Reactive oxygen species inhibited by titanium oxide coatings

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Abstract: Titanium is a successful biomaterial that possesses good biocompatibility. It is covered by a surface layer of titanium dioxide, and this oxide may play a critical role in inhibiting reactive oxygen species, such as peroxynitrite, produced during the inflammatory response. In the present study, titanium dioxide was coated onto silicone substrates by radio-frequency sputtering. Silicone coating with titanium dioxide enhanced the breakdown of peroxynitrite by 79%. At physiologic pH, the peroxynitrite donor 3-morpholinosydnonimine-N-ethylcarbamide (SIN-1) was used to nitrate 4-hydroxyphenylacetic acid (4-HPA) to form 4-hydroxy-3-nitrophenyl acetic acid (NHPA). Titanium dioxide-coated silicone inhibited the nitration of 4-HPA by 61% compared to aluminum oxide-coated silicone and 55% compared to uncoated silicone. J774A.1 mouse macrophages were plated on oxide-coated silicone and polystyrene and stimulated to produce superoxide and interleukin-6. Superoxide production was measured by the chemiluminescent reaction with 2-methyl-6-[p-methoxyphenyl]-3,7-dihydroimidazo[1,2-a]pyrazin-3-one (MCLA). Titanium dioxide-coated silicone exhibited a 55% decrease in superoxide compared to uncoated silicone and a 165% decrease in superoxide compared to uncoated polystyrene. Titanium dioxide-coated silicone inhibited IL-6 production by 77% compared to uncoated silicone. These results show that the anti-inflammatory properties of titanium dioxide can be transferred to the surfaces of silicone substrates. © 2003 Wiley Periodicals, Inc. J Biomed Mater Res 66A: 396–402, 2003

Key words: titanium dioxide; silicone; anti-inflammatory; oxide coating; peroxynitrite

INTRODUCTION

Inflammatory response is part of a general pattern of recovery and wound healing that leads to eventual acceptance of a foreign material placed in the body.[1] This pattern of events typically leads to fibrotic encapsulation of the implant. Prolonged inflammatory responses can have the consequence of more intense tissue reactions requiring extrusion of the implant.[2]

The reactive oxygen species peroxynitrite ($OONO^-$) has been shown to play a role in inflammation. Peroxynitrite is formed from superoxide ($O_2\cdot^-$) and nitric oxide ($NO\cdot$),[3] and it is a potent oxidant capable of a wide range of reactions.[4–6] Peroxynitrite directly induces colonic inflammation in rats[7] and has been demonstrated to be present in the inflamed guinea pig ileum.[8] Peroxynitrite was found to be produced by acute inflammation from edema induced in the hind paws of rats.[9]

Clinical studies also provide evidence that peroxynitrite is produced during inflammation. The blood serum and synovial fluid from patients with the inflammatory joint disease rheumatoid arthritis were found to contain 3-nitrotyrosine markers, indicating peroxynitrite formation while body fluids from normal patients contained no detectable 3-nitrotyrosine. Similarly, no 3-nitrotyrosine markers were detected in body fluids from patients with osteoarthritis, a largely non-inflammatory joint disease.[10] It is important to note that it has been reported that 3-nitrotyrosine markers for peroxynitrite also have been observed at the interface membrane of hip implants suffering from aseptic loosening, which is characterized by local inflammation.[11,12]

Previously, it was shown that titanium dioxide is capable of inhibiting the reactivity of peroxynitrite.[13]

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Titanium dioxide was shown to enhance the breakdown of peroxynitrite and inhibit the nitration reactions of peroxynitrite at physiologic pH levels. Titanium surfaces retained the ability to inhibit peroxynitrite even in the presence of 10% fetal bovine serum, fibrinogen, and bicarbonate.

Others have shown that a surface with the ability to breakdown reactive oxygen species can improve the biocompatibility of polymers. Polyethylene implants coated with superoxide dismutase mimics showed a notable decrease in capsule thickness compared to uncoated controls.[14] These results indicate that superoxide, a precursor of peroxynitrite, plays a role in the inflammatory response to biomaterial surfaces.

The objective of this study was to determine if the ability to inhibit reactive inflammatory species also could be imparted to a polymer by coating the surface with a thin film of titanium dioxide. Such coatings may lead to improvement in biocompatibility and mitigation of the inflammatory response of implants.

MATERIALS AND METHODS

Sample preparation

Oxide-coated silicone elastomer samples were fabricated using radio frequency (RF) plasma magnetron sputtering. The silicone substrates were cut from sheets of non-reinforced, translucent silicone sheeting (SF Medical, Hudson, MA) and were 1.5 mm in thickness.

Deposition rates of the oxide layer were calibrated using film deposition on quartz substrates under conditions similar to those on silicone substrates. The thickness of the oxide layer was measured with a Dektak IIa (Digital Instruments, Santa Barbara, CA). The typical range of thickness for oxide coatings was 100–200 nm.

The continuity of the coatings was probed using energy-dispersive X-ray spectroscopy (EDS) in a mapping function (Oxford Instruments X-ray spectrometer, Concord, MA). Elemental information was obtained from points on the surface of the sample corresponding to the scanning electron microscope (SEM) image (Cambridge 360 SEM LEO Electron Microscopy, Thornwood, NY). No preferential location of elements was seen, indicating there was no disruption of continuity of the coating within the resolution of the instrument.

Peroxynitrite degradation rates

Peroxynitrite was synthesized using a quenched-flow reactor system.[15] The breakdown of peroxynitrite over substrate samples was monitored by the decrease in absorbance at 302 nm using a DU 640 Beckman spectrophotometer.

Nitration of phenol (4-HPA) by peroxynitrite donor

Peroxynitrite has a half-life of 1.9 s at physiologic pH.[16] The short half-life makes experiments difficult in this pH range. This problem was circumvented through the use of 3-morpholinosydnonimine-N-ethylcarbamide (SIN-1) (Alexis Chemicals, San Diego, CA). SIN-1 slowly decomposes to release NO and superoxide at physiologic pH, which then react to form peroxynitrite.[4,15]

Peroxynitrite is highly reactive and can nitrate phenolic residues, such as tyrosine.[15] It nitrates 4-hydroxyphenylacetic acid (4-HPA) to form 4-hydroxy-3-nitrophenyl acetic acid (NHPA). NHPA absorbs at 432 nm, and its concentration was calculated by measuring the change of absorbance at pH 6.0–6.5 and pH 10.0–10.5.[17]

Solutions of PBS buffer (Irvine Scientific, Irvine, CA) with 0.5 m*M* of 4-HPA (Aldrich, Milwaukee, WI) were placed over coated silicone substrates in airtight containers. After autoclaving, SIN-1 was added to yield a final concentration of 5 m*M*. A second oxide-coated silicone substrate was placed over the first sample, trapping the solution between the two samples. The containers were sealed and placed in the dark in 37°C incubators for 14 days.

Surface interaction with superoxide from activated macrophages

Mouse macrophages from the cell line J774A.1 (ATCC, Manassas, VA) were plated on oxide-coated silicone substrates lining the bottoms of petri dishes. The cells grew to form a monolayer that adhered to the coated surfaces. Oxide coatings at the thickness deposited were transparent, allowing for spectrometry assays.

The macrophages were grown in DMEM on the oxide-coated silicone substrates contained in sterile petri dishes (Irvine Scientific, Irvine, CA) with 5% fetal calf serum (Hyclone) and 1 m*M* of sodium pyruvate (Aldrich). The cells were stimulated to produce superoxide by addition of 15 μg/mL of phorbol 12-myristate 13-acetate (PMA). Superoxide production was measured by the chemiluminescent reaction with 2-methyl-6-[p-methoxyphenyl]-3,7-dihydroimidazo[1,2-α]pyrazin-3-one (MCLA).[18,19] MCLA is two orders of magnitude more specific for superoxide detection than lucigenin or luminol.[20]

Macrophages were incubated in a solution of PBS with MCLA (1.5 μ*M*). The cells were stimulated with PMA (15 μg/mL) and placed in a receptacle with an attached photomultiplier tube and photon counter, allowing measurement of the resulting chemiluminescence. The receptacle was located in a darkroom and was equipped with a temperature control system that maintained the cells at 37°C.

Cytokine production from activated macrophages

Mouse J774A.1 macrophages were plated on silicone substrates with titanium dioxide coatings. Aluminum oxide-coated silicone served as controls. The macrophages were

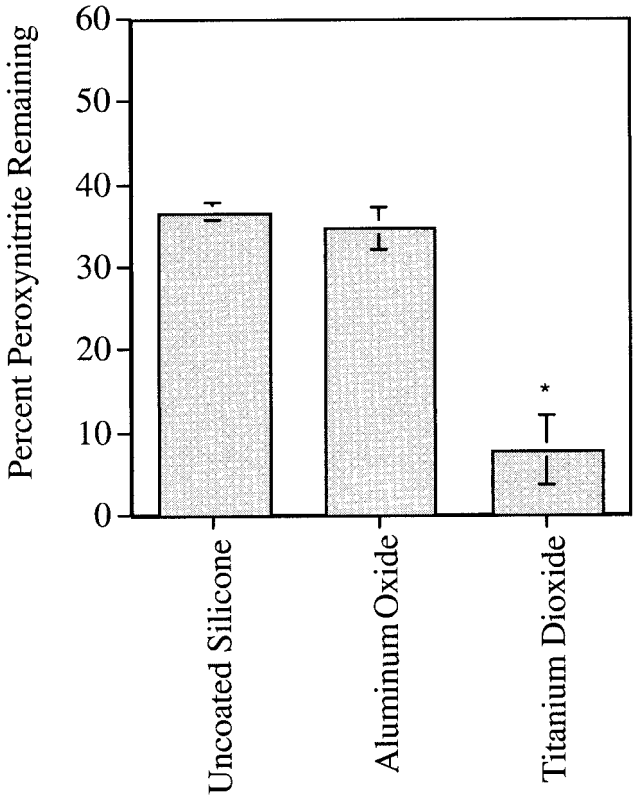

Figure 1. Decomposition of peroxynitrite after 6 h. Peroxynitrite measured by absorbance at 302 nm. Solution pH = 13.4 [*$p < 0.01$ compared to plain and aluminum coated silicone (Student–Newman–Keuls test)]. Error bars given as Standard Error of the Mean.

stimulated with PMA (15 μg/mL), and IL-6 levels were measured by ELISA (R&D Systems; Minneapolis, MN).

RESULTS

Figure 1 shows the decomposition of peroxynitrite in solution (pH = 13.4) after 6 h of exposure to silicone substrates with different oxide coatings. There was a 79% increase in the decomposition of peroxynitrite in solutions exposed to titanium dioxide-coated silicone compared to uncoated silicone and a 77% increase compared to aluminum oxide-coated silicone.

Experiments at physiologic pH (7.4) were conducted using solutions of 4-HPA with SIN-1 as a peroxynitrite donor. Solutions were exposed to silicone substrates with different oxide coatings during the breakdown of SIN-1 to peroxynitrite. Titanium dioxide-coated silicone exhibited a decrease in nitration of 4-HPA to NHPA compared to aluminum oxide-coated silicone and uncoated silicone (Fig. 2). There was a 61% decrease in the nitration of 4-HPA with titanium dioxide-coated silicone compared to aluminum-coated silicone and a 55% decrease compared to uncoated silicone.

Superoxide is a precursor to peroxynitrite and produced by stimulated macrophages.[21] Figure 3 compares the chemiluminescence from stimulated macrophages plated on various substrates with different oxide coatings. The signal values are 5000 s after stimulation and normalized to the initial baseline signal.

Macrophages were plated on uncoated silicone and titanium dioxide-coated silicone [Fig. 3(A)]. Cells plated on titanium dioxide-coated substrates exhibited a decrease in their chemiluminescent signals after stimulation. There was a 55% decrease in signals from cells on titanium dioxide-coated silicone compared to uncoated silicone. Macrophages also were plated on uncoated polystyrene and titanium dioxide-coated polystyrene [Fig. 3(B)]. Macrophages on titanium dioxide-coated polystyrene exhibited a 165% decrease in their chemiluminescent signals after stimulation compared to uncoated polystyrene.

Macrophages were plated on uncoated and oxide-coated silicone and stimulated to produce interleukin-6 (Fig. 4). There was a 77% decrease in the production of IL-6 from stimulated macrophages cultured

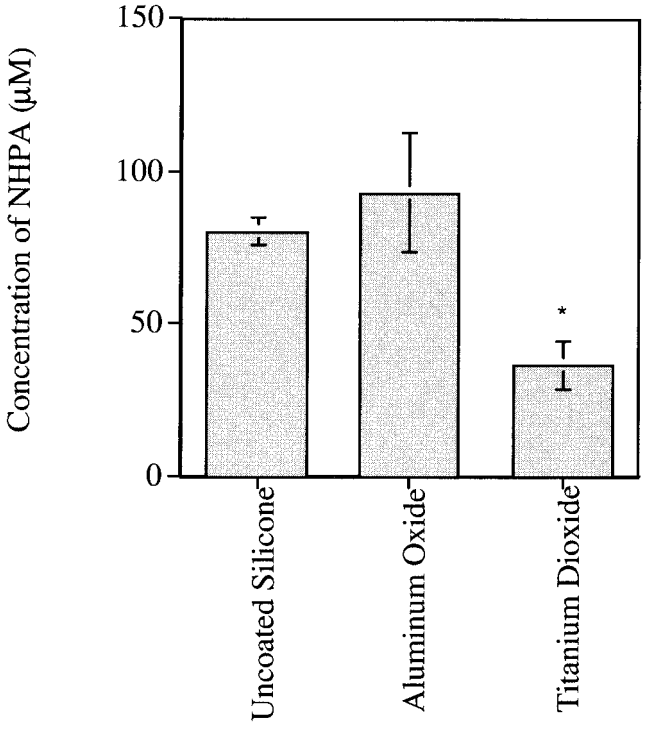

Figure 2. Nitration of 4-hydroxyphenylacetic acid (4-HPA) by 3-morpholinosydnonimine (SIN-1) breakdown in the presence of silicone with different oxide coatings. Concentration of nitrated 4-HPA was measured by absorbance at 432 nm. Samples measured after 14 days of SIN-1 breakdown. [*$p < 0.05$ vs. aluminum oxide coating (Student–Newman–Keuls test) and $p < 0.05$ vs. silicone control (Student–Newman–Keuls test)]. Error bars given as Standard Error of the Mean.

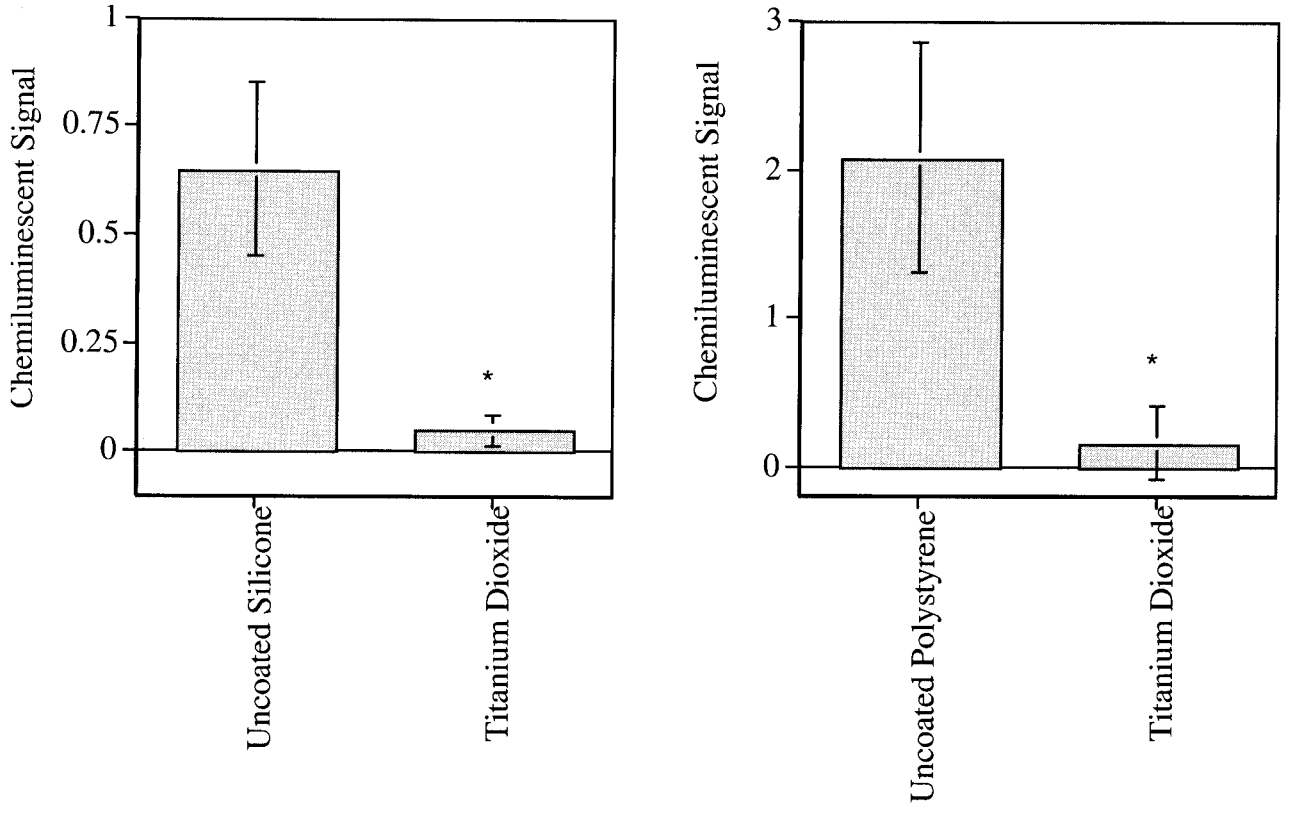

Figure 3. Chemiluminescent signal produced by J774.1A murine macrophages after stimulation by PMA. Macrophages cultured on A) silicone polymer substrates with and without titanium oxide coatings [*$p < 0.05$ vs. Silicone (Student's t test)] and B) tissue cultured treated plastic dishes with and without amorphous titanium oxide coatings [*$p < 0.1$ vs. culture dish (Student's t test)]. Values are normalized ratios of luminescent signal after 5000 s to baseline signal. Error bars given as Standard Error of the Mean.

on titanium dioxide-coated silicone compared to uncoated silicone controls. Cytokine production was less than half that of aluminum oxide-coated silicone.

DISCUSSION

These results show that coatings of titanium dioxide applied to silicone substrates can result in the decrease in the reactivity and production of inflammatory mediators such as peroxynitrite and superoxide.

A few studies have compared the biocompatibility of titanium and polymer implants. Titanium particulates showed less initial reaction when injected into rats than particulates comprised of polymethylmethacrylate.[22] Titanium implants showed less inflammatory response compared to polyethylene implants when placed in normal and arthritic joints of rats.[23] Leukocytes associated with the surface of titanium implants in rats were less responsive to stimulation than were leukocytes from polytetrafluoroethylene implants.[24] Plugs of titanium implanted in the abdominal walls of rats were seen to become integrated with the surrounding soft tissues without inflammation, in contrast to plugs comprised of the polymers Teflon and Delrin, which induced chronic inflammation and never fully integrated with the tissue.[25]

Titanium is widely used as an implant material with excellent clinical results. The exact mechanisms of its superior performance in the biologic environment currently are unknown. Upon exposure to air, titanium readily forms a stable surface layer of oxide that consists predominantly of titanium dioxide, TiO_2.[26] After insertion of the implant, recruited inflammatory cells encounter this oxide layer of the titanium, and it has been proposed that the oxide layer plays a fundamental role in tissue response.[27]

Titanium dioxide is known to catalytically break down hydrogen peroxide, the product of the superoxide dismutase-catalyzed reaction of superoxide.[28] It also is known that TiO_2 can act as a catalyst in reactions involving reactive oxygen species.[29,30] Crystalline TiO_2 powder has been examined as a photocatalyst for the purification of water.[31] Hydroxyl radicals that initiate oxidation of hydrocarbons to carbon dioxide, water, and water-soluble organics are involved in these reactions.[32–34] These findings indicate that

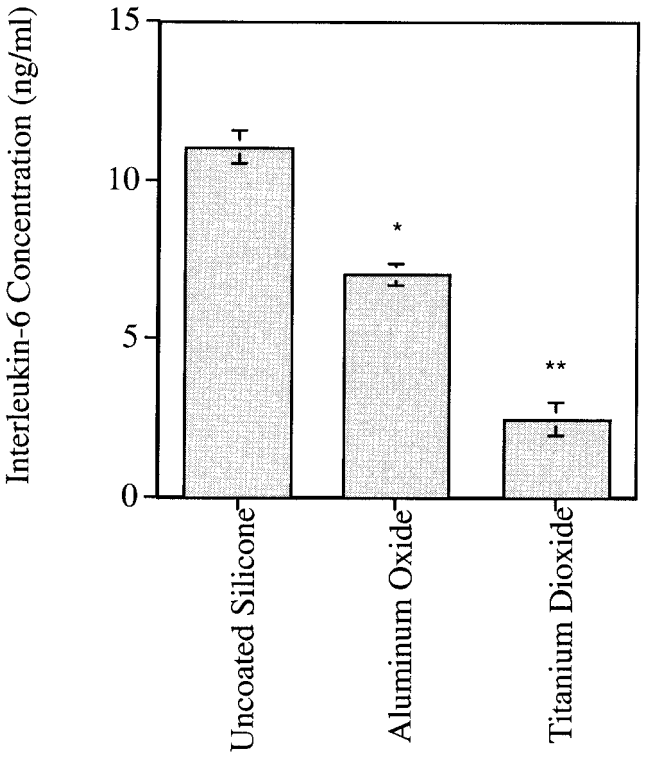

Figure 4. Interleukin-6 production by stimulated J77A.1 macrophages cultured on silicone substrates with different oxide coatings. [*$p < 0.005$ vs. aluminum oxide (Student's t test)]. Error bars given as Standard Error of the Mean.

titanium oxide can act as a catalyst in reactions involving free-radical species.

The observation has been made that the tissue around titanium implants, although not inflamed, takes on a bluish discoloring.[35] This discoloring results from nonabrasive leaching of the metal into the surrounding tissue. The bluish color of titanium complexes caused by leaching indicates that the titanium is complexed in the Ti(III) (3+ valence) state.[35,36] The exact mechanism for the leaching is unknown, but in spite of this behavior, the discoloring is regarded as harmless, and it does not affect the biocompatibility of titanium.

It has been suggested that this leaching phenomenon may be due to an interaction with reactive oxygen species produced during the inflammatory response.[36] Such reactive species would interact with the surface of the titanium implant that is covered with a titanium oxide layer.

Catalytic materials have high surface areas and possess surface-active sites. The chemical reactivity of metal oxide surfaces has been shown to be related directly to the coordination environment of the surface cations[37] and to be most affected by oxygen deficiency.[38] The oxygen defect concentration is controlled by aliovalent doping or annealing in a reducing atmosphere. Catalytic oxidation reactions can be described by a general redox mechanism:

$$\text{catalyst–O} + \text{reductant} \rightarrow \text{catalyst} + \text{reductant–O} \quad (1A)$$

$$\text{catalyst} + \text{oxidant–O} \rightarrow \text{catalyst–O} + \text{oxidant} \quad (1B)$$

This results in the transfer of oxygen from the catalyst to the reductant and then from the oxidant back to the catalyst. Surface lattice oxygen in metal oxide catalysts participates in these reactions. A similar catalytic reaction scheme has been suggested for the observed breakdown of hydrogen peroxide by titanium oxide.[39] The possibility exists that other reactive oxygen species could undergo these reactions as well.

A neighboring pair of titanium dioxide [$Ti(IV)O_2$] sites could serve as the oxygen-donating catalyst in Equation (1A). Superoxide could serve as the reductant, with two free radicals donating their extra electrons to reduce the two titanium dioxide sites to a single titanium sesquioxide [$Ti(III)_2O_3$] site, with the resulting generation of oxygen and water. Peroxynitrite is known to undergo two electron oxidation reactions,[5] and this species could serve as the oxidant in Equation (1B). Substituting these species into Equations (2A) and (2B) gives the following reactions:

$$Ti(IV)O_2 + 2O_2\cdot^- + 2H^+ \rightarrow Ti(III)_2O_3 + O_2 + H_2O \quad (2A)$$

$$Ti(III)_2O_3 + OONO^- \rightarrow 2\ Ti(IV)O_2 + NO_2^- \quad (2B)$$

Furthermore, in Equation (2B) hydrogen peroxide also could be substituted for peroxynitrite, resulting in the breakdown of hydrogen peroxide and the formation of $2Ti(IV)O_2$ sites.[39]

These reactions provide a pathway through which titanium oxide can directly remove superoxide, which agrees with the results shown in Figure 3. Additionally, this same mechanism provides the means for titanium oxide to remove the reactive superoxide product, peroxynitrite, as shown in Figures 1 and 2.

The mechanism requires the formation of Ti(III) in the oxide, and this may account for the bluish discoloring around titanium implants caused by Ti(III), as observed *in vivo*. Although the mechanism is catalytic, with Ti(III) returning to the Ti(IV) valence state, it is conceivable that excess Ti(III) may be formed by superoxide produced during the inflammatory response, resulting in Ti(III) leaching out into the surrounding tissue.

Other mechanisms through which titanium oxide could catalytically break down reactive species also are possible. Peroxynitrite can undergo similar reactions as superoxide.[17,40–42] Given the fact that peroxynitrite can undergo reactions in place of the superoxide, it is natural to hypothesize that peroxynitrite

may interact with the titanium oxide surface layer in a similar manner.

Figures 3 and 4 show that superoxide production and cytokine production of stimulated macrophages, respectively, were reduced in the presence of titanium dioxide surfaces. This is consistent with reports demonstrating antioxidant activity affecting the cellular redox state and, in turn, mediating proinflammatory cytokine gene expression.[43] Therefore, we hypothesize that degradation of reactive oxygen species by titanium dioxide surfaces leads to reduced oxidant stress in macrophages, and then to decreased production of cytokines such as interleukin-6.

Polyethylene implants coated with superoxide dismutase mimics showed a notable decrease in capsule thickness compared to uncoated controls.[14] This is consistent with the reported direct link between superoxide and the production of proinflammatory mediators, including cytokines, through two transcriptional activators, NF-κB and AP-1.[43] These results agree with the results shown in Figure 4, which indicate that species such as superoxide, a precursor of peroxynitrite, play a role in the inflammatory response to biomaterial surfaces. Interestingly, this group concluded that peroxynitrite was a likely mediator of the proinflammatory effects of superoxide in the implant environment.

CONCLUSIONS

The results of these presented experiments suggest that a crucial difference between titanium and polymer implants may be the ability of titanium to inhibit reactive oxygen species, which results in the mitigation of the inflammatory response. This may explain the differences in tissue responses at an implant.

Polyethylene surfaces coated with superoxide dismutase mimics enhance the breakdown of superoxide at the surface of the implant,[14] but such mimics can convert superoxide only to hydrogen peroxide. Hydrogen peroxide is a reactive species and the enzyme catalase is required to further break hydrogen peroxide down to water and oxygen to prevent damage to surrounding tissue. Titanium dioxide coatings have the advantage of catalytically breaking down hydrogen peroxide[28] as well as promoting the breakdown of superoxide and peroxynitrite.

These experimental findings offer the potential of combining the range of physical characteristics and cost effectiveness of silicone and other polymers with the biocompatibility exhibited by titanium, resulting in a new and novel class of biomaterials for use in implant applications.

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