**QUEEN'S
UNIVERSITY
BELFAST**

Creating an antibacterial surface on beta TNZT alloys for hip implant applications by laser nitriding

Donaghy, C. L., McFadden, R., Kelaini, S., Carson, L., Margariti, A., & Chan, C-W. (2020). Creating an antibacterial surface on beta TNZT alloys for hip implant applications by laser nitriding. *Optics and Laser Technology*, 121, [105793]. <https://doi.org/10.1016/j.optlastec.2019.105793>

Published in:
Optics and Laser Technology

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
[Link to publication record in Queen's University Belfast Research Portal](#)

Publisher rights

© 2019 Elsevier Ltd.

This manuscript version is made available under the CC-BY-NC-ND 4.0 license <http://creativecommons.org/licenses/by-nc-nd/4.0/>, which permits distribution and reproduction for non-commercial purposes, provided the author and source are cited.

General rights

Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.

Creating an antibacterial surface on beta TNZT alloys for hip implant applications by laser nitriding

*Clare Lubov Donaghy^a, Ryan McFadden^a, Sophia Kelaini^b, Louise Carson^c, Andriana Margariti^b, *Chi-Wai Chan^a

^a School of Mechanical and Aerospace Engineering, Queen's University Belfast, Belfast BT9 5AH, UK

^b Centre for Experimental Medicine, Queen's University Belfast, Belfast, BT9 7BL, UK

^c School of Pharmacy, Queen's University Belfast, Belfast, BT9 7BL, UK

*Corresponding authors: Clare Lubov Donaghy cdonaghy38@qub.ac.uk; Chi-Wai Chan c.w.chan@qub.ac.uk

Highlights

- Fibre laser nitriding in open air conducted on beta titanium alloy TNZT
- Surface roughness (in microscale) increased and distinct laser induced topography created
- Water contact angle significantly decreased post laser nitriding
- Nitrided surfaces led to significant antibacterial effect but offered no particular advantage to MSC response

Abstract

The National Joint Registry reported that the main causes for hip implant revision surgery include aseptic loosening, infection and adverse soft tissue reaction to particulate debris. There is a great need to improve the implant properties which can be achieved through a combined solution of beta titanium alloy (TNZT) with low elastic modulus and laser surface nitriding to improve mechanical properties and biological response. While titanium nitride (TiN) possesses good biocompatibility and remarkable antibacterial properties; its effectiveness as a coating on Ti-35Nb-7Zr-6Ta has not been investigated in relation to stem cell response and antibacterial capability. TNZT surfaces were laser-nitrided in incremental power, specifically 35, 40 and 45 W. Investigation included surface roughness and topography in microscale (WLI and SEM), microstructure (XRD) and wettability (water contact angle). Biological studies of the laser-nitrided surfaces included *in vitro* culture for 24 hours using mesenchymal stem cell (MSC) fluorescence staining and *Staphylococcus aureus* (*S. aureus*) Live/Dead staining. Sample groups consisted of control base metal (BM), laser-nitrided at 35 W (LT35), 40 W (LT40) and 45 W (LT45). Results revealed that laser nitriding generates significantly rougher surfaces (R_a value of BM was 199 nm, LT35 was 723 nm, LT40 was 458 nm and LT45 was 1180 nm) with distinctive surface features ($R_{sk} < 0$ and $R_{ku} > 3$). Surfaces after laser nitriding, regardless of laser power, can be tailored to become hydrophilic ($27 - 34^\circ$). Fibre laser nitriding can be used to create antibacterial surface patterns on TNZT in a high power regime. A laser power of 45 W proved to be the most effective in this study, creating an overlapping crescent shape which becomes more obvious with increasing power. To summarise, laser-nitrided surfaces led to a significant antibacterial effect but offered no particular advantage to MSC response.

Keywords: mesenchymal stem cell (MSC), antibacterial performance, TNZT, beta titanium, fibre laser nitriding

1. Introduction

According to the National Joint Registry (NJR), the total number of hip replacements recorded since 2003 in England, Wales and Northern Ireland almost totals one million[1]. Titanium and its alloys, mainly Ti-6Al-4V, have been used for load-bearing implant applications for decades although limitations such as wear, corrosion, aseptic loosening and infection are prevalent and result in the need for implant failure and revision surgeries. The failure of the hip implant ultimately leads to a revision surgery but the path from failure to revision is due to a number of issues which have been widely expressed in literature[2].

Firstly, wear and corrosion are known to be the cause leading to debris generation. The debris can be solubilised in blood and secreted through urine or it can accumulate in the tissue. If it accumulates in tissue this can lead to inflammation and tissue damage in the short term while long term effects can be more damaging[3]. Apart from the debris generation, implant failure can also occur due to fibrous encapsulation whereby the implant is encased in surrounding tissue within sufficient *in situ* anchorage. Equally inflammation can be severe enough to require revision surgery. There is a need for biomaterials and surface modification techniques that can provide the optimal resistance to infection and this requires an understating of the complex interactions at the bone-implant interface. Biomaterial-associated infections due to *Staphylococcus aureus* and *Staphylococcus epidermis* account for two-thirds of implant related infections[4] and of the revision surgeries reported by the NJR 13,885 were due to infection[1].

Relating to mechanical properties, poor strength and fracture toughness can lead to implant fracture. A major reason for failure, which has been addressed in previous work[5], is a mismatch of elastic modulus between bone and implant leading to stress shielding and bone resorption. A sensible solution to tackle this problem is using a material which has a closer modulus to the human bone. Beta Ti alloys, particularly the Ti-Nb-Zr-Ta (TNZT) system, have drawn the highest attention among other potential candidates due to its lowest elastic modulus

among the beta Ti family and meanwhile contains no toxic elements. However, poor wear behaviour is the main concern when applying TNZT system to form the load-bearing components (i.e. femoral stem) of the hip implants.

In response to these issues, an assortment of surface modifying techniques have been implemented and developed for the improvement of wear and corrosion properties (i.e. reducing the amount of debris generation by coating implant surfaces with hard and anti-corrosion layers), osseointegration (i.e. encouraging more bone cell attachment by creating a rougher surface on an implant) and antibacterial properties (i.e. reducing bacterial attachment and biofilm formation using coatings contained antibacterial agents or surface features).

Existing surface modification methods include plasma spraying, sand blasting, physical or chemical vapour deposition (PVD or CVD), to name a few, see **Table 1** (supplementary material) for the list of surface modification methods and associated advantages and disadvantages. Compared to these methods, laser surface nitriding (LSN) has been shown to be an effective method to modify the material surface as well as significantly increase the surface hardness and corrosion resistance without altering the bulk properties[6–8]. It is a clean, fast and highly repeatable process as well as can be performed in an open atmosphere[9]. The improved surface properties post laser nitriding are due to the formation of a titanium nitride (TiN) layer. TiN has been proven to be a very hard and anti-corrosive material and intrinsically biocompatible due to its ability to inactivate bacterial biofilms[10,11]. The antibacterial effect is believed to be due to a correlation between the decrease in surface free charge carriers (i.e. responsible for electrostatic bacterial interaction with the surface) and increase in thickness of TiN coatings[12].

Fibre laser nitriding has been used successfully on TNZT in a low energy regime (i.e. no surface melting occurred) to improve wear and corrosion resistance[13], and the nitriding process was

performed in a gas chamber which greatly limits the practicality to treat a free-form or curved shape implant surfaces. The present study aims at using fibre laser nitriding in a higher energy regime and under the open air condition in which surface melting occurs. The effects of changes in surface roughness and topography as well as wettability, as a consequence of fibre laser nitriding of TNZT, on the early human MSC response and bacterial attachment of *S. aureus* were investigated.

2. Materials and methods

2.1 Materials

The Ti-35Nb-7Zr-6Ta alloy used in this study was sourced from American Element, USA as a large plate that was 250 mm² and 3 mm thick. It was wire cut into smaller plates (30 × 40 mm) using electrical discharge machining (EDM). Sample preparation prior to laser nitriding involved standard metallographic preparation using silicon carbide (SiC) polishing to 1000 grit, to remove any defects from the manufacturing process and pre-existing oxide layer. Before material preparation and experimental work the samples were ultrasonically cleaned for 10 minutes in acetone and then rinsed with deionised water and dried to remove any loosely bound contaminants. All experiments were completed with a sample size of $n = 3$ while, $n = 4$ was used for bacterial attachment.

2.2 Fibre laser nitriding

Laser nitriding was performed using a 200 W automated continuous wave (CW) fibre laser system (MLS-4030) integrated by Micro Lasersystems BV (Driel, The Netherlands) and produced by SPI Lasers UK Ltd (Southampton, UK) with a wavelength of 1064 nm. Samples were irradiated with the laser beam using the following parameters: scanning speed of 25 mm/s, 1.5 mm stand-off, 100 µm spot size, shielded with high purity N₂ (pressure range 5 – 7 bar) in open at three different laser powers, 35 W, 40 W and 45 W. Laser-nitrided samples are noted

as LT35, LT40 and LT45 while the untreated control group consists of base metal samples (1000 grit SiC finish) and are noted as BM. Samples were wire cut into 6 mm diameter discs for all studies but plates were used for bacterial attachment. Samples for biological cultures were cleaned in acetone twice for 1 hour, deionised water for 30 minutes in an ultrasonic bath and then air-dried in a fume hood followed by autoclave sterilisation (Prestige Medical, UK); 121°C and 1.5 bar for 20 minutes.

2.3 Surface roughness and topography

White light interferometry (WLI) (Talysurf CCI 6000, UK) was used to capture the surface roughness and profiles of the untreated and laser-nitrided surfaces (perpendicular to the laser track orientation). The following parameters were used to assess the roughness: arithmetic mean (R_a), maximum profile height (R_z), surface skewness (R_{sk}) and surface kurtosis (R_{ku}). Values were assessed based on the 2D profile 1.2 mm² scan areas. Surface topography was captured using scanning electron microscopy (SEM) (FlexSEM 1000, Hitachi, UK) to image the surface features on the laser-nitrided samples. Images were captured using a 20 kV beam at various magnifications in the backscattered electron compositional (BSE-COMP) mode detection.

2.4 Phase identification

X-ray diffractometry (XRD) using the PANalytical X'Pert Pro MPD (PANalytical, UK) was used to identify the phase of the untreated and laser-nitrided surfaces. XRD was performed in the 2-theta (2θ) range between 10° – 90° with a copper-K α radiation source (40 kV and 40 mA) and nickel filter operated at fixed slit (1/2°), 10° anti-scatter slit and 0.02 step size.

2.5 Surface wettability

Surface wettability was assessed at room temperature using the sessile drop method and images were captured with a video-based contact angle analyser (FTA 200, First Ten Angstroms Inc.,

USA) to measure the contact angle of a liquid drop of deionised water on the untreated and laser-nitrided samples. Droplet profiles were captured using the FTA32 video software and the testing liquid was controlled using a microlitre syringe to produce 7- μ l drops. Images were captured perpendicular to the laser track orientation.

2.6 Biological attachment

Cell and bacterial attachment were assessed at 24 hours and performed as described in the author's previous study[5]. Mesenchymal stem cell attachment was assessed using direct immunofluorescent staining with α SMA and DAPI while *Staphylococcus aureus* attachment was assessed using fluorescent live/dead staining. Briefly, an 18 hour culture of *S. aureus* (ATCC 6538) was adjusted to approximately 1×10^6 cfu/ml (as verified by viable count) in Mueller Hinton broth. Sterile TZNT samples were placed in individual wells of a sterile 6-well plate, submerged in the inoculum, and incubated in a gyratory incubator for 24 h at 37°C. After this time, samples were removed from the bacterial culture and washed three times in sterile phosphate buffered saline to remove loosely adherent planktonic bacterial cells. Sessile, surface-adhered bacteria were stained using Live/Dead® BacLight™ (Molecular Probes) in order to distinguish viable (stained green) from non-viable bacterial cells (stained red), and also to quantify the extent of bacterial adherence. Stained samples were examined using fluorescence microscopy. Twenty-four random fields of view were captured per sample, and image analysis was conducted using ImageJ software (NIH, USA).

2.7 Statistical analysis

The statistical significance of the data were expressed as mean $\pm$ standard error (SE) and were tested and compared by one-way ANOVA using Prism software (GraphPad Prism Software Version 7, USA). A significant difference was calculated, with a *p*-value of ≤ 0.05 considered significant; (*) *p* < 0.05; (**) *p* < 0.01; (***) *p* < 0.001.

3. Results

3.1 Surface roughness by WLI and topography by SEM

The 3D and 2D profiles of the untreated and laser-nitrided surfaces, captured by WLI, are shown in **Figure 1**. The BM sample has the typical variation in surface roughness as a result of the metallographic SiC polishing process. The laser-nitrided samples had an overlapping crescent effect evident in **Figure 1 (A)** and **(B)**. The roughest regions are prominent on the LT35 sample within the overlapping crescent shape while on LT40 it appears to be at the edges of the overlapping tracks. The overlap becomes more obvious as the laser power increases; LT45 had the roughest regions at the edges of some, but not all, track edges, which are indicated by the black and red locations of the 3D and 2D scans, respectively.

Figure 1. Laser nitriding creates an overlapping crescent pattern which becomes more obvious with increased laser power. WLI surface profiles **(A)** 3D (1.208 mm² scan area) and **(B)** 2D (1.2 mm² scan area) for the untreated and laser-nitrided samples with the colour scale to the left of each scan. **(C)** Surface roughness results; R_a , R_z , R_{sk} and R_{ku} , extracted from 2D profile.

The surface roughness results, specifically R_a , R_z , R_{sk} and R_{ku} , which were based on the 2D profiles are shown in **Figure 1 (C)**. The R_a values range from the lowest 199.3 nm on BM and

increases on the laser-nitrided samples. There is not an increasing trend as the laser power increases; LT40 had the lowest Ra among the laser-nitrided samples (458.4 nm) followed by LT35 with 722.8 nm and LT45 has the highest Ra with 1180.2 nm, significantly higher than BM. The Rz values display the same pattern as the Ra values, with the lowest maximum profile height belonging to the untreated BM surface (1900.3 nm) followed by LT40 (4368.7 nm) while LT35 and LT45 were 7057 and 11152.3 nm, respectively. Laser nitriding significantly increased the Ra and Rz in comparison to BM due to the intricate topography created by the laser.

Surface skewness (i.e. spikes; $R_{sk} > 0$ or valleys; $R_{sk} < 0$) and kurtosis (i.e. peaks; $R_{ku} > 3$ or flat; $R_{ku} < 3$) values of the untreated and laser-nitrided samples were used as quantification parameters. There is a decreasing trend in R_{sk} while the R_{ku} values are quite similar, as shown in **Figure 1 (C)**. The BM had the largest R_{sk} (1.229) and this drops significantly to -0.92 for the LT35 surface and almost halves to -0.518 for LT40 and almost halves again for LT45 (-0.289). The laser-nitrided surfaces are flat in comparison with BM. All R_{ku} values are > 3 with BM being the highest at 5.749 by a small margin and slightly decreasing with laser power. The similar trend follows but in the negative direction of LT40 being the highest among the laser group at 5.142 followed by LT35 (5.109) and LT45 (4.145). All the laser-nitrided surfaces had a $R_{sk} < 0$ and $R_{ku} > 3$, showing that they are not as spiky as their argon counterparts[5] and have peaks, correlating with the higher roughness values.

SEM images of the BM and laser-nitrided surfaces are shown in **Figure 2**. The overlapping crescent effect shown in **Figure 1 (A)** and **(B)** is clearly visible in the SEM images. The typical variation of the topography is visible on the BM surface and on LT35 (most notable at $\times 1500$ magnification) and partly LT40 in between the laser tracks and is not visible on the LT45 surface due to the complete overlapping of the laser tracks. This overlap becomes more obvious with increasing power as shown in the $\times 150$ magnification images. There are more detailed

features present on the edges of the surfaces nitrided at lower power, LT35 and LT40, shown by the fanned dendritic pattern (as indicated by solid white arrows in **Figure 2** at $\times 3000$ magnification) along the overlapped crescents, showing the effects of the melt pool created by the laser. The ripple effect is not seen on the laser-nitrided samples although there are lamellae (as indicated in **Figure 2** by dashed white arrows at $\times 1500$ magnification) seen emanating from the centre of the crescent shapes.

Figure 2. Distinct surface topography created by laser nitriding. SEM images at various magnifications of the base metal and laser-nitrided TNZT surfaces. Magnifications shown on vertical and scale bars are shown for each magnification. Dashed white arrows indicate fanned dendritic pattern emanating from crescent centre. Solid white arrows indicate lamellae, a feature of laser nitriding.

3.2 Phase identification by XRD

Phases present in the untreated and laser-nitrided surfaces were analysed with XRD (**Figure 3**). Blue dot is indicative of phase present on all surfaces. Each surface has a peak present at $\sim 38.5^\circ$ β (110), $\sim 56^\circ$ β (200) and $\sim 69^\circ$ β (211); all of which are associated with beta phase and due to the presence of Nb and Zr (beta stabilising elements), alpha phases are not present, as phase formation is suppressed. BM has a prominent sharp peak ($\sim 56^\circ$ β (200)) with two smaller peaks present ($\sim 38.5^\circ$ β (110) and $\sim 69^\circ$ β (211)). TiN was detected and the crystallographic plane shifts to $\sim 43^\circ$ TiN (200) after laser nitriding. There is a prominent crystallographic plane shift post laser nitriding with the same three peak pattern present in all the laser-nitrided samples, in decreasing peak sharpness; TiN (200), β (110), and TiN (111). β (220).

Figure 3. Prominent three peak pattern present in laser-nitrided samples. XRD patterns of the untreated and laser-nitrided samples. Scanning angles of 2 theta (2θ) range between $10^\circ - 90^\circ$. Intensity (a.u.) shown on y-axis.

3.3 Surface wettability by contact angle

The water contact angles of the untreated and laser-nitrided surfaces are presented in **Figure 4**. The results show that BM and the whole laser-nitrided group are hydrophilic with the latter being notably more hydrophilic, as shown in **Figure 4 (A)**. In comparison with the BM, the contact angle of the laser-nitrided samples decreases approximately by two-thirds. There is significant difference between all samples ($p < 0.001$), except for LT35 and LT40 where there was no significant difference, as shown in **Figure 4 (B)** and **(C)**. The contact angle results have shown that laser nitriding with any laser power can be used to create a hydrophilic surface.

Figure 4. Laser nitriding created a hydrophilic surface. **(A)** Representative images of liquid water drop on each surface. **(B)** Graph of mean water contact angles and SE measured on the untreated and laser-nitrided surfaces calculated using ImageJ. Error bars are the SE of n = 3. **(C)** Tabulated water contact angles.

3.4 Cell attachment response

Cell attachment was assessed with fluorescently stained MSCs using α SMA and DAPI at 24 hours to analyse cell morphology. At the early time point it is clear cells behaved differently on the laser-nitrided surfaces compared to the BM surfaces, as shown in **Figure 5**. MSCs display the same morphology and behaviour on BM and tissue culture plastic (TCP) as in a previous study[5]. The cells are spindle shaped and stretch in no particular direction (as indicated by white arrows in the $\times 200$ magnification images) due to the random metallographic process and the characteristics of TCP. At 24 hours on all the laser-nitrided surfaces the MSCs appear to be in abundance due to the number of nuclei seen at $\times 50$ magnification, although the cells appear to have burst leaving a halo of debris. It is evident that the surface characteristics are not ideal for cells and causes cell death.

Figure 5. Laser nitriding offers no particular advantage to MSC response. Merged fluorescence images of MSCs stained at 24 hours at $\times 50$, $\times 100$ and $\times 200$ magnification. Red represents α SMA actin fibres; Blue represents DAPI nuclei stain. Scale bars are shown for each magnification. Representative images of coverage on whole entire surface. White arrows indicate cells spreading in no particular direction.

Cell ID and nuclei count are used to quantitatively analyse the cell attachment at 24 hours, as shown in **Figure 6**. The nuclei count shows that there is no significant difference between samples but the cell ID is much lower on the laser-nitrided samples. The cell ID on the BM surface is significantly different to all laser-nitrided groups at the 24 hour time point. BM and the lowest laser power surface, LT35, have a significance of $p < 0.01$ while this significance increases with the remaining two groups ($p < 0.001$). There is no significance within the laser-nitrided group. The notable decrease in cell ID on the laser-nitrided surfaces in comparison with BM, supports the qualitative results, where only the nuclei remains with a halo of cell debris.

Figure 6. Laser nitriding causes decrease in cell integrated density. MSC attachment quantification at 24 hours on untreated and laser-nitrided samples. **(A)** Graph of cell integrated density based on α SMA. **(B)** Graph of nuclei count based on DAPI. Data based on nine images per surface using ImageJ software.

3.5 Bacterial attachment

The bacterial attachment results are shown in **Figure 7**, where analysis was performed using fluorescence images and quantitatively using bacteria coverage and live-to-dead ratio (i.e. ratio between green and red stained cells) to determine which surfaces, if any, had elicited reduced biofilm coverage and/or an antibacterial response. There were more green stained *S. aureus* cells present on the BM surface with some micro-colonies, the beginning of biofilm formation (as indicated by white arrows in **Figure 7 (A)**). Laser nitriding was able to effectively produce a surface where bacteria were significantly less capable of forming biofilms in the early stages of attachment (i.e. 24 hours) in comparison to BM. Quantification results show that bacteria coverage on BM was significantly different from LT40 and LT45 ($p < 0.01$ and $p < 0.001$, respectively), see **Figure 7 (B)**. Within the laser-nitrided group there was a significant difference between LT35 and LT45 ($p < 0.01$). The live-to-dead bacteria ratio on all samples showed a stepping decrease, see **Figure 7 (C)**.

Figure 7. Laser nitriding at higher power significantly decreases bacterial adherence. **(A)** Merged (red and green) live/dead stained fluorescence images of *S. aureus* bacteria at 24 hours. Scale bar = 50 μm . Representative images of coverage on whole entire surface. White arrows indicate micro-colony formation. **(B)** Total bacteria coverage (sum of red and green fluorescence); SE of $n = 24$. **(C)** Live-to-dead ratio (average red and green fluorescence); SE of $n = 24$. Data calculated using ImageJ software.

4. Discussion

It has been widely reported that the two main surface attributes which can be improved, using laser nitriding, are increased resistance to wear and corrosion[7,13]. Poor tribological behaviour can cause adverse reaction to particulate debris and results in the need for revision surgery according to the NJR. The tribological effects were investigated in a previous study[7], while the work detailed in this paper focuses on the investigation of surface topographical characteristics effects on biological response, namely initial MSC attachment and bacterial attachment.

Bacterial adherence results demonstrate an antibacterial effect after applying fibre laser nitriding to the surface. The mechanisms of biofilm formation on an implant involve a process that is initiated through three fundamental steps: initial adherence, colonisation and biofilm formation. Under DLVO[14] (a widely accepted theory in determining early stages of bacterial attachment) surface energy and subsequently surface wettability play an important role in determining the initial bacterial attachment. It has been reported that laser nitriding can reduce bacterial attachment[8], with the hydrophilic nature of the laser-nitrided surfaces inhibiting the attachment of hydrophobic *S. aureus* cells, as previously reported in literature[15]. The significantly lower number of bacteria, after 24 hours, on the laser-nitrided surfaces, compared to BM, proposes that fibre laser nitriding would create a less hospitable surface for the bacteria, (see **Figure 7**), resulting in reduced viability, in comparison with the previous results[5].

The effect of using a nitrogen shielding environment during the laser nitriding creates a vastly different surface in comparison with the previously reported argon samples[5]. The relationship between bacterial attachment and surface roughness is complex and not completely understood according to reports in the literature[16–18]. However it has been reported that nanoscale surface features help minimise infection[19–21] and enhance osseointegration[22], whilst microscale features have more of an effect on the hydrodynamic environment as the bacteria approached the surface[23,24]. Roughness values alone are not enough to correlate to bacterial early bacterial attachment and needs to be considered alongside topography and physico-chemical effects[25,26]. Overall fibre laser nitriding significantly increased *Ra* and *Rz* in microscale, compared to BM, due laser-induced topography, with a five-fold increase in *Ra* and *Rz*. The LT45 group had the highest *Ra* and *Rz* while the SEM images show the unique laser induced surface topography in microscale. Nanoscale features could be present but are beyond the resolution of the SEM used for characterisation in this study.

A unique surface pattern appears, post fibre laser nitriding, which overlaps more as the laser power increases. The prominent overlapping crescent effect on the laser-nitrided samples as shown in **Figure 1 (A)** and **(B)**; the roughest regions of which are prominent within the overlapping crescent shape on LT35, while on LT40 it appears to be at the edges of the overlapping tracks. The unique surface features on the lower power surfaces, LT35 and LT40, is evident from the characteristic trait of a fanned dendritic pattern along the overlapped crescents, showing the effects of the melt pool created by the laser, as shown in **Figure 2** at $\times 1500$ magnification. LT45 has the roughest regions at the edges of some, but not all, track edges (as indicated by the black and red locations of the 3D and 2D scans, respectively). The ripple effect is not present on the laser-nitrided samples, as previously seen in the laser-treated TNZT[5] under the argon shielding environment, although there is lamellae (puckered effect) seen emanating from the centre of the crescent shapes. Similar lamellae protruding in the radial

directional, described by Kloosterman et al., was seen on the solidification front of the laser melt pool, increasing with laser power as the presence of ripples decreases[27].

Fibre laser nitriding significantly enhanced the hydrophilicity of the surface (contact angle range; 27 – 34°) in comparison with the BM contact angle (83°); with a decrease of approximately two-thirds (**Figure 4**). As hydrophilicity increases the bacterial attachment decreases (as indicated by the bacterial coverage measurements in **Figure 7**). LT35, the lowest power, has a contact angle of 31.82° and bacterial coverage of 3.87% while at a higher power, LT45, the contact angle is 27.06° but the bacterial coverage and significantly decreased to 0.72%. *S. aureus* has been described as hydrophobic in the literature[28] and has a water contact angle of over 70°. This could suggest that the decreased hydrophobicity of the laser-nitrided samples compared to BM is a contributing factor to the reduced bacterial adhesion and subsequent biofilm coverage. Hydrophilic surfaces resist protein adsorption while hydrophobic surfaces support the opposite, favouring protein adsorption[29]. As cells directly interact with this protein layer the proteinaceous film is crucial in subsequent cell adhesion and cellular response[30]. *S. aureus* has been observed to preferentially localise fibronectin-binding protein in literature[31,32]. This could potentially mean that a result of the hydrophilic surface reducing protein adsorption, *S. aureus* has less such surface proteins with which to preferentially bind. Perhaps the hydrophilicity in combination with the surface topography elicits a poor cellular response but an excellent antibacterial effect. At 24 hours on all the laser-nitrided surfaces the MSCs appear to be in abundance due to the number of nuclei seen at $\times 50$ magnification, although the cells appear to have burst leaving a halo of debris, as shown in **Figure 5**. It is evident that the surface characteristics are not ideal for cells and causes cell death. There is no significant difference within the laser-nitrided group. The notable decrease in cell ID (**Figure 6**) on the laser-nitrided surfaces as compared to BM, supports the qualitative results, where only the nuclei remains with a halo of cell debris. Cells typically respond with

proteolytic burst when proteins are strongly adsorbed (hydrophobic surface) onto the substrate[30], although the opposite occurred in the results with the laser induced hydrophilic surfaces causing cell death.

There is another notable preferential crystallographic plane shift post fibre laser nitriding with the same three prominent three peak pattern present in all the laser-nitrided samples, in decreasing peak sharpness; TiN (200), β (110), and TiN (111), shown in **Figure 3**. The same TiN peaks were found in the previous study[7] using Grade 2 and 5 titanium alloys, the presence of which is due to the controlled nitrogen rich environment. Additionally TiN (220) is found at $\sim 63^\circ$, TiN (311) at $\sim 73^\circ$ and TiN (222) at $\sim 78^\circ$, similar to the findings reported by Kami[33]. The presence of TiN, as shown by XRD, may be a contributing factor in the antibacterial effect, as seen in literature[5,34–36], contributing to the improved antibacterial performance.

The results have shown that fibre laser nitriding within the reported laser power range, namely 35, 40 and 45 W can be used to create an antibacterial surface. Such antibacterial effect is believed to be a consequence of the fibre laser nitriding process altering the surface roughness, topography (i.e. presence of unique laser created surface features) and wettability (i.e. surfaces become hydrophilic).

5. Conclusions

The beta TNZT alloy modified using fibre laser nitriding has been shown to be a suitable candidate for hip implant applications. The laser-nitrided TNZT implant possesses improved antibacterial performance as well as wear and corrosion resistance, which are the main concerns in the implant design. The major findings of the fibre laser nitriding results are summarised below:

- Fibre laser nitriding can be used to create distinct surface patterns on TNZT in a high power regime, with the laser power of 45 W being the best performer;
- Laser-nitrided surfaces, regardless of laser power, can be tailored to become hydrophilic ($27 - 34^\circ$);
- Laser-nitrided samples showed only beta Ti and TiN phases (the sharpest and hence most crystalline being, TiN (200), and TiN (111)).
- Laser nitriding produced a rougher surface (Ra value of BM was 199 nm, LT35 was 723 nm, LT40 was 458 nm and LT45 was 1180 nm) with unique laser-induced surface features ($R_{sk} < 0$ and $R_{ku} > 3$) potentially encouraging the antibacterial effect.

To further summarise, the bacterial attachment results indicated that that all the laser-nitrided samples drastically reduced bacterial attachment with LT45 being the ideal condition to modify TNZT to produce the greatest significant reduction in bacterial coverage.

Acknowledgements: Dr Anna Krasnodembskaya's group at the Centre for Experimental Medicine (Queen's University Belfast) for the provision of MSCs.

Funding: This work was supported by research grants from the Queen's University Belfast, awarded to C-WC and LC (D8201MAS, D8304PMY). The cell work was funded by Biotechnology and Biological Sciences Research Council (BBSRC) and British Heart Foundation (BHF) grants awarded to AM.

Conflicts of Interest: The authors declare no conflict of interest.

Author Contributions: Clare Lubov Donaghy (CLD), Ryan McFadden (RM), Sophia Kelaini (SK), Louise Carson (LC), Andriana Margariti (AM), Chi-Wai Chan (C-WC)

CLD: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing

RM: collection and/or assembly of data, data analysis and interpretation, manuscript writing

LC and AM: provision of study material, manuscript writing, financial support

SK: provision of study material

C-WC: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, financial support, final approval of manuscript

Author Contributions: Conceptualization, C.L.D. and C.-W.C.; methodology, C.L.D., L.C. and A.M.; formal analysis, C.L.D., R.M. and C.-W.C.; investigation, C.L.D.; resources, R.M., S.K., L.C., A.M. and C.-W.C.; data curation, C.L.D. and C.-W.C.; writing—original draft preparation, C.L.D. and C.-W.C.; writing—review and editing, C.L.D., R.M.F., L.C., A.M. and C.-W.C.; visualization, C.L.D. and C.-W.C.; supervision, A.M. and C.-W.C.; project administration, C.-W.C.; funding acquisition, L.C., A.M. and C.-W.C.

References

- [1] National Joint Registry, National Joint Registry for England, Wales and Northern Ireland 15th Annual Report, 2018.
- [2] M. Geetha, A.K. Singh, R. Asokamani, A.K. Gogia, Ti based biomaterials, the ultimate choice for orthopaedic implants - A review, *Prog. Mater. Sci.* 54 (2009) 397–425. doi:10.1016/j.pmatsci.2008.06.004.
- [3] Y. Wang, Y. Yan, Y. Su, L. Qiao, Release of metal ions from nano CoCrMo wear debris generated from tribo-corrosion processes in artificial hip implants, *J. Mech. Behav. Biomed. Mater.* 68 (2017) 124–133. doi:10.1016/J.JMBBM.2017.01.041.
- [4] W.F. Oliveira, P.M.S. Silva, R.C.S. Silva, G.M.M. Silva, G. Machado, L.C.B.B. Coelho, M.T.S. Correia, *Staphylococcus aureus* and *Staphylococcus epidermidis* infections on implants, *J. Hosp. Infect.* 98 (2018) 111–117. doi:10.1016/J.JHIN.2017.11.008.
- [5] C.L. Donaghy, R. McFadden, G.C. Smith, S. Kelaini, L. Carson, S. Malinov, A. Margariti, C.-W. Chan, C.L. Donaghy, R. McFadden, G.C. Smith, S. Kelaini, L.

- Carson, S. Malinov, A. Margariti, C.-W. Chan, Fibre Laser Treatment of Beta TNZT Titanium Alloys for Load-Bearing Implant Applications: Effects of Surface Physical and Chemical Features on Mesenchymal Stem Cell Response and Staphylococcus aureus Bacterial Attachment, *Coatings* 2019, Vol. 9, Page 186. 9 (2019) 186–206. doi:10.3390/COATINGS9030186.
- [6] H.C. Man, Z.D. Cui, X.J. Yang, Analysis of laser gas nitrided titanium by X-ray photoelectron spectroscopy, *Appl. Surf. Sci.* 199 (2002) 293–302. <https://www.sciencedirect.com/science/article/pii/S0169433202008668> (accessed May 3, 2019).
- [7] C.-W. Chan, S. Lee, G.C. Smith, C. Donaghy, Fibre laser nitriding of titanium and its alloy in open atmosphere for orthopaedic implant applications: Investigations on surface quality, microstructure and tribological properties, *Surf. Coatings Technol.* 309 (2017) 628–640. doi:10.1016/j.surfcoat.2016.12.036.
- [8] A. Cunha, A.-M. Elie, L. Plawinski, A.P. Serro, A.M. Botelho Do Rego, A. Almeida, M.C. Urdaci, M.-C. Durrieu, R. Vilar, Femtosecond laser surface texturing of titanium as a method to reduce the adhesion of Staphylococcus aureus and biofilm formation, *Appl. Surf. Sci.* 360 (2016) 485–493. doi:10.1016/j.apsusc.2015.10.102.
- [9] A. Cunha, O.F. Zouani, L. Plawinski, A.M. Botelho Do Rego, A. Almeida, R. Vilar, M.-C.C. Durrieu, Human mesenchymal stem cell behavior on femtosecond laser-textured Ti-6Al-4V surfaces., *Nanomedicine.* 10 (2015) 725–39. doi:10.2217/nnm.15.19.
- [10] G. Brunello, P. Brun, C. Gardin, L. Ferroni, E. Bressan, R. Meneghello, B. Zavan, S. Sivoilella, Biocompatibility and antibacterial properties of zirconium nitride coating on titanium abutments: An in vitro study, *PLoS One.* 13 (2018) 1–17. doi:10.1371/journal.pone.0199591.
- [11] B. Groessner-Schreiber, M. Hannig, A. Duck, M. Griepentrog, D.F. Wenderoth, Do different implant surfaces exposed in the oral cavity of humans show different biofilm compositions and activities?, *Eur. J. Oral Sci.* 112 (2004) 516–522. doi:10.1111/j.1600-0722.2004.00171.x.
- [12] Y.L. Jeyachandran, S.K. Narayandass, The Effect of Thickness of Titanium Nitride Coatings on Bacterial Adhesion, *Trends Biomater. Artif. Organs.* 24 (2010) 90–93. <http://www.sbaoi.org> (accessed May 7, 2019).
- [13] C.-W. Chan, S. Lee, G. Smith, G. Sarri, C.-H. Ng, A. Sharba, H.H.-C. Man, Enhancement of wear and corrosion resistance of beta titanium alloy by laser gas alloying with nitrogen, *Appl. Surf. Sci.* 367 (2016) 80–90. doi:10.1016/j.apsusc.2016.01.091.
- [14] M. Hermansson, The DLVO theory in microbial adhesion, *Colloids Surfaces B Biointerfaces.* 14 (1999) 105–119. doi:10.1016/S0927-7765(99)00029-6.
- [15] C.W. Chan, L. Carson, G.C. Smith, A. Morelli, S. Lee, Enhancing the antibacterial performance of orthopaedic implant materials by fibre laser surface engineering, *Appl. Surf. Sci.* 404 (2017) 67–81. doi:10.1016/j.apsusc.2017.01.233.

- [16] B.D. Hatton, Antimicrobial coatings for metallic biomaterials, in: *Surf. Coat. Modif. Met. Biomater.*, Woodhead Publishing Limited, 2015: pp. 379–392.
- [17] K. Anselme, P. Davidson, A.M. Popa, M. Giazzon, M. Liley, L. Ploux, The interaction of cells and bacteria with surfaces structured at the nanometre scale, *Acta Biomater.* 6 (2010) 3824–3846. doi:10.1016/J.ACTBIO.2010.04.001.
- [18] R.J. Crawford, H.K. Webb, V.K. Truong, J. Hasan, E.P. Ivanova, Surface topographical factors influencing bacterial attachment, *Adv. Colloid Interface Sci.* 179–182 (2012) 142–149. doi:10.1016/J.CIS.2012.06.015.
- [19] S. Rigo, C. Cai, G. Gunkel-Grabole, L. Maurizi, X. Zhang, J. Xu, C.G. Palivan, Nanoscience-Based Strategies to Engineer Antimicrobial Surfaces, *Adv. Sci.* 5 (2018) 1–13. doi:10.1002/advs.201700892.
- [20] M. Michalska, F. Gambacorta, R. Divan, I.S. Aranson, A. Sokolov, P. Noirot, P.D. Laible, Tuning antimicrobial properties of biomimetic nanopatterned surfaces., *Nanoscale.* 10 (2018) 6639–6650. doi:10.1039/c8nr00439k.
- [21] A. Jaggesar, H. Shahali, A. Mathew, P.K.D. V. Yarlagadda, Bio-mimicking nano and micro-structured surface fabrication for antibacterial properties in medical implants, *J. Nanobiotechnology.* 15 (2017) 64. doi:10.1186/s12951-017-0306-1.
- [22] M. Kaur, K. Singh, Review on titanium and titanium based alloys as biomaterials for orthopaedic applications, *Mater. Sci. Eng. C.* (2019). doi:10.1016/J.MSEC.2019.04.064.
- [23] P. Halder, M. Nasabi, F.J.T. Lopez, N. Jayasuriya, S. Bhattacharya, M. Deighton, A. Mitchell, M.A. Bhuiyan, A novel approach to determine the efficacy of patterned surfaces for biofouling control in relation to its microfluidic environment, *Biofouling.* 29 (2013) 697–713. doi:10.1080/08927014.2013.800192.
- [24] Y.K. Lee, Y.-J. Won, J.H. Yoo, K.H. Ahn, C.-H. Lee, Flow analysis and fouling on the patterned membrane surface, *J. Memb. Sci.* 427 (2013) 320–325. doi:10.1016/J.MEMSCI.2012.10.010.
- [25] V. Carniello, B.W. Peterson, H.C. van der Mei, H.J. Busscher, Physico-chemistry from initial bacterial adhesion to surface-programmed biofilm growth, *Adv. Colloid Interface Sci.* 261 (2018) 1–14. doi:10.1016/J.CIS.2018.10.005.
- [26] S. Shaikh, S. Kedia, D. Singh, M. Subramanian, S. Sinha, Surface texturing of Ti6Al4V alloy using femtosecond laser for superior antibacterial performance, *J. Laser Appl.* 31 (2019) 022011. doi:10.2351/1.5081106.
- [27] A. Kloosterman, J.T.. De Hosson, Microstructural Characterization of Laser Nitrided Titanium, *Scr. Met. Materialia.* 33 (1995) 567–573. <https://core.ac.uk/download/pdf/12884489.pdf> (accessed April 11, 2019).
- [28] N. Mitik-Dineva, J. Wang, V.K. Truong, P. Stoddart, F. Malherbe, R.J. Crawford, E.P. Ivanova, Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus Attachment Patterns on Glass Surfaces with Nanoscale Roughness, *Curr. Microbiol.* 58 (2009) 268–273. doi:10.1007/s00284-008-9320-8.

- [29] P. Tengvall, How Surfaces Interact with the Biological Environment, in: *Bio-Implant Interface Improv. Biomater. Tissue React.*, CRC Press, 2003: pp. 285–303.
- [30] C. González-García, M. Cantini, J. Ballester-Beltrán, G. Altankov, M. Salmerón-Sánchez, The strength of the protein-material interaction determines cell fate, *Acta Biomater.* 77 (2018) 74–84. doi:10.1016/J.ACTBIO.2018.07.016.
- [31] S.K. Lower, S. Lamlertthon, N.N. Casillas-Ituarte, R.D. Lins, R. Yongsunthon, E.S. Taylor, A.C. DiBartola, C. Edmonson, L.M. McIntyre, L.B. Reller, Y.-A. Que, R. Ros, B.H. Lower, V.G. Fowler, Jr., Polymorphisms in fibronectin binding protein A of *Staphylococcus aureus* are associated with infection of cardiovascular devices., *Proc. Natl. Acad. Sci. U. S. A.* 108 (2011) 18372–7. doi:10.1073/pnas.1109071108.
- [32] A.W. Buck, V.G. Fowler, R. Yongsunthon, J. Liu, A.C. DiBartola, Y.-A. Que, P. Moreillon, S.K. Lower, S.K. Lower, Bonds between fibronectin and fibronectin-binding proteins on *Staphylococcus aureus* and *Lactococcus lactis*., *Langmuir.* 26 (2010) 10764–70. doi:10.1021/la100549u.
- [33] P. Kami, Surface Modifications to Enhance the Wear Resistance and the Osseo-Integration Properties of Biomedical Ti-Alloy, University of North Texas, 2013. https://digital.library.unt.edu/ark:/67531/metadc500110/m2/1/high_res_d/thesis.pdf (accessed April 11, 2019).
- [34] M. Griepentrog, I. Haustein, W.-D. Mü, K.-P. Lange, H. Briedigkeit, U. Berthold, G. Bel, B. Größner-Schreiber, Plaque formation on surface modified dental implants, *Clin. Oral Impl. Res.* 12 (2001) 543–551. <https://onlinelibrary.wiley.com/doi/pdf/10.1034/j.1600-0501.2001.120601.x> (accessed August 17, 2018).
- [35] A. Scarano, M. Piattelli, G. Vrespa, S. Caputi, A. Piattelli, Bacterial adhesion on titanium nitride-coated and uncoated implants: an in vivo human study, *Oral Implantol.* 29 (2003) 80–85. <https://www.ncbi.nlm.nih.gov/pubmed/12760451> (accessed August 17, 2018).
- [36] R.P. van Hove, I.N. Sierevelt, B.J. van Royen, P.A. Nolte, Titanium-Nitride Coating of Orthopaedic Implants: A Review of the Literature, *Biomed Res. Int.* 2015 (2015) 1–9. doi:10.1155/2015/485975.