Enhanced Osteoblast Proliferation and Hydroxyapatite Formation on Surface Modulated Silicon Nitride

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INTRODUCTION: Hydroxyapatite (HAp) is the fundamental mineral providing structure to all living vertebrates. Its zwitterionic-like surface chemistry and stoichiometric flexibility are essential for osteogenic function[1] and few natural or synthetic materials can emulate its unique characteristics. Prior studies suggested that Si_3N_4's surface chemistry and defect structure could be modified to exhibit zwitterionic behavior similar to natural HAp,[2, 3] which in turn might lead to enhanced osteoconductive performance. This *in vitro* study examined the effect of various Si_3N_4 surface treatments on osteoblast proliferation and hydroxyapatite formation.

METHODS: Fully dense and polished silicon nitride disk samples (Ø12.7 mm x 1 mm, 20 nm R_a, Si_3N_4, *MC*$^{2®}$, Amedica Corporation, Salt Lake City, UT, USA) were subjected to three separate post-densification treatments including HF etching (5% HF solution for 45 s), high-temperature nitrogen annealing (1400°C for 30 min under 1−2 psi of filtered N_2 gas), and thermal oxidation (1070°C for 7 h in air). The various Si_3N_4 surfaces were examined by scanning electron microscopy, elemental analyses, and cathodoluminescence and Raman spectroscopy.[4] Cell proliferation was assessed by seeding each sample with 5 x 10^5/ml of human osteosarcoma cells (SaOS-2) within well-plates in an osteogenic medium which consisted of Dulbecco's modified Eagle medium (DMEM) supplemented with ≈50 μg/mL ascorbic acid, about 10 mM β-glycerol phosphate, and approximately 100 nM dexamethasone in ≈10 wt.% fetal bovine calf serum. The samples were incubated for 7 days. Results were assessed by laser scanning microscopy and 3D image analyses. The presence of the receptor activator of NF-kB ligand (sRANKL) was used to test for the propensity to form osteoclasts using an ELISA kit (R&D Systems, Minneapolis, MI, USA); and Insulin-like growth factor 1 (IGF-1) was used as a probe for cell proliferation and differentiation efficiency by a separate ELISA kit (RayBio; RayBiotech, Inc., Norcross, GA, USA), both according to manufacturer's instructions. Statistical analyses were performed according to the unpaired Student's t-test or by one-way Analysis of Variance (ANOVA). A p value <0.05 was considered statistically significant. Identical tests for cell proliferation and HAp formation were performed on a commercially available titanium-alloy (Ti6Al4V) biomaterial.

RESULTS: Results are provided in Figure 1(a)~(c) for each treatment condition and biomaterial. The volume of hydroxyapatite formed on each of the tested surfaces (as measured by 3D laser scanning microscopy) is shown in Figure 1(a). The N_2-annealed samples (N_2A) showed the greatest affinity for cell adhesion, proliferation, and HAp formation, with up to a 40% increase in comparison to the as-fired Si_3N_4 (AF). HAp growth was even more dramatic when compared to the other tested materials, with increases of ~64%, ~86%, and ~100%, for the oxidized (Ox), Ti-alloy, and HF-etched materials (HF), respectively. Differences between the N_2A samples and the remaining tested materials were significant, whereas the only other statistical difference was between the AF and Ti-alloy conditions. However, the Ti-alloy showed a statistically higher amount of free SRANKL than all of the Si_3N_4 treatments (*cf.*, Figure 1(b)), indicating a greater amount of osteoclastic activity. This suggests that the *in vitro* environment for Si_3N_4 was more conducive to osteoblastic function. Additionally, the IGF-1 concentrations (*cf.*, Figure 1(c)) did not statistically differ among the tested Si_3N_4; but they were significantly higher than the Ti alloy. IGF-1 regulates cell metabolism and bone remodeling. Higher concentrations stimulate cells to proliferate and differentiate. These results were consistent with the free sRANKL data, suggesting that Si_3N_4 is an effective bone repair biomaterial.

DISCUSSION: The enhanced cell proliferation and HAp formation on particularly the N_2A samples corresponded to the presence of an off-stoichiometry SiYAlON phase at its surface which was rich in yttrium and aluminum cations and nitrogen vacancies, with Si^{4+} and N^{3-} partially replaced by Y^{3+} or Al^{3+} and O^{2-}, respectively. An increase in V_N^{3+} was enhanced by the formation of negatively charged Al_{Si}^- or Y_{Si}^- substitutional defects, which also resulted in the abundant formation of the N−N bonds, N^{4+}. Silanol groups (SiOH) at the surface deprotonate at homeostatic pH creating a dipolar electrostatic environment in a regular zwitterionic-like array. Consequently, although different chemically, Si_3N_4's surface structure can be critically compared to the positively- and negatively-charged functional groups found in native HAp. For HAp, charged pairs of calcium ions (positive sites) and clusters of six charged oxygen atoms (negative sites) are associated with crystal phosphates, These groups are distributed in a dipolar pattern which give its surface zwitterionic character. It has been noted that the bioactive nature of HAp is in large measure due to this type of surface chemistry which repels bacteria while enhancing mammalian cell adhesion and motility. Similarly, Si_3N_4 appears to possess a zwitterionic surface that provides improved osteoconductivity and bacteriostasis.

SIGNIFICANCE: The engineered surface properties of Si_3N_4 demonstrate that alterations of its lattice defects promote proliferation of osteoblasts and the subsequent generation of natural hydroxyapatite. Surface modulation of Si_3N_4 illustrates the concept that engineering of atomically defective biomaterials can lead to remarkable osteoconductive characteristics.

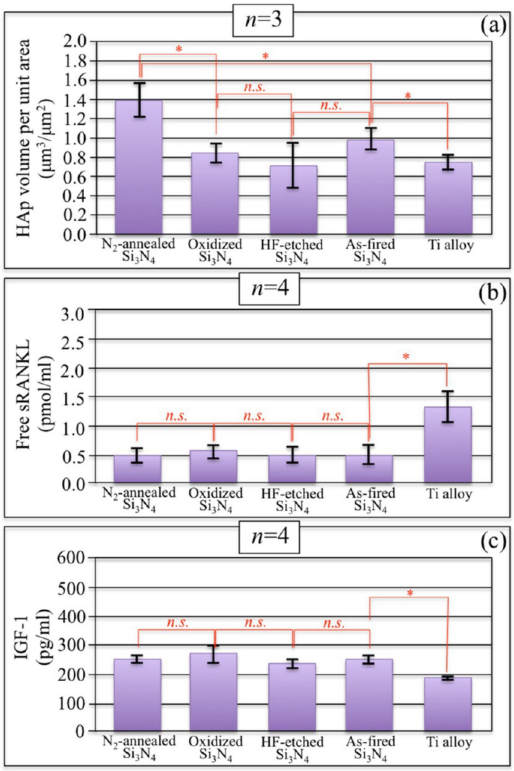

Figure 1. (a) Hydroxyapatite formation as measured by 3D laser scanning microscopy. (b) Propensity for osteoclast formation as measured by concentration of free sRANKL. (c) Efficiency of cell proliferation and differentiation as measured by IGF-1 concentration.

REFERENCES:

1 I. Izquierdo-Barba, *et al.*, *Acta Biomater.*, (2016).
2 R.M. Bock, *et al.*, *Acta Biomater.*, **26** 318–330 (2015).
3 W. Zhu,*et al.*, *J. Phys. Chem. C*, **119** [6] 3279–3287 (2015).
4 G. Pezzotti, *et al.*, *ACS Biomater. Sci. Eng.*, **2** [7] 1121–1134 (2016).