

Enhanced Osteoblast Adhesion, Proliferation and Differentiation on Nanocrystalline Diamond Coatings

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Abstract:

Osteoblast (OB, bone forming cell) functions on diamond are critical for considering the use of anti-abrasive diamond coatings on orthopedic implants. In this study, OB functions including adhesion (up to 4 hrs), proliferation (up to 5 days) and differentiation (up to 28 days) on various diamond coatings were investigated. Two kinds of diamond coatings (nano- and submicron-crystalline diamond) were fabricated through microwave plasma enhanced chemical vapor deposition and characterized by scanning electron microscopy (SEM) and atomic force microscopy (AFM). Surface roughness and hydrophobicity of the diamond coatings increased dramatically as grain size grew. These coatings were tested for OB adhesion and proliferation by counting adherent cells after incubation of 4hrs up to 5 days. OB differentiation on diamond coatings after incubation from 1 to 3 weeks was investigated by measuring total intracellular protein synthesis, alkaline phosphatase activity, and calcium deposition. Results demonstrated enhanced OB functions on nanocrystalline diamond coatings compared to submicron grain size coatings. The long-term (up to 3 weeks) functions of OB on nanocrystalline diamond coatings were promoted compared to submicron diamond, polished silicon and glass coverslips. In summary, these results provided insights into the application of diamond coatings in orthopedics, which can potentially improve wear problems of current implants and prolong their lifetime.

Keywords: Osteoblast; adhesion; proliferation; differentiation; nanocrystalline diamond

1. Introduction

An increasing demand for total hip and knee replacements requires improvements in durability and biocompatibility properties from today's orthopedic implants. In order to prolong the 10-15 year lifetime of current implants and reduce the need for revision surgeries, promoting bone cell functions at the bone-implant interface and fostering resistance to biochemical (erosion) and mechanical (especially wear) reactions are of great importance. Along these lines, it has been reported that diamond is one of the most robust materials for orthopedic applications to date, however, bone cell functions on diamond remain unclear.

Recent researchers have revealed several promising approaches to improve bone cell interactions on implants by introducing nanoscale features [1]. This rationale has been also applied to diamond coatings on orthopedic implants. Specifically, nanocrystalline diamond (NCD) coatings have been investigated for their promise in orthopedic applications and recent studies have demonstrated their strong potential. For example, Amaral *et al.* [2] fabricated NCD on a Si₃N₄ substrate and demonstrated improved osteoblast (OB, bone forming cells) proliferation and synthesis of OB differentiation markers, like alkaline phosphatase and extracellular matrix calcium mineralization, compared to polystyrene tissue culture plates. In addition, diamond with combined micron and nano features promoted osteoblast proliferation the most compared to polystyrene tissue culture plates after 3 days [3].

However, a thorough study concerning both short and long-term functions of OB (specifically, adhesion, proliferation and differentiation) has not been completed on diamond of various micron to nanometer surface features. The present work revealed a systematic investigation on OB functions from 4 h up to 3 weeks on both NCD with grain sizes <100 nm and submicron diamond (SMD) with grain sizes 100-600 nm. Interactions between diamond surface properties and OB functions were also related here.

2. Materials and Methods

Diamond coatings on polished silicon were fabricated through microwave plasma enhanced chemical-vapor-deposition (MPCVD) using Ar-H₂-CH₄ plasma. The concentration of CH₄ was kept at 1 vol.% and H₂ varied from 5 vol.% to 20 vol.% for NCD and SMD, respectively. The silicon substrates were seeded with nano diamond powders with average sizes around 50 nm before growth. The growth pressure and temperature were 140 torr and 800 °C, respectively, with a growth time of 2 h.

The surface topography of NCD and SMD was examined by scanning electron microscopy (SEM, LEO 1530VP, Zeiss, field emission gun at 4 kV) and atomic force microscopy (AFM, AutoProbe, Park Scientific Instrument, silicon nitride tips, contact mode at a scanning speed of 1 Hz). The root mean square (RMS) roughness of the coatings was calculated from AFM data based on 10 × 10 μm scans. Contact angles on diamond surfaces were measured through the sessile drop shape method.

Human osteoblasts (OB; CRL-11372, ATCC, population number between 10 and 15) were used for adhesion, proliferation and differentiation tests. For the adhesion assays, the cells were seeded at a density of 3500 cells/cm² on NCD, SMD, polished silicon and microscope glass coverslips (CG) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). After incubating in a CO₂ humidified incubator (5% CO₂, 37°C) for 4 h, the cells

were washed three times with phosphate buffering solution (PBS) and the adherent cells were fixed with 10% Formalin (Fisher Scientific, Co.) and stained by DAPI (Sigma Aldrich Co.). The stained cells were counted under a fluorescence microscope (FM, Axiovert 200M, Zeiss) to calculate OB adhesion density (cell per unit area of the substrate). For OB proliferation tests, the procedures were similar, except that the seeding density of OB was 3000 cells/cm² and the cells were cultured up for 1, 3 and 5 days.

For OB differentiation tests, OB were seeded at a density of 10⁵ cells/cm² and were cultured in DMEM supplemented with 10% FBS, 1% P/S, 50 µg/ml L-ascorbate (Sigma) and 10 mM β-glycerophosphate (Sigma). The cells were incubated at 5% CO₂ and at 37 °C for 7, 14, and 21 days. Medium was replaced every second day. At the end of each prescribed time period, supernatant medium was removed and the remaining OB were lysed using distilled water and three freeze-thaw cycles. This protocol only removes intracellular as well as cell membrane-bound proteins and does not remove proteins contained in the extracellular matrix [4]. Total protein content in the cell lysates was determined spectrophotometrically using a commercially available kit (Pierce Chemical Co.) following the manufacturer's instructions. For the measurement of intracellular alkaline phosphate (ALP) activity, cell lysates were analyzed by a commercial ALP activity detection kit (Upstate Cell Signal Solutions, USA) and details can be found elsewhere [5]. The ALP activity was normalized by total intracellular protein synthesis and substrate surface area. For the calcium deposits, OBs were removed from substrates (specifically, diamond coatings as well as silicon and CG controls) after 21 days of incubation and the substrates were treated with 0.6 M HCl overnight at room temperature. The amount of calcium present in the acidic supernatant was quantified using a commercially available kit (Pointe Scientific Co., MA) and following manufacturer's instructions [5]. The amount of calcium deposited was normalized over the substrate surface areas.

3. Results and Discussion

Surface properties of the diamond coatings

NCD coatings fabricated with 5% H₂ revealed spherical grains with sizes between 30~100 nm and nanoscale roughness with RMS roughness values of 19.8nm (Fig.1). In contrast, SMD coatings fabricated with 20% H₂ had a wide range of grain sizes from 100nm to 600 nm and consequently a much higher RMS roughness value (59.5 nm) than NCD (Fig.1). A previous study demonstrated that diamond grain sizes increase dramatically as the H₂ concentration increases during the MPCVD process [6]. This trend agrees with the topographical results mentioned above.

Contact angle measurements revealed a small difference between NCD (88.7±6.8°) and SMD (99.5±5.8°). NCD had a smaller contact angle although both of them were very hydrophobic surfaces for biological applications.

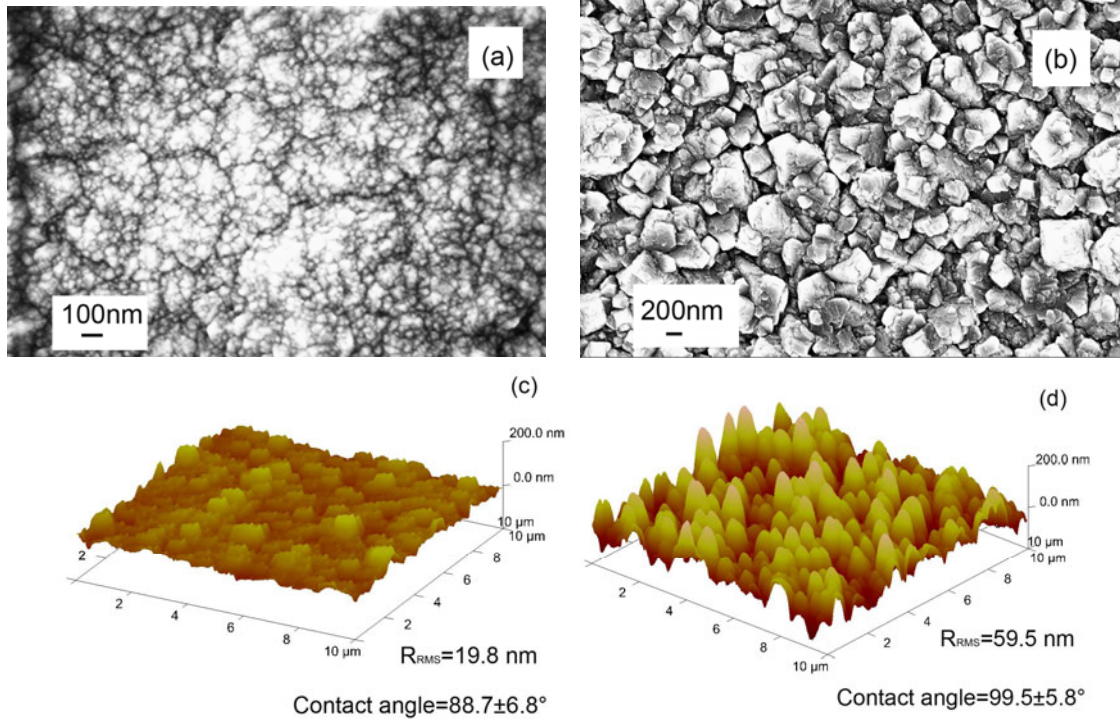


Fig.1 SEM and AFM images of NCD (a and c) and SMD (b and d).

OB adhesion and proliferation on the diamond coatings

The results of OB adhesion and proliferation on NCD and SMD are shown in Fig. 2(a) and (b), respectively. NCD promoted OB adhesion more than SMD and the average adherent OB density on NCD was almost twice as that on SMD. This could be attributed to the lower contact angle on NCD compared to SMD, indicating a more hydrophilic surface than SMD and resulting in enhanced protein adsorption favorable for cell adhesion [7]. However, it was noticed that OB adhesion on both diamond coatings were much poorer than on silicon and CG controls, which could be explained by the more hydrophilic properties of silicon and CG (contact angles $\sim 30^\circ$). For all the time periods up to 5 days, OB proliferated more on NCD compared to SMD. After 5 days, OB proliferation on both diamond coatings was higher than on silicon controls, although it was still much lower than on CG. These results indicated that NCD promoted OB proliferation compared to SMD and silicon controls.

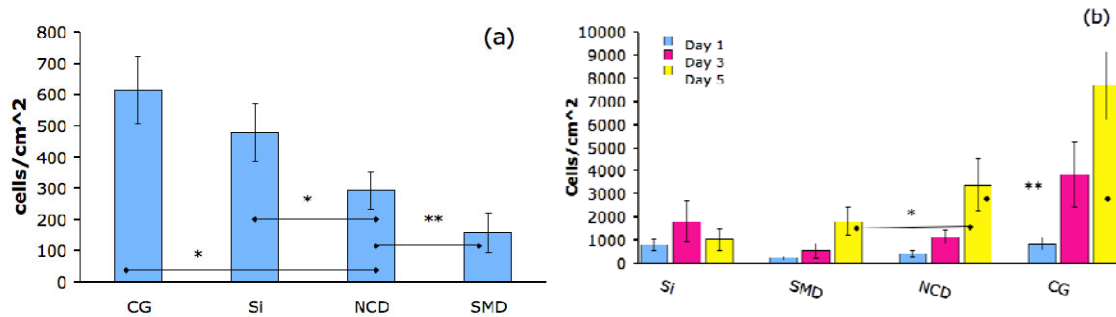


Fig. 2 OB adhesion (a) and proliferation (b) on NCD and SMD coatings. Data=mean value \pm standard deviation; n=9; *p<0.01, **p<0.05

OB differentiation on the diamond coatings

The results of total intracellular protein synthesis on diamond coatings are shown in Fig. 3. After 21 days, OB on NCD synthesized more intracellular protein than on silicon and CG, and slightly more than on SMD although the difference was not significant. The results of ALP activity on diamond coatings are revealed in Fig. 4. OB on NCD showed significantly enhanced ALP activity than on SMD and CG, indicating possibly more OB differentiation on NCD than on SMD and CG. This result was further confirmed by the enhanced calcium deposition of OB on NCD, as shown in Fig. 5. All the OB differentiation makers demonstrated that NCD enhanced OB differentiation more than SMD for almost all of the time periods. NCD significantly promoted the OB functions (ALP activity and calcium deposition, which are important indicators of OB differentiation) after 21 days compared to CG and silicon control.

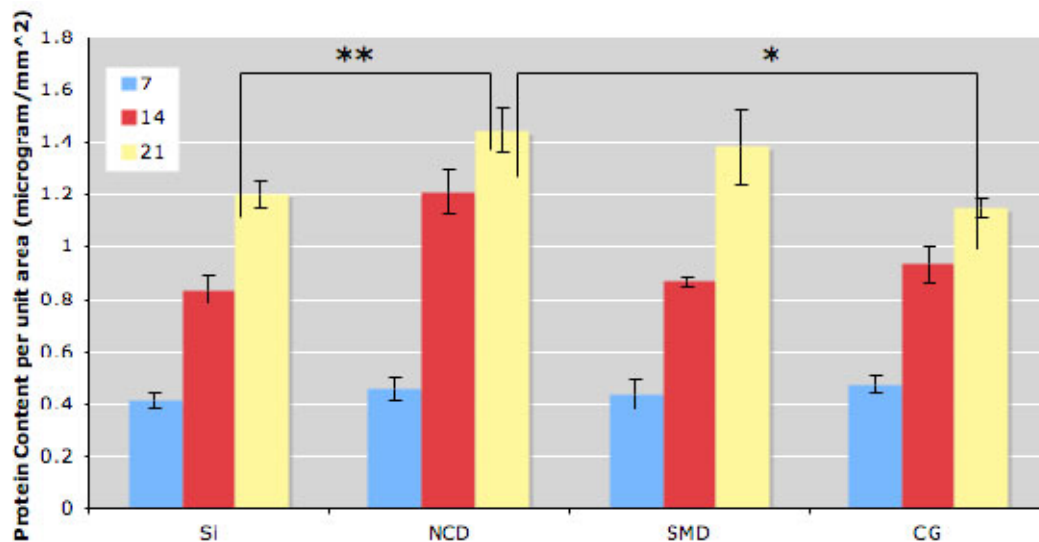


Fig. 3 Total intracellular protein synthesis by OBs on diamond coatings. Data=mean value \pm SEM; n=9; *p<0.05, **p<0.07

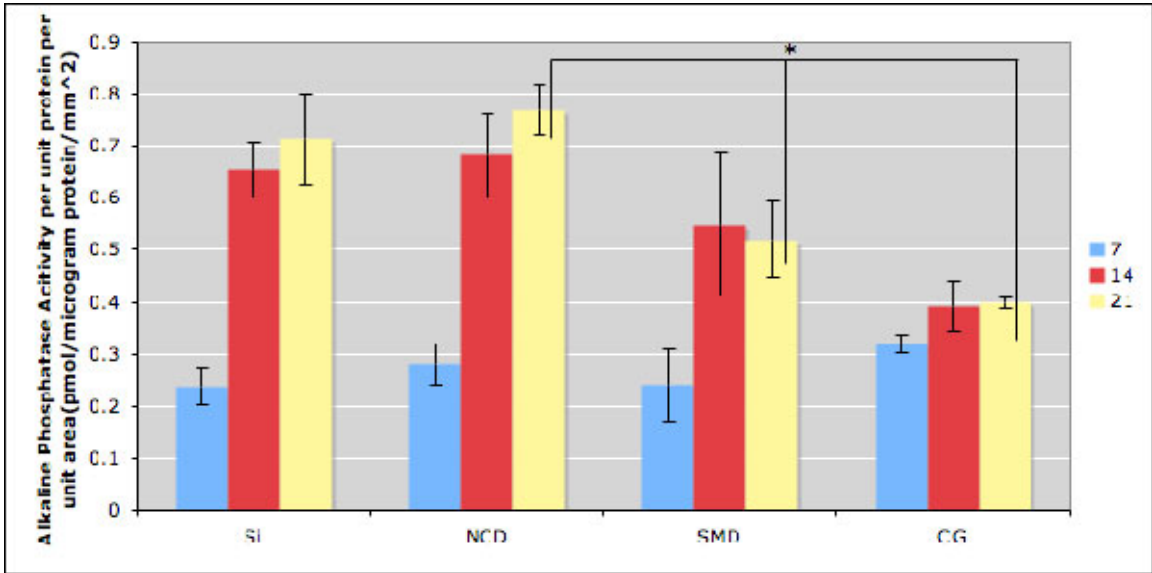


Fig. 4 ALP activity of OBs on diamond coatings. Data=mean value \pm SEM; n=9; *p<0.05.

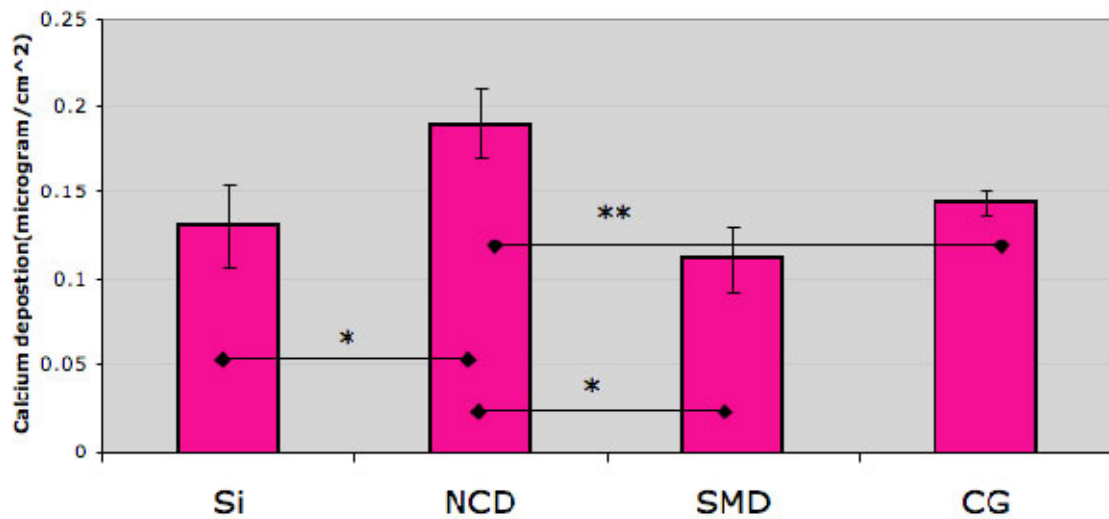


Fig. 5 OB calcium deposition on diamond coatings after 21 days. Data=mean value \pm SEM; n=9; *p<0.03, **p<0.09.

4. Conclusions

Both short-term functions (adhesion and proliferation) and long-term functions (total protein synthesis, ALP activity, and calcium deposition) of OBs were promoted on NCD compared to SMD. This could be correlated to the nanoscale roughness and less hydrophobic properties of the NCD coatings. NCD coatings also promoted OB differentiation compared to silicon and CG after long time periods (2~3 weeks), although OB adhesion and proliferation were less on NCD. These results provided important information for applying of diamond coatings on orthopedic implants.

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