Monitoring metabolic reactions in *Staphylococcus epidermidis* exposed to silicon nitride using *in situ* time-lapse Raman spectroscopy

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Abstract. *Staphylococcus epidermidis* (*S. epidermidis*) is one of the leading nosocomial pathogens, particularly associated with periprosthetic infections of biomedical implants. Silicon nitride (*Si₃N₄*), a nonoxide biomaterial widely used in spinal implants, has shown bacteriostatic effects against both gram-positive and gram-negative bacteria; however, the physicochemical interactions between *Si₃N₄* and bacteria yet remain conspicuously unexplored. *In situ* time-lapse Raman spectroscopic experiments were conducted by exposing *S. epidermidis* for 12, 24, and 48 h to *Si₃N₄* substrates to understand the evolution of bacterial metabolism and to elucidate the ceramics antimicrobial behavior. The Raman probe captured an initial metabolic response of the bacteria to the adverse chemistry of the *Si₃N₄* surface, which included peroxidation of membrane phospholipids and protein structural modifications to adjust for survivorship. However, beyond 24 h of exposure, the Raman signals representing DNA, lipids, proteins, and carbohydrates showed clear fingerprints of bacterial lysis. Bands related to biofilm formation completely disappeared or underwent drastically reduced intensity. Bacterial lysis was confirmed by conventional fluorescence microscopy methods. Spectroscopic experiments suggested that a pH change at the *Si₃N₄* surface induced variations in the membrane structure and d-alanylation of teichoic acids in its peptidoglycan layer. Concurrent stimulation of peptidoglycan hydrolase (i.e., an enzyme involved with autolysis) ultimately led to membrane degradation and cellular death. An additional finding was that modulating the *Si₃N₄* surface by increasing the population of amine groups improved the efficiency of the substrate against *S. epidermidis*, thus suggesting that optimization of the near-surface (alkaline) conditions may be a viable approach to bacterial reduction. © 2018 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.23.5.056002]

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1 Introduction

One of the clinical research goals during the last decade has been the clarification of staphylococcal pathogenesis mechanisms as they relate to implanted biomaterials for joint prostheses, catheters, heart valves, and pacemakers. The pathogen which represents a common source of infections on indwelling medical devices is *Staphylococcus epidermidis*, a gram-positive bacterium that permanently resides on human skin and in mucosa membranes. *S. epidermidis* is capable of adhering to synthetic biomaterial surfaces and forming a stable biofilm leading to persistent infections. During biofilm formation, bacterial cells secrete extracellular polymeric substances that include lipids, proteins, polysaccharides, and others; these protect the bacterial colony from external attack. Accordingly, treating biofilm-related infections is extremely difficult. Actions of the immune system can be ineffective and eradication of the pathogen often requires extreme doses of antibiotics. Biofilm-encased bacteria are up to a 1000 times more resistant to bactericidal drugs than their planktonic predecessors. In addition, for patients with compromised immune systems, the release of bacteria from the biofilm often triggers remote secondary site infections. One possible way of preventing periprosthetic infections is to design biomaterials with physical or chemical barriers that are capable of inherently resisting bacterial surface adhesion. One candidate material with such properties is *Si₃N₄*, a nonoxide ceramic whose surface can be chemically and topographically altered. Previous tests conducted by Bock et al. verified the antibacterial behavior of this bioceramic. They compared the bacteriostatic characteristics of different biomaterials used in medical...
devices against *S. epidermidis* and *Escherichia coli* (*E. coli*). Their results showed a pronounced reduction in bacteria adhesion and live bacteria with increased exposure to Si$_3$N$_4$.

Raman spectroscopy on living bacteria has been employed to follow its metabolism and to understand the chemical interactions between bacterial cells and biomaterials.\textsuperscript{10-12} This *in situ* spectroscopic technique is useful in identifying and observing specific time-dependent biochemical changes in the composition and activity of prokaryotic cells. Neugebauer et al.\textsuperscript{10} succeeded in characterizing *S. epidermidis* with respect to its metabolic functions using different spectroscopic techniques; Samek et al.\textsuperscript{11} analyzed the formation of *S. epidermidis* biofilm by labeling characteristic Raman bands in its spectrum. The metabolism of gram-negative *Porphyromonas gingivalis* was also clarified in a recent paper before and after its inoculation onto various Si$_3$N$_4$ substrates.\textsuperscript{12}

In this study, the *in situ* metabolic activity, cellular growth, and lysis of *S. epidermidis* were examined at different time points under the chemical and physical influences of various Si$_3$N$_4$ substrates. Comparative data were also acquired on two other popular biomaterials: polyetheretherketone (PEEK) and a titanium alloy (Ti). *In situ* Raman spectroscopy provided physico-chemical insights into the interaction of *S. epidermidis* colonies with these various biomaterials, from which a mechanistic understanding of Si$_3$N$_4$'s bacteriostatic behavior was proposed.

## 2 Materials and Methods

### 2.1 Investigated Biomaterials

The Si$_3$N$_4$ samples were produced by Amedica Corporation (Salt Lake City, Utah) using conventional ceramic fabrication techniques.\textsuperscript{13} In brief, 90 wt. % Si$_3$N$_4$ powder (Ube SN E-10, Ube City, Japan) was mixed with 6 wt. % yttrium oxide (Y$_2$O$_3$, Grade C, H. C. Starck, Munich, Germany) and 4 wt. % aluminum oxide (Al$_2$O$_3$, SA8-DBM, Baikowski/Malakoff, Charlotte, North Carolina) followed by comminution and spray drying. The Y$_2$O$_3$ and Al$_2$O$_3$ additives served as sintering aids. Disk samples (Ø12.7 × 1 mm) were dry-pressed, debinderized, and sintered in a nitrogen atmosphere at temperatures in excess of 1700°C and further densified by hot-isostatic pressing at >1650°C under a N$_2$ gas pressure of >200 MPa. The resulting dense Si$_3$N$_4$ samples consisted of a two-phase microstructure—acicular β-Si$_3$N$_4$ grains and a grain boundary phase of amorphous or crystalline yttrium aluminum oxynitride (i.e., SiYAlON).\textsuperscript{14} The resulting disks were designated as “untreated” or “as-fabricated” Si$_3$N$_4$. A subset of these disks was further subjected to post densification annealing in a nitrogen atmosphere (~1.1 bar) at 1400°C for 30 min. Following this heat treatment, the surface density of amines relative to hydroxyl groups increased\textsuperscript{15} and a fraction of SiYAlON glass migrated to the sample’s surfaces. Comparative testing was also performed on dimensionally identical samples of PEEK (ASTM D6262, Ketron® PEEK 1000, Quadrant EPP USA, Inc., Reading, Pennsylvania, distributed by McMaster-Carr, Santa Fe Springs, California) and a Ti-alloy (ASTM F136, Ti6Al4V-ELI, distributed by Vincent Metals, Minneapolis, Minnesota). The PEEK and Ti-alloy samples were used as negative and positive controls, respectively.

### 2.2 Bacterial Culture

*S. epidermidis* (14990\textsuperscript{6}ATCC\textsuperscript{TM}) was cultured at the Kyoto Prefectural University of Medicine using a brain heart infusion (BHI) Agar medium. The initial $1.8 × 10^{10}$ CFU/ml was subsequently diluted to $1 × 10^6$ CFU/ml using a phosphate-buffered saline solution to mimic ion blood concentrations. Then, 100 μL of bacteria solution were transferred to separate Petri dishes containing the BHI agar. All samples were sterilized by UV before inoculation with the bacteria. Incubation at 37°C took place under aerobic conditions for three time-points: 12, 24, and 48 h, respectively.

### 2.3 Laser Microscopy

The surface morphology of the studied substrates, which can affect the primary interaction between the implant body and the environment, was characterized with respect to its microscopic topography. Average roughness, $R_a$, for the different surfaces was acquired using a confocal scanning laser microscope (Laser Microscope 3D & Profile measurements, Keyence, VKx200 Series, Osaka, Japan) capable of high-resolution optical images with depth selectivity. The average roughness was calculated as the mean value over 36 images randomly acquired on each sample using 150x magnification.

### 2.4 Fluorescence Microscopy and Microbial Viability Assay

After exposure to the different material surfaces, bacterial samples were observed using a fluorescence microscope (BZ-X700; Keyence, Osaka, Japan). To facilitate their examination, the bacteria were stained with different solutions: propidium iodide (PI; Dojindo, Kumamoto, Japan), 5(6)-carboxyfluorescein diacetate (CFDA; Dojindo, Kumamoto, Japan), and 4',6-diamidino-2-phenylindole (DAPI; Dojindo, Kumamoto, Japan). PI’s red color highlighted dead or injured bacteria; CFDA’s green color revealed living bacteria; and DAPI, which binds to DNA, verified cell nuclei locations. The staining protocol consisted of adding 1 μL of DAPI, the PI solution, and 15 μL of CFDA solution to the samples, and then incubating for 5 min at 37°C. After removing the buffer, the cells were stained and analyzed under the fluorescence microscope. The exposure time for each image was 5 s under an 80-W metal halide lamp. After exposures of 12 and 48 h, bacterial metabolism was examined using a colorimetric assay (Microbial Viability Assay Kit-WST, Dojindo, Kumamoto, Japan). This assay employed a colorimetric indicator (WST-8), which produces a water-soluble formazan dye upon reduction in the presence of an electron mediator. The amount of the formazan dye generated is directly proportional to the number of living microorganism. Solutions were analyzed using microplate readers (EMax, Molecular Devices, Sunnyvale, California) via an optical density (OD) method for living cells.\textsuperscript{16}

### 2.5 In Situ Raman Spectroscopy

Time-lapse Raman spectra were collected after exposure of cultured *S. epidermidis* to two different Si$_3$N$_4$ samples, a Ti-alloy, and silica glass control (Silanized Slides; DAKO, Denmark). Spectra for bacteria on PEEK samples could not be obtained due to the high fluorescence emitted from the PEEK material. Spectra were collected using a confocal Raman microscope (LabRAM HR800; Horiba/Jobin-Yvon, Kyoto, Japan), which employed a single monochromator connected to an air-cooled CCD detector (Andor DV420-0E322; 1024 × 256 pixels). The excitation radiation was provided by a coherent helium-neon lamp emitting at 633 nm with a power of ~10 mW at the sample.
surface. A 600 gr/mm grating and a D.03 holographic notch filter were employed with a 100× objective lens. A 100-mm cross slit and a 200-mm confocal pinhole were employed. The Raman spectra were acquired with an exposure time of 2 s in each of four different spectral ranges. Average Raman spectra for each substrate were calculated from 15 measurements at different arbitrary locations. Raman spectral acquisition and preprocessing of raw data (i.e., baseline subtraction, smoothing, normalization, and fitting) were carried out using commercially available software (LabSpec, Horiba/Jobin-Yvon, Kyoto, Japan and Origin 8.5, OriginLab Co., Northampton, Massachusetts). Baseline subtraction of all the spectra has been obtained according to the polynomial method using a polynomial function of degree 8. Fitting was performed using Gaussian/Lorentzian functions.

2.6 Statistical Analysis

All data were expressed as mean values ± one standard deviation and were analyzed for their statistical significance ($p \leq 0.05$) using the Student’s $t$ test.

3 Results

3.1 Topography of Substrate Surfaces

Laser microscopy images and related three-dimensional (3-D) plots for different substrates are shown in Fig. 1. Untreated Si$_3$N$_4$ [Fig. 1(a)] exhibited a peculiar topology with the presence of protruding acicular grains interspersed into an oxide intergranular matrix. The N$_2$-annealed Si$_3$N$_4$ surface [Fig. 1(b)] presented a similar acicular grain structure but also displayed increased areal fractions of the intergranular phase that was expressed from the bulk during the thermal treatment.$^{15}$ Figures 1(c) and 1(d) show the surfaces of the Ti-alloy and PEEK, respectively.

The surface topology of the Ti-alloy was regular and homogeneous without the presence of secondary phases; the surface of PEEK appeared to be the smoothest of the tested materials. The average roughness, $R_a$, for these samples ($n = 4$ each) is compared in Fig. 2 together with a statistical analysis of observed differences. The N$_2$-annealed Si$_3$N$_4$ presented the highest roughness ($0.40 \pm 0.04$ μm) followed by the untreated Si$_3$N$_4$ ($0.32 \pm 0.02$ μm). The PEEK surface possessed the lowest roughness ($0.26 \pm 0.03$ μm), whereas the topology of the Ti-alloy ($0.29 \pm 0.04$ μm) was not significantly different from untreated Si$_3$N$_4$.

3.2 Microbial Viability Assays and Fluorescence Microscopy

As shown in Figs. 3(a) and 3(b), bacterial cell viability was verified at 12 and 48 h, respectively. After 12 h of exposure, the N$_2$-annealed Si$_3$N$_4$ showed the highest OD value ($3.12 \pm 0.044$) indicating the largest number of living bacteria among the studied materials. The Ti-alloy had the lowest OD value ($1.38 \pm 0.02$), whereas the PEEK ($2.14 \pm 0.06$) and untreated Si$_3$N$_4$...
had intermediate OD values. OD values between the tested materials were significantly different ($p < 0.05$) with the exception of the PEEK and untreated Si₃N₄. However, a noteworthy variation in bacterial growth occurred at the 48-h time-point. The highest OD value belonged to PEEK (0.87/C6 0.09), whereas the N₂-annealed Si₃N₄ displayed the lowest OD value (0.59/C6 0.02). Again, a comparison between untreated Si₃N₄ (0.73/C6 0.02) and the Ti-alloy (0.69/C6 0.04) resulted in a minimal statistical difference. After 48 h, all samples were statistically different ($p < 0.05$).

Note that the OD values measured for all samples were lower at 48 h than at 12 h. This was likely a consequence of the two sets of experiments being performed on independent samples, rather than representing time-series events.

At 24 h of exposure, fluorescence micrographs were obtained for all samples after first staining them with PI, CFDA, and DAPI markers, which colored dead bacteria (red), live bacteria (green), and nuclei (blue), respectively. Results are shown in Fig. 4 with a constant magnification of 20X for all samples. For the silicon nitride substrates, a relatively high amount of bacteria was detected [cf. DAPI blue-stained micrographs in Figs. 4(a) and 4(b)] for both the untreated and N₂-annealed samples, respectively. However, PI red-stained areas associated with dead bacteria clearly indicated that significant lysis had occurred, and it was more evident on the N₂-annealed material. Red stains were also detected on the Ti-alloy [Fig. 4(c)], with its surface presenting a relatively low density of bacterial cells. Micrographs on PEEK [Fig. 4(d)] confirmed no antimicrobial behavior for this material. There were no apparent dead bacteria and a high number of living cells over the entire sample’s surface, indicating significant bacteria proliferation.

### 3.3 Raman Spectroscopy Results

#### 3.3.1 Labeling Raman emissions from control samples

Figure 5 shows average Raman spectra collected on living S. epidermidis bacteria cultured on a silica glass control substrate. Three spectra are shown as a function of exposure time (12, 24, and 48 h; as labeled in the inset) in the spectral region between 300 and 3500 cm⁻¹. All the observed bands belonged to molecular species within S. epidermidis. The spectra were divided into four main regions, labeled zones I to IV, and individual bands were assigned according to their physical origin and vibrational modes in the cellular compounds (cf. Fig. 5 and Tables 1–3).

In zone I, between 600 and 900 cm⁻¹, a set of bands related to DNA and RNA dominated the Raman spectrum. In particular, the band at 720 cm⁻¹ was attributed to adenine containing species, whereas bands at 735 and 782 cm⁻¹ were related to a mode of adenine ring breathing in DNA and DNA phosphodiester stretching, respectively. Additional bands in this region were located at 820 and 840 cm⁻¹, the former corresponding to out-of-plane ring breathing modes of tyrosine and the latter to the C–O–C stretching vibration of 1,4 glycosidic link in carbohydrates.

Zone II at 900 to 1500 cm⁻¹ was characterized by a series of emission bands from amides, lipids, and carbohydrates. Two
main bands in this region, at 1092 and 1125 cm\(^{-1}\), are related to C─O─C antisymmetric stretching in aliphatic esters and glycosidic link of carbohydrates, and proteins, and lipids, respectively.\(^{22}\) Additional bands at 1253 and 1313 cm\(^{-1}\) are related to C─N stretching in amide III and twisting of saturated CH\(_2\) moieties in membrane lipids, respectively.\(^{23,24}\) Another prominent band at 1442 cm\(^{-1}\) corresponds to bending of saturated CH\(_2\) in polysaccharides and membrane lipids.\(^{24,25}\)

Zone III between 1500 and 1850 cm\(^{-1}\) contained one α-helix-related band (C═O stretch) at 1600 cm\(^{-1}\),\(^{18}\) but it was dominated by the β-sheet-related C═O stretching emission of amide I at 1680 cm\(^{-1}\).\(^{26}\)

Zone IV between 2800 and 3100 cm\(^{-1}\) contained three main bands at 2880 cm\(^{-1}\) (symmetric stretching of CH\(_3\) in lipids and fatty acids\(^{27}\), 2934 cm\(^{-1}\) (symmetric and antisymmetric C-H stretching of CH\(_2\) and CH\(_3\) in lipids, proteins, and carbohydrates\(^{28}\)), and 2970 cm\(^{-1}\) (assigned to CH\(_3\) symmetric stretching in lipids and fatty acids\(^{29}\)).

Looking at the time-lapse spectra collected on the control substrate revealed a slight intensity decrease of the triplet at 2881, 2934, and 2970 cm\(^{-1}\) and of the amide I band at 1670 cm\(^{-1}\) at 24-h exposure as compared with 12 h. However, the spectrum at 48 h did not show significant variations as compared with 24 h or any significant decreases in band intensities. Rather, DNA bands of phosphodoxy groups and adenine at 1092 and 729 cm\(^{-1}\) experienced higher intensities after 48 h than after 24 h. Another noted difference was the amide I band at 1680 cm\(^{-1}\). It was slightly more intense at 48 h compared with 24 h. A similar trend was observed for protein and carbohydrate bands at 836 and 858 cm\(^{-1}\); these were completely absent after 24 h but were clearly visible after 48 h. The evolution of these bands as a function of time on the silica substrate was utilized as a standard for the metabolic activity of S. epidermidis. It was used to establish meaningful criteria for judging
changes in the bacteria’s metabolism upon its exposure to the test samples. It should be noted that bands related to proteins and carbohydrates actually change with the formation of a biofilm, whereas DNA-related bands are barometers of bacterial cell proliferation and well-being.

### 3.3.2 Raman emissions from *S. epidermidis* on different substrates

Normalized Raman spectra of the bacteria as a function of exposure time on the different substrates in zone I are shown in Figs. 6(a)–6(c) for 12, 24, and 48 h, respectively. As shown in Table 1, 10 distinct bands were found and a number of features became visible in their time dependencies. In comparison with the control sample, the emission at 720 cm$^{-1}$ (labeled as band 4 and related to phosphatidylserine) tended to disappear for both untreated and N$_2$-annealed Si$_3$N$_4$. In contrast, this band underwent a decrease in intensity at >24 h for bacteria exposed to the Ti-alloy. In the main triplet of bands 5 to 7, only the phosphodiester band 7 completely disappeared with elapsed time for both Si$_3$N$_4$ substrates, whereas the DNA-related band 6 increased in relative intensity for both Si$_3$N$_4$ samples. Band 7 persisted up to 24 h with a relatively strong intensity for the Ti-alloy substrate. Band 8 tended to disappear upon exposure of the bacteria to untreated Si$_3$N$_4$, whereas it showed an increase at 24 h and then a reduction at 48 h in the case of N$_2$-annealed Si$_3$N$_4$. The trend found for band 8 in bacteria exposed to the Ti-alloy substrate was similar to that observed for N$_2$-annealed substrate. Band 10 (ring breathing in trypsin) was quite strong for bacteria exposed to both types of materials, and Ti-alloy. Although in the case of glass control, the two substrates, whereas the DNA-related band 6 increased in relative intensity for both Si$_3$N$_4$ samples. Band 7 persisted up to 24 h with a relatively strong intensity for the Ti-alloy substrate. Band 8 tended to disappear upon exposure of the bacteria to untreated Si$_3$N$_4$, whereas it showed an increase at 24 h and then a reduction at 48 h in the case of N$_2$-annealed Si$_3$N$_4$. The trend found for band 8 in bacteria exposed to the Ti-alloy substrate was similar to that observed for N$_2$-annealed substrate. Band 10 (ring breathing in trypsin) was quite strong for bacteria exposed to both types of Si$_3$N$_4$ substrates, whereas it almost disappeared after 48 h on the Ti-alloy.

Spectral emissions in zone II [Figs. 7(a)–7(c) for 12, 24, and 48 h exposures, respectively] are shown in Table 2. They included nine bands labeled as bands 11 to 19. In this zone, a clear difference was noted in the trend of the phenylalanine band 13 at ~1004 cm$^{-1}$ for the glass control, the two Si$_3$N$_4$ materials, and Ti-alloy. Although in the case of glass control, this band maintained a moderate relative intensity with elapsing exposure in comparison with neighboring bands, its intensity was quite pronounced for both of the Si$_3$N$_4$ substrates and almost null for the Ti-alloy. Bands 17 to 19, which are contributed by membrane lipids, showed an abnormal increase in their relative intensities for *S. epidermidis* at intermediate exposures to the N$_2$-annealed substrate. A similar trend, although less pronounced, was observed for untreated Si$_3$N$_4$ and Ti-alloy. The two bands sensitive to lipid conformation, namely bands 14 and 15, showed a clear inverse trend in their relative intensity upon exposure to untreated Si$_3$N$_4$ and the Ti-alloy as compared with the glass control. This change consisted of a significant increase in the latter band, which can be interpreted as a lipid population with a trans versus a gauche configuration.

Amide I dominated zone III [Figs. 7(a)–7(c); Table 3] with its main feature being band 21 (C=O stretching emission at 1680 cm$^{-1}$). This band followed the same trend observed for the lipid-related bands 17 to 19 in zone II, namely a significant enhancement at intermediate exposure times for both Si$_3$N$_4$-based substrates, but it was more pronounced for

### Table 1 Band labeling, assignment, and related references in spectral zone I.

<table>
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<tr>
<th>Band</th>
<th>Assignment</th>
<th>Frequency (cm$^{-1}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Skeletal vibrations in tyrosine</td>
<td>638</td>
<td>17, 18</td>
</tr>
<tr>
<td>2</td>
<td>Ring vibrations in poly-deoxyadenosine</td>
<td>650</td>
<td>14, 15</td>
</tr>
<tr>
<td>3</td>
<td>Ring breathing in guanine (DNA)</td>
<td>670</td>
<td>14, 15</td>
</tr>
<tr>
<td>4</td>
<td>Phosphatidylserine</td>
<td>720</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>Ring breathing adenine (DNA)</td>
<td>735</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>Ring breathing in tryptophan (DNA)</td>
<td>760</td>
<td>17, 18</td>
</tr>
<tr>
<td>7</td>
<td>Phosphodiester</td>
<td>782</td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td>Out-of-plane ring breathing in DNA</td>
<td>820</td>
<td>17, 18</td>
</tr>
<tr>
<td>9</td>
<td>COC 1,4 glycosidic link in carbohydrates</td>
<td>840</td>
<td>16</td>
</tr>
<tr>
<td>10</td>
<td>Ring breathing in trypsin (DNA)</td>
<td>850</td>
<td>14</td>
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</table>

### Table 2 Band labeling, assignment, and related references in spectral zone II.

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<th>Band</th>
<th>Assignment</th>
<th>Frequency (cm$^{-1}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>C–O–C glycosidic linkage of carbohydrates</td>
<td>937</td>
<td>19</td>
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<tr>
<td>12</td>
<td>C–C stretching in $\beta$-sheet</td>
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<td>13</td>
<td>Ring breathing in phenylalanine</td>
<td>1004</td>
<td>17, 18</td>
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<td>14</td>
<td>C–C gauche stretching in membrane lipids</td>
<td>1092</td>
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<td>15</td>
<td>C–C trans stretching in membrane lipids</td>
<td>1125</td>
<td>19</td>
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<tr>
<td>16</td>
<td>C–N stretching amide III in $\beta$-sheet</td>
<td>1253</td>
<td>20</td>
</tr>
<tr>
<td>17</td>
<td>Twisting of saturated CH$_2$ in membrane lipids</td>
<td>1313</td>
<td>21</td>
</tr>
<tr>
<td>18</td>
<td>Symmetric stretching of O–C–O in COO$^-$</td>
<td>1400</td>
<td>22</td>
</tr>
<tr>
<td>19</td>
<td>Bending of saturated CH$_2$ in membrane lipids</td>
<td>1442</td>
<td>21</td>
</tr>
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</table>
the N$_2$-annealed material. An enhancement in the relative intensity of band 21 was also observed for the Ti-alloy as compared with glass control. However, no significant differences were noted as a function of exposure time up to 48 h.

### 4 Discussion

Mechanisms of bacterial adhesion onto and interaction with biomaterial surfaces are complex and depend on many factors, such as morphological and chemical features of the surfaces. Surface topography plays a fundamental role in bacteria/substrate interaction affecting both adhesion and biofilm formation. In general, rough surfaces promote adhesion and colonization. For example, Yoda et al. tested the bacteriostatic behavior of different substrates with controlled surface roughness with respect to *S. epidermidis*. They showed that adhesion and proliferation were greatly favored on rougher materials. However, nanorough

![Normalized and deconvoluted Raman spectra in spectral zone I from *S. epidermidis* exposed to different substrates (cf. labels in the inset to each column) for (a) 12 h, (b) 24 h, and (c) 48 h.](image1)

![Normalized and deconvoluted Raman spectra in spectral zones II and III from *S. epidermidis* exposed to different substrates (cf. labels in the inset to each column) for (a) 12 h, (b) 24 h, and (c) 48 h.](image2)
surfaces have an opposite effect. Inspired by nature, the nanostructured surfaces of cicada wings have led to the development of engineered antimicrobial features on various biomaterials including titanium, polyurethane, and PEEK. Additionally, as demonstrated by two independent studies, the nanofeatures of untreated Si$_3$N$_4$ proved to be more resistant to bacterial adhesion and biofilm formation than chemically identical substrates that were either machined or polished.

In this study, the N$_2$-annealed Si$_3$N$_4$ presented the highest $Ra$ value among the tested materials (cf. Fig. 2). It also showed the highest amount of living cells after 12 h according to microbial viability tests (cf. Fig. 3(a)). Conversely, the PEEK substrate had the smoothest surface among the tested substrates, but the amount of living bacteria on its surface after 12 h was comparable with the untreated Si$_3$N$_4$ and Ti-alloy despite the fact that these latter materials experienced substantially higher $Ra$ values. The situation after 48 h was found completely reversed with the N$_2$-annealed Si$_3$N$_4$ possessing the lowest quantity and the PEEK having the highest amount of living bacteria among the tested samples. These data suggest that the bacteriostatic behavior of the tested surfaces cannot simply be interpreted using surface roughness data alone. Surface chemistry needs to be taken into consideration as well. The chemical reactions at the surface of Si$_3$N$_4$ were discussed in a previous study. They suggested the existence of a diffusion-limited thermodynamic driving force to convert silicon nitride into silicic acid and ammonia (NH$_3$). Release of NH$_3$ from the ceramic surface was experimentally demonstrated by a significant increase in local pH (i.e., from ~5.5 to ~8.5).

As indicated by prior research, highly alkaline environments may delay or prevent bacterial adhesion and biofilm formation. For instance, Hamadi et al. investigated the adhesion of Staphylococcus aureus to glass at high pH values and observed that bacteria strongly adhered in the pH range 4 to 6, whereas adhesion was weak under both highly acidic (pH ≈ 2 to 3) and alkaline conditions (pH > 7).

Raman spectroscopy provided further insight into the bacteria’s metabolism by showing the time-dependent variations of its molecular structure after contact with the different substrates. A significant reduction in the intensity of its DNA-related bands in spectral zone I (e.g., bands 2, 3, 7, and 8) after 48 h of exposure to both types of Si$_3$N$_4$ and the Ti-alloy substrates was an important indicator of its metabolic degradation. Escoriza et al. studied the time-lapse Raman response of bacteria during their proliferation and found that bands related to nucleic bases typically showed high intensities at the beginning of bacterial growth, but their intensities slowly decreased with time. In the current experiments, a similar initial trend coupled with an abrupt decrease at 48 h was observed. This sudden decrease strongly suggests that both Si$_3$N$_4$ materials and the Ti-alloy possess inherent antibacterial mechanisms.

Alterations of phospholipid-related and protein-related bands were also observed, which indicated major irreversible modifications to the bacteria’s membrane and protein structures. The spectral bands at 1125 cm$^{-1}$ (band 15) and 1442 cm$^{-1}$ (band 19) monitor the polysaccharides for intercellular adhesion and biofilm formation. These bands initially increased but then conspicuously disappeared after exposure to N$_2$-annealed Si$_3$N$_4$ for 48 h. This trend can be interpreted as disruption of the biofilm after its initial formation at intermediate times. An important marker for proteins is band 13 at 1004 cm$^{-1}$ related to vibrational modes in phenylalanine. However, this band tended to disappear only for bacteria exposed to the Ti-alloy. The strong band 17 (at 1313 cm$^{-1}$) reflects twisting vibrations of saturated CH$_2$ bonds in membrane lipids. When comparing the relative intensity of this band to that of phenylalanine (band 13), a significant enhancement was noticed after 24 h of exposure to both types of Si$_3$N$_4$ surfaces, followed by a clear intensity reduction after 48 h. The intensity enhancement of bands 17 was interpreted as acidification of the membrane lipids. However, bacteria have an ability to control the biophysical properties of the phospholipids in their membranes in the presence of an external physiological stress and this was observed via Raman spectroscopic monitoring in the current experiments. Following the work of Nostro et al., the bacterial behavior observed on the Si$_3$N$_4$ substrates was interpreted as a reaction to the increase in local pH. This reaction reduced the bacteria’s initial adhesion capability and subsequently led to the impairment of biofilm maturation. An alkaline pH directly influences bacterial attachment because it affects the degree of D-alanylation of lipoteichoic acid and wall teichoic acid, which are the most abundant polyanions in cytoplasmic membrane lipids and are linked to peptidoglycan. Another important aspect of...
the change in pH is its impact on enzymes related to autolysis of prokaryotic cells. A pH change favors activation of autolysin leading to peptidoglycan hydrolyses, which strongly alters peptidoglycan, turn over. When hydrolyase is activated, peptidoglycans are degraded and the osmotic balance of the bacterial cell is lost with the cell turning from a respiring to a depolarized status. Ammonium moieties released from the Si₃N₄ surface then become free to flow into the cytoplasmic environment because the structural integrity of the cell wall is compromised. This process ultimately results in cell lysis. Figure 8 shows a schematic model of *S. epidermidis* exposed to a silicon nitride surface with a time-dependent increase in local pH. A process of uncontrolled autolysis is envisaged, which modifies the peptidoglycan wall structure and increases the susceptibility of the membranes to molecular moieties liberated by the substrate, thereby destabilizing the cell.

5 Conclusion

To clarify the origin of Si₃N₄’s bacteriostatic behavior, changes within gram-positive *S. epidermidis* were studied after its exposure to substrates consisting of two Si₃N₄ variants, a Ti-alloy and a glass control for time-points up to 48 h. Previously reported results were confirmed using microbial viability assays and fluorescence microscope analyses. Then, in situ Raman spectroscopy was employed to monitor the metabolic variations of the bacteria at different exposure times. These variations were interpreted based on the vibrational responses of their biological DNA. The Raman spectra showed a decrease in the band intensities related to biofilm formation and DNA after 48 h suggesting the initiation of lysis. The Raman data agreed with the results derived from the biological tests. Based on these observations and in comparison with previously published literature, the metabolic changes in *S. epidermidis* were attributed to a pH drift toward alkaline values on the Si₃N₄ surfaces. This drift modified the behavior of enzymes and cellular compounds, limiting its adhesion and increasing the permeability of the cell walls. In conclusion, this study demonstrated that the surface chemistry of Si₃N₄ may be useful in inhibiting periprosthetic infections by hindering biofilm formation and bacterial proliferation.

Disclosures

Competing financial interests: Giuseppe Pezzotti is a consultant to Amecida Corporation, and Bryan McEntire works at Amecida Corporation.

References


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