Antibacterial Activity of Cold Plasma–Treated Titanium Alloy

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ABSTRACT: Titanium and titanium alloys are widely considered successful biocompatible materials because titanium implants become easily connected, both structurally and functionally, to biological systems. Antibacterial activity for medical implants could be an interesting property to prevent inflammation of the implant site. In this study, cold plasma treatments were performed on a titanium surface and the antibacterial activity of treated samples was tested against Escherichia coli 32. Some plasma parameters were varied to find the best antibacterial conditions. Samples showing higher germicidal activity were obtained in several experimental set-ups, and the antibacterial activity was detected at same level 16 days after the plasma treatment. A correlation between antibacterial effect and plasma-treated titanium surface energy also was studied.

KEYWORDS: antibacterial activity, cold plasma, titanium, titanium dioxide

I. INTRODUCTION

It is well known that titanium is a successfully biocompatible material. Over the past 30 years, the development of new processing methods has expanded the use of titanium in biomedical devices. In fact, continual improvements in device design, surface treatments, and clinical implantation techniques have led to well-accepted, titanium prosthetic joints, surgical splints, stents and fasteners, dental implants, dental crowns, and partial denture frameworks.

Titanium biocompatibility of dental implant permits close apposition of physiological fluids, proteins, and hard and soft tissues to the metal surface. This process, whereby living tissue and an implant become structurally and functionally connected, is called osseointegration.

Microbial colonization on metallic and ceramic implant materials has been reported in in vitro and in vivo tests. Because commercial pure titanium, used in the biomedical industry, does not have antibacterial activity, there is a probable risk of plaque formation on its surface, but it has been reported that anatase titanium oxide, which forms on the surface of the implant, has some germicidal activity. Antibacterial activity of titanium implants could be a useful property to maintain plaque-free surfaces exposed to the oral cavity, preventing peri-implantitis. Some electrochemical techniques to coat the titanium surface with an antibacterial layer have been studied, but they are harmful to the environment and require long treatment. In this study, an attempt was made to
use a short and environmentally friendly cold plasma treatment\(^5\) to impose antibacterial activity to titanium.

Plasma is defined as a quasineutral ionized gas. It is constituted by particles—including photons, electrons, positive and negative ions, atoms, free radicals, and excited or nonexcited molecules—in permanent interaction. Some kinds of plasma may have a general oxidative effect on the cell surface layers of microorganisms, inhibiting growth.

On the other hand, titanium oxide is a semiconductor, and its photocatalytic activity can be activated by exposure to sunlight or a ultraviolet (UV) rays. The antimicrobial effect of titanium oxide, when exposed to UV light, has been proven; this oxide is utilized as disinfecting and depolluting material for surface coatings in many applications. The irradiation of titanium dioxide (TiO\(_2\)) with UV light promotes electrons from the valence band to the conduction band, leaving a positively charged hole. The electrons and holes migrate and, at the surface, react to form highly reactive oxygen species, which can oxidize organic compound or cells on the TiO\(_2\) surface, resulting in the death of the microorganism. Unfortunately, UV irradiation causes fast decay. Only a few scientists have studied TiO\(_2\) antibacterial activity that is not activated by UV light. Liu et al.\(^6\) revealed non-UV-based TiO\(_2\) germicidal activity, which is useful for disinfecting surfaces in public areas.

Antibacterial activity of nonthermal plasmas are industrially interesting because they are nonpollutant and do not require expensive set-up.\(^7\) This study was designed to investigate the antibacterial effect of cold plasma–treated titanium surfaces using a model system involving *Escherichia coli*; some other preliminary studies will be reported.

**II. MATERIALS AND METHODS**

Commercially pure, grade 2 titanium sheets for biomedical application were purchased from Titanium Consulting and were cut into slides (20 × 20 × 1 mm). Groups of 6 slides were treated with cold plasma using an internal, parallel plate, capacitive reactor (diameter of the electrodes, 25 cm; distance between electrodes, 3 cm; reactor wall and electrodes were made of stainless steel AISI 316L). The frequency of the power source was 13.56 MHz, and it can deliver a power of 1000 W. Gas used for the plasma treatment was of commercial purity. Before the plasma treatments, the plasma reactor was evacuated to a pressure of approximately 0.04 mbar for 30 minutes.

An initial batch of tests was performed to discover if and when the titanium surface acquires antibacterial proprieties. Some plasma parameters were varied: gas nature (oxygen [O\(_2\)], air), pressure, feed gas flow rate, electrical power, and duration of treatment. A second batch of tests was used to test the durability of antibacterial activity 16 days after plasma treatment and after 3, 24, and 72 hours of immersion in the plate assays.

X-ray analysis of treated samples was performed using a Philips PV1710 spectrometer.

Contact angles were obtained using the sessile drop method with a Dataphysics OCA-20 contact angle analyzer. The drop image was processed by an image analysis system, which calculated both the left and right contact angles from the image of the drop with an accuracy of ±0.1%. Two test liquids were used as a probe for surface free
energy calculations: (1) distilled water, (2) di-iodomethane (purchased from Sigma). The data for surface tension components of the test liquids are given in Table 1.\textsuperscript{8,9} All measurements were made at 25°C. The surface tension was calculated according to the Owens-Wendt-Rebel-Kaelble equation. Untreated samples were cleaned ultrasonically in ethanol for 5 minutes before contact angle measurement. Five measurements were taken for each sample, and 3 samples were used for each experimental set-up.

\textit{Escherichia coli} 32 was isolated from fresh meat samples, characterized by DNA sequencing, and kept in sterile environment. All experiments were performed aseptically per normal procedure in Microbiology Laboratories. The strain was cultivated daily in triptone soy broth (Oxoid) supplemented with yeast extract 0.5% at 37°C overnight. Two treatment methods were performed: a drop method and an immersion method.

For the drop method, 10 µL of an \textit{E. coli} 32 overnight culture, diluted in quarter strength Ringer’s solution (Oxoid) to reach about $5 \times 10^7$ cells/mL, were placed onto a treated titanium slide (or untreated as control) and incubated at room temperature in a humid chamber to ensure the drop did not dry. Viable staining was carried out immediately before the incubation and after 30 minutes, 1 hour, and 3 hours.

In the immersion method, one plasma-treated titanium slide was immersed in 10 mL of an \textit{E. coli} 32 overnight culture, diluted in quarter strength Ringer’s solution to reach about $10^4$ cells/mL, and incubated at room temperature for 3, 24, and 72 hours in slight agitation. During incubation the titanium slide was immersed completely in the cell suspension. The untreated titanium slide was used as control. Cell survival was determined by counting colony-forming units (CFUs). Each experiment was carried out in triplicate.

\textit{E. coli} 32 cells were counted directly on the titanium slide using a LIVE/DEAD \textit{Bac}Light Bacterial Viability Kit (Molecular Probes Inc., Eugene, OR), according to the procedure described by Ercolini et al.\textsuperscript{10} A stock solution of the 2 fluorochromes was prepared with 0.7 µL of SYTO 9 and 1 µL of propidium iodide in 330 µL of sterile deionized water. After the viable staining, samples were observed using a Nikon Eclipse E400 epifluorescence microscope (Nikon, Tokio, Japan) equipped with a UV lamp and a 100× magnification objective. After incubation in a humid chamber, as previously described, 6 µL of fluorochrome stock solution were applied to 10 µL of treated cells directly on the titanium slide and incubated in the dark for 15 minutes at room temperature. The titanium slides were used for microscopic counting. Untreated \textit{E. coli} 32 was analyzed in each determination as control. Enumeration of cells was performed randomly by counting 30 microscopic fields. The number of green cells (with undamaged membrane) and red cells (with damaged membrane) was calculated as follows:

<table>
<thead>
<tr>
<th>Liquid</th>
<th>Surface Free Energy $\sigma$ (mJ/m$^2$)</th>
<th>Polar Component (mJ/m$^2$)</th>
<th>Dispersion Component (mJ/m$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water ($H_2O$)</td>
<td>72.8</td>
<td>51.0</td>
<td>21.8</td>
</tr>
<tr>
<td>Di-iodomethane ($CH_2I_2$)</td>
<td>50.8</td>
<td>2.3</td>
<td>48.5</td>
</tr>
</tbody>
</table>
\[ N = \frac{n}{a \times V} \]

where \( N \) is the cells per milliliter; \( n \) is the average number of cells per microscopic field (based on 30 fields); \( a \) is the microscopic field area (in millimeters squared); and \( V \) is the volume of the sample.

After the incubation periods reported above for the immersion method, 1 mL of cell suspension was diluted 10-fold, immediately and serially, in quarter strength Ringer’s solution. An aliquot of 100 µL of each dilution was spread on triptone soy agar (Oxoid) supplemented with yeast extract 0.5% and incubated at 37°C for 16 hours. Antibacterial activity was expressed as percentage of cell survival after 3 and 24 hours of contact. Each experiment was performed in triplicate in 3 different trials.

**III. RESULTS AND DISCUSSION**

Counting the CFUs revealed that titanium slides treated with air treatments for all tested conditions of plasma formation had no effect on *E. coli* 32 cells. In fact, the percentage of living cells after incubation was 100%, and the results were maintained after 3 and 24 hours of incubation (Table 2).

Titanium slides treated with \( \text{O}_2 \) plasma (Table 2) affected *E. coli* 32 cells differently, depending on conditions used for plasma formation. Treatments 5, 11, 12, and 13 had no effect on *E. coli* 32 cells. Plasma creation by treatments 6, 7 and 8 (\( \text{O}_2 \) flow, 20 sccm; pressure, 0.145 mbar; electrical power, 200 W) varied in duration at 2.5, 3.5, and 5 minutes on each side, respectively, and resulted in different survival percentages. Treatment 6 halved the *E. coli* 32 population; treatment 7 caused a decrease in *E. coli* 32 survival (33%) after 24 hours of incubation. Intense *E. coli* 32 growth inhibition was detected after 3 hours of incubation only when plasma was created using treatment 8. In this case, the *E. coli* 32 survival percentage calculated after 3 hours of incubation was 30%. The inhibitory effect was much more intense after 24 hours of incubation, when survival of cells was only 23%. Using treatment 9, *E. coli* 32 survival calculated after 24 hours of incubation was 24%, whereas no effect on *E. coli* 32 cells was detected after 3 hours. Treatment 14 caused a decrease in survival percentage (42%) after 3 hours of incubation. This treatment had a strong effect on *E. coli* 32 cells after 24 hours of incubation, determining a survival rate of 1%. The same effect was registered after 3 hours of incubation by putting *E. coli* 32 cells in contact with titanium slides treated by treatment 15, which had the highest electrical power. \( \text{O}_2 \) plasma formation at 0.125 mbar (treatment 10) had no considerable effect on *E. coli* 32 cells (77% of survival after 24 hours of incubation).

Viable staining of *E. coli* 32 was performed by using a LIVE/DEAD BacLight Bacterial Viability Kit. Titanium slides treated by cold plasma did not any have any effect on *E. coli* 32 membranes; the red cells were never observed and the number of green cells remained constant. The same results were found after 3 and 24 hours of incubation at all tested conditions for plasma formation, but bacteria were no longer able to form
colonies, as shown by the CFU counting method.

Titanium oxide spontaneously forms on a titanium surface in the presence of even trace amounts of any form of oxygen\(^\text{11}\). Crystalline TiO\(_2\) phases are the focus of interest because of their antibacterial properties compared with amorphous TiO\(_2\) films. Toku et al.\(^\text{12}\) found that O\(_2\) concentration in the reaction mixture influences TiO\(_2\) formation because higher O\(_2\) concentration aids the attainment of optimal conditions for growth of crystalline TiO\(_2\) films. In our experimental conditions, when performing plasma treatment using air, we did not detect any decrease in \textit{E. coli} 32 survival. Instead, higher \textit{E. coli} 32 growth inhibitions were obtained when plasma was created in the presence of O\(_2\).

Table 2 confirms that duration of treatment plays an important role in the effectiveness of treatments (see treatments 6–8), whereas little or no effect is made by pressure (see treatments 8 and 10). When titanium slides were treated for 3 and 24 hours, the percentages of \textit{E. coli} 32 survival were 100% for all treatments except treatments 6 and 7, which show 50% and 33% survival, respectively. These results are consistent with our hypothesis that oxygen concentration in the reaction mixture using air allows the formation of amorphous TiO\(_2\), whereas cold plasma treatment on titanium using O\(_2\) allowed the formation of anatase TiO\(_2\) and subsequently produced slides with antibacterial activity against \textit{E. coli} 32. However, our test, using RX spectra (data not shown), does not confirm the presence of an anatase layer on plasma treated samples; this may be due to its thinness.

Among titanium slides showing germicidal activity, those obtained using a duration of 5- and 10-minute plasma treatments with 300- and 500-W electrical power show high antibacterial activity. Data shown in Table 2 confirm that duration of treatment plays an important role in the effectiveness of treatments (see treatments 6–8), whereas little or no effect is made by pressure (see treatments 8 and 10). When titanium slides were

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Gas</th>
<th>Flow (sccm)</th>
<th>Electrical Power (W)</th>
<th>Pressure (mbar)</th>
<th>Time (min)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>air</td>
<td>20</td>
<td>50</td>
<td>0.116</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>air</td>
<td>20</td>
<td>50</td>
<td>0.2</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>air</td>
<td>20</td>
<td>50</td>
<td>0.5</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>air</td>
<td>50</td>
<td>50</td>
<td>0.116</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>O(_2)</td>
<td>20</td>
<td>100</td>
<td>0.145</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>O(_2)</td>
<td>20</td>
<td>200</td>
<td>0.145</td>
<td>2.5 per side</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>O(_2)</td>
<td>20</td>
<td>200</td>
<td>0.145</td>
<td>3.5</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>O(_2)</td>
<td>20</td>
<td>200</td>
<td>0.145</td>
<td>5</td>
<td>30*</td>
</tr>
<tr>
<td>9</td>
<td>O(_2)</td>
<td>20</td>
<td>300</td>
<td>0.145</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>O(_2)</td>
<td>20</td>
<td>200</td>
<td>0.125</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
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<td>O(_2)</td>
<td>10</td>
<td>50</td>
<td>0.09</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>O(_2)</td>
<td>20</td>
<td>50</td>
<td>0.3</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>13</td>
<td>O(_2)</td>
<td>60</td>
<td>50</td>
<td>0.3</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>14</td>
<td>O(_2)</td>
<td>100</td>
<td>300</td>
<td>0.145</td>
<td>42*</td>
<td>1*</td>
</tr>
<tr>
<td>15</td>
<td>O(_2)</td>
<td>100</td>
<td>500</td>
<td>0.145</td>
<td>5</td>
<td>1*</td>
</tr>
</tbody>
</table>

*Significance <0.05, calculated by t-test.
treated with cold plasma (O₂ flow rate, 20 sccm; pressure, 0.145 mbar), electrical power was varied to observe how this condition during plasma formation affected titanium antibacterial properties. O₂ plasma treatment performed at a low electrical power of 50–100 W and at different gas flow rates and pressures (independent of treatment duration) produced titanium slides that were not able to reduce E. coli 32 growth. Increasing electrical power, bacterial survival was 33% on titanium slides at 200 W after 5 minutes of immersion (treatment 8), 24% at 300 W after 3 minutes of immersion (treatment 9), and 43% and 1% at 500 W after 10 minutes and 24 hours of immersion, respectively (treatment 15). This clearly shows that electrical power plays a fundamental role. Obviously, the best results were obtained by combining a long treatment time and high electrical power (treatments 14 and 15), but it should be noted that, compared with other treatment times used in other applications or in some industrial procedures (e.g., metallization of plastic or silicon cold plasma dry etching), the treatment times used in this study could be considered a “short” treatment.

To test the durability of E. coli growth inhibition due to cold plasma treatments, specimens treated as in conditions 14 and 15 (called 14b and 15b; see Table 3), were kept at room temperature in a sterile environment and tested 16 days after plasma treatments. Results showed that titanium slides were still effective against E. coli 32 cells (1% of survival) after 24 hours and 72 hours (1 or 2% of survival).

To evaluate whether a relationship exists between the antibacterial behavior of plasma-treated samples and surface energy, we have measured the surface energy on O₂ plasma-treated specimens the day after the treatments. The surface energy of the nontreated specimen was calculated as a reference (total surface energy is 45 mN/m with a dispersive part of 17 mN/m and a polar part of 28 mN/m).

The total surfaces energy was increased of about 45% by the plasma treatments (Fig. 1); there is no substantial difference between 300- and 500-W treatments. In both cases, the surfaces become more hydrophilic with increasing polar shares, that is, the contact angle of water droplets falls because of the better wetting. Polar interactions due to dipoles also had much higher bonding energies than dispersion forces; therefore, one can also expect high shared polar energy to lead to better spreading and, in some cases, good adhesion.

The plasma treatments in O₂ gas changed the polar part of surface energy more than

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### TABLE 3. Effect on Percentage of E. coli Survival of Some Types of Cold Plasma Treatments on Commercially Pure Grade 2 Titanium Sixteen Days After the Plasma Treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Gas</th>
<th>Flow (sccm)</th>
<th>Electrical Power (W)</th>
<th>Time (min)</th>
<th>Days Since Treatment</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14b</td>
<td>O₂</td>
<td>100</td>
<td>300</td>
<td>10</td>
<td>16</td>
<td>100 1 2</td>
</tr>
<tr>
<td>15b</td>
<td>O₂</td>
<td>100</td>
<td>500</td>
<td>5</td>
<td>16</td>
<td>50 1 1</td>
</tr>
</tbody>
</table>

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the dispersive part. The dispersion component seemed to be not influenced greatly by
the power and duration of the plasma field (Fig. 2); in fact, it was quite constant around
24 mN/m.

The polar component of total surface energy (Fig. 3) was increased significantly by
the plasma treatment, although in this case there did not seem to be a significant differ-
ence between the 300- and 500-W treatments.

Considering the preliminary results obtained in the experimental characterization of
the antibacterial activity of treated samples, there seems to be no relationship between
the former and surface energy. Experimental data seem to show that higher total surface
energy is reached at a 100-W “short” treatment, whereas increasing power is deleterious
for the ability of samples to be wetted. More experimental data are necessary to try to
find a relationship between antibacterial activity and cold plasma treatments.

IV. CONCLUSIONS

Cold plasma treatment is an effective technology to show the antibacterial activity of
commercial purity grade 2 titanium against *E. coli* 32. The antibacterial activity of ti-
tanium treated with cold plasma exerted different efficacies, depending on different
conditions for plasma formation. Best results were registered by using O\textsubscript{2} at 500 W of
electrical power and a treatment duration of 5 minutes. The same level of antibacterial
activity imposed on titanium detected on the day after treatment was detected 16 days
FIGURE 2. The dispersive part of the surface energy of plasma-treated and untreated commercially pure grade 2 titanium specimens.

FIGURE 3. The polar part of surface energy of plasma-treated and untreated commercially pure grade 2 titanium specimens.
after treatment.
There seemed to be no relationship between the antibacterial activity of titanium and its surface energy. The plasma treatment in O\textsubscript{2} gas increased the surface energy of titanium specimens, and the polar component was influenced by the duration and electrical power of treatment.

To observe the antimicrobial spectrum and the interaction between the cold plasma and the antibacterial activity of the titanium slides, further studies are required. Our results suggest that cold plasma created on titanium surfaces could be a promising technique for treating medical devices because it is an inexpensive and toxic-free treatment. Moreover cold plasma–treated titanium samples do not require any other activation step to dispatch antibacterial activity.

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