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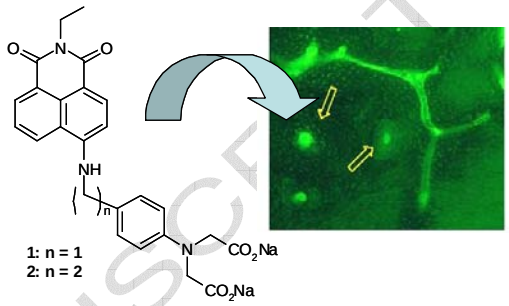

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LETTERS

Fluorescence imaging of bone cracks (microdamage) using visibly emitting 1,8-naphthalimide-based PET sensors

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Abstract—The ability of two 4-amino-1,8-naphthalimide (**1** and **2**) and anthracene (**3**) based photoinduced electron transfer (PET) sensors, previously developed in our laboratory, to function as selective imaging agents for exposed Ca(II) in bone cracks, using fluorescence microscopy imaging is described. While the emission from **3** is masked by the autofluorescence arising from the (bovine) bone matrix, both **1** and **2**, emitting in the green, are able to clearly identify areas of damaged bone.

The occurrence of micro-cracks in bones has profound biological effects, as they play a role in the normal turnover process as well as in the adaptive behaviour of bone.[1,2] The repercussions of bone-damage depends on how the structure-function relationship is affected by damage.[3] Therefore, it is very important to be able to characterise bone damage and to obtain a thorough understanding of the factors responsible for the various mechanical, structural and functional properties of bone.[1,4] Three independent methods are generally used to analyse microdamage in bone. These are: a) mechanical characterisation based on property degradation; b) real-time characterisation by using acoustic emission and Raman spectroscopy and c) physical characterisation using histological, and histomorphometric methods.[5] However, these methods have significant drawbacks, as one of the main problems with bone imaging is that the mixture of organic matrix and crystalline hydroxyapaptite makes it very difficult to distinguish the contrast agent from the (healthy) surrounding bone.[6] Consequently, their currently exists a real need for developing a targeted approach to bone analysis and imaging.

We have developed several examples of novel contrast agents for bone structure analysis.[7] These systems were synthesized in a few high yielding steps, where exposed Ca(II) sites in the bone matrix were targeted, using phenyliminodiacetate as a Ca(II) chelator, linked *via* an amide to a triiodo benzene skeleton. Using bovine bone samples and computer tomography (CT), their potential use as selective CT imaging agents was explored.[8] The idea of achieving a more targeted approach to such imaging using fluorescence reagents was also investigated with some success, using commercially available dyes such as calcium orange and fluo-3.[9] However, the ability to detect selectively microdamage, either on the surface, or within the bone-matrix, using easily synthesised and highly targeted fluorescent sensors/imaging agents, that have high affinity for exposed Ca(II) sites and emit within the visible region has, to the best of our knowledge, not yet been satisfactorily achieved.

As part of our ongoing research programme into the development of luminescent and colorimetric sensors,[10,11] the PET sensors **1-3**, shown in Figure 1, were prepared.[12,13]

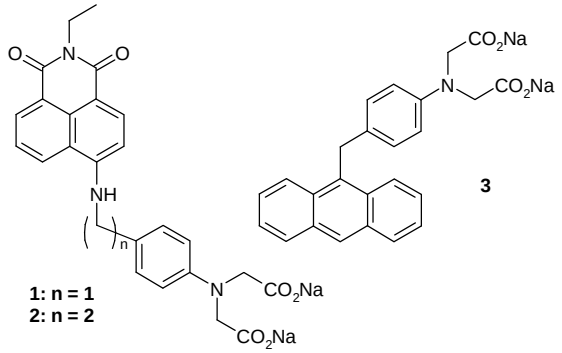

Figure 1. Structures of PET sensors **1-3** employed in the current study.

These structures are based on the use of the *fluorophore-spacer-receptor* model developed by de Silva *et al*.[14] where the phenyliminodiacetate receptor used in the above CT-contrast agents was employed. In competitive aqueous

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solution, these sensors showed very different ion selectivity, where the long-wavelength emitting sensors **1** and **2** (arising from their Internal Charge Transfer (ICT) excited state), which only differ in the length of the alkyl spacer unit, displayed excellent selectivity and sensitivity towards physiological concentrations of free Zn(II). The anthracence-based PET sensor **3**, showed excellent selectivity for Cd(II) at pH 7.4, which demonstrated that the ion-selectivity of such PET sensors is highly dependent, not only on the structure of the receptor, but also on the nature of the fluorophore employed. As the hydroxyapatite matrix of bone possesses gel-like properties and has the ability to incorporate substances containing carboxylates, we decided to investigate the ability of compounds **1-3** to label micro-cracks or bone-scratches. The carboxylate groups in **1-3** should be able to interact with the chemical components of the damaged bone lattice, providing selective labelling of any cracks.

The syntheses of **1-3** have previously been reported by us and in the case of the 4-amino-1,8-naphthalimide based sensors **1** and **2**,[12] these were formed in a few steps, involving incorporation of the iminodiacetate moiety on the aniline using ethyl bromoacetate, followed by hydrolysis. In the case of **3**, the iminodiester was prepared in a single-step by Friedel-Crafts alkylation using 9-chloro-methylanthracene, in high yield, followed by hydrolysis of the esters.[13]

We envisaged that within the bone structure, these sensors could potentially bind to exposed Ca(II) sites *via* the iminodiacetate moiety, which would 'switch-off' the PET quenching process from the receptor to the fluorophore, with concomitant enhancement in the fluorescence of these structures. All of the scratch tests were performed on bone specimens using senosrs **1-3**, where 5 mm straight lines were scratched on the surface of a bovine bone sample. The bone samples were then dipped into a 10^{-4} M buffered pH 7.4 solution of each PET sensor in an individual vial, which was placed under vacuum (50 mmHg) for intervals of 5, 15, 30 and 60 minutes, respectively. All the specimens were washed using deionised water, with the aim of removing any excess sensor and examined using epifluorescence microscopy. The results showed that no significant changes were observed in the emission arising from the sensor-treated bone after 15 min.

All the sensors were shown to be able to bind selectively to exposed Ca(II) sites within the scratches generated on the surface of the bones as demonstrated in Figure 2. A blue emission arising from the bone surface (autofluorescence) was visible for all the examples, and in the case of **3** (Figure 2c), masks the emission arising from the anthracene excited state. In contrast, the green emission arising from the naphthalimide-based sensors **1** or **2** is clearly visible from the background, Figures 2a and 2b, respectively. This indicates that both of these sensors were able to label the entire scratch without affecting the surrounding bone area.

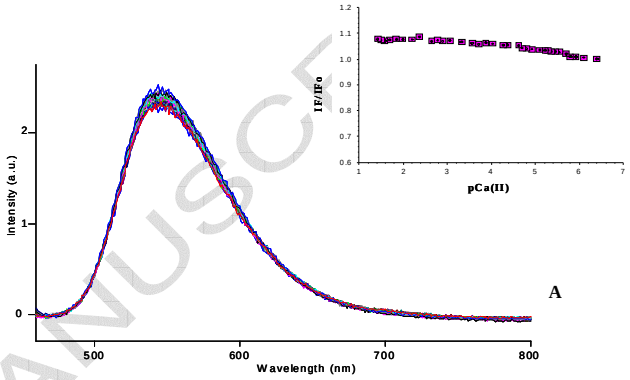

Figure 3. Fluorescence emission response of **1** upon titration with Ca(II) in buffered pH 7.4 solution, upon excitation at 442 nm. *Insert* The emission intensity plot at 550 nm vs. –log [Ca(II)].

Compounds **1-3** are all PET sensors, and in order to show noticeable emission, it is necessary to inhibit the PET process. Solution evaluation of **1** (Figure 3) and **2**, demonstrated that the emission was not "switched-on" in solution for either of these sensors, and was only slightly affected in the presence of up to a 10^{-2} M concentration of Ca(II), Figure 3 (see inset). However, the results observed from the bone scratch tests, demonstrate that the emission is clearly visible (or "switched-on"). Therefore it is reasoned that scratches give rise to exposed free Ca(II) vacancies in the bone lattice facilitating some form of binding. We have previously demonstrated, by using

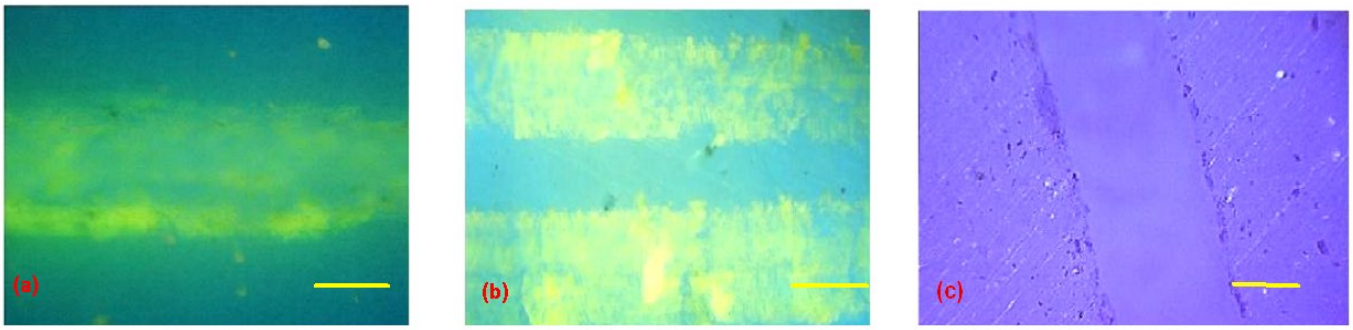

Figure 2. Scratched bone surface labelled with (a) **1**, (b) with **2** and (c) **3**; at 10 fold magnification using UV epifluorescence (excitation at 365 nm). Yellow bar = 100 μm

Energy Dispersive X-ray (EDX) analysis.[8] Such differences, between solution and solid state results have previously been reported, and are often thought be due to the ability of such sensors to adsorb, or bind, differently to their targeted analyte within different media, or the packing or organization within the media.[15] The changes seen in Figure 2, clearly show that only the scratched areas give rise to the green emission arising from the two PET sensors, hence, we assign this to direct binding of the sensors to the exposed Ca(II) sites within this area. Concomitantly, this blocks any PET from the electron-rich receptor to the excited stated of the naphthalimide component, as upon binding, the oxidation potential of the sensor is increased, giving rise to the intense imaging observed.[16]

To investigate further the ability of **1** and **2** to function as imaging agents, their ability to label, and hence image, the internal structure of the bone was also studied using a penetration test. As bone is a complex material that consists of canals, Haversian systems, canaliculi and resorption cavities, the ability of these sensors to penetrate the bone matrix are of considerable significance in understanding this complex morphology.[1] To achieve this, bone samples were prepared, where the bone samples were immersed in a 10^{-4} M solution of the sensors, and placed under vacuum (50 mmHg) for 24 hours. Transverse sections were then cut from each sample using a diamond saw, cleaned and polished with emery paper, and washed with deionised water before the samples were imaged using epifluorescence microscopy, by observation of the changes at both 365 and 546 nm. The results obtained from the labelling with **1**, are shown in Figure 4.

It is clear from Figure 4, that the internal structure of the bone, comprising osteons and interstial lamellae, are clearly imaged when viewed under green epifluorescence (546 nm), Figure 4b. It can be reasoned that these components may contain suitable vacancies (e.g. free Ca(II) sites) to facilitate binding to the receptor and inhibiting PET

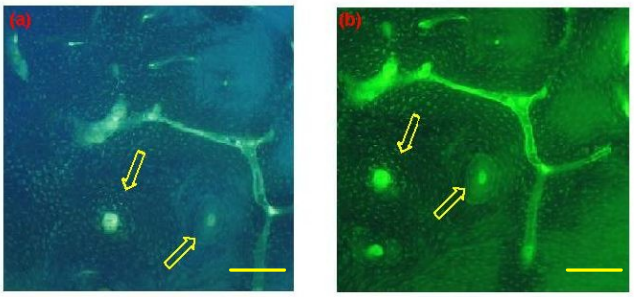

Figure 4. Transverse section of the bone labelled with **1**, viewed in (a) UV epifluorescence (365 nm) and (b) green epifluorescence microscopy (546 nm excitation); yellow arrow show osteons with its lacunae, Haversian canal and canaliculi. Bar = 100 μm

interaction with the sensor in a similar manner to that observed in the scratch test. Hence, these results demonstrate the ability of **1**, to selectively label the components of bone matrix, although these sensors do not show any significant binding ability to Ca(II) in solution as discussed above.

In summary, we have demonstrated that three PET sensors **1-3**, previously developed in our laboratory, possessing phenyliminodiacetate receptors can bind to areas within bone structures which are known to contain exposed Ca(II). While the emission from sensor **3**, which emits in the blue, was masked by the auto-fluorescence from the bone matrix, the binding of both **1** and **2**, gave rise to significant fluorescence. This emission, arising from the 4-amino-1,8-naphthalimide moiety, was only visible at the scratched areas of the bone specimens, demonstrating selective imaging of these areas. The ability of these sensors to label and image the internal structure of the bone was also examined. The results presented herein, clearly demonstrate the use of PET sensors in fluorescence imaging of bone structures. We are in the process of further evaluating the use of such targeting PET sensors as fluorescent imaging agents for bone structure analysis.

Acknowledgements

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