixed, paraffin-embedded and sectioned (8 µm) from an athymic nude mouse injected first with IRDye 800CW BT (green, indicated 1st), followed 42 days later with IRDye 680 BT (red, indicated 2nd). The image was captured at 20X magnification. TB = trabecular bone; CB = cortical bone. Look-up table indicators are inset on panels A-D and F.
istered increasing concentrations of the agent intravenously and imaged 24 h later. As expected, fluorescence signal was minimal in animals receiving only a vehicle injection (Figure 4A). Increases in signal intensity were evident in all views upon injection of 1, 2, or 3 nmol IRDye® 800CW BT, shown in representative dorsal views of the skull and vertebrae (Figures 4B, 4C and 4D, respectively). Higher resolution imaging of a skull from an animal given 2 nmol of the agent, with signal-dampening skin removed, revealed the fine detail of bone morphology in the skull (Figure 4E), illustrating the potential efficacy of the agent for detecting gross morphological changes in skeletal integrity. We imaged mice up to six weeks post-injection of a single 2 nmol intravenous dose of IRDye 800CW BT and saw that the deposition and fluorescence intensity of this agent will persist for this period at levels nearly identical to those measured after 24 h (Figure 4F). The overall signal intensities are slightly lower at this time point, but the signal-to-background ratios measured for various regions are consistent over time (not shown).

**Microscopic quantification of bone loss following ovariectomy**

To examine the potential utility of IRDye 800CW BT as a measure of bone remodeling, we performed both static and dynamic histomorphometric analysis. Female athymic nu/nu mice undergoing sham surgeries were compared with OVX mice, representing a reliable model for osteoporotic bone loss. In the first group of animals, a single injection of IRDye 800CW BT was administered 8 weeks after surgery. Animals were sacrificed 24 h later and tissues were harvested for sectioning. Consistent with successful ovariectomy, uterine weights in OVX nu/nu mice were 24% lower when compared with the sham-treated group. No effect of treatment was noted for femur or tibia weights; however, differences in cortical bone morphology were readily detected between sham (Figures 5A and 5B) and OVX (Figures 5C and 5D) longitudinal femur sections. Animals that had been ovariectomized 8 weeks earlier had significantly less label present in the cortical bone than did those that had received sham treatment (Figure 5C compared with 5A). A 21-µm Odyssey® scan of a femur cross-section showed that both cortical and trabecular bone tissues were labeled by IRDye 800CW BT (Figure 5E). When representative sections from sham-treated and OVX animals were deparaffinized and evaluated microscopically at 60X magnification, the morphology and labeled probe deposition indicated an increase in cortical bone depth for the sham-treated group (cf. Figures 5B and 5D). Dynamic histomorphometry was performed on a second group of mice in which IRDye 800CW BT (shown in green, labeled as the 1st deposited front) was administered 42 days prior to an injection of IRDye 680 BT (shown in red, labeled 2nd). Microscopic analysis of longitudinal femoral sections revealed a distinct span between mineralization fronts that incorporated each agent (Figure 5F). These results collectively demonstrate that the administration of these NIR bone labeling reagents can facilitate histomorphometric characterization of cortical bone status in both static and dynamic assay contexts, which is essential to the examination of bone remodeling mechanisms.

**DISCUSSION**

Visualization, quantification and monitoring of dynamic changes in bone tissue afford many insights into effects of dietary, mechanical, and disease influences on normal bone turnover. In this study, we identified and validated a novel bone targeting compound that is highly specific for mineralized bone and can be dynamically incorporated into bone during periods of remodeling. These properties are compatible with long-term, stable conjugation to the NIR fluorophores IRDye 800CW and IRDye 680, and allow the labeled compound to persist in mouse bone with no loss of fluorescence signal over a period of many weeks. The potential for dual-wavelength imaging following alternate injections of IRDye 800CW BT and IRDye 680 BT further advances the utility of this targeting agent, since its incorporation can be examined histomorphometrically to visualize changes in local bone architecture that occurred over an extended period in the animal.

Our efforts to generate an effective bone-binding agent using calcein proved to be problematic because of an unstable chemical linkage chemistry between calcein and the NIR fluorophore that allowed the dye to uncouple from the calcein moiety and clear through the kidneys. Although the attachment occurred via an amide bond, which is normally a very stable linkage, neighboring carboxylic acid groups in the calcein molecule promoted hydrolysis of the amide under relatively mild conditions. Long-term storage of the probe in solution was also compromised by lability of the Ar-CH2-N linker in calcein, which conjoins the fluorescent dye portion of the molecule to its carboxyl chelating groups. Chemical labeling of calcein yielded a mixture of products (e.g., various isomers, imide linkage to IRDye 800CW
rather than amide) that were not fully characterized due to extensive heterogeneity and variable stability among the components. The linkage chemistry in the tetracycline derivative, which we have termed the BT agent, was designed to avoid these issues. Stability of attachment between the IRDye® 800CW fluorophore and BT was significantly improved. We demonstrated that the chemical conjugation had the advantage of eliminating the inhibitory or antibiotic activity of the unconjugated BT, thereby ensuring there would be no interference of the BT conjugate arising from its antibiotic properties or with tetracycline-inducible gene expression systems used in animal studies. In addition, high signal-to-noise ratios were achieved with IRDye 800CW BT because there was relatively little non-specific binding in non-bone tissue, and because the 800 nm optimal detection wavelength is a region of low inherent tissue auto-fluorescence in the animal.

We selected an animal model of osteoporosis as a relevant pathological context in which to evaluate the capacity of IRDye 800CW BT conjugate to be a measurable indicator of bone mineralization. After women reach menopause (between 45 and 55 years of age) bone resorption may begin to exceed bone formation, resulting in diminished overall quantity of mineralized bone and an increased risk for the development of osteoporosis. The OVX rodent is the most commonly used animal model of osteoporosis [21,26]. The disease condition is simulated by reduction in the animal’s circulating estrogens, which has been shown to affect bone mass significantly [27]. Although rats are more frequently used, athymic nu/nu (T-lymphocyte-deficient) mice have also demonstrated trabecular and cortical bone loss following ovariectomy [28,29]. We were initially interested in determining whether ovariectomy and subsequent loss of mineralized bone would be detectable by noninvasive imaging. However, in the time course over which our analysis was performed, we were not able to see reproducible differences in total animal or regional skeletal fluorescence using either images from the intact animals or from the excised femora. Nonetheless, when bones were sectioned and examined histomorphometrically, we were able to clearly observe significant mineralized bone loss that could be measured both by reduced intensity of the IRDye 800CW BT fluorescence signal and by the obvious morphological changes in the mineralization front.

An additional component of our validation studies was the introduction of alternating fluorophore deposition by conjugating the BT to two different NIR fluorophores and injecting them at two different time points. Dual labeling discrete layers of mineralizing bone in this manner was intended to facilitate temporal evaluation of bone formation [30], which has particular application for monitoring tissue-engineered bone remodeling in metabolic or systemic diseases, for example. Here we effectively labeled the changing mineralization front in normal growing nude mice by administration of IRDye 800CW BT and IRDye 680 BT 6 wks apart. Fluorescent microscopic analysis of sectioned bones clearly revealed the temporally separated deposition of our labels as spatially distinct layers of uniquely colored fluorescence. These results are similar to a previously published demonstration of a dynamic histomorphometric analysis after tetracycline/calcein double labeling in ganciclovir-treated mice [31]. The use of identical targeting agents conjugated to different fluorophores, however, has the advantage of eliminating potential concerns about agent deposition being the sole result of differing binding affinities between the targeting agents. This would be particularly significant if multiple successive bone deposition layers were analyzed as a measure of rates of change in bone growth or loss.

It is worth noting that tetracyclines have been shown to inhibit osteoclast formation in rats [32]. These findings could be perceived as contraindicative for use of the label we describe in studies of dynamic bone remodeling processes. However, subtle differences in the actual tetracycline derivative structures give rise to significantly varied inhibitory and/or anabolic effects. A key factor affecting the outcome is that the dose required for imaging of bone mineralization in an animal is multifold lower than a standard therapeutic dose of tetracycline. Imaging in the NIR allows us to further reduce doses because of the minimal background fluorescence at this wavelength.

In summary, our results support use of BT, conjugated to NIR fluorophores with strong signal intensity, for anatomic orientation and skeletal definition in the noninvasive context. Additionally, targeting of BT to mineralized bone in either a static or dynamic assay can be used to facilitate further investigation of bone remodeling mechanisms and pathologies, where such markers are essential to visualize osseointegration of implants or to assess healing of small bone defects. Possibilities are also open for dual probe analyses with agents specific to bone and tumor tissue.
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