Electronic Supporting Information

Smart nanoprobes for the detection of alkaline phosphatase activity during osteoblast differentiation

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Materials.

Gold (III) chloride trihydrate, calcium hydroxide, phosphoric acid, potassium carbonate, (3-aminopropyl) trimethoxy silane (APTMS), tetrakis (hydorxymethyl) phosphonium chloride (THPC), β-glycerophosphate and L-ascorbic acid were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Indocyanine green was obtained from Tokyo Chemical Industry (TCI) Co. Phosphate-buffered saline (PBS: 10 mM, pH 7.4), alpha minimum essential medium (α-MEM) and fetal bovine serum (FBS) were purchased from Gibco. All other chemicals and reagents were of analytical grade.

Synthesis of gold nanoparticles-conjugated fluorescent-hydroxyapatite

Fluorescent-hydroxyapatite synthesis

Fluorescent-hydroxyapatite (FHAp) was synthesized by chemical precipitation and a hydrothermal technique according to a previously published protocol with some modifications.[1] An aqueous solution of phosphoric acid (0.3 M, 10 mL) with indocyanine green (ICG) (1 mg) was added drop wise to a vigorously stirred aqueous solution of calcium hydroxide (0.25 M, 21 mL) at a rate of 1 mL/min at 60 °C. The pH of the suspension was then adjusted to pH 7.0. After aging, the FHAp was purified and collected by repeated washing with distilled water and centrifugation at 5,000 rpm for 30 minutes (repeated 3-cycles). The final FHAp product was obtained by freeze-drying.

Gold nanoparticle-conjugated fluorescent-hydroxyapatite synthesis

The gold nanoparticles were prepared by the reduction of 1.0 wt % gold (III) chloride trihydrate (2 mL) in the

presence of the tetrakis (hydorxymethyl) phosphonium chloride (THPC, 12 μL) and sodium hydroxide (0.5 mL, 1 M) solution as a reducing agents with rapid stirring for 7 min at room temperature.[2] FHAp (50 mg) and 3-aminopropyltrimethoxysilane (APTMS) (100 μL) were then mixed with 10 mL of distilled water at 70 °C for 3 hours to prepare aminated FHAp, which was purified by centrifugation at 13,000 rpm for 5 min and re-dispersed in distilled water in triplicate. The presence of amine groups on the surface of FHAp was confirmed using Fourier transform-infrared spectroscopy (FT-IR) and X-ray photoelectron spectroscopy (XPS). To prepare gold nanoparticle-conjugated fluorescent-hydroxyapatite (AuFHAp), the aminated FHAp (10 mg) was mixed with an excess of gold nanoparticles (5 mL) whereby the amine groups were used as binding sites for the gold nanoparticles.

Characterization

We confirmed the characteristics of FHAp and AuFHAp using various analytical method. Their morphologies were confirmed using a high-resolution transmission electron microscope (HR-TEM) (TECNAI G2 F30) and a scanning electron microscope (SEM) (Quanta FEG MK2). Their optical properties, absorbance and fluorescence intensity were measured using a UV-Vis spectrophotometer (DU®800 Spectrometer) and a fluorescence spectrometer (FS-2). Additionally, the crystallinity and loading ratio (%) of gold in the AuFHAp were analyzed using X-ray diffraction (D/MAX-2500) at 298 K and a thermogravimetric analyzer (TGA) (TGA N-1000).

Alkaline phosphatase (ALP) activity assay

The assay was performed by mixing 5μL of alkaline phosphatase (ALP) (Sigma®) (1 mg/mL) with FHAp and AuFHAp at various concentrations. The mixtures were measured at 810 nm using a fluorescence spectrometer. In addition, the inhibition of ALP activity was tested using Levamisole as an ALP-inhibitor. ALP was pre-incubated with different concentrations of the inhibitor at 25 °C. After 2 min, the inhibitor pre-treated ALP solution was added to 500 μL of AuFHAp (0.3 mg/mL) and the ALP activity was confirmed by measuring fluorescence intensity at 810 nm.

Cell culture and osteoblast differentiation

Mouse pre-osteoblast-like MC3T3-E1 cells were obtained from the American Type Culture Collection (ATCC®). MC3T3-E1 cells were grown in alpha minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum and 1% antibiotics in a 37 °C humidified atmosphere containing 5% CO_2. The MC3T3-E1 cells were prepared by the addition of 5 mM β-glycerophosphate and 0.2 M L-ascorbic acid as differentiation inducing agents to the growth medium to induce osteoblast differentiation. Unless indicated otherwise, the osteoblast culture medium was replaced every 3 days.

Biocompatibility tests

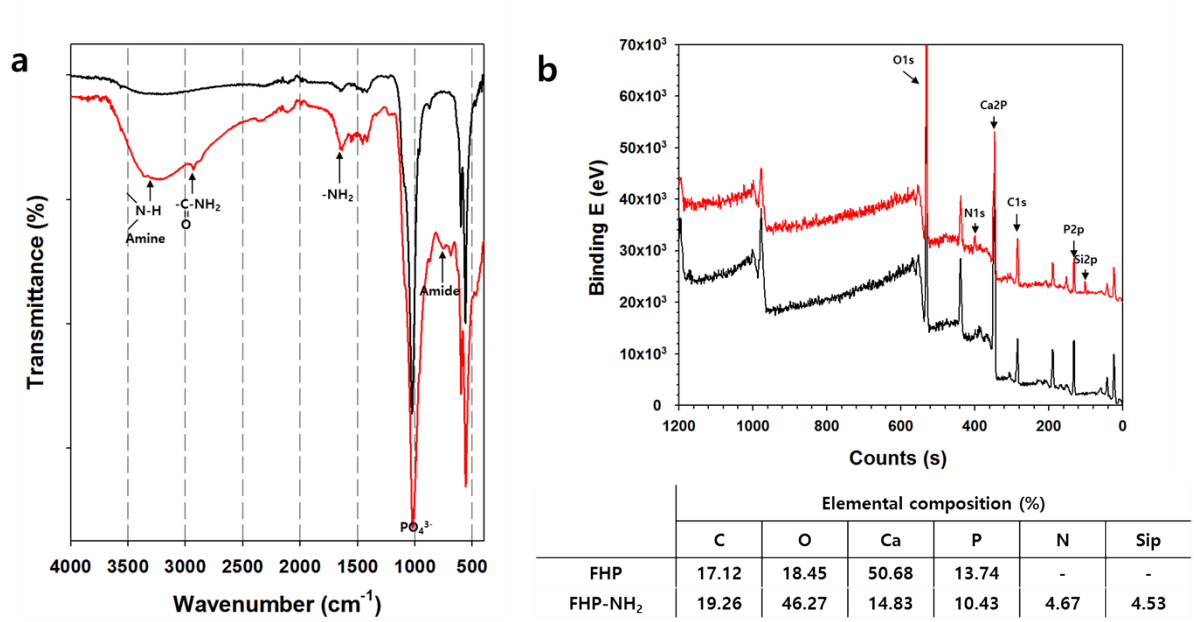
The cytotoxic effect of AuFHAp against pre-osteoblast and osteoblast cells was evaluated by measuring the inhibition of cell growth using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (1 x 10^4 cells/well) were plated in a 96-well plate at 37 °C overnight. The cells then were treated with various concentrations of AuFHAp for 24 hours. After completion of the treatment, the cells were washed with PBS and the MTT assay was performed, in which yellow tetrazolium salt was reduced to purple formazan crystals in metabolically active cells. The relative percent cell viability was determined as the ratio of formazan intensity in viable cells treated with AuFHAp to the intensity in non-treated (control) cells. Cell viability was normalized to non-treated cells, which were considered to be 100% viable. Similarly, the tests for biocompatibility of FHAp were performed in the same manner as a control.

***In vitro* Alkaline phosphatase activity assay**

MC3T3E-1 cells were differentiated for 21 days under osteoblast differentiation conditions and assessed for alkaline phosphatase (ALP) activity. First, the cells were harvested in PBS. They were then pelleted and lysed by sequential freeze/thaw cycles in radio immune precipitation (RIPA) lysis buffer. For each reaction, 1×10^5 cells was adjusted to a final volume of 10 µL in E1 lysis buffer and then added to the FHAp and AuFHAp (200 µL of 0.3 mg/mL). Afterward, the mixtures were measured for fluorescence at 810 nm (λ_{ex}: 790 nm) using a fluorescence spectrometer. As a control, alkaline phosphatase activity in the same cell conditions was detected with an ALP detection kit (Sigma chemical Co.) using 4-Methylumbelliferyl phosphate disodium as a substrate according to a manufacturer's instructions. In addition, for detection of ALP activity in living cells, the cell were seeded at a density of 1×10^3cells/well in 8-well plates in osteoblast culture medium at 37 °C and then cultured for 21 days. At predetermined time points, cells were treated with AuFHAp and FHAp and further incubated for 24 h. The cells were then washed 3-times with PBS and stained by Hoechst to label their nuclei. Fluorescence images were obtained using a fluorescence microscope.

1 H. J. Lee, H. W. Choi, K. J. Kim and S. C. Lee, *Chem. Mater.* 2006, **18**, 5111-5118.

2 N. R. Jana, L. Gearheart and C. J. Murphy, *Langmuir*, 2001, **17**, 6782-6786.



	Elemental composition (%)					
	C	O	Ca	P	N	Sip
FHP	17.12	18.45	50.68	13.74	-	-
FHP-NH_2	19.26	46.27	14.83	10.43	4.67	4.53

Figure S1. (a) FT-IR spectra for FHAp (black) and amine-functionalized FHAp (red). (b) The elemental surface composition (%) obtained by XPS.

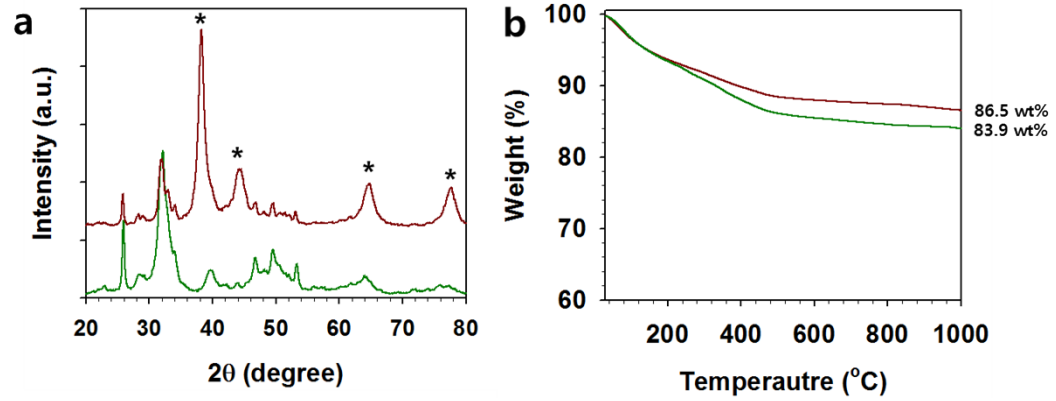


Figure S2. (a) X-ray differentiation (XRD) patterns and (b) thermogravimetric analysis (TGA) of FHAp (green) and AuFHAp (dark red).

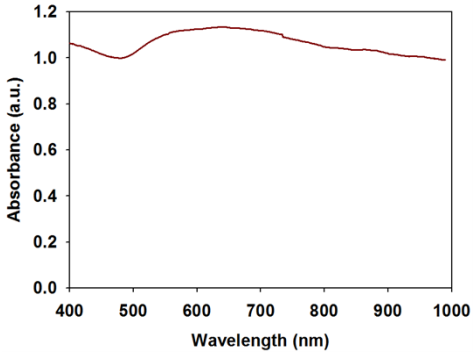


Figure S3. The absorption spectrum of AuFHAp.

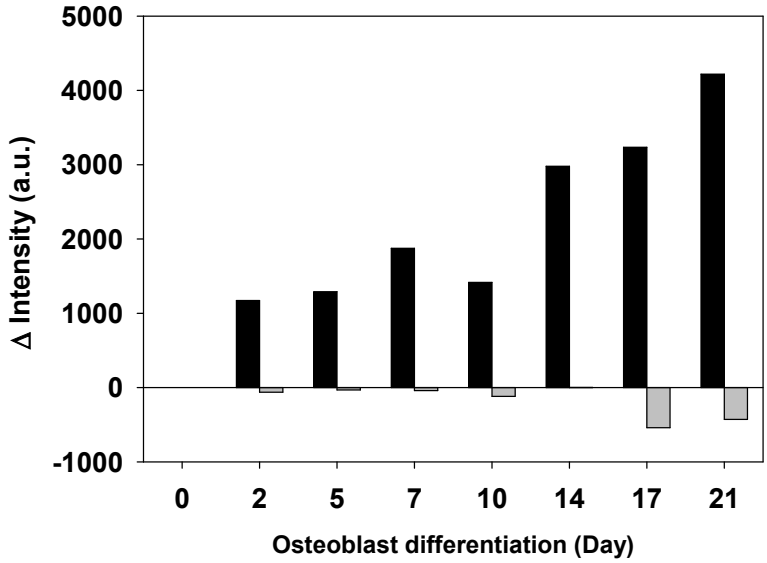


Figure S4. Measurement of alkaline phosphatase (ALP) activity using the commercial ALP detection kit (Sigma®) for pre-osteoblasts (gray; MC3T3-E1 cells) and osteoblasts (black; differentiation inducing agents-treated MC3T3-E1 cells) during osteoblast differentiation (ΔIntensity = Intensity – $\text{Intensity}_{0\text{day}}$).

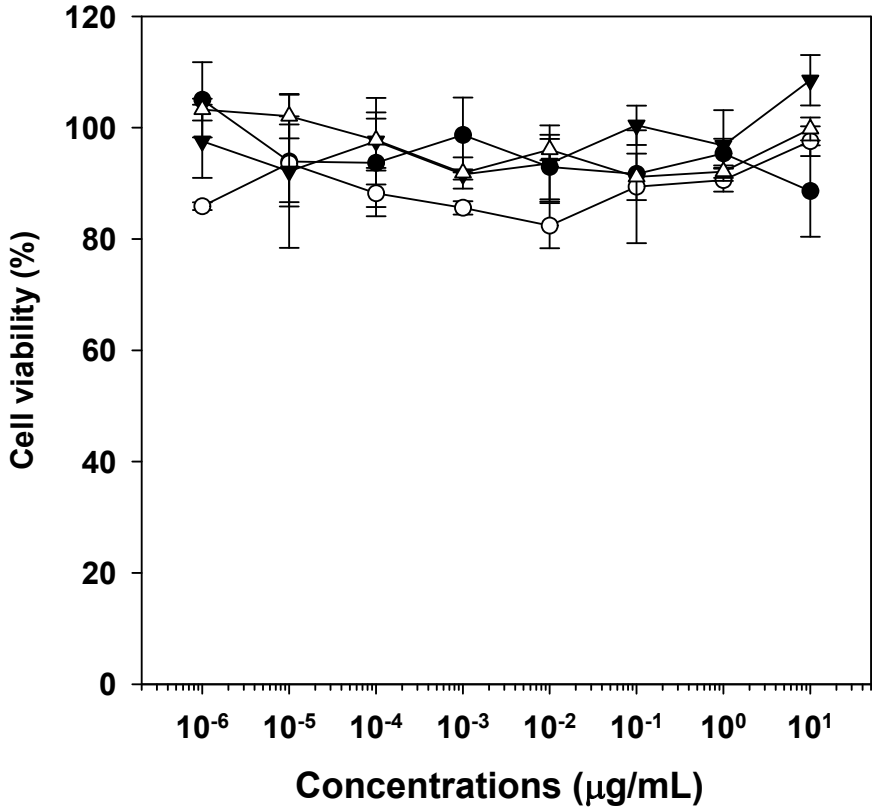


Figure S5. MC3T3E-1 (pre-osteoblast) and MC3TE-1 cell viability under differentiation inducing agents (Osteoblast) treated with FHAp and AuFHAp (●: AuFHAp-treated osteoblast, ○: AuFHAp-treated pre-osteoblast, ▼: FHAp-treated osteoblast, △: FHAp-treated pre-osteoblast).

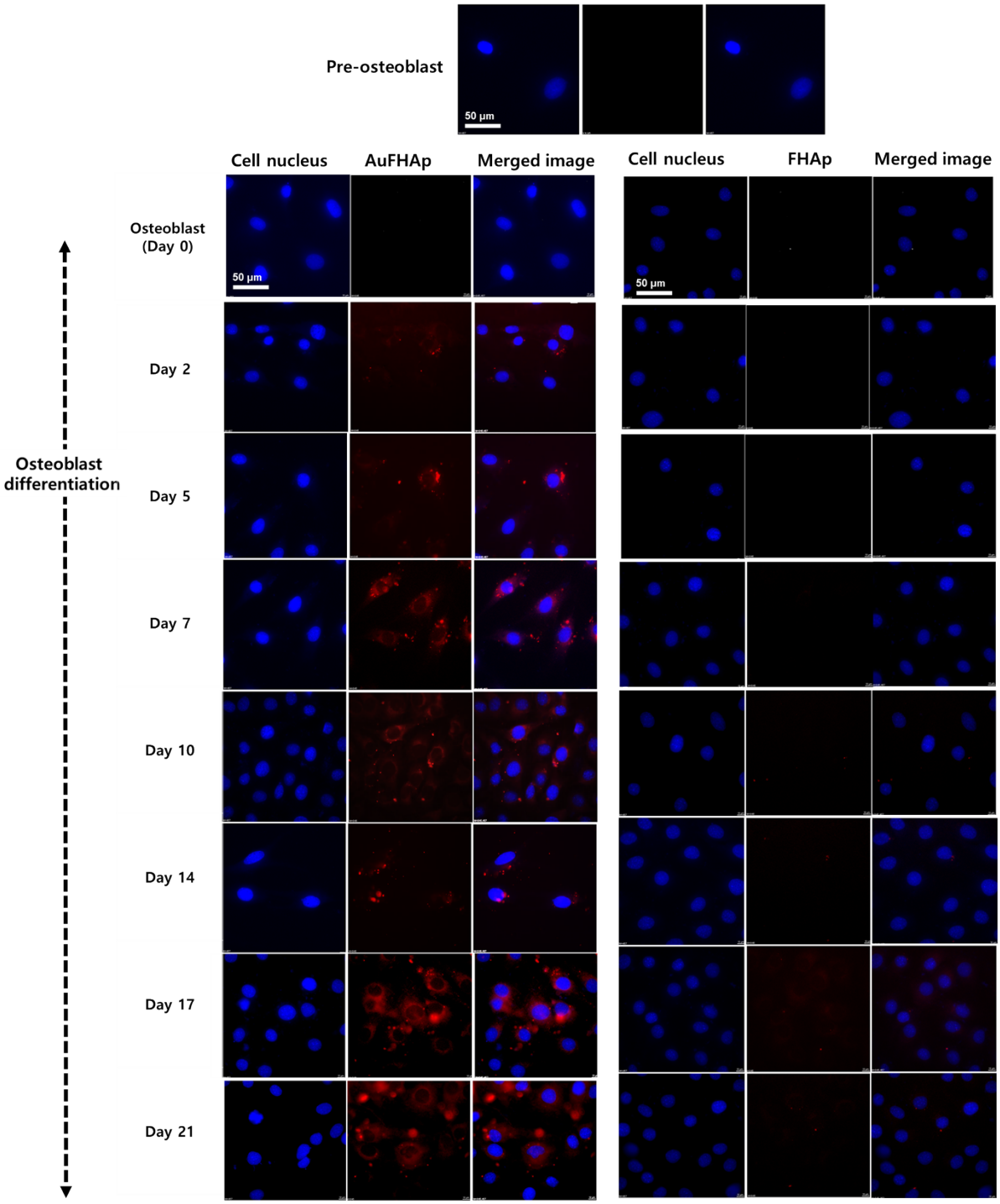


Figure S6. Detection of alkaline phosphatase (ALP) activity in osteoblasts using AuFHAp and FHAp during osteoblast differentiation. Merged show the overlay of a blue filter for cell nuclei (Hoechst 33324) and a red filter for indocyanine green fluorescence.