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Real-Time Activity Bioassay of Single Osteoclasts using a Silicon Nanocrystal - Impregnated Artificial Matrix

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Keywords: (Osteoclast, silicon nanocrystal, resorption, fluorescence, resorption assay)
Abstract

The lack of an in vitro real-time osteoclast (OC) activity assay has hampered mechanistic studies of bone resorption. We have developed such an assay, employing a hydroxyapatite matrix impregnated with alkyl-capped silicon nanocrystals, which is capable of monitoring the time-course of resorption by single osteoclasts. Resorption of the matrix by OC releases the nanocrystals, which are internalised by the cell and detected as an increase in OC luminescence. Our particular choice of nanocrystals was motivated by their bright pH-independent luminescence, proportional to concentration, and by their rapid uptake without cytotoxicity. In this in vitro assay, OCs are inhibited by calcitonin (CT) and methyl-β-cyclodextrin (MCD), and stimulated by RANKL in the expected manner. The kinetics of the assay exhibit a lag phase representing cell attachment and commencement of resorption processes, followed by a growth of cell luminescence intensity and the whole time-course is satisfactorily described by the logistic equation.

1. Introduction

Osteoclasts (OC) are bone cells with the unique and important ability to perform extracellular bone dissolution. They play a critical role in the pathogenesis of a number of diseases, including osteoporosis.[1-3] At present there is no single assay for OC activity that provides real-time data and can be applied at the single cell level; this greatly hampers the study of OC biology and the development of therapies for disease states in which the OC is important. In this report we demonstrate that an artificial matrix impregnated with silicon quantum dots provides the basis for such an assay.
The osteoclast-bone slice assay, also known as the ‘pit’ assay, and its various modifications have been the cornerstone of many studies of osteoclast biology. However, these assays are cumbersome, insensitive, retrospective and are at best semi-quantitative.\textsuperscript{[4-5]} The pit assay is also slow, because it relies on microscopic examination of resorption pits. A variety of real-time assays have also been used that measure one or more of the products generated by OC during resorptive activity, such as superoxide anion,\textsuperscript{[6,7]} proton pump activity,\textsuperscript{[8]} calcium generation\textsuperscript{[9]} or enzyme release.\textsuperscript{[10]} There has also been a quantitative description of components of in vitro morphometric changes in OC, which have been shown to have a relationship with cell activity.\textsuperscript{[11]} An important disadvantage of these assays is that they estimate only one aspect of OC activity, which may or may not relate to the overall resorptive activity of these cells. An improved method uses calcium phosphate and a fluorescent polyanion and determines the dye concentration released into the supernatant by the action of the OC;\textsuperscript{[12]} this assay is the closest in concept to that reported here, however it requires the experimenter to wait for the dye to accumulate in the supernatant in order to detect the resorptive activity and the times reported are of the order of days.

Motivated by the demonstrations of transcytosis of resorption products by fluorescence microscopy,\textsuperscript{[1,2]} and our observations of the rapid cellular internalization of luminescent SiNCs,\textsuperscript{[13]} we have developed an assay, which is easy to use, rapid, and allows the real-time resorption assay of a single OC. This assay also facilitates studies of the kinetics of resorption. The assay is
based on an artificial hydroxyapatite / collagen I matrix containing a luminescent tracer. As the OC resorbs the matrix, the tracers (SiNCs) are released, internalised by the cell and observed in a confocal fluorescence microscope. The assay therefore determines the overall resorption activity of the OC, as in the pit assay, but with the sensitivity speed and convenience of fluorescence microscopy. In principle, many fluorescent dyes could be employed as the tracer; however there are important criteria the dye must satisfy to ensure the quality of the assay. In our previous studies we have shown that alkyl-capped silicon nanocrystals (SiNCs, Fig. 1.) have appropriate properties for such applications: they are insoluble in water and do not leach from the matrix, they have bright luminescence that is proportional to their concentration and there is no evidence of the self-quenching effects typical of molecular dyes. Silicon nanocrystals have advantages over other types of quantum dot for long duration experiments because they cannot leach heavy metal ions. Direct bonding of alkyl chains to the surface Si atoms via hydrosilation chemistry is also known to dramatically enhance the stability of Si nanostructures and these materials have been extensively characterised. Alkyl-capped SiNCs are also rapidly internalised by various cell types with evidence of lower cytotoxicity than CdSe-based materials and are actively investigated for biological applications.

2. Results and Discussion

First we describe the preparation of the assay matrix and its characterization by fluorescence and atomic force microscopy. Next, we show that the alkyl-capped SiNCs are internalized by OC progenitor cells without any acute toxicity.
cytotoxic effects nor any effect on osteoclastogenesis. We then demonstrate that the rate of uptake of SiNCs reflects the rate of resorption and is not limited by the kinetics of endocytosis by comparing the rate of uptake of SiNCs dispersed in the medium with the uptake of SiNCs from the assay matrix. Finally, we show that the assay is sensitive to known inhibitors and stimulants of the OC.

2.1. Preparation of SiNC-impregnated hydroxyapatite/collagen 1 matrix

Figure 2 shows a schematic of the assay principle and a comparison with experiments measuring the internalization by the OC of SiNCs diffusing freely in the medium. The basic concept of the assay is that the luminescent SiNCs are impregnated in the HA/collagen 1 matrix and are only released and internalised by the OC upon resorption of the matrix. In order for the assay to be successful, the matrix and the luminescent tracer must satisfy several requirements: the matrix should be sufficiently flat that optical microscopy of adhering OCs is possible, the SiNCs should not show any toxicity over the time period of the assay, the SiNCs must not leach from the matrix independent of the action of the OC and the luminescence of SiNCs in the OCs should be quantifiable and distinguishable from the matrix itself.

The matrix was prepared by pressing a pellet with an HA : collagen 1 in a ratio of 4 : 1 by weight. The pellet had a total mass of 0.6g and contained 20 µg of SiNCs (with a C_{11}-alkyl capping layer). AFM images of the pressed pellet (Figure 3a) showed a root mean square surface roughness of about 30 nm. This is sufficiently small for optical microscopy of OCs to be technically
straightforward on these surfaces. A uniform distribution of SiNCs in the matrix is clearly desirable for the assay; however the hydrophobic nature of the SiNCs means they have a tendency to aggregate. The problem of uneven distribution of SiNCs across the surface was solved by dispersing SiNCs, collagen I and HA in dichloromethane (DCM) prior to pressing the disc. DCM, though cytotoxic, has a high vapour pressure and is rapidly and completely removed during the process of pressing the disc under vacuum. The luminescence of the matrix itself is shown in Figure 3b and, although a small number of hotspots due to aggregation of SiNCs are observed (e.g., center of image), it is straightforward to locate areas of the matrix where the distribution is uniform and where the action of OCs can be assayed.

2.2. Absence of acute cytotoxicity of SiNCs in OC progenitor cells

The physicochemical and biological properties of our preparation of alkyl-capped SiNCs, such as intense luminescence, core and total diameters, extent of solubility and lack of acute cytotoxicity in various human cell lines have been detailed elsewhere.[13-15, 18] Primary OC do not divide in culture. Screening for long term cytotoxicity following variable durations of exposure to alkyl-SiNCs was carried out using a murine cell-line RAW264.7 (OC progenitor cells) possessing phenotype characteristics of monocytes and macrophages. We also examined the rate of uptake of alkyl-capped SiNCs by the RAW264.7 cell line and monitored the efficiency of osteoclastogenesis. Osteoclastogenesis was carried out using RAW264.7 and RANKL (50 ng mL−1)[28] (characterization data in supporting information). The maximum intracellular internalization of SiNCs by RAW264.7 cells was seen after 15 min (Figure 4a) of exposure to the
nanocrystals in the culture medium. Figures 4b & 4c show a confocal luminescence spectral image and a spectrum confirming that the observed luminescence is due to the SiNCs. This exposure to SiNCs was found to have no subsequent effect on cell morphology, cell proliferation or efficiency of osteoclastogenesis over a 12 day period (Figure 4d).

2.3. OC activity assay using SiNC-impregnated matrix

Primary OCs were isolated from rat long bone and characterized by size, morphology, TRAP and surface αvβ3-integrin staining, and responsiveness to calcitonin (data in supporting information and below). The OC were settled on the SiNC-impregnated matrix for 30 min. Observation of OC by optical microscopy indicated that 30 min was sufficient for the cells to adhere to the matrix and therefore the t = 0 point on the real-time assay figures corresponds to this time. Although such a value is somewhat arbitrary, all that is required for the assay is to choose a consistent starting point.

The focal plane of the fluorescence microscope was adjusted using white light illumination to observe the matrix surface and OCs. After locating a cell, the 488 nm line of an argon ion laser was used to excite luminescence from a selected OC on top of the matrix. A confocal microscope was used in our studies to provide the best discrimination of the luminescence from the cell from the background due to the SiNCs present in the matrix, but the assay could also be carried out using an epifluorescence microscope, albeit with larger baseline luminescence. As the assay relies on the observation of the luminescence of SiNCs released from the matrix by resorption and
internalization, the rate limiting step could be either the resorption or the endocytosis of the SiNCs. In order to demonstrate that the assay actually determines resorption, we compared the time course of uptake of SiNCs dispersed in culture medium with the rate of increase of luminescence owing to resorption of the SiNC-impregnated matrix (see Figure 2). OCs on glass coverslips endocytose SiNCs suspended in culture medium, whilst OCs on matrix endocytose SiNCs released in the resorptive hemivacuole by osteoclastic activity. The time-course of uptake of SiNCs dispersed in culture medium by primary OC adhering to glass substrates was substantially the same as the uptake by osteoclast-like cells produced by the action of RANKL on RAW264.7 (Figure 4a) and responded to known inhibitors and stimulants in the expected manner (supporting information). These data were satisfactorily fit by the Levenberg-Marquardt method using a simple exponential growth (first order kinetics) model (equation (1)). In the case of primary OC, the fitted parameters were \( k = 5.8 \pm 0.9 \times 10^{-2} \text{ min}^{-1} \) and (s.e.m., \( n=8 \)) and for the generated OC \( k = 1.5 \pm 0.3 \times 10^{-1} \text{ min}^{-1} \) (s.e.m., \( n=7 \)). In contrast, the luminescence increase for cells on the assay matrix is not at all well described by first order kinetics, because there is a distinct time-lag before significant increase in luminescence is observed (Figures 5 & 6). The data can however be modelled by the logistic equation (2). The different shape of the curves reflects the time delay before SiNCs are released by resorption of matrix; there is no comparable delay in the endocytosis of SiNCs from culture medium. This can be observed clearly by comparing the uptake from medium after 5 min in Figure 3a (about 50% maximum) with the uptake after 5 min from the discs at 5 min in Figure 5a (negligible).
Internalisation of SiNCs from medium: \[ I = I_0 + I_\infty \left[ 1 - \exp(-kt) \right] \] (1)

Internalisation of SiNCs from assay matrix: \[ I = I_0 + I_\infty \left[ \frac{1}{1 + \exp(-k|t-\tau|)} \right] \] (2)

In equations (1) and (2), \( k \) is the first order rate constant describing uptake of SiNCs by resorption of the matrix and endocytosis (from assay matrix) or by endocytosis alone (glass substrate experiments). \( I_0 \) is the baseline intensity at \( t=0 \) which was only significant for the assay experiments, \( I \) stands for the measured luminescence intensity at time \( t \) and \( I_\infty \) is the limiting value of luminescence intensity above the baseline (\( I_0 \)) at long times. In equation (2) \( \tau \) represents a lag time before resorption occurs. The values of \( I_0 \) are not particularly informative because they depend on the precise amount of SiNCs present in the field of view and the background intensity of luminescence. \( I_\infty \) will depend on the particular cell under observation and the local concentration of SiNCs, but a large value of \( I_\infty \) does indicate extensive resorption because it represents the increase in luminescence intensity. The rate constants, \( k \), are characteristic of the uptake process and, by the assumptions of the models, independent of SiNC concentration. The accuracy of these assumptions is borne out by the fit of the models to the data (Table 1). It is clear from the shape of the curves that the data for the resorptive activity on the matrix cannot be fitted by a simple first order growth model, but the fit to the logistic equation (1) is acceptable.
The data (Table 1 & Figure 5) show a clear effect of the inhibitors on the total amount of luminescence observed; Calcitonin and MCD were inhibitory whilst RANKL showed stimulation (increased \( I_\sigma \)) of SiNC-associated luminescence seen in OC cultured on SiNC-impregnated artificial matrix (Figures 5 & 6). Control experiments showing the absence of luminescence for non-resorbing cells are included in supporting information. Calcitonin causes a small net decrease in luminescence, which is really an effect on the cell morphology - the luminescence intensities are obtained by integrating over the cell and therefore a decrease in cell area shows up as a decrease in luminescence - one should not therefore interpret the rate constant as representing a rate of expulsion of SiNCs. Nevertheless, it is very clear that CT abolishes resorptive activity as expected; CT is a potent and selective inhibitor of OC and this result also confirms that the resorptive action of the OC is crucial to the luminescence data observed in the assay. The rate constants in the case of RANKL and the control are not significantly different, however the total increase in observed luminescence \( I_\sigma \) is about 75% greater in the presence of RANKL. Interestingly, the lag-times \( t \) are not significantly different between the control and RANKL – this suggests that the processes responsible for the lag between adherence of the cell and resorption are not modulated by RANKL, although the overall extent of resorptive activity is. The rate of SiNC uptake is greatly suppressed (\( k < 0.01 \) min\(^{-1}\)) in the presence of 10 mM MCD, a known inhibitor of cholesterol-mediated endocytosis. The MCD data is also important because it shows that simple leaching of the SiNCs from the matrix into the confocal volume is insignificant. In summary, the assay shows a clear ability to
observe the effects of stimulants and inhibitors of OC activity in single cells in real-time over a period of about 30 min.

3. Conclusions
Measurements of osteoclast functional activity by in vitro assays have a wide range of important applications in biomedical research in general, and bone and mineral metabolism in particular. The present assay measures the real resorptive activity, which has many intricate and complex components, as against estimating just one aspect of OC activity. Furthermore, this assay allows estimation of the activity of single as well as multiple cells, compared to pit assays in which the activity of a number of cells is estimated and the precise number of OC involved is uncertain. The importance of the activity assay is that it is not retrospective and can be used in experimentation where there is a requirement to screen for rapid response to stimuli. Such assays are needed to investigate aspects of OC biology that have not been possible due to the insensitive and often cumbersome methods currently available. The present SiNC-based activity analysis, by allowing real-time estimation of OC activity, opens up opportunities for studying hitherto unknown aspects of OC activity.

4. Experimental Section
Biochemical reagents: Filipin, fluorescein isothiocyanate, salmon thyrocalcitonin, methyl-β-cyclodextrin, RANK Ligand (receptor activator of NF-κB ligand), parathyroid hormone, sodium tartrate dihydrate (Sigma ultra), were all purchased from Sigma-Aldrich (UK) and used as-received.
Mouse monoclonal [BV3] to integrin Alpha V Beta 3 catalogue No/ab7166 were obtained from Abcam (UK). The secondary antibody employed was FITC-conjugated goat anti-mouse (catalogue no; 62-6111, diluted 1:500, Zymed). Goat serum containing 15 mmol/NAN3 Catalogue No; X0907 was obtained from Dako (UK). 4’,6- diamidino-2-phenylindole DAPI was obtained from Invitrogen (UK). MTT (3-(4,5- dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) was obtained from Promega (UK).

*Cells and cell culture media*: Cell culture media were used are DMEM, Ham's F-10 and RPMI-1640 were purchased from Invitrogen Life Technologies (UK). Trypsin-EDTA, foetal calf serum (FCS), penicillin/streptomycin (10000 IU mL⁻¹) were purchased from ICN Chemical Company (UK). Phosphate buffered saline (1xPBS) was purchased from Lonza (UK). Poly-L-lysine coated microscope slide and glass cover slip 22 mm were obtained from Sigma Aldrich (UK). Plasticware, namely tissue culture plates (6, 12, 24, and 96 wells), 25 and 75 cm² vented cell culture flasks, as well as cryogenic vials were obtained from Corning/Costar Ltd (UK).

*Preparation of matrix impregnated with Alkyl-SiNCs*: The preparation of the alkylated SiNCs with C₁₁ (undecyl) capping layers used here has been discussed in more detail elsewhere.[14-16] The matrices used in the assay were prepared by pressing a powder of hydroxyapatite/collagen type 1 into a disc. Hydroxyapatite (HA) was ground with collagen 1 powder in a 1 : 4 ratio by weight (total mass 0.6 g). SiNCs (20 µg) were added and the mixture was dispersed in dichloromethane (DCM) with stirring to ensure a uniform
distribution of SiNCs through the HA/collagen 1 matrix. The DCM was allowed to evaporate at room temperature and the mixture loaded into a stainless steel pellet press (id 1.5 cm) and air was removed from the powder using a rotary pump. The optimum applied force to press the powder was 20 tons for a duration of 15 min; this produced discs 1 mm thick and 1.5 cm in diameter. These discs were found to be stable, as judged by retention of the initial smooth surfaces in the optical microscope, in aqueous solution or cell-culture medium for up to 24h. Lower applied forces produced discs that showed evidence of pitting or disaggregation upon exposure to aqueous solutions for long periods.

Osteoclast isolation, osteoclastogenesis and cytotoxicity screening: Primary and generated OC were isolated from neonatal Wistar rats, between 2 to 4 days old, by an established method[9] or generated from RAW 264.7 using RANKL (50 ng mL\(^{-1}\).[28] RAW264.7 is a murine macrophage-like cell line – Abelson leukemia virus transformed cells derived from BALB/c mice.

Osteoclast isolation: Primary OCs were mechanically harvested from the long bones of the rats that had been sacrificed by cervical dislocation. The unwanted tissue and skin were removed and the long bones of the rats were curetted into 1 ml of pre-warmed HEPES-buffered medium 199 containing 10% FCS. The OCs were allowed to adhere to appropriate matrix by culturing the cell suspension under standard conditions (5% CO\(_2\) in air in a humidified incubator at 37\(^\circ\)C) for 60 min. After 40 minutes, the non-attached cells were rinsed away. Cellular debris was removed by washing the preparation.
vigorously with pre-warmed phosphate buffer comprising 145 mM NaCl, 4.5 mM KCl, 5 mM NaH$_2$PO$_4$, 10 mM HEPES, 1 mM MgCl$_2$, 6 mM glucose and adjusted to pH 7 with NaOH.[9]

*Osteoclastogenesis*: RAW 264.7 monocytes, a murine macrophage-like cell line, were cultured on sterile 22 mm diameter glass coverslips in 6-well tissue culture-treated plates under standard conditions with RPMI-1640 containing 2 mM L-glutamine, 10% FCS and antibiotics/antimycotics (50 units mL$^{-1}$ penicillin, 50 mg mL$^{-1}$ streptomycin and 1% ketoconodazole). When the cells reached approximately 60% confluence, they were differentiated from RAW264.7 by culture in the presence of RANKL for 12 days (RPMI-1640, supplemented with 10% HIFBS, 100 units mL$^{-1}$ penicillin, 100 mg mL$^{-1}$ streptomycin and 50 ng mL$^{-1}$ RANKL).[29]

*Cell Proliferation Assay*: Cells growing in a 75 cm$^2$ cell-culture flask (Corning, UK) were harvested by trypsinization (0.05% trypsin, 0.02% ethylenediaminetetraacetic acid (EDTA)) and equal volumes of cells were aliquoted into 24-well cell-culture plates (Corning, UK) containing DMEM supplemented with 10% FCS. The cells were incubated at 37°C for 24h to allow cell attachment and were prepared for experiments by overnight serum starvation in medium containing 0.5% FCS (1 mL). Thereafter, cells were exposed to either NCs suspended in 2 µL ether (Sigma) or (controls) were exposed to vehicle (2 µL ether) alone. The 2 µL ether/SiNCs is injected rapidly with sonication if necessary to disperse the SiNCs as a lyophobic sol.[15] All
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experiments (n=8) were carried out for 2, 4, and 24h and experiments were repeated on at least three different occasions. Cell wells containing 1 mL of DMEM medium supplemented with 10% FCS alone served as control experiments. The control and test plates were then treated and analyzed under identical conditions allowing paired comparison of the corresponding wells. At the end point of each exposure time (2, 4, or 24 h), cell viability was estimated using cell proliferation assay by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Promega) following the manufacturer’s protocol. Briefly, serum-starved cells exposed to NCs or vehicles were incubated with MTT solution (1 mg mL\(^{-1}\)) in NM for 2h in a 5% CO\(_2\) incubator. The MTT solution was discarded and acidic isopropanol was added to the cells and shaken for 30 min to allow complete solubilization of the colored product. The absorbance was read at 560 nm, blanking on control wells.

Confocal Laser Scanning Microscopy and Microspectroscopy: The LEICA TCS SP2 system with a LEICA DM IRE2 microscope having an Argon/Krypton Laser (Leica TCS SP2, Spectral Confocal and Multiphoton Microscope, Leica Microsystems Ltd., Milton Keynes, UK) was used in the assay experiments. This system allows the imaging of a single focal plane as well as a series of planes—horizontal or vertical. The microscope is a true point-scanning system with theoretical maximum x-, y- and z-resolution. Observations were made using a HCX PLAPOCS 40.0T1.25 oil immersion objective lens.

A CRM200 confocal Raman microscope (Witec GmbH, Ulm, Germany) was used to obtain luminescence spectral images. The 488 nm line of an argon ion laser
provided the excitation light and the emitted and/or scattered light passed through a Raman edge filter to remove elastically scattered light. The filtered light was collected by a multimode optical fiber that also served as the confocal pinhole. The lateral spatial resolution of the instrument is close to the diffraction limit, that is, about 250 nm. The collected light was analyzed by a spectrograph with typical settings of 150 lines mm\(^{-1}\) (grating), an integration time of 0.1 s per pixel, and 256 × 256 pixels for each image.

*Atomic force microscopy:* Atomic force microscopy (AFM) imaging was performed in air using a Digital Instruments Dimension/Nanoscope V system (Veeco Inc., Metrology group) and NanoProbe tips (Model TESPW; Veeco Inc) made of silicon. All of the AFM images were obtained in tapping mode at a scan rate of 0.71 Hz, over scan areas of 100 mm\(^2\) and the resonant frequency of the tip was about 250 kHz.

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**Author contributions**

HKD, MAB and BRH designed the study, HKD and BRH wrote the paper. NHA, HKD & MAB carried out the assays and preparation of the matrix and SAFS contributed probe microscopy and analysis. All authors edited and commented on the manuscript.
Supporting information

1. Osteoclastogenesis data; 2. Characterisation of primary osteoclasts;
3. Assay controls; 4. Effect of inhibitors & stimulants on uptake of SiNCs from
the medium by OC; 5. Fluorescence images showing uptake of SiNCs from
medium by RAW264.7 cells and 6. Atomic force microscopy of SiNCs on mica
with size (diameter) distribution analysis.

Competing financial interests
The authors declare no competing financial interests

References


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Figure 1. Diagram showing the structure of the silicon nanocrystals employed in the assay. The Si core is known to be crystalline and is responsible for the luminescence (18). The alkyl monolayer caps the core and slows down the corrosion of the core by water/oxygen.
Figure 2. A schematic diagram showing (a) the basic concept of the assay in which the OC releases SiNCs from the matrix and then internalizes them by the process of resorption, and contrasting this with (b) the direct uptake by the OC of SiNCs dispersed in the culture medium.

Figure 3. A disc of artificial matrix (15 mm diameter and 1 mm thickness) comprising hydroxyapatite impregnated with alkyl-SiNCs as used for osteoclast resorption assays. (a) The smoothness of the matrix surface is demonstrated by atomic force microscopy (the grayscale corresponds to a height of 140 nm and the rms roughness over the field of view was 30 nm). (b) The uniformity of the distribution of SiNCs over the matrix surface is shown by fluorescence microscopy ($\lambda_{\text{ex}} = 488 \text{ nm}; \ 550 < \lambda_{\text{em}} < 650 \text{ nm}$). The image is in false color and indicates luminescence intensity; see fig 4(c) for the spectrum of the luminescence.
Figure 4. Screening for long term cytotoxicity following variable durations of exposure to alkyl-SiNCs was carried out using the mouse macrophage/monocytic cell-line RAW264.7, a progenitor cell of the OC. (a) The maximum internalization of alkyl-SiNCs by RAW264.7 was seen within 15 minutes of exposure. (b) A 3-D intracellular confocal luminescence image from alkyl-SiNCs within OC progenitor RAW264.7 cells (the colourscale indicates luminescence intensity), and (c) an intracellular photoluminescence spectrum of alkyl SiNCs which shows that the luminescence spectrum of the particles after cellular internalization is unchanged (c.f. Refs 13, 14). (d) The 15 min exposure of RAW264.7 to SiNCs had no significant effect on the long-term viability of the cells, and RANKL-induced osteoclastogenesis. Osteoclastogenesis efficiency and cell survival was estimated by counting cell number and generation of multinucleate (≥3 nuclei) TRAP positive cells. Exposure to SiNCs had no effect on cell proliferation, the mean ± S.D. cell number at day 3, 6 and 12 for control and tests were 15 ± 8 vs. 15 ± 7; 35 ± 10 vs. 33 ± 8; and 45 ± 7 vs. 49 ± 4. All experiments ranged between n = 5 to 8 and were repeated at least on three different occasions.

Figure 5. The change in luminescence intensity with time of single primary OCs cultured on pressed hydroxyapatite matrix, comprising collagen 1 and...
hydroxyapatite impregnated with alkyl-capped SiNCs. Control indicates the absence of any stimulant/inhibitor. RANKL is a known stimulant, MCD inhibits cholesterol-dependent internalization and CT is a potent, specific OC inhibitor.
Figure 6. Luminescence images at different time points of an OC on SiNC-impregnated hydroxyapatite/collagen 1 matrix during a typical control assay. "Control" means an OC resorption assay in the absence of inhibitors and stimulants. The image is in false color and indicates luminescence intensity; see fig 4(c) for the spectrum of the luminescence. (a) 5 min; (b) 10 min; (c) 15 min; (d) 20 min and (e) 25 min.

Table 1. Parameters of primary OC resorptive activity performed in vitro using SiNC-impregnated pressed hydroxyapatite/collagen 1 matrix. The parameters were extracted from a non-linear regression using the method of least squares and the logistic equation (1) as a model function for the luminescence-time curves (Figure 5). Values are reported as mean ± sem estimated from the covariance matrix obtained from the Levenberg-Marquardt non-linear regression and also replicate experiments (n in brackets). $I_\infty$ is the total increase in luminescence intensity above baseline as $t \to \infty$, $\tau$ is the delay time in the logistic equation and $k$ is the rate constant.

<table>
<thead>
<tr>
<th></th>
<th>$I_\infty$</th>
<th>$\tau$ / min</th>
<th>$k$ / min$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inhibitor or stimulant (n=7)</td>
<td>21.2 ± 2.1</td>
<td>19.9 ± 1.0</td>
<td>0.18 ± 0.010</td>
</tr>
<tr>
<td>Calcitonin (n=2)</td>
<td>-5.4 ± 0.45</td>
<td>10.4 ± 1.5</td>
<td>(0.35 ± 0.044)*</td>
</tr>
<tr>
<td>RANKL (n=3)</td>
<td>36.9 ± 1.7</td>
<td>21.6 ± 0.9</td>
<td>0.15 ± 0.006</td>
</tr>
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</table>

[a] This rate constant does not have the same biological interpretation as the others in the table, because the luminescence intensity decreases ($I_\infty<0$) in the presence of calcitonin. The origin of this effect is explained in the text.
Osteoclasts are the cells which resorb bone and are important in disease states such as osteoporosis. A real-time assay for the activity of single osteoclasts is presented. The assay utilises luminescent silicon quantum dots in a hydroxyapatite matrix; resorption of the matrix by the cell releases the dots, which are rapidly internalised and detected by confocal fluorescence microscopy.

TOC Keyword: Resorption, silicon quantum dots, osteoclast, assay

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Real-Time Activity Bioassay of Single Osteoclasts using a Silicon Nanocrystal-Impregnated Artificial Matrix
Supporting information for:

Real-Time Activity Bioassay of Single Osteoclasts using a Silicon Nanocrystal - Impregnated Artificial Matrix

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Osteoclastogenesis - characterization data.

2. Characterisation of primary osteoclasts.

3. Assay controls.

4. Effect of inhibitors & stimulants on uptake of SiNCs from the medium.

5. Fluorescence images showing uptake of SiNCs from medium by RAW264.7 cells.

6. Histogram of SiNC diameters and AFM images
1. Osteoclastogenesis data.

Figure S1a. TRAP staining of osteoclast-like cells generated from RAW264.7 after 12 days.

Figure S1b. Immunostaining with anti $\alpha_\beta_3$ integrin mouse monoclonal primary Ab, the detection utilized a rat anti mouse Ab (FITC-labelled).
2. Characterization of primary OC isolated from rat long bones.

**Figure S2a.** TRAP staining of a primary osteoclast.

**Figure S2b.** DAP (blue) stain demonstrating multinucletarity and immunostaining for $\alpha_v\beta_3$ integrin (green)

**Figure S2c.** An osteoclasts on the assay matrix, characterized by surface $\alpha_\beta_3$ integrin immunostaining.
3. Assay controls.

Figure S3. Control experiments for the assay – fluorescence images (dark background) and corresponding reflected light optical images (bright background). (a) A negative control comprising OC on an identical matrix to the assay, but containing no SiNCs. (b) & (c) Non-resorbing cells (RAW264.7 and Hela cells) were placed on SiNC-impregnated matrix and resorption was determined by measuring the luminescence intensity of the cells - as expected, no fluorescence is observed. (d) Positive control showing an OC on a glass slide internalizing SiNCs that were added to the culture medium. (e) Osteoclast placed on the SiNC-impregnated matrix after 1h. The fluorescence images are in false color and represent intensity, but do not reflect the actual color of the SiNC luminescence.
4. Effect of inhibitors & stimulants on uptake of SiNCs from the medium by OC.

**Figure S4.** Uptake of SiNCs dispersed in the medium by primary OC in the presence of various stimulants (PTH, RANKL) and inhibitors (MCD, CT, Filipin). MCD and Filipin are known to inhibit cholesterol-mediated endocytosis pathways. CT is a potent selective inhibitor of the OC. The control indicates no stimulant/inhibitor was added to the medium.
5. Fluorescence images showing uptake of SiNCs from medium by RAW264.7 cells.

**Figure S5.** Fluorescence images of RAW264.7 cells at different time points after exposure to SiNCs dispersed in the culture medium. (a) control (no SiNCs present, only vehicle = 2 µL ether); (b) optical image corresponding to (a); (c) - (g) with SiNCs added to the medium. (c) 5 min; (d) 10 min; (e) 15 min; (f) 30 min; (g) 60 min. The color scale is a false color and shows the intensity, not the true color of the SiNC luminescence.
6. Histogram of SiNC diameters and AFM images.

**Figure S6.** Particle size distribution of the SiNCs by atomic force microscopy on a mica substrate. (a) AFM image of SiNCs on mica. The area is 10 x 10 microns and the colourscale corresponds to 10 nm. Individual particles are observed as well as some aggregates (larger, bright features); (b) Histogram of particle diameters obtained by line section analysis of AFM images. The mean is 5.2 nm and the standard deviation is 1.1 nm. 104 particles were analysed from 5 images.