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Giemsa as a fluorescent dye for mineralizing bone-like nodules *in vitro*

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Abstract

Giemsa was first used as a fluorescent dye for mineralized bone and cartilage in tissue sections. The aim of this study was to establish the use of Giemsa as a fluorescent dye for mineralizing bone-like nodules produced in cell cultures. Osteoblasts were grown under mineralizing conditions for 14 days, producing typical bone-like nodules. Upon staining with Giemsa stock solution for 1 min, the mineralizing nodules could be selectively visualized emitting intense green and red fluorescence when observed under blue and green illumination, respectively. The textural details of the nodules were clearly observed under fluorescence microscopy, allowing to identify regions with different degrees of mineralization. The mineralized nature of the nodules was confirmed using von Kossa's method, Alizarin Red S staining and x-ray mapping for Ca and P in a scanning electron microscope, showing a strong correlation between the mineralizing and the fluorescent nodules. The selective fluorescence was related to the mineral phase, being absent in decalcified samples. The use of Giemsa as a fluorescent dye for mineralizing bone-like nodules presents a simple alternative method to quickly analyze biomineralization assays *in vitro* under fluorescence microscopy, particularly in the biological evaluation of biomaterials.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Cell culture systems are often used as a model to study bone mineralization *in vitro* (Boskey and Roy 2008). Different cell types, such as osteoblasts (primary cultures and cell lines), osteogenic progenitors and mesenchymal stem cells, when induced to differentiation under proper culture conditions can promote matrix mineralization in a process similar to natural bone formation (Bonewald *et al* 2003, Midura *et al* 2004,

Barragan-Adjemian *et al* 2006, Hoemann *et al* 2009). Several techniques have been used to identify, analyze and quantify mineralizing bone-like nodules *in vitro* (Boskey and Roy 2008), among which the von Kossa staining for phosphate deposits, and Alizarin Red S staining for calcium deposits are the most widely used staining methods (Barragan-Adjemian *et al* 2006). They are essential tools in the initial and overall analysis of the cultures, although both staining procedures themselves are not sufficient to characterize the mineralizing nodules produced *in vitro* as a bone-like matrix (Bonewald

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et al 2003). In order to confirm bone-like properties *in vitro*, ultrastructural analyses using scanning and transmission electron microscopy (Nanci *et al* 1996) and atomic force microscopy (Barragan-Adjemian *et al* 2006) may be applied. Moreover, the mineral phase may be investigated using x-ray diffraction, Fourier transform infrared spectroscopy and nuclear magnetic resonance (Kuhn *et al* 2000).

Giemsa staining is routinely used in hematology, histology, and in the identification of Plasmodium parasites in malaria (Barcia, 2007). This stain produces the Romanowsky–Giemsa effect, based on the combination of the anionic dye Eosin Y and the cationic dye Azure B (Wittekind 1983). The use of Giemsa as a fluorescent dye was first established for mineralized bone and cartilage in tissue sections (Bradbeer *et al* 1994). The simple one-step method described a previously unrecognized differential staining effect of Giemsa, which allowed the selective visualization of the mineralized matrix under fluorescence microscopy. Other fluorescent dyes, such as Calcein and Xylenol Orange, were previously used to analyze mineralizing nodules *in vitro* (Hale *et al* 2000, Uchimura *et al* 2003, Wang *et al* 2006). However, the establishment of Giemsa as a fluorescent dye for mineralizing bone-like nodules may present a quick alternative method for osteogenic cell differentiation studies *in vitro*, especially when the traditional staining methods are not ideal, such as on the surface of titanium disks (Goto *et al* 2003).

2. Materials and methods

The cells used in this study were F-OST osteoblasts, previously isolated from murine femurs and shown to form spontaneously mineralizing nodules in culture (Balduino *et al* 2005). The ultrastructure and mineral phase of the matrix assembled in F-OST osteoblast cultures was recently characterized, presenting many bone-like features (Querido *et al* 2011). The cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (FBS) and antibiotics (100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin) at 37 °C in a humidified 5% CO₂ incubator. For the biomineralization assay, cells were seeded at a density of 1.3×10^4 cells cm⁻² on 13 mm² glass coverslips in 24 well plates and, once semi-confluence was reached, cultured for 14 days in the presence of 50 µg mL⁻¹ ascorbic acid and 10 mM β-glycerophosphate to accelerate matrix and mineral formation (Boskey and Roy 2008).

At the end of culture, cells were rinsed with phosphate buffered saline (PBS) and fixed in ice-cold methanol at about −18 °C for 20 min, followed by three 5 min washes with PBS. The cultures were stained with Giemsa stock solution for 1 min and washed thoroughly with distilled water. The samples were air-dried and observed in an Olympus IX71 fluorescence microscope equipped with an Olympus DP71 CCD camera and a filter wheel. For green fluorescence analysis, a 450–490 nm excitation filter (blue illumination) and a 500–530 nm emission filter were used, typically for fluorescein isothiocyanate (FITC), for example. For red fluorescence analysis, a 520–560 nm excitation filter (green illumination) and a 580–620 nm emission filter were used,

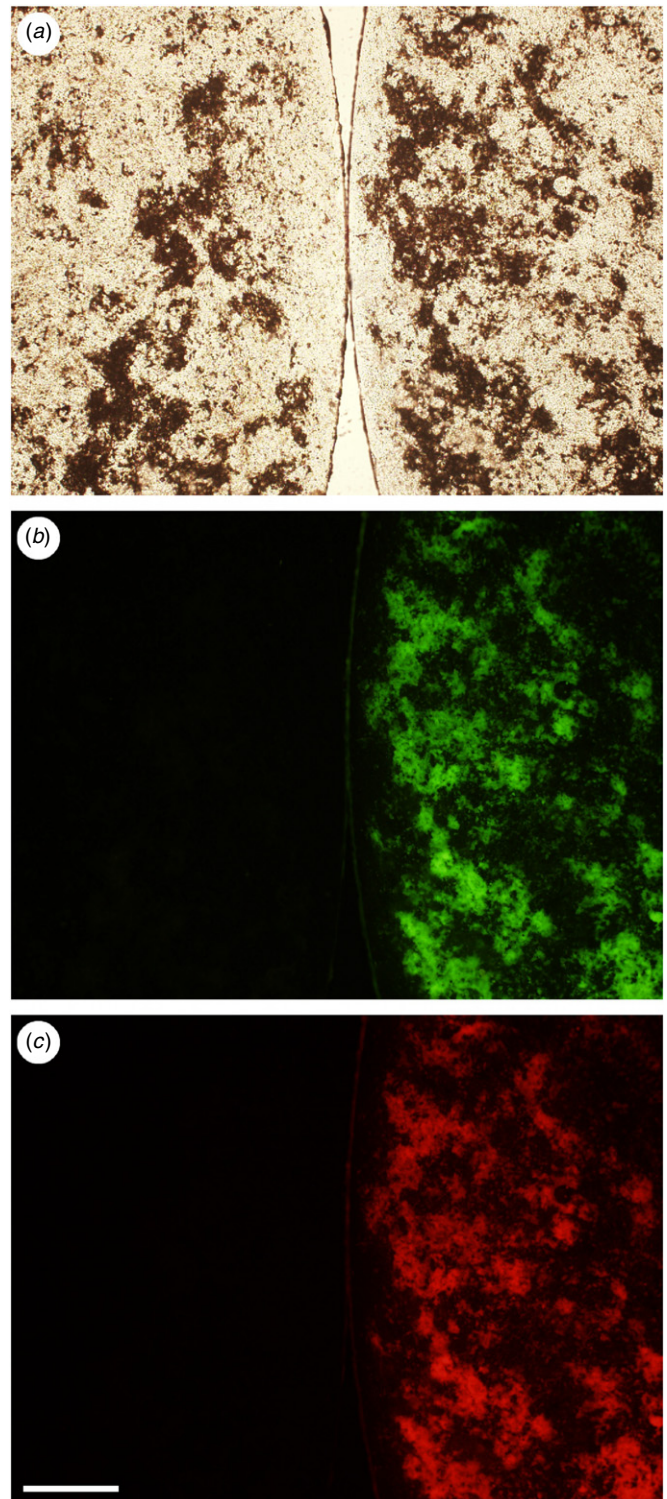


Figure 1. General analysis of the cultures. The sample on the left was unstained and the sample on the right was stained with Giemsa stock solution for 1 min. The same field is presented in all images: (a) brightfield micrograph (mineralizing nodules in both samples present similar aspect), (b) fluorescence micrograph under blue illumination (note the intense green fluorescence of the mineralizing nodules upon staining with Giemsa) and (c) fluorescence micrograph under green illumination (note the intense red fluorescence of the mineralizing nodules upon staining with Giemsa). Scale bar: 0.5 mm.

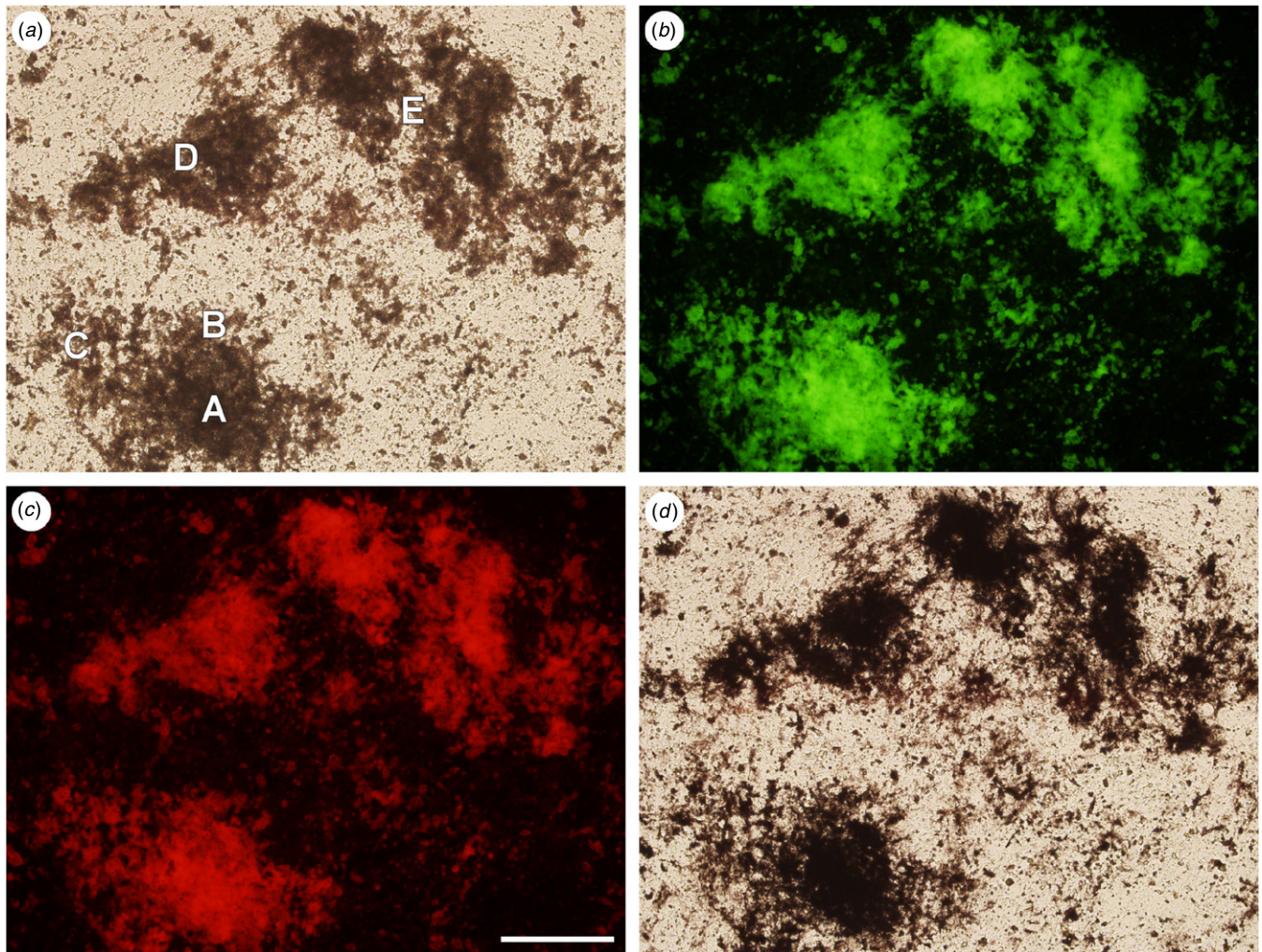


Figure 2. Analyses of the mineralizing nodules. The sample was stained with Giemsa and the same field is shown in all images. (a) Brightfield micrograph. Note the texture of the nodules, presenting dense mineralized centers (region A) and less mineralized peripheries (region B), and different degrees of mineralization (region A compared to region C) and fusion with near neighbors (region D compared to region E). (b) Fluorescence micrograph under blue illumination. (c) Fluorescence micrograph under green illumination. The textural details of the mineralizing nodules can also be observed under fluorescence microscopy. (d) Brightfield micrograph of the sample further stained using von Kossa's method. Note the strong correlation between the fluorescent nodules and the black stained mineralizing nodules. Scale bar: 0.25 mm.

typically for tetramethylrhodamine isothiocyanate (TRITC), for example. In order to decalcify the cultures prior to Giemsa staining, the samples were incubated into 10% EDTA solution in distilled water overnight at about 6 °C, followed by five 30 min baths with the same solution and washes with distilled water.

The von Kossa staining method was used to reveal phosphate deposits in black after Giemsa staining. Briefly, the samples were exposed to a 2% silver nitrate solution under sunlight for 20 min and rinsed with distilled water. Unreacted silver salts were dissolved using a 5% sodium thiosulfate solution and washed off. Alizarin Red S staining was used to show calcium deposits in red after Giemsa staining. Briefly, the samples were subjected to a 2% Alizarin Red S solution pH 4.2 for 10 min and rinsed with distilled water.

Energy dispersive x-ray spectroscopy (EDS) elemental mapping analysis was performed to show the distribution of Ca and P in the cultures. The samples were analyzed in a

JEOL JSM-6460LV scanning electron microscope at 20 kV equipped with a Noran EDS system.

Further analysis was performed by scanning electron microscopy (SEM) backscatter imaging. Briefly, cells were cultured on 13 mm² glass coverslips as described above and fixed in 2.5% glutaraldehyde for 60 min, followed by three 10 min washes with 0.1 M sodium cacodylate buffer. The samples were dehydrated in 10 min baths with a graded ethanol series (35%, 50%, 70%, 80%, 90% and twice with 100% ethanol) and critical point-dried using CO₂ in a Balzers CPD 020. The samples were sputter-coated with gold in a Bal-Tec SCD 050 and observed in an FEI Quanta 250 microscope at 25 kV.

3. Results

The osteoblast cultures achieved the formation of typical mineralizing bone-like nodules, identified as heavy deposits

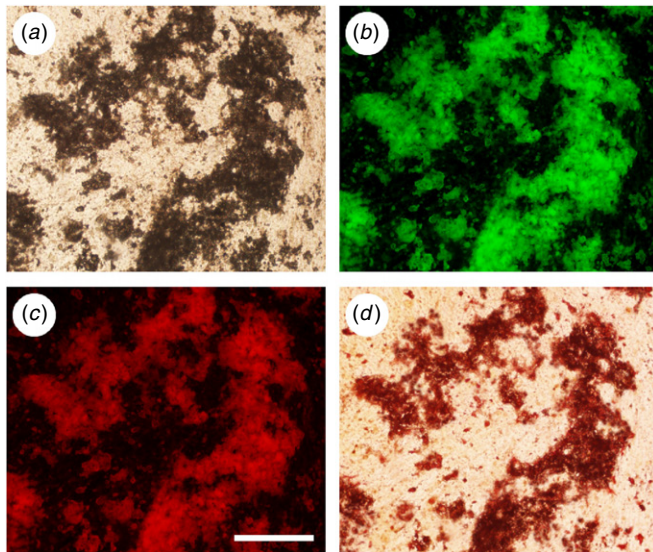


Figure 3. Control series with Alizarin Red S staining. The sample was stained with Giemsa and the same field is shown in all images: (a) brightfield micrograph, (b) fluorescence micrograph under blue illumination, (c) fluorescence micrograph under green illumination and (d) brightfield micrograph of the sample further stained with Alizarin Red S. Note the strong correlation between the fluorescent nodules and the red stained mineralizing nodules observed in the previous figures. Scale bar: 0.25 mm.

under brightfield microscopy (figure 1(a)). When stained with Giemsa, the mineralizing nodules were seemingly unstained under brightfield microscopy, presenting the same aspect as the unstained samples (figure 1(a), right plate); however, they could be selectively visualized emitting intense green (figure 1(b), right plate) and red fluorescence (figure 1(c), right plate) when observed under blue and green illumination, respectively. No fluorescence was noticed in the unstained samples: neither emitting green fluorescence (figure 1(b), left plate) nor red fluorescence (figure 1(c), left plate).

The mineralizing nodules observed under brightfield microscopy presented a distinct texture, with dense mineralized centers (figure 2(a), region A) and less mineralized peripheries (figure 2(a), region B). Moreover, they could be seen with different degrees of mineralization (figure 2(a), region A compared to region C) and fusion with near neighbors (figure 2(a), region D compared to region E). These textural details were also revealed under fluorescence microscopy, emitting green (figure 2(b)) and red fluorescence (figure 2(c)). The intensity of the observed fluorescence presented a strong correspondence to the distinct texture of the mineralizing nodules seen under brightfield microscopy.

To confirm that the Giemsa-stained nodules visualized under fluorescence microscopy were in fact mineralized, the samples were stained using von Kossa's method after analyzed for Giemsa staining, and the same regions were observed under brightfield microscopy. A strong correlation could be noticed between the fluorescent nodules and the mineralizing nodules stained using von Kossa's method (figure 2(d)). Similar results were obtained with Alizarin Red S staining (figures 3(a)–(d)). The observed fluorescence pattern also presented a strong

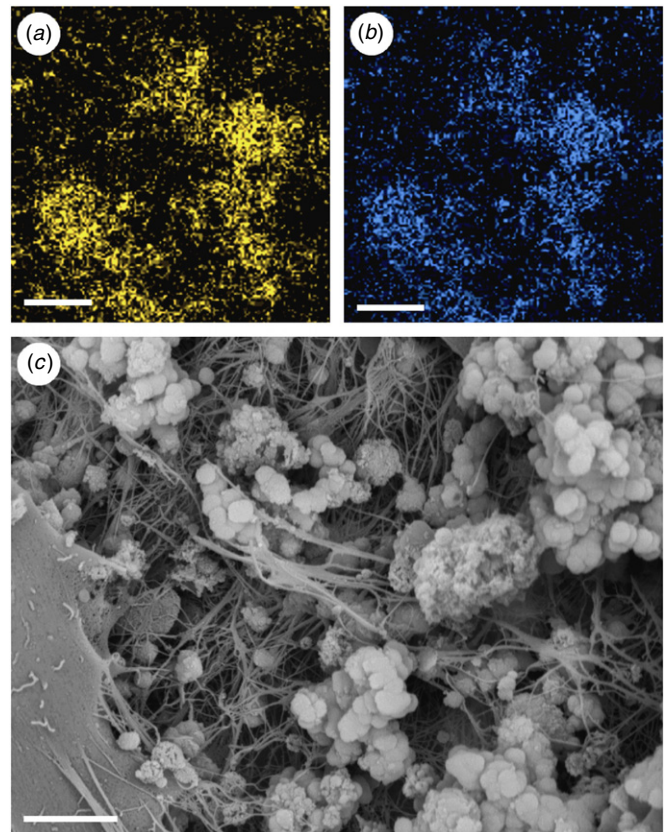


Figure 4. EDS and SEM analyses of the mineralized cultures. (a) EDS Ca mapping, (b) EDS P mapping (note the strong correlation between the distribution of Ca and P in the cultures and the fluorescence pattern) and (c) SEM backscatter micrograph. The matrix presented many mineralized globular accretions and arrays of collagen fibrils. Scale bars: for (a) and (b) is 0.15 mm and for (c) is 5 μ m.

correlation with the distribution of Ca and P in the cultures, obtained using EDS elemental mapping analysis on a scanning electron microscope (figures 4(a) and (b)).

The mineralizing nodules were further investigated by SEM backscatter imaging (figure 4(c)). The matrix produced in culture comprised numerous globular accretions and arrays of poorly oriented collagen fibrils, often found beneath the cell layers. The formation of hydroxyapatite was mainly associated with the globular accretions, which seem to be related to the matrix vesicles described in bone mineralization (Anderson 2003). A detailed characterization of the ultrastructure and mineral phase of the bone-like matrix produced in F-OST osteoblast cultures can be found elsewhere (Querido *et al* 2011).

To evaluate if the mineral phase was the matrix component being observed under fluorescence microscopy, cultures were decalcified prior to Giemsa staining. A regular sample stained with Giemsa was used as a positive control (figure 5(a), right plate). After decalcification, the mineral deposits were no longer seen on brightfield microscopy (figure 5(a), left plate), and no fluorescence was observed: neither emitting green fluorescence (figure 5(b), left plate) nor red fluorescence (figure 5(c), left plate). In line with this observation, synthetic hydroxyapatite could be stained with Giemsa, presenting

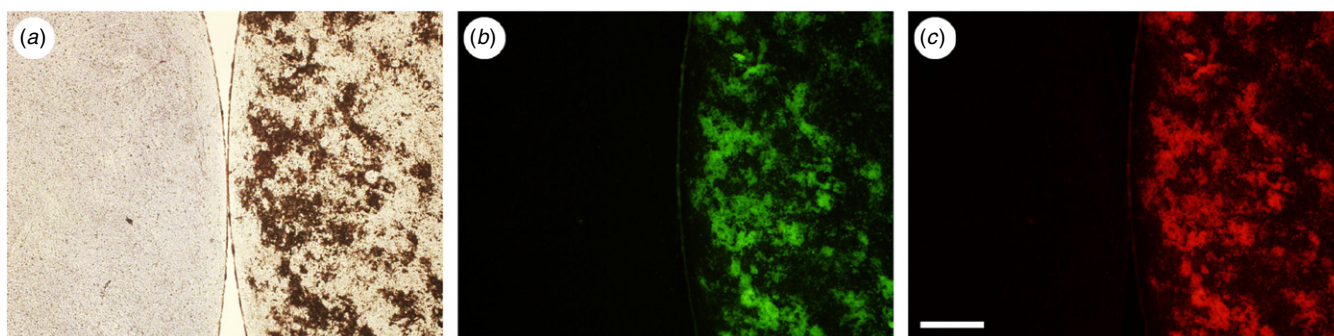


Figure 5. Analysis of the decalcified cultures. The sample on the left was decalcified prior Giemsa staining. A regular sample stained with Giemsa is presented on the right and the same field is shown in all images: (a) brightfield micrograph (note the absence of mineralizing nodules on the decalcified sample), (b) fluorescence micrograph under blue illumination and (c) fluorescence micrograph under green illumination (note the lack of fluorescence exhibited after decalcification). Scale bar: 0.5 mm.

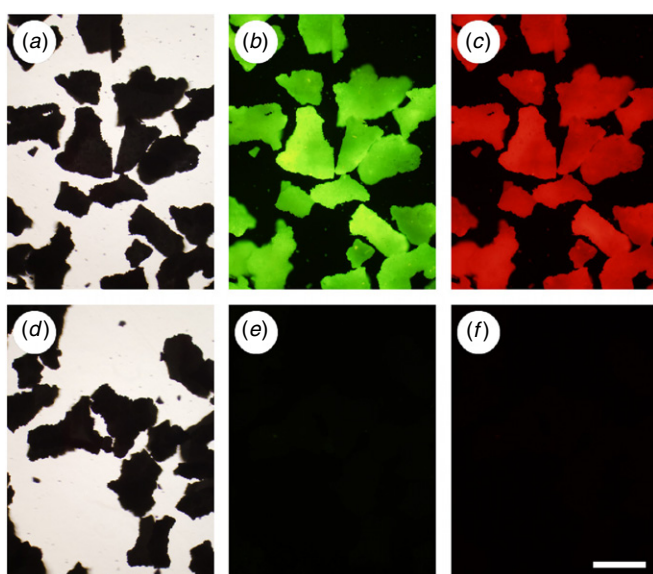


Figure 6. Analysis of Giemsa staining on synthetic hydroxyapatite: (a)–(c) sample stained with Giemsa stock solution for 1 min and (d)–(f) unstained sample. The same field is presented for each series. ((a) and (d)) Brightfield micrographs. The mineral is shown as dark (opaque) particles. ((b) and (e)) Fluorescence micrographs under blue illumination. ((c) and (f)) Fluorescence micrograph under green illumination. Scale bar: 0.25 mm.

a similar fluorescence effect as described on mineralizing nodules (figures 6(a)–(f)).

4. Discussion

Our results indicate that Giemsa can be used as a fluorescent dye for mineralizing bone-like nodules *in vitro*. The results obtained in this study are in agreement with the previously reported use of Giemsa as a fluorescent dye for mineralized bone and cartilage (Bradbeer *et al* 1994). Similar to tissue sections, the mineralizing nodules assembled in osteoblast cultures were selectively visualized under fluorescence microscopy upon staining with undiluted Giemsa stock solution for 1 min. The nodules could be observed emitting intense green and red fluorescence, although the latter

seemed slightly less clear. The use of fluorescent dyes was shown especially useful to analyze the mineralizing nodules produced in cell cultures performed on biomaterials, such as titanium disks (Goto *et al* 2003) and three-dimensional hydrogel scaffolds (Hamada *et al* 2008).

We describe in this study the establishment of a simple method to quickly analyze biomineralization in cell culture systems. This method can be used as an alternative to traditional staining (von Kossa, Alizarin Red S) and fluorescent dyes (Calcein, Xylenol Orange). An important observation herein described was on the textural details of the mineralizing nodules observed under fluorescence microscopy, which was not explored in studies with Calcein and Xylenol Orange (Hale *et al* 2000, Uchimura *et al* 2003, Wang *et al* 2006). The texture of the mineralizing nodules stained with Giemsa was clearly observed emitting green and red fluorescence, allowing to identify and distinguish regions with different degrees of mineralization.

The mineral phase was the only matrix component selectively visualized under fluorescence microscopy. A corresponding staining effect was described in bone samples, and an explanation was proposed (Bradbeer *et al* 1994). The observed fluorescence arises from the binding of the fluorescent dye Eosin Y to the mineral. However, Eosin Y is not specific to mineral and, when used alone, causes several tissue structures to be unselectively visualized under fluorescence microscopy. In contrast, the cationic dyes Azure B and Methylene Blue, which do not bind to the mineral, show no fluorescence properties. The structures that do bind to the cationic dyes, as well as those highly eosinophilic, would not show fluorescence (de Carvalho and Taboga 1996). Therefore, the selective fluorescence effect caused by Giemsa staining may be related to the faint presence of Eosin Y and the absence of Azure B and Methylene Blue bonded to the mineral phase.

In conclusion, the use of Giemsa as a fluorescent dye provides a quick, straightforward alternative method to selectively visualize the mineralizing bone-like nodules assembled in cell culture systems. This procedure may be especially useful in the initial and overall evaluation of *in vitro* biomineralization assays onto opaque biomaterials surfaces and of the newly formed mineralized tissue surrounding scaffolds used as grafts for bone engineering.

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