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# Comparing biocompatibility of IBAD-produced nano-films of titanium oxide with orthopaedic grade titanium

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## ABSTRACT

Titanium (Ti) is the material of choice for orthopaedic applications because it is biocompatible and encourages osteoblast growth. The present study was devised to compare the osteoblast functions on nano-crystalline titanium oxide films produced by an ion beam assisted deposition (IBAD) technique with the osteoblast functions on micro-crystalline orthopaedic-grade titanium. To characterize the biocompatibility of these surfaces, we have studied cell adhesion, growth, and differentiation on different samples. Cell responses to surfaces were examined using the human osteosarcoma cell line (SAOS-2) with osteoblastic properties. To compare cell adhesion and growth, DAPI-stained nuclei were counted using ImageJ and Metamorph software. Immunofluorescence staining was also applied to monitor actin stress fiber shapes in order to compare cell morphology and actin remodeling at focal adhesion sites on the surfaces. Then, alizarin red assay was used to detect calcium compounds on surfaces which is an indication of successful in-vitro bone formation. Our experimental results indicated that nano-crystalline TiO<sub>2</sub> is superior to micro-crystalline Ti in supporting growth, adhesion, and proliferation. Also, significantly more calcium deposition was observed on the nano-surfaces.

## Introduction

Annually, more than one million Americans undergo joint replacement surgeries (JRS), and by 2030, the projected number will exceed more than four million. Unfortunately, 10-20% of arthroplasty procedures will need revision surgery within 15-20 years (1). Thus, there is a dire need to improve implants used in JRS so they remain well-fixed and last longer. It has been shown that osseointegration of orthopaedic implants is dependent on different surface properties that can be modified in a way to increase host cell adhesion and growth. The objective of this study was to evaluate nano-crystalline implant coatings produced by the ion beam assisted deposition (IBAD) technique with superior osseointegration and mechanical properties. In this regard, the present study examines the adhesion, survival and growth of osteoblast-like cells on the engineered nano-crystalline TiO<sub>2</sub> produced by the IBAD technique, and compares it with orthopaedic grade Ti.

## IBAD technique

In this process, a nano-crystalline film is deposited on a substrate with the combination of physical evaporation and concurrent ion beam bombardment in a high vacuum environment. The nano-crystalline film produced by this method has a 3 to 70 nm grain size with combined properties of hardness and wettability.

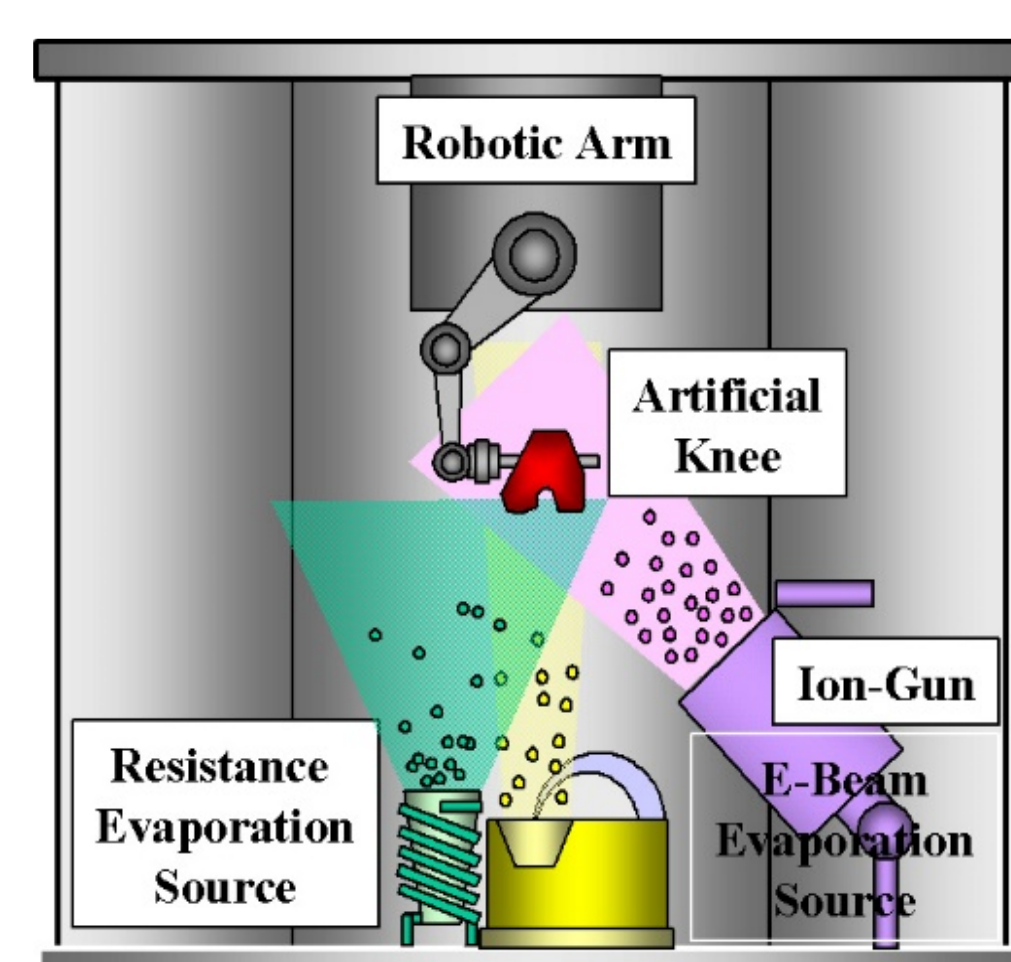


Figure 1. IBAD schematic (2)

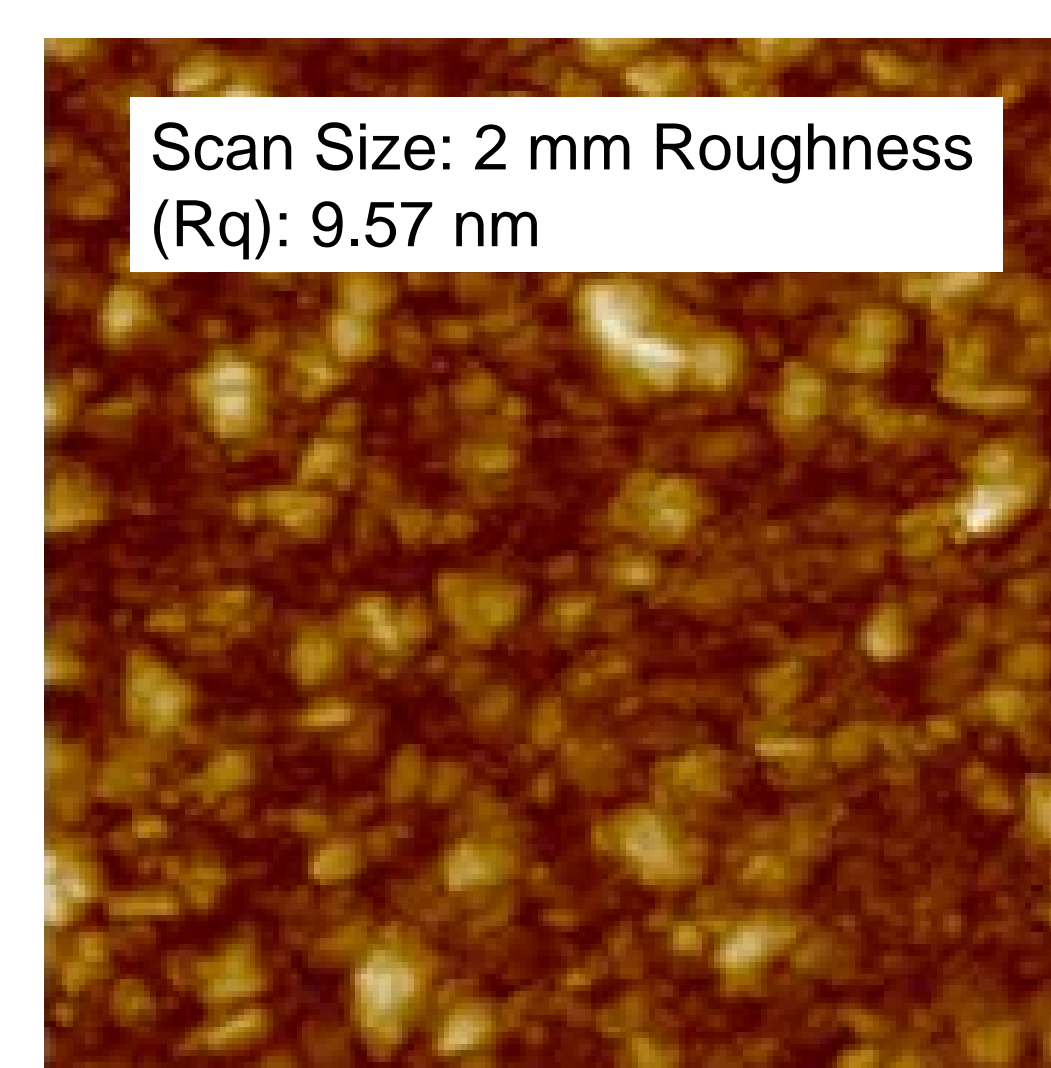


Figure 2. Atomic force microscopy (AFM) image of nano-structure TiO<sub>2</sub> film produced by the IBAD technique

## Cell Culture

A human osteoblast-like cell line (SAOS-2) was used for this study. SAOS-2 (ATCC) cells are late mature cells and are grown in McCoy's 5A medium (ATCC) supplemented with 15% fetal bovine serum (FBS) and 1% gentamycin (Invitrogen) in a humidified 5% CO<sub>2</sub> atmosphere at 37° C. Besides the worldwide availability, other advantages of using this human osteosarcoma cell line are its similarity to human osteoblast cells, high matrix mineralization capacity, and a known cytokine and growth factor profile (3).

## Immunofluorescence techniques

To study the effect of the surface properties on cell adhesion and proliferation, immunofluorescence techniques were applied. DAPI (4', 6-Diamidino-2-Phenylindole, Dihydrochloride) is a stain that emits blue fluorescent color when it binds to AT regions in DNA. Actin is a protein that forms microfilaments (the major components of the cytoskeleton), and actin staining is commonly used to determine the structure of the cytoskeleton.

### Long-term adhesion:

SAOS-2 cells were monitored and compared for adhesion to the nano-crystalline TiO<sub>2</sub> and medical grade Ti substrates. Different numbers of cells were seeded on the substrates (10000, 25000, and 50000 cells) and the number of adherent cells were determined by nuclei quantification with DAPI after 48 hours using ImageJ software. Figure 3 shows a greater number of DAPI-stained cells on nano-crystalline TiO<sub>2</sub> compared to micro-crystalline Ti, which indicates more adhesion and growth on nano-surfaces.

Morphology and adherence of SAOS-2 cells on nano-crystalline TiO<sub>2</sub> and biomedical grade Ti were also visualized at 50000 initial cell seeding by fluorescence microscopy. Figure 4 shows cell morphology on different substrates. Besides the higher numbers of the cells on nano-surfaces, cells look more spread and organized compared to micro-crystalline Ti.

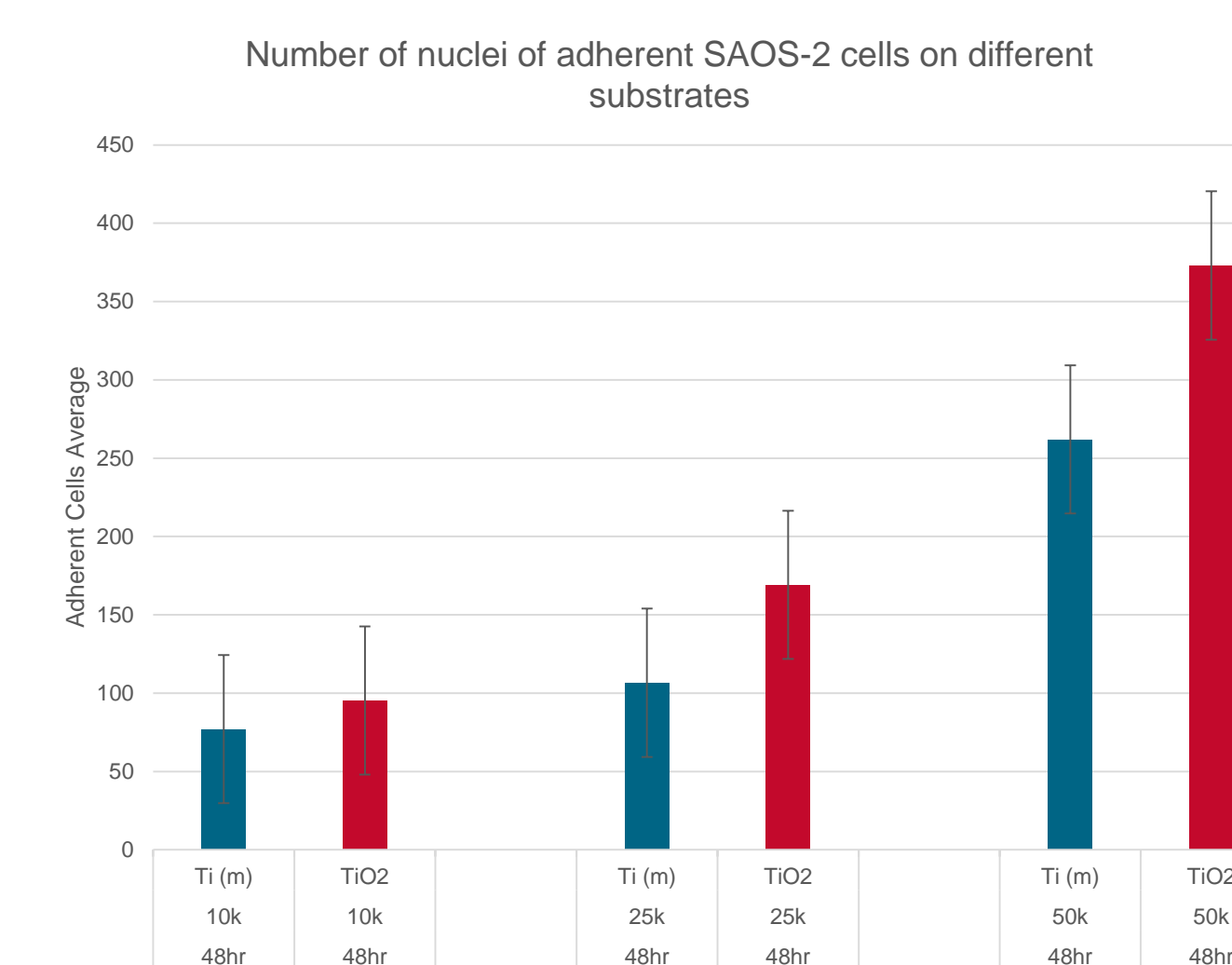
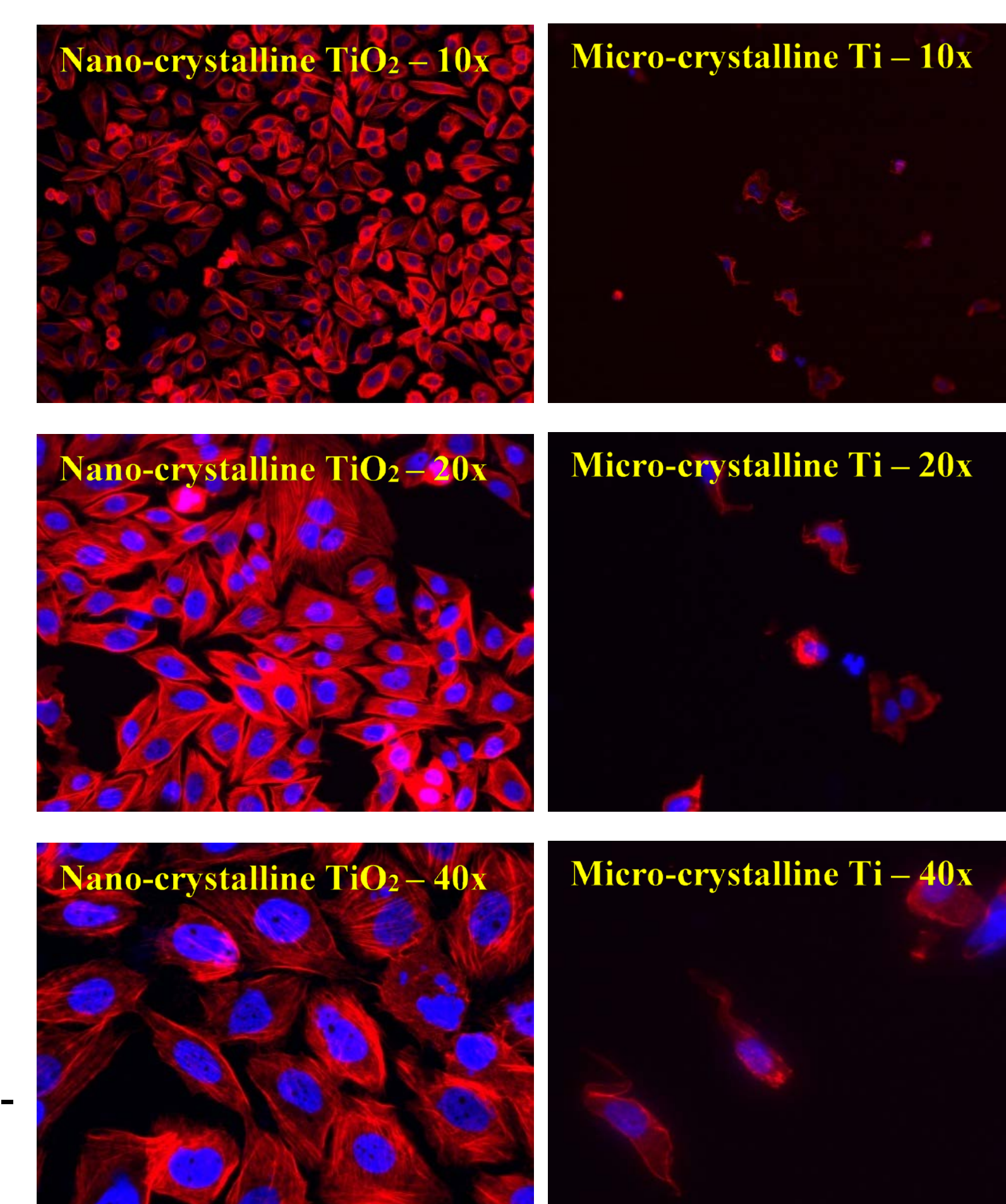


Figure 3. Average number of cells in 10 randomly selected fields at 10X zoom

Figure 4. Morphology of adherent SAOS-2 cells on typical fields of nano-crystalline TiO<sub>2</sub> and micro-crystalline Ti at 10X, 20X and 40X zoom.



### Short-term adhesion vs. long-term adhesion:

100000 cells were seeded to each sample. The number of adherent osteoblasts was measured by counting DAPI-stained nuclei after 2 hours and 48 hours using Metamorph software. The number of cells initially attached to nano TiO<sub>2</sub> was higher compared to Ti. Moreover, after 48 hours, there was a greater number of cells on nano-crystalline TiO<sub>2</sub> compared to micro-crystalline Ti.

### Adherent cell proliferation:

50000 cells were seeded to each sample, then incubated for 24 hours. DAPI-stained nuclei were visualized to compare the numbers and patterns of adherent cells. Additionally, actin stress fiber patterns were examined to compare actin remodeling at focal adhesion sites on the surfaces. Although there was variability in different areas of biomedical grade Ti, overall, fewer cells were observed adhering to micro-crystalline Ti. Besides DAPI staining, actin stress fiber shapes were also monitored because by only observing nuclei, it is impossible to know if cells are healthy and prolific on the surface or not. Most of the adherent cells on the nano-crystalline TiO<sub>2</sub> were bigger more spread.

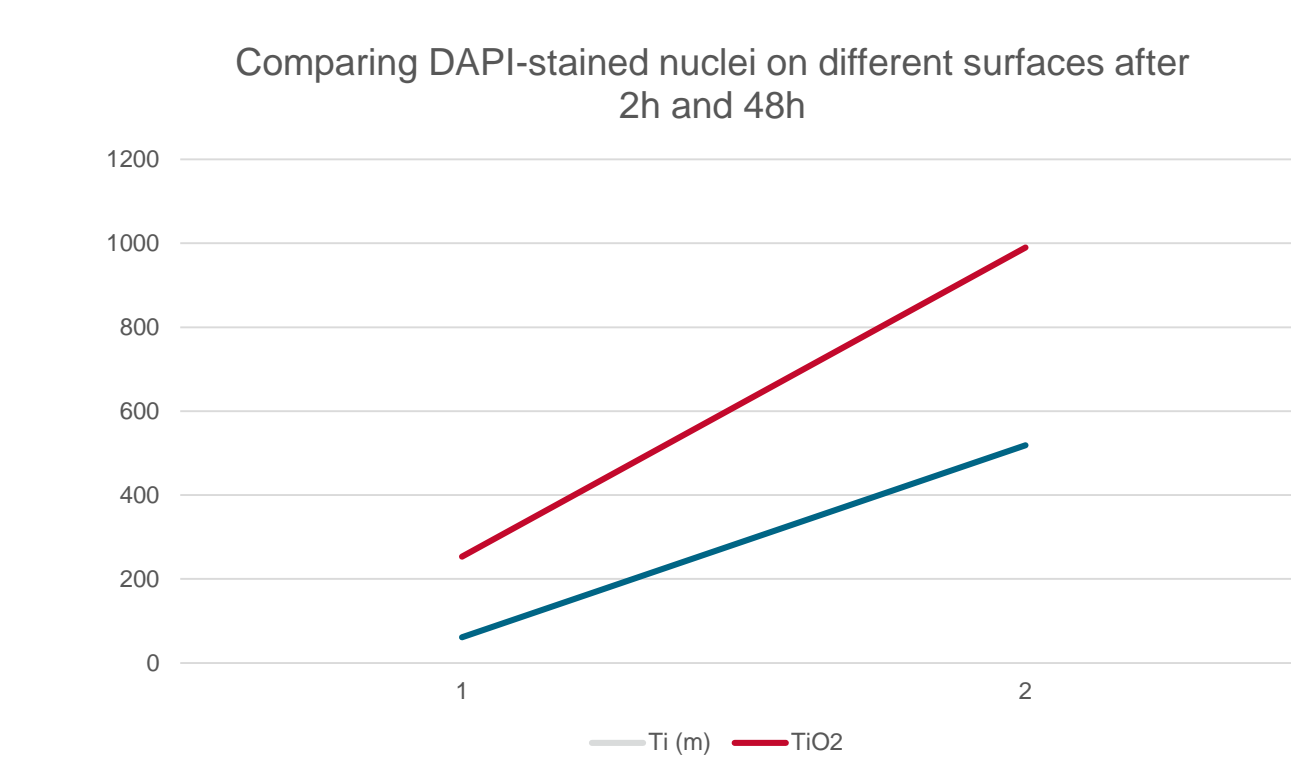


Figure 5. Average number of cells in 1x1 mm<sup>2</sup> area selected at the middle of the samples at 10X zoom

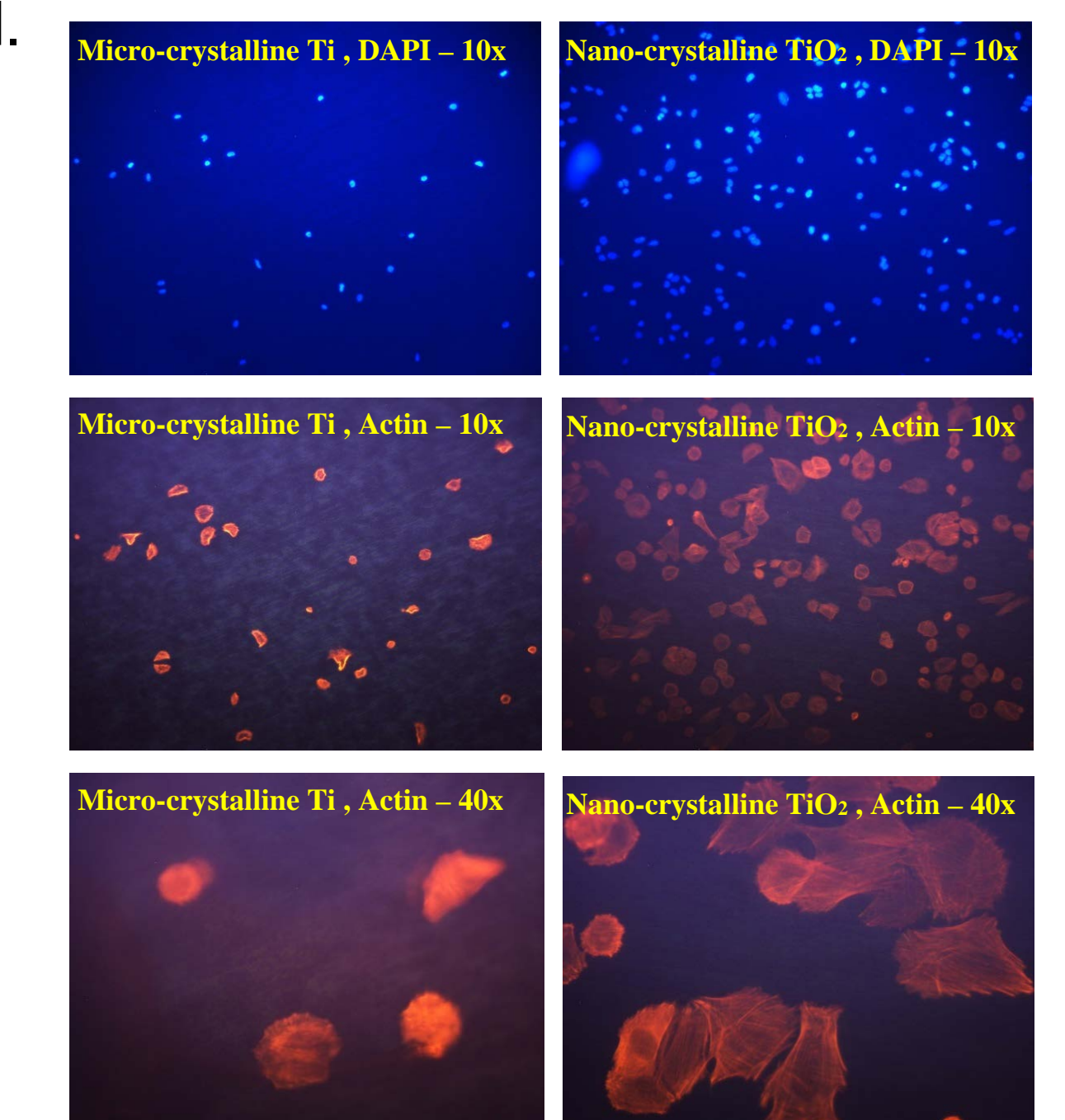


Figure 6. Fluorescent microscopy images of adherent SAOS-2 cells on typical fields of nano-crystalline TiO<sub>2</sub> and micro-crystalline Ti at 10X, and 40X zoom

## Alizarin red assay

For determination of calcium deposition rates, as an indication of successful in-vitro bone formation, alizarin red quantification was applied after 7 and 14 days which showed a significant difference between nano TiO<sub>2</sub> and orthopaedic grade Ti.

Table 1 indicates the significantly greater calcium deposition on nano-engineered TiO<sub>2</sub> compared to nano-crystalline Ti and orthopaedic grade Ti after 7 and 14 days.

Day of experiment	Substrates	Alizarin red concentration average	Kruskal-Wallis p-value
Day 7	Ti (m)	4.1 ± 0.4	0.02
	TiO <sub>2</sub>	8 ± 0.8	
Day 14	Ti (m)	1.9 ± 1	0.03
	TiO <sub>2</sub>	8.2 ± 3.2	

## Conclusion

This study showed that nano-crystalline TiO<sub>2</sub> is superior to micro-crystalline Ti in cell adhesion, proliferation, and calcium deposition. Thus, enhanced bone formation ability from those surfaces is expected. It also indicated that surface topography, which was altered by the IBAD technique, affects cell interactions and therefore, plays a crucial role in biocompatibility of nano-engineered surfaces. Therefore, improving the quality of surface oxide, i.e. fabricating stoichiometric oxides, as well as nano-engineering the surface topology is crucial for increasing the biocompatibility of Ti implant materials.

## References

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