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# Preliminary Approach of Real-Time Monitoring In Vitro Matrix Mineralization Based on Surface Plasmon Resonance Detection

Shin Ae Kim,[1] Sumit Das,[1] Howon Lee,[1] Junghoon Kim,[1] Yun Mi Song,[2] In Sook Kim,[2] Kyung Min Byun,[3] Soon Jung Hwang,[2,4] Sung June Kim[1]

[1]School of Electrical Engineering and Computer Science, Seoul National University, Seoul 151-742, Korea; tel.: +82-2-880-1812; fax: +82-2-882-4158; e-mail: kimsj@snu.ac.kr

[2]Dental Research Institute, Seoul National University, Seoul 110-749, Korea

[3]Department of Biomedical Engineering, Kyung Hee University, Yongin 446-701, Korea

[4]Department of Oral and Maxillofacial Surgery, School of Dentistry, BK 21 2nd Program for Craniomaxillofacial Life Science, Seoul National University, Seoul 110–749, Korea

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**ABSTRACT:** Matrix mineralization is a terminal process in osteoblast differentiation, and several approaches have been introduced to characterize the process in tissues or cultured cells. However, an analytical technique that quantitates in vitro matrix mineralization of live cells without any labeling or complex treatments is still lacking. In this study, we investigate a simple and enhanced optical method based on surface plasmon resonance (SPR) detection that can monitor the surface-limited refractive index change in real-time. During monitoring MC3T3-E1 cells in vitro culture every 2 days for over 4 weeks, the SPR angle is shifted with a greater resonance change in cells cultured with osteogenic reagents than those without the reagents. In addition, the SPR results obtained have a close relevance with the tendency of conventional mineralization staining and an inductively coupled plasma-based calcium content measure. These results suggest a new approach of a real-time SPR monitoring in vitro matrix mineralization of cultured cells.

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**KEYWORDS:** osteoblast cell; mineralization; real-time monitoring; surface plasmon resonance; bone formation

Osteogenic cell cultures have been widely used to study the fields of bone biology for applications to skeletal implants, including the areas of orthopedic and dental clinics (Diduch et al., 1993). While various factors in bone cells can be employed to observe the bone-forming process, the course of osteogenesis is generally characterized by the synthesis of a mineralized matrix (Chung et al., 1992; Diduch et al., 1993). Here, biological mineralization means the process by which calcium and phosphate are deposited between collagen fibrils of extracellular matrix as a form of calcium hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$, Chung et al., 1992; Dorozhkin and Epple 2002). Because obtaining a densified bone is important for implantation in clinical applications, it is necessary to monitor the status of the mineralization process in real-time.

Recently, several detection methods for mineralization have been introduced: scanning electron microscopy (SEM), energy-dispersive X-ray microanalysis (EDX), X-ray diffraction (XRD), inductively coupled plasma (ICP) spectrometer, histology employing von Kossa and Alizarin red S staining, and labeling with fluorescent dyes (Chou et al., 2005; Chung et al., 1992; Diduch et al., 1993; Wang et al., 2005). Although these conventional approaches have demonstrated meaningful results, their critical drawbacks can limit the potential use in practical applications. For example, the SEM, EDX, and XRD methods are known to have limitations in quantitative analysis (Basdra and Komposch 1999; Chou et al., 2005; Wang et al., 2005). ICP is a quantitative and sensitive analysis technique, but it is somewhat time-consuming due to the pretreatment procedure required to get rid of organic residues (Wang et al., 2005). In particular, these methods demand complicated and expensive facilities that make them inappropriate for real-time and in situ analysis. Further, as staining and labeling methods are harmful to live cells, they are intrinsically destructive (Wang et al., 2005).

Correspondence to: Sung June Kim

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Moreover, despite its rapid and sensitive detection, labeling can suffer from restrictions of monitoring time and troublesome residuum (Bonewald et al., 2003). Therefore, for effective and long-term detection of the bone-forming process, it is highly required to develop a quantitative, rapid, non-destructive, and label-free sensing technique.

Since the introduction of surface plasmon resonance (SPR), which is an electromagnetic phenomenon occurring on a thin metal film, SPR-based optical biosensors have been extensively explored (Liedberg et al., 1995). In principle, SPR biosensors measure a refractive index change at a dielectric-metal interface. SPR technique is quantitative, sensitive, fast, and label-free and thus, it has been used to detect the kinetics of surface-immobilized biomolecules (Kim et al., 2009; Peterson et al., 2009). Also, these properties enable SPR sensors to detect cell activities because cellular metabolism is accompanied by a small change in the membrane-localized refractive index (Kim et al., 2008; Zhang et al., 2009). However, to our knowledge, long-term and continuous SPR measurement of live cells has not been reported yet, while 1-day observation of the protein deposition occurring at vascular muscle cells was reported (Peterson et al., 2009).

Accordingly, the purpose of this study is to demonstrate an interesting possibility of detecting the osteogenic differentiation process from collagen accumulation to mineral deposition of live osteoblast cells. To achieve this, the SPR substrate was developed to observe the optical changes caused by cell differentiation. Moreover, as a proof study, the experimental results obtained were validated by conventional analytical techniques based on ICP spectrometer and staining methods. From our results, we intend to present the potential for quantitative detection of osteogenic processes and the possibility of implementing long-term live cell monitoring for clinical and cytometric analyses.

The plasmonic gold substrates were loaded into a custom-made SPR system to monitor osteoblast differentiation in real-time. A schematic diagram of the SPR measurement system is introduced in Figure 1. A SPR sensor chip with a 50-nm-thick gold film on a BK7 glass slide was modified for use as a recording chamber using a Teflon ring (Kim et al., 2008). We formerly verified the biocompatibility of the sensor chip by 1-month neuron culture. A low-power He-Ne laser of $\lambda = 632.8$ nm was used as a light source. The laser beam is collimated before striking the prism, allowing the beam to be focused on the gold surface with a limited spot size of $\sim$1 mm in diameter. Our SPR setup was based on motorized rotation stages for angle-scanning measurements with a nominal angular resolution at 0.001°. During the osteoblast differentiation experiments, SPR curves were measured at the range of incidence angle from 73.5° to 82.5° with a resolution of 0.01°. The reflected light was detected by an amplified Si photodetector (PDA100A-EC; Thorlabs, Newton, NJ) with a gain of 30 dB. The angle-scanning signals were recorded and time-labeled by a single data acquisition board. The initial version of this SPR platform exhibited a minimally measurable refractive index change of about $10^{-5}$.

MC3T3-E1 preosteoblast cells were cultured in α-MEM supplemented with 10% heat-inactivated FBS and 1% penicillin streptomycin. The cells were grown in a humidified atmosphere of 5% $CO_2$ at 37°C. Osteogenic medium consisted of α-MEM-based culture medium with 10 mM β-glycerol phosphate and 50 μM ascorbic acid. Also, recombinant human BMP-2 was added at a concentration of 100 ng/mL in the medium. Under these conditions, MC3T3-

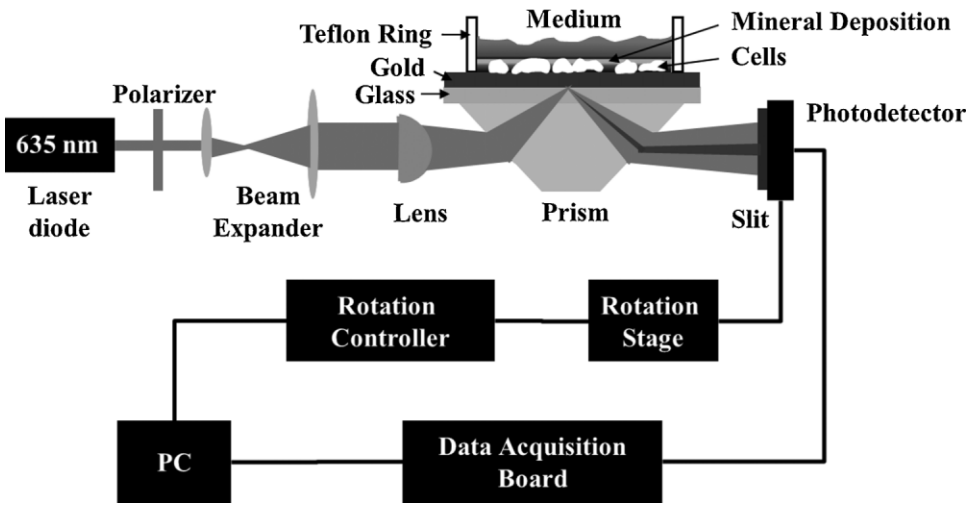


**Figure 1.** Schematic of the angle-scanning SPR system. The experimental setup is based on the attenuated total reflection configuration in which an incident beam is coupled through a BK7 prism on a glass slide. And the SPR substrate containing cells and media is developed to observe the resonance angle changes using motorized rotation stage. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

E1 cells were observed for 4 weeks on the SPR sensor chip by plating in the cell density of $2 \times 10^4$ cells/cm$^2$. For a comparison study, two experimental groups with and without differentiation reagents were designed. The reagents in the osteogenic media included β-glycerol phosphate, ascorbic acid, and BMP-2. Before seeding the cells, the sensor chip was prepared by cleaning with sonication in acetone, 70% ethanol, and Milli-Q water and by UV-irradiation to remove any dust or organics on the surface. All media were changed every 3 days.

For ICP measurement, the cultured samples were fixed in 10% formaldehyde and dried. The samples were then placed in 10 mL of 60% nitric acid solution. Nitric acid was used primarily in the preparation of inorganic sample types, as it is useful in the destruction of organics. Subsequently, the solution was stirred and heated above 150°C. After dissolving organic components, the solution was carefully collected and diluted to 10 mL with DI water. Finally, calcium concentration was determined using an ICP optical emission spectrometer (730-ES; Varian, Palo Alto, CA).

von Kossa staining is used to qualitatively estimate the phosphate content in the cultured cells. Cells were cultured for 4 weeks with or without osteogenic reagents as described above. The cultured cells in 24-well plates were stained every week to assess the mineralized matrix. First, the cells were washed with PBS, fixed in 10% formaldehyde solution for 12 h, and washed in deionized water (DI water). The fixed cells were incubated with 5% silver nitrate (Kojima Chemicals Co., Sayama, Japan) solution for an hour in the dark and rinsed with DI water. The cells were treated with sodium-carbonate formaldehyde solution for 2 min, and then incubated with staining solution (1% potassium ferrocyanide trihydrate and 9% sodium thiosulfate) for 20 min after washing in DI water. Finally, the stained cells were washed in DI water and left to air-dry to obtain images using a digital camera.

Before the SPR monitoring with two experimental groups, conditions of MC3T3-E1 cells in the osteogenic medium after growing on a gold film were observed by microscope for 4 weeks. While not shown here, cell proliferation and confluence terminated within a week and were subsequently followed by inhibition of proliferation and division due to the post-confluence effect (Quarles et al., 1992; Sudo et al., 1983). During proliferation, the cell population tended to increase slowly until the bone-forming process began. And then, significant amounts of collagen and alkaline phosphatase accumulated as extracellular matrix in mature cultures and the matrix was mineralized. To examine the kinetics of osteogenic differentiation of the MC3T3-E1 preosteoblasts non-destructively, we monitored the SPR signals every 2 days for 4 weeks.

Time-dependent resonance angle shifts during differentiation of the MC3T3-E1 cells are shown in Figure 2. To determine the SPR angles and their shifts from the experimental data, third-order polynomial fits were used as presented in Figure 2a and b. When the cells were cultured in the medium with no osteogenic reagents, the SPR angle increased monotonically from $0.03^\circ \pm 0.02^\circ$ on day 1 to $6.04^\circ \pm 0.44^\circ$ on day 28. Note that, while multiple sites in a given sample were measured by spatial translation to ensure consistency and thereby to reduce variation, the standard error was relatively large because the cell distribution was not perfectly uniform on the gold substrate. Also, based on the beam spot size and penetration depth of surface plasmons, overall intra- and extra-cellular changes occurring in the vicinity of the sensor surface, ~100 nm in thickness at $\lambda = 633$ nm (Brekhovskikh 1980), were measured effectively and about 160 cells in the course of differentiation were estimated to participate in individual SPR experiments.

The results in Figure 2c show that both the increase in cell population and the creation of by-products associated with osteogenic differentiation caused a corresponding refractive index change on a gold film, leading to a significant SPR angle shift. In particular, the data obtained from the osteogenic medium showed a steeper resonance shift ranging from $0.03^\circ \pm 0.02^\circ$ (day 1) to $8.19^\circ \pm 0.59^\circ$ (day 28). This enhanced plasmon shift by more than 30% was attributed to the effect of the osteogenic reagents which accelerate the formation of mineral substances inside the MC3T3-E1 cells. In other words, because facilitating reagents can effectively invigorate cellular activities, more bone-forming materials were produced and driven closer to the sensor surface, transporting a higher mass, and yielding a larger refractive index contrast. Using linear regression analysis, a relationship between the time-dependent differentiation and resonance angle shift was determined. The correlation coefficient ($R$), where $R$ denotes the degree of the linearity, was equal to 0.9675 and 0.9793 for the cultures without and with osteogenic reagents, respectively, implying a fairly linear detection of the bone-forming process. Note that, for wide resonance change larger than 1°, the amount of SPR shift generally shows a nonlinear response due to the dispersion relation which indicates nonlinearity between refractive index change and SPR angle (Shumaker-Parry et al., 2004; Weiger et al., 2010). However, since our intention in this study is only to demonstrate the conceptual influence of an overall time-dependent variation of the cultured cells on the SPR angle shift, a simplified linear model was considered and a more complex analysis for accurate quantification will be performed in our subsequent study.

Next, to analyze the cell differentiation quantitatively, ICP technique was employed to measure the amount of calcium, a key indicator of mineralization, formed in the MC3T3-E1 cells by processing 1 mg of the cultured samples (Fig. 3). For both cultures with and without osteogenic reagents, calcium content gradually increased with the bone-forming process. Of importance is that the results obtained from the osteogenic medium clearly show a drastic improvement in calcium content. This improvement implies that the MC3T3-E1 cells can be differentiated and mineralized more efficiently with the help of osteogenic reagents. The overall

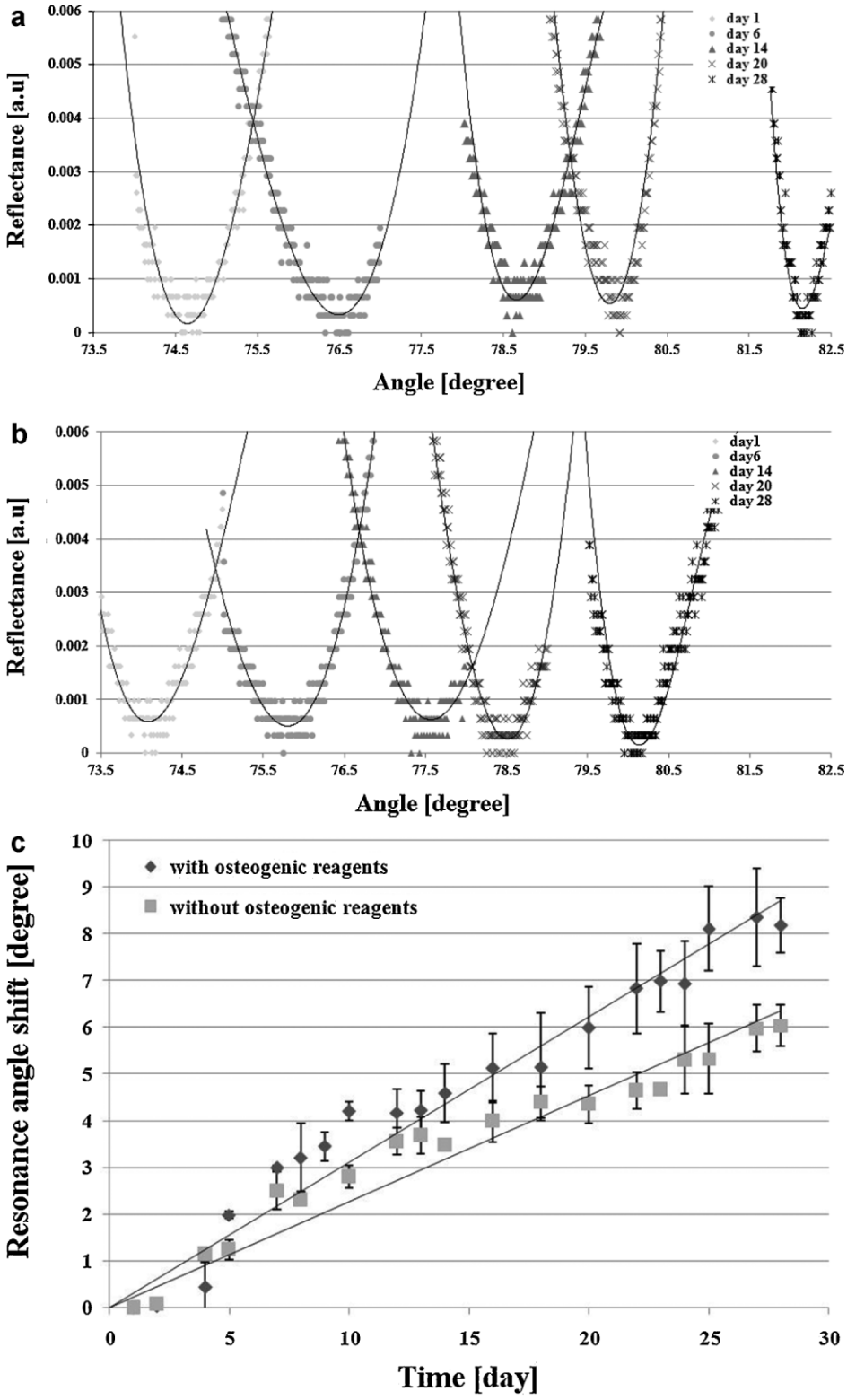


**Figure 2.** Reflectance curves of the samples (**a**) without and (**b**) with osteogenic medium. The measured data are fitted by third-order polynomial to determine SPR angles and their shifts. **c**: Linear regression analyses of time-dependent SPR angle shift for MC3T3-E1 cells cultured with (dark-gray diamonds, $n=3$, $R=0.9793$) and without (bright-gray cubes, $n=4$, $R=0.9675$) osteogenic reagents.

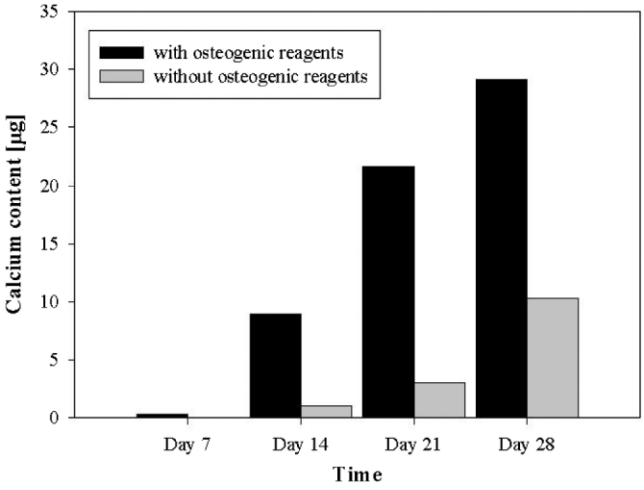


**Figure 3.** ICP spectrometer results to detect calcium content for 4 weeks with and without osteogenic reagents. The vertical axis shows the calcium content (in μg) in 1 mg of cells.

trends in Figure 3 are in good agreement with the SPR characteristics in Figure 2c. Although it is not simple to estimate accurate contribution of the amount of calcium to an SPR angle shift without any supplement of selective detection techniques, it is found from the results in Figures 2 and 3 that growing SPR angle shifts for the two groups and their net contrast resemble a time-dependent qualitative tendency of ICP measure. Moreover, an acceleration effect of osteogenic medium on the enhancement of differentiation is commonly shown for both SPR and ICP results.

It is interesting to note that no production of calcium was observed until the seventh day, especially for the cells cultured in the medium without osteogenic reagents. For the cells with the reagents, the calcium content was also very little at the first week, demonstrating that the matrix formation and mineralization did not play a role in the initial stage of osteogenesis. In this period, it seems that only cell proliferation might happen as differentiation begins in earnest when proliferation is diminished (Shahdadfar et al., 2005; Wang et al., 2005). Due to the cell proliferation occurring in all cultures regardless of the presence of the osteogenic reagents, the results at the first week exhibited no significant difference in resonance shift for the two groups, as shown in Figure 2c. On the other hand, the contrast in plasmon shift became prominent thereafter and the calcium content increased rapidly with the assistance of osteogenic reagents. As inferred from the increases in calcium content in Figure 3, although the MC3T3-E1 cells can be differentiated and mineralized by nature, osteogenic medium with osteogenic reagents can contribute to a rapid mineralization.

As another verification study for validating the SPR measure of osteoblast differentiation, mineral deposition was observed using conventional von Kossa staining every 7 days. In Figure 4, von Kossa staining of 7-, 14-, 21-, and 28-day samples of the MC3T3-E1 cells displayed a change in the deposited sediments of the mineralized matrix. On the day 7, mineral formation was rarely found in both groups, in accord with the imperceptible production of calcium at the first week in Figure 3. After a week, however, the staining images showed a denser deposition in the samples with osteogenic reagents than for the cells without the reagents. The overall change of staining color caused by mineral deposition was found to be highly correlated with the trend of calcium content shown in Figure 3. It is also important to find that, contrary to the conventional staining methods used for verification (Bonewald et al., 2003), SPR detection can provide unique advantages, such as rapid, non-destructive, and quantitative detection for the osteogenic differentiation.

Finally, our SPR approach to detect mineralization may have a limitation in selectivity because we did not attach any

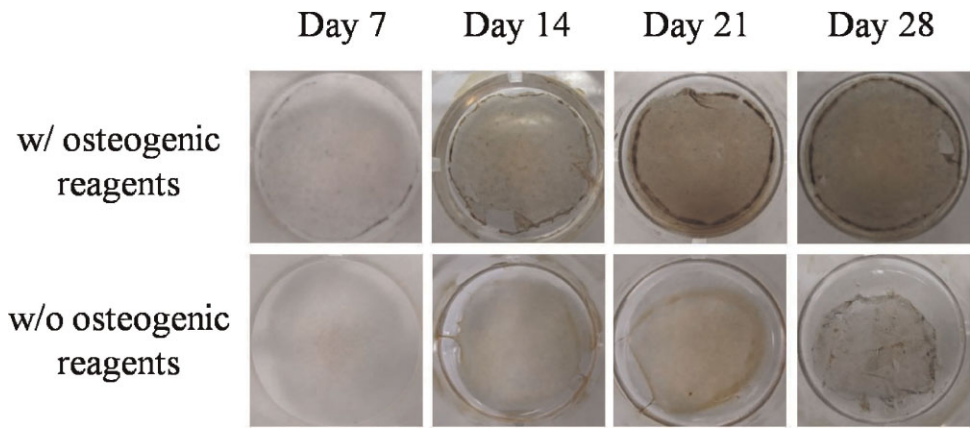


**Figure 4.** von Kossa staining of mineralized cells cultured with (upper row) or without (lower row) osteogenic reagents in the culture medium. From second week, the staining color of the upper samples is considerably darker than for the lower cells because of the effect of the differentiation-promoting reagents. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

bound ligands on the gold surface, and thus, it was not feasible to discriminate differentiation phase and any specific mineralization substance. To achieve a selective detection in mixed mineral compositions, the use of tagging agents or carriers with selective affinity to the target analytes of interest would be required. Interestingly, selective adsorptions of specific ions in aqueous solutions on gold surfaces have been demonstrated with self-assembled monolayers (Chah et al., 2004) and enzyme-linked immunoassays (Cao and Sim 2007). As a result, it is expected that surface treatments or tagging agents will enable to not only overcome the limitation on selectivity but also improve sensitivity by means of external labels providing additional mass.

In summary, we have demonstrated that a SPR biosensor may provide a non-destructive, label-free, and rapid detection of osteoblast differentiation. In especial, SPR technique is advantageous for in vitro observation with no requirement of complex treatment or external labeling. From the SPR experiments, the results presented a successful real-time monitoring of osteoblast differentiation with a large dynamic range. Moreover, the trend in SPR angle shift was well consistent with the results of conventional ICP and staining methods. Finally, non-destructive and continuous SPR detection is expected to realize the possibility of implementing long-term live cell monitoring for clinical and cytometric analyses.

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