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Development of Osteoblast Colonies on New Bioactive Coatings

J.-G. Legoux, F. Chellat, R.S. Lima, B.R. Marple, M.N. Bureau, H. Shen, and G.A. Candelieri

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The aging baby boomer population coupled with an increase in life expectancy is leading to a rising number of active elderly persons in occidental countries. As a result, the orthopedic implant industry is facing numerous challenges such as the need to extend implant life, reduce the incidence of revision surgery, and improve implant performance. This paper reports results of an investigation on the bioperformance of newly developed coating-substrate systems. Hydroxyapatite (HA) and nano-titania (nano-TiO₂) coatings were produced on Ti-6Al-4V and fiber reinforced polymer composite substrates. In vitro studies were conducted to determine the capacity of bioactive coatings developed to sustain osteoblast cells (fetal rat calvaria) adherence, growth, and differentiation.

As revealed by scanning electron microscopy (SEM) observations and alkaline phosphatase activity, cell adhesion and proliferation demonstrated that HA coatings over a polymer composite are at least as good as HA coatings made over Ti-6Al-4V substrate in terms of osteoblast cell activity. Nano-TiO₂ coatings produced by high-velocity oxyfuel (HVOF) spraying led to different results. For short-term cell culture (4.5 and 24 h), the osteoblasts appeared more flattened when grown on nano-TiO₂ than on HA. The surface cell coverage after seven days of incubation was also more complete on nano-TiO₂ than HA. Preliminary results indicate that osteoblast activity after 15 days of incubation on nano-TiO₂ is equivalent to or greater than that observed on HA.

Keywords bioactive coatings, hydroxyapatite, nano titania, osteoblast adhesion, polymer composite substrate, Ti-6Al-4V substrate

1. Introduction

One of the largest successes of modern medicine is the total hip replacement surgery. Presently, this procedure has one of the highest success rates among surgical interventions, only second to the appendix removal procedure. However, the lifetime of the prosthesis itself is still limited to 10 to 20 years, which means that for numerous patients a revision surgery will become mandatory. This need for replacement arises for a number of reasons including aseptic loosening of the prosthesis. This can be caused by the formation of wear particles at articular joints and by the difference in stiffness between the bone and the metallic prosthesis, leading to the phenomena called stress shielding and implant migration. To overcome this problem, two solutions can be envisioned. The first implies design of more biomimetic prostheses, with bone-matching stiffness. A novel design based on polymer composite materials of total hip replacement prosthesis is under development (Ref 1). One of the key characteristics of

this biomimetic prosthesis is its hydroxyapatite (HA) coating, which permits osseointegration (integration with the bone). Thermally sprayed HA coatings are already used successfully for metallic implants (Ref 2), but such coatings have yet to be developed for polymer composites due to quite challenging heat management and adhesion concerns. The second approach is related to the development of new coating systems. Regarding this aspect, nanostructured titania (TiO₂) coatings have demonstrated interesting potential. The goal of this paper is to evaluate both coating systems with regard to their response to osteoblast (fetal rat calvaria) cells. Other properties for the nano-TiO₂ coating and its optimization are described in more detail in a companion paper (Ref 3).

2. Materials and Methods

2.1 Substrate Materials

Coatings were produced on two types of substrates, a titanium alloy (Ti-6Al-4V) that is widely used for hip prostheses and a polyamide 12/carbon fiber (PA12/CF) composite used for a novel design of hip prostheses (patent pending). To improve the coating adhesion and heat resistance, a 100 μm layer made of twin-screw-extruder compounded PA12/HA was overmolded onto composite substrates (patent pending).

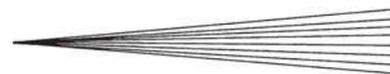
2.2 Bioactive Coatings

Two types of coatings over two different substrates were produced: a plasma sprayed HA coating and a high-velocity oxyfuel (HVOF) nano-TiO₂ coating on both polymer composite and Ti-based substrates.

2.2.1 HA Coating. A bioactive HA powder (Captal 30, Plasma Biotol Ltd, Tideswell, UK) was used for depositing HA

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coatings. Granulometry testing on the initial HA powder (LS Particle Size Analyzer, Beckman Coulter, Fullerton, CA) indicated a number-average diameter (sum of particle diameters/number of particles) of 33 μm . The HA coatings were produced using atmospheric plasma spray.

2.2.2 Nano-TiO₂ Coating. Titania feedstock used in this work (VHP-DCS, Altair Nanomaterials Inc., Reno, NV) exhibited a nominal particle size range from 5 to 20 μm . Each feedstock particle was formed via the agglomeration of individual nanostructured TiO₂ particles smaller than 100 nm. The feedstock powder was thermally sprayed via the HVOF technique using an oxypropylene-based torch (Diamond Jet 2700-hybrid, Sulzer Metco, Westbury, NY).

The coatings were sprayed on grit-blasted substrates to roughen the surface prior to spraying. During the spraying process on the substrates a cooling system (air jets) was applied to reduce the coating temperature, which was monitored using a pyrometer. The maximum surface temperature was approximately 240 °C for the Ti-6Al-4V substrates and 130 °C for the PA12/CF substrates. Detailed information about coating deposition, microstructural characterization, and mechanical property evaluation of this biomedical coating can be found in a companion paper contained in this journal (Ref 3).

2.3 Osteoblast Isolation and Seeding

Osteoblasts were isolated from the calvariae of 21-day-old Spargue Dawley rat fetuses by sequential collagenase digestion as described by Bellows et al. (Ref 4). The cells were then plated in T-75 flasks in a Dulbecco's modified Eagle medium containing 10% of fetal bovine serum. After 24 h, the adhered cells were washed with phosphate buffer saline to remove dead cells and other debris, then detached using 0.01% trypsin in phosphate buffer saline. The resuspended cells were counted and seeded on the different disc-shaped material surfaces previously placed in the six-well culture plates at 2×10^4 cells/well in an osteogenic medium (growth medium containing 50 mg/mL of ascorbic acid, 10 mM Na-b-glycerophosphate, and 1% antibiotics). The cells were incubated at 37 °C in a humidified atmosphere consisting of 95% O₂ and 5% CO₂ and allowed to grow for 4.5 h, 1, 7, and 15 days. For these periods, the medium was changed three times per week.

2.4 Osteoblast Adherence, Growth, and Differentiation on Materials

Scanning electron microscopy (SEM) observation was used to determine the adherence, morphology, and growth of the osteoblasts on the different coatings after 4.5 h, 1, 7, and 15 days. At the end of each incubation period, the cells were rinsed in phosphate buffer pH 7.2, fixed in a 0.089 M phosphate buffer solution containing 2.5% glutaraldehyde and 2.5 mM magnesium chloride, pH 7.2 for 3 h. The samples were then rinsed in 0.1 M phosphate buffer, postfixed in 1% osmium tetroxide for 1 h, washed in distilled water three times, and then dehydrated in a graded series of ethanol solutions (70 through 100% dry ethanol). Specimens were then treated with mixtures consisting of 75:25, 50:50, 25:75, and 0:100 ethanol:amyl acetate. The samples were dried by the critical-point drying method, sputter-coated by gold/palladium and observed using a scanning elec-

tron microscope (Hitachi, Model S-4700, manufacturer, Hitachi Science Systems, Ibaraki, Japan).

2.5 Alkaline Phosphatase Activity

The osteoblast phenotype of cells cultured on different surfaces was determined by enzymatic alkaline phosphatase activity test after 15 days. Before staining, coated samples (with attached cells) were rinsed once with cold PBS, then the cells were fixed in 10% cold neutral buffered formalin for 15 min, rinsed with distilled water, and then left in distilled water for 15 min. A fresh mixture constituted of 10 mg Naphthol AS MX-PO4 in 400 μL N,N-dimethylformamide, 50 mL distilled water, 50 mL of 0.2 M Tris-HCl pH 8.3, 60 mg red violet LB salt was used for alkaline phosphatase staining. The alkaline phosphatase staining mixture was placed on the coated samples covered with the fixed cells and incubated for 1 h at room temperature. All the chemicals were purchased from Sigma-Aldrich Chemical Company (Oakville, Canada). The different material discs were then removed from the wells, rinsed in tap water, drained and air dried, and then photographed. The alkaline phosphatase positive signal was quantified with Imagine J software. For normalization, the background color was subtracted by setting a threshold.

3. Results and Discussion

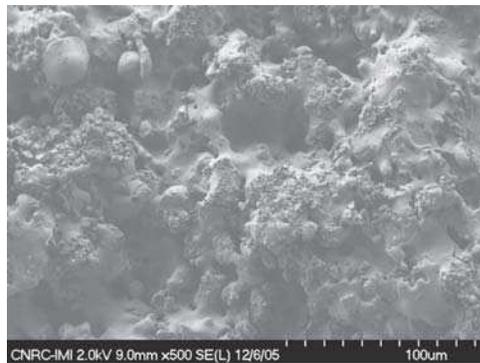
3.1 Coating Surfaces Characterization by SEM

Prior to cell culture experiments, HA and nano-TiO₂ coatings are quite different, as shown in Fig. 1. Both coating surfaces are constituted of smooth zones formed from the solidification of a liquid and rougher areas constituted either of unmelted or "oversprayed" material. The HA coating is considerably rougher than the nano-TiO₂ as can be expected when comparing a HVOF coating made with fast and small particles with plasma sprayed coatings made from relatively large and slow particles.

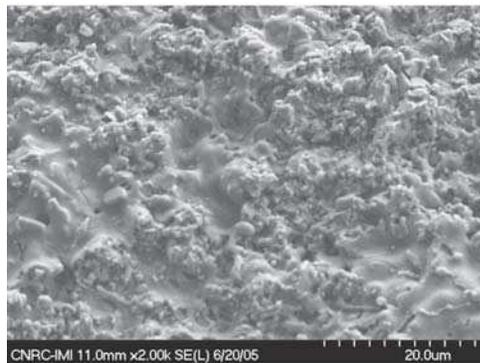
3.2 Cell Adhesion and Growth

After 4.5 h of incubation the coating surfaces were examined by SEM. Typical cell morphology is presented in Fig. 2 for cells attached to HA and nano-TiO₂ coatings. It can be noted that the two coating topologies are quite different, the plasma sprayed HA coating being rougher than the HVOF nano-TiO₂ that exhibits a smoother aspect caused by flattening of semimolten particles impinging on the surface at high velocity. Osteoblasts found on the nano-TiO₂ (Fig. 2b) are well flattened on the surface and have started to spread, whereas osteoblast cells do not appear to follow the contour of the HA coating surface (Fig. 2a). Similar elongated osteoblast cells not in close contact with the HA surface have been reported (Ref 5). Also, osteoblast cells were more difficult to locate on the HA surface, which might be related to a slower initial adhesion (Ref 6), but also to the difficulty of locating cells on a rougher surface (e.g., cells at bottom of valleys). It should also be noted that the HA coating surface was modified during its immersion in the culture media. Similar phenomena of dissolution and reprecipitation of apatite on coating surfaces have been reported (Ref 7, 8).

Images of cells after one day of incubation are presented in Fig. 3. As observed previously for 4.5 h, cell morphology on HA



(a)



(b)

Fig. 1 Typical surface morphology for (a) HA coatings and (b) nano-TiO₂ coatings

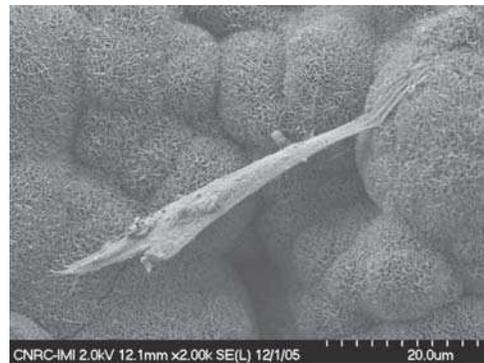
and nano-TiO₂ coatings is quite different. Cells remained with an elongated shape on the HA coating; they had a close to circular shape on nano-TiO₂. Interestingly, osteoblast cells attached to the HA surface appeared partly covered by mineral concretion, probably some HA precipitated from the culture media. Indeed, it has been widely demonstrated that while immersed in body fluid, plasma sprayed HA coatings undergo several modifications, with some phases dissolved while a layer is formed on the surface (Ref 7). It is possible in this case that the precipitation process took place as well on the cell surface. With time, cells become incorporated into the reprecipitated HA matrix.

After 7 days, cells spread to the complete substrate surfaces (Fig. 4). However, while they remain elongated and penetrate the HA coating structure (Fig. 4a), they cover the entire surface on TiO₂ coatings (Fig. 4b). Also apparent from Fig. 4(a) is the smoother HA surface after 7 days of incubation in culture media when compared with the shorter time (Fig. 2a and 3a).

After 15 days, osteoblast cells are also covering the entire surface of HA coatings (Fig. 5). It has been reported that solubilization of calcium phosphate phases might decrease cell adhesion early after the initial cell exposition, but this was compensated by a noticeable acceleration later in the process (Ref 9).

3.3 Alkaline Phosphatase Activity and Cell Differentiation

After 15 days of incubation the number of cells was characterized by the staining of the alkaline phosphatase, which pro-



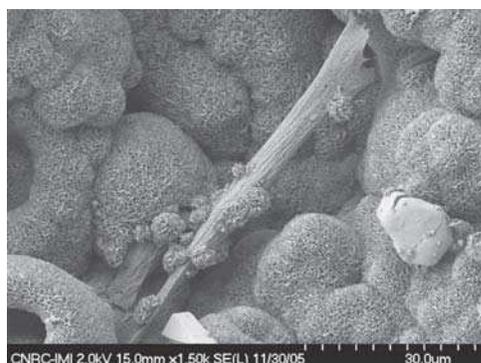
(a)



(b)

Fig. 2 Cell attachment after 4.5 h: (a) HA on PA12/CF substrate; (b) nano-TiO₂ on PA12C substrate

duce a red color. Figure 6 presents the substrate after staining. All surfaces were almost completely covered by red stained cells. Osteoblast morphology remains more elongated on the HA coatings (Fig. 5) compared with that developed on nano-TiO₂ coatings. It is difficult to evaluate the difference between white HA coatings and dark gray nano-TiO₂ coatings mainly due to the difference in contrast. To quantify the coating response, color image analysis was performed on samples after proper normalization to take into account the divergence in contrast caused by the substrate color. Results are presented by Fig. 7. The nano-TiO₂ coating exhibits the highest intensity, followed closely by HA coating on the polymer composite substrate, and finally HA coatings over Ti-6Al-4V substrate. These results indicate that even though no morphological difference was seen between the different substrates, the cell activity as defined by the alkaline phosphatase staining shows a difference. The reason for this difference is not explained at this point. However, it should be highlighted that the staining for alkaline phosphatase activity, as performed in this study, does not allow for a precise quantification. Also the large variation in the data especially for the HA coating over the Ti-6Al-4V substrate would justify further study. The quantification of alkaline phosphatase activity is very important especially for the rougher HA coating where only the stained surface is visible, whereas the cells embedded in the valleys or in the reprecipitated apatite are not shown. A more detailed and a quantifiable means of measurement will be required to investigate quantitatively the alkaline phosphatase activity in the future.



(a)



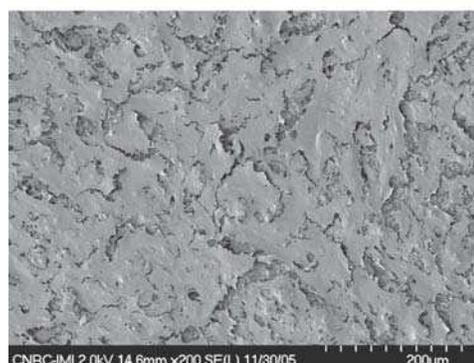
(b)

Fig. 3 Osteoblast morphology after 1 day: (a) HA on Ti-6Al-4V; (b) nano-TiO₂ on Ti-6Al-4V

The interpretation of the observed differences of the osteoblast initial adhesion and proliferation for the two coatings can be quite complex. Differences in coating chemistry may play a role; it has been shown that for titanium alloys, chemistry (i.e., pure Ti, Ti-6Al-4V, TiNb₁₃Zr₁₃, TiNb₃₀) (Ref 10) induces different responses. Also titanium oxide formed by laser heating might have a different effect than native oxide. However, other studies show no effect of the surface chemistry (Ref 6, 11). Another important factor linked to the cell adhesion is the wettability of the surface, which can have either a direct effect on adhesion by promoting the cell contact with the surface or an indirect one by promoting the protein unfolding at the surface (Ref 12-14). The effect of protein type on cell adhesion is certainly very important. For example, it has been shown that vitronectin, fibronectin, or osteopontin have an important effect on cell adhesion (Ref 10, 15). Transmembranous integrins might play a role in the signal transduction from the environmental milieu up to the cell nucleus leading to an appropriate cell response such as proliferation rates or morphology. This signal seems to be the consequence of the interaction of various molecules and growth factors (Ref 16). Regarding the effect of surface roughness, it has been shown that osteogenicity was enhanced by increasing surface roughness (Ref 17). However, it was shown that for samples with the highest roughness, cells were less adhesive, which was attributed to an effect of confinement of cells at the bottom of deep holes, leading to early decease and detachment. This might be related only to in vitro experiments. Other studies have related this apparent negative effect of high surface rough-



(a)



(b)

Fig. 4 Osteoblast surface after 7 days: (a) HA on PA12C; (b) nano-TiO₂ on PA12C



Fig. 5 Surface of the HA coating on PA12C substrate after 15 days of incubation

ness mainly to the fact that at the cell level the surface appears to be flat (Ref 18).

It is beyond the scope of this paper to determine what exact biomechanisms predominate in this study. Aside from their chemistry, three major differences can be identified between the HA and nano-TiO₂ coatings studied here:

- *Surface roughness*: the HA coatings are considerably rougher than the nano-TiO₂ coatings.
- *Crystallite size*: this peculiar microstructure of the nano-TiO₂ coatings contains well dispersed “nano-zones.”

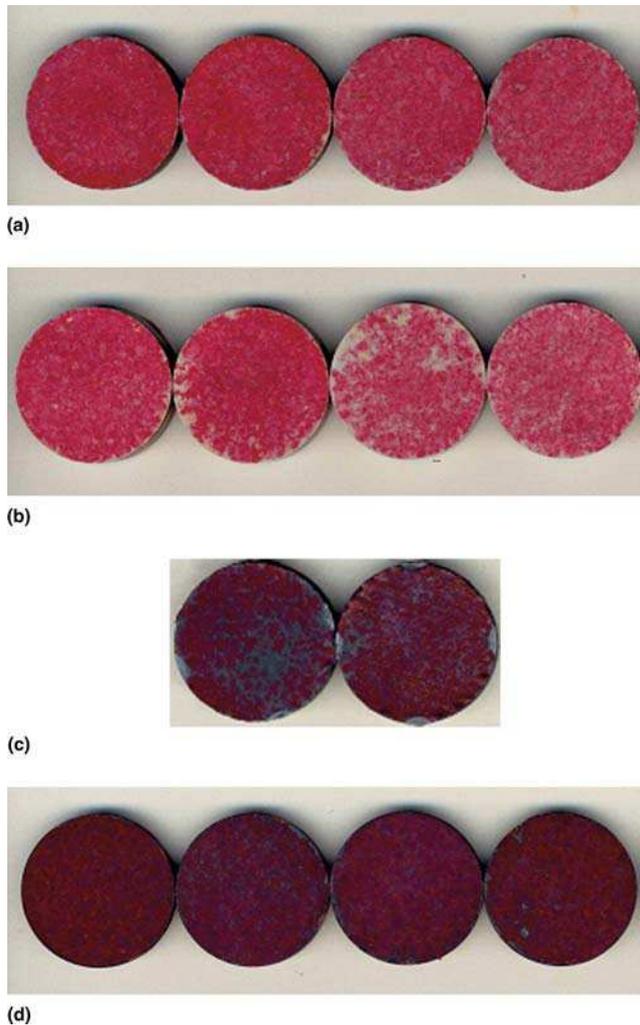


Fig. 6 Alkaline phosphatase stained sample surfaces after 15 days of growth. (a) HA coating on PA12C. (b) HA coating on Ti-6Al-4V. (c) Nano-TiO₂ coating on PA12C (only two replicate coatings tested). (d) Nano-TiO₂ coating on Ti-6Al-4V

- **Chemical stability:** higher stability of the nano-TiO₂ coatings compared with the dissolution and reprecipitation behavior of the HA coating.

The early stage of cell adhesion was characterized by direct observation of the coating surfaces, which can be more difficult on rough surfaces. The presence of nanostructured surfaces on the nano-TiO₂ coatings may help the unfolding of adhesion proteins, which results in very good growth and alkaline phosphatase activity values. At the same time, as cell adhesion involves surface adsorption phenomena, it is reasonable to think that these phenomena would be slower or even inhibited on a surface that is undergoing major transformation (both dissolving and reforming itself). This may explain why after a longer exposure time (7 days), when the surface became more stable, cell proliferation accelerated, reaching both a high degree of covering and of alkaline phosphatase activity.

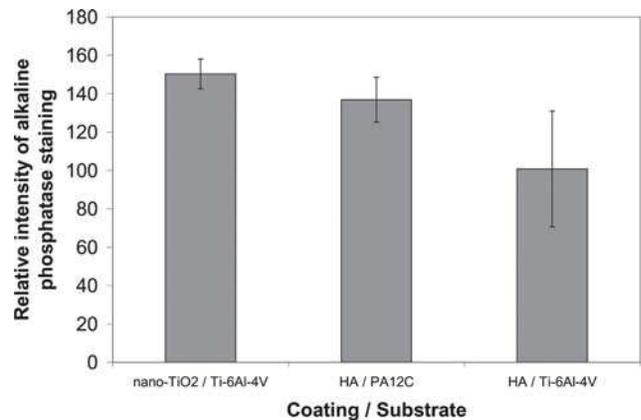


Fig. 7 Intensity of the alkaline phosphatase staining for different coatings

4. Conclusions

The current study demonstrates that nano-TiO₂ as well as HA coatings support the attachment, growth, and expression of the osteoblastic phenotype of the cells as assessed by the alkaline phosphatase activity assay.

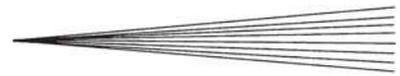
Results on cell adhesion and proliferation have demonstrated that hydroxyapatite coatings on a polymer composite are at least as good as HA coatings deposited on a Ti-6Al-4V substrate, in terms of osteoblast cell activity.

Nano-TiO₂ coatings produced by the HVOF technique behave differently when compared with HA coatings. Preliminary osteoblast cell culture revealed that the activity of the cells after 15 days of incubation is equivalent or superior to that observed on hydroxyapatite coatings.

The intrinsic structural and chemical differences of each type of coating led to some important differences in the attachment behavior of the cells and their morphological appearance (elongated versus flattened). Further planned studies may allow relating the effect of the coating structure and composition on the rate of osteoblast adherence and growth, as well as the differentiation stage as a function of time.

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