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Reduction of bacterial adhesion on titanium-doped diamond-like carbon coatings

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ABSTRACT

A range of titanium doped diamond-like carbon (Ti-DLC) coatings with different Ti contents were prepared on stainless steel substrates using a plasma-enhanced chemical vapour deposition technique. It was found that both the electron donor surface energy and the surface roughness of the Ti-DLC coatings increased with increasing Ti contents in the coatings. Bacterial adhesion to the coatings was evaluated against *Escherichia coli* WT F1693 and *Pseudomonas aeruginosa* ATCC 33347. The experimental data showed that bacterial adhesion decreased with the increases of the Ti content, the electron donor surface energy and surface roughness of the coatings, while the bacterial removal percentage increased with the increases of these parameters. The Ti-DLC coatings reduced bacterial attachment by up to 75% and increased bacterial detachment from 15 to 45%, compared with stainless steel control.

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KEYWORDS

Bacterial adhesion; DLC coatings; surface energy; contact angle; biomaterials

Introduction

The high incidence of infections caused by the use of metallic biomedical implants and devices, including bone fracture fixation pins and hip and knee replacement, has a severe impact on human health and health care costs (Bociaga et al. 2015; Percival et al. 2015; Ceroni et al. 2016; Dunne et al. 2017). Prevention of bacterial adhesion on the surfaces of medical devices will have a major impact in preventing medical device-related infections. An effective and desired approach reducing bacterial adhesion is to alter the surface properties of the medical devices and to make them less attractive for bacteria using surface coating techniques. For biomedical application the coatings should have both good biocompatible and mechanical properties. Diamond-like carbon (DLC) coatings have been attracting a great deal of interest for biomedical devices due to their excellent properties such as biocompatibility, haemocompatibility, low friction and chemical inertness (Maegawa et al. 2016). DLC is an excellent base coating to be alloyed with different elements. Incorporation of selected elements into DLC has emerged as an innovative approach to add unique functional properties to DLC coatings, thus opening up a range of new potential applications for biomedical devices (Dearnaley

and Arps 2005; Marciano et al. 2010; Maegawa et al. 2016; Swiatek et al. 2016). The incorporation of Si, F, N or Ag into DLC reduced bacterial adhesion (Liu et al. 2008; Marciano et al. 2010; Su et al. 2010; Cloutier et al. 2016). Moreover, Ti coatings improved the antibacterial property (Dunne et al. 2017). Recently, the incorporation of Ti into DLC has been shown to be an effective method to improve tribological properties (Bayóna et al. 2015). However no research has been reported on the influence of Ti-doped DLC coatings on bacterial adhesion.

In this study, a range of Ti-doped DLC coatings with different Ti contents were prepared on a stainless steel 316L substrate using a plasma-enhanced chemical vapour deposition technique. The influence of Ti contents in the coatings on bacterial adhesion was investigated with *Escherichia coli* and *Pseudomonas aeruginosa* which are commonly associated with infections of medical devices and implants (Rădulescu et al. 2016).

Materials and methods

Preparation of Ti-DLC coatings

In order to investigate the effect of Ti contents in DLC coatings on bacterial adhesion, a range of Ti-DLC

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coatings with different Ti contents (1.3, 1.8 and 3.2 at.%, respectively) were produced on stainless steel 316L plates (25 mm × 26 mm × 1 mm) by a magnetron sputter system consisting of a Ti target combined with plasma-enhanced chemical vapour deposition (PECVD). The average coating thickness was controlled around 1 µm. The Ti contents in the Ti-DLC coatings were determined by Energy Dispersive X-ray analysis (EDX) (Philips XL 30, Eindhoven, Netherlands). A Dimension 3000 atomic force microscope (AFM Digital Instruments, Santa Barbara, CA, USA) was employed to characterise the surface topography of the coatings with a lateral resolution of 1–2 nm and a vertical resolution of 0.01 nm. Crosscut tests and abrasion resistance tests with water and sand at 5 bar under angle of 45° for 0.5 min were performed in order to evaluate the coating adhesion strength to the substrate.

Contact angle and surface energy

The surface energy of a solid surface gives a direct measure of intermolecular or interfacial attractive forces. van Oss (1994) divided the total surface energy (γ_2^{TOT}) of a solid into two components, Lifshitz-van der Waals (LW) apolar component (γ_2^{LW}) and Lewis acid/base polar component (γ_2^{AB}). The γ_2^{AB} is further divided into an electron donor (γ_2^-) component and an electron acceptor (γ_2^+) component. The contact angle on the coatings was measured using the sessile drop method with a Dataphysics OCA-20 contact angle analyser (DataPhysics Instruments GmbH, Filderstadt, Germany) with distilled water (W), diiodomethane (Di) and ethylene glycol (EG) as the test liquids, respectively. The surface energy components of the coatings were calculated using the van Oss approach (van Oss et al. 1994) using the contact angle data. The details of contact angle measurements and surface energy calculations were described in a previous study (Liu and Zhao 2011). In order to investigate bacterial adhesion mechanism, the surface energy of biofilms was also measured. To measure the contact angle of a biofilm, *E. coli* WT F1693 or *P. aeruginosa* ATCC 33347 were grown on a glass surface in bacterial suspension of 10⁶ CFU ml⁻¹ for over 10 days with tryptone soya broth (TSB; Oxoid®, Cheshire, UK) as the growth medium. Using a microscope it was confirmed that a high density biofilm had formed on the glass slide at this time. The biofilm coated glass slide was removed from the bacterial suspension and was dried in air until it maintained a stable water contact angle. The contact angles of the living biofilm were then further measured by using the contact angle analyser with diiodomethane and ethylene glycol, respectively (Liu and Zhao 2011).

Bacterial adhesion and removal

E. coli WT F1693 and *P. aeruginosa* ATCC 33347 were obtained from the Institute of Infection and Immunity,

Nottingham University, UK. Both *E. coli* and *P. aeruginosa* are microorganisms that frequently cause infections related to medical devices and implants. Each strain was sub-cultured and preserved in 15% glycerol in TSB (Oxoid®) as frozen stock at –80°C. For all adhesion tests, tryptone soya agar (TSA) plates were streaked out with a loop from the frozen stock and grown overnight at 37°C. A single colony was inoculated in 20 ml TSB and grown statically overnight at 37°C. From this culture 500 µl were further inoculated into 100 ml TSB in a conical flask and grown in a shaker-incubator at 37°C and 250 rpm. The culture was grown to mid-exponential phase (corresponding to an optical density absorbance at 600 nm of 0.5–0.7), since cells harvested in this growing phase have shown the best adhesion to a solid surface. Two ml of *E. coli* or *P. aeruginosa* were harvested by centrifugation at 2,500 rpm for 5 min at –4°C, washed once in sterile distilled water and resuspended in the 500 ml PBS (pre-warmed to 37°C) at a 10⁶ CFU ml⁻¹ concentration.

For bacterial adhesion assays, six replicate samples of each coating were immersed in a glass tank containing 500 ml of the suspension of *E. coli* or *P. aeruginosa* (10⁶ CFU ml⁻¹), incubated on a shaker (20 rpm) at 37°C for 1 h. Each sample was dipped twice vertically in a glass vessel containing 130 ml sterile distilled water with a custom-made automated dipper apparatus under a constant speed of 0.028 m s⁻¹ with corresponding shear stress of 0.014 N m⁻² in order to remove loosely attached bacteria. In order to observe the number of cells microscopically, the bacteria on the samples were stained using LIVE/DEAD BacLight Kit L 13152 for (Molecular Probes Europe BV, Leiden, The Netherlands) 15 min and then observed under a fluorescence microscope (OLYMPUS BX 41, Tokyo, Japan) and counted using Image Pro Plus software (Media Cybernetics, Rockville, MD, USA). Then the dead, live and total numbers of adhered bacteria on each sample were determined. For each sample, six measurements on the six different areas of the sample were made. The total measurements for each type of coating were 36.

To assess the adhesion strength of the attached bacteria, each sample was dipped a further 20 times vertically in the glass vessel containing 130 ml sterile distilled water at 37°C and a constant shear stress of 0.014 N m⁻². Each test sample was stained and then observed under the fluorescence microscope to determine the remaining bacteria. Then the bacterial removal rate *R* (%) for the each sample was determined:

$$R = (A - L)/A \times 100\% \quad (1)$$

where *A* is total number of adhered bacteria before dipping process and *L* is the remaining bacteria after dipping process.

Statistical analysis by *t*-test

The *t*-test is perhaps the most widely used method for comparing two independent groups of data. Each group is normally distributed around its respective mean value, and the two groups have the same variance. The sole difference between the groups is that their means may not be the same. The *t*-statistic is defined as:

$$t = \frac{\bar{x} - \bar{y}}{S \sqrt{1/n + 1/m}} \quad (2)$$

where \bar{x} is the sample mean of data in the first group x_i , $i = 1, 2, \dots, n$; \bar{y} is the sample mean of data in the second group, y_j , $j = 1, 2, \dots, m$; and S is the pooled sample standard deviation, estimating the standard deviation assumed identical in both groups:

$$S = \sqrt{\frac{(n-1)s_x^2 + (m-1)s_y^2}{n+m-2}} \quad (3)$$

The sample variances of both groups, s_x^2 and s_y^2 are used to estimate S .

$$s_x^2 = \frac{1}{n-1} \sum_{i=1}^n (x_i - \bar{x})^2 \quad (4)$$

$$s_y^2 = \frac{1}{m-1} \sum_{j=1}^m (y_j - \bar{y})^2 \quad (5)$$

The *t*-test was used as statistical analysis for evaluating the anti-microbial ability of coated and control surfaces. The conventional 0.05 level was considered to reflect statistical significance.

Results

Surface characterisation

The Ti contents in the three Ti-DLC coatings were determined by Energy Dispersive X-ray analysis, which were 1.3, 1.8 and 3.2 at.%, respectively. The surface topography and roughness of the coatings were measured with an AFM. The roughness for each surface was an average of six measurements at different position with surface area of $5 \times 5 \mu\text{m}$. Figure 1a, b, c and d show the AFM images of pure DLC coating, 1.3%Ti-DLC coating, 1.8%Ti-DLC coating and 3.2%Ti-DLC coating, respectively. Both pure DLC coating and the Ti-DLC coatings consisted essentially of hemispherical rounded hillock units, and their crystallites were distributed densely and homogeneously. Table 1 indicates that the surface roughness increases slightly with increasing Ti content in the DLC coatings. The results of the crosscut tests and abrasion resistance

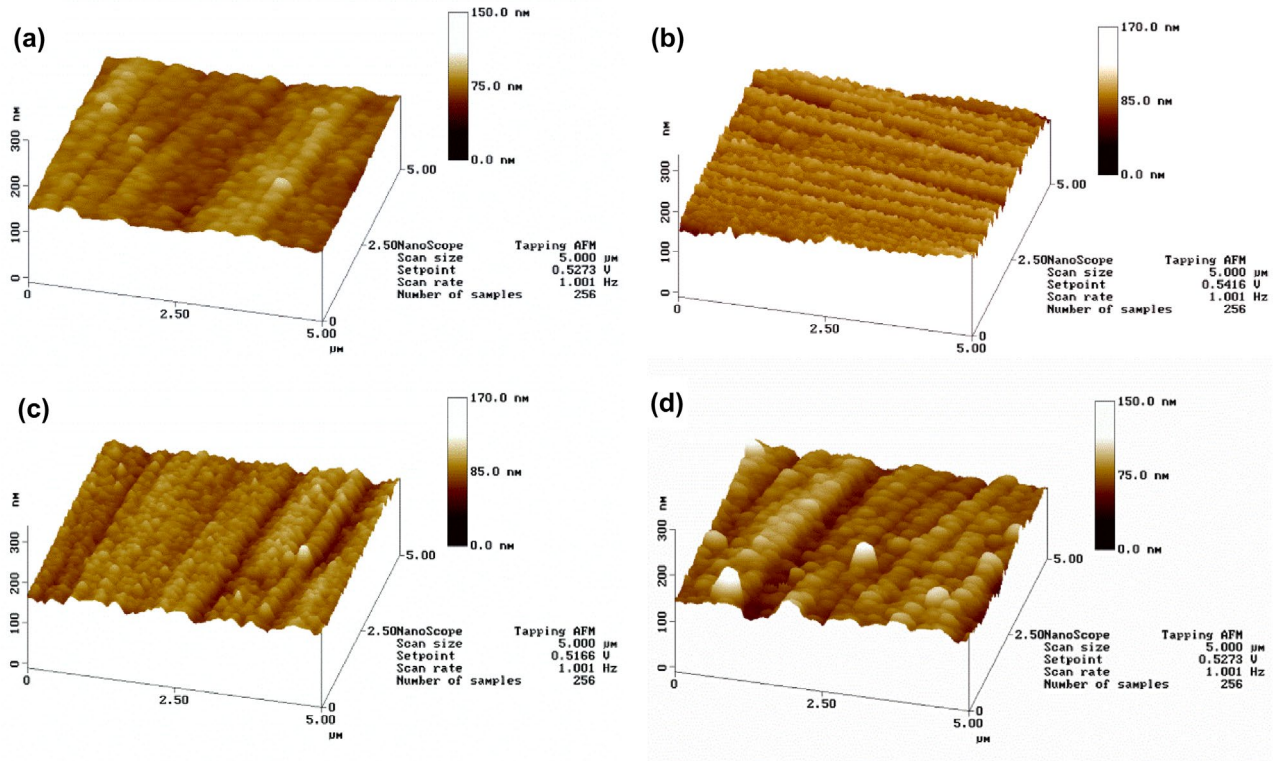


Figure 1. AFM images of (a) DLC coating; (b) 1.3%Ti-DLC coating; (c) 1.8%Ti-DLC coating; (d) 3.2%Ti-DLC coating.

tests showed that the Ti-DLC coatings had very good adhesion to the substrates and very good resistance to abrasion.

Contact angle and surface energy component

Table 2 shows the contact angle and surface energy components of coatings used in this investigation, including polished stainless steel, DLC coating, 1.3%Ti-DLC coating, 1.8%Ti-DLC coating and 3.2%Ti-DLC coating. The results showed that the γ_2^{LW} component of the Ti-DLC coatings almost kept constant, about 44 mJ m⁻²; while the γ_2^- component of the Ti-DLC coatings increased significantly with increasing Ti content in the coatings and the γ_2^+ component of the coatings was almost zero. Table 2 also shows the contact angle and surface energy components of *E. coli* and *P. aeruginosa* biofilms used in this investigation.

Bacterial adhesion and removal

Figure 2 shows the attachment of *E. coli* cells on stainless steel, DLC coating and Ti-DLC coatings. Due to exposure to air for 15 min, ~50% of the bacteria on the Ti-DLC coatings were dead. Both pure DLC coating and the Ti-DLC coatings performed much better than stainless steel against bacterial attachment. The 3.2% Ti-DLC performed best, which reduced total bacterial adhesion by 75% and 66%, respectively, compared with stainless steel and pure DLC coating. Figure 3 clearly shows that the number of adhered bacteria (live, dead and total bacteria) decreased with Ti content increasing with very high correlation coefficients ($R^2=0.96-0.98$). The statistical analysis by *t*-test indicated that there was a significant statistical difference between the Ti-DLC coatings and stainless steel in reducing *E. coli* adhesion ($p \leq 0.05$).

Table 1. Ti content and surface roughness Ra.

Coatings	Ra by AFM (nm)
DLC	4.7±0.4
1.3%Ti-DLC	7.9±0.9
1.8%Ti-DLC	10.6±1.2
3.2%Ti-DLC	17.3±1.4

Table 2. Contact angle and surface energy components of coating samples and bacteria.

Coatings orbacteria	Contact angle θ [°]			Surface energy components [mJ m ⁻²]				
	θ^W	θ^{Di}	θ^{EG}	γ^{LW}	γ^+	γ^-	γ^{TOT}	γ_2^{LW}/γ_2^-
Polished steel	79.6±3.0	32.1±1.5	45.2±1.5	43.33	0.15	4.15	44.90	10.44
DLC	77.2±3.0	31.6±1.5	39.5±1.5	43.56	0.35	4.47	46.06	9.74
1.3%Ti-DLC	78.9±1.9	27.1±1.0	54.6±0.2	44.11	0.00	5.72	44.11	7.71
1.8%Ti-DLC	75.2±0.3	29.6±2.9	47.4±0.3	44.39	0.00	7.97	44.39	5.57
3.2%Ti-DLC	72.8±0.3	27.4±0.2	50.0±0.9	44.40	0.00	9.83	44.40	4.52
<i>E. coli</i>	17.2±1.3	78.0±2.1	18.6±0.6	18.52	3.05	70.01	47.75	–
<i>P. aeruginosa</i>	78.0±2.5	55.4±2.2	72.4±2.6	31.20	0.66	7.43	35.66	–

During the dipping process, some bacteria were removed from the coatings. Figure 4 shows that the numbers of remaining bacteria (live, dead and total bacteria) decreased with Ti content increasing. Figure 5 shows that the removal percentage of *E. coli* cells from stainless steel, the DLC coating and the Ti-DLC coatings after dipping process increased with increasing Ti content in the DLC coating. The removal percentage of live, dead and total bacteria on stainless steel was only about 15%; while the bacterial removal percentage on the 3.2% Ti-DLC coatings increased to about 45%. The statistical analysis by *t*-test indicated that there was a significant statistical difference between the Ti-DLC coatings and stainless steel in the removal rates of *E. coli* cells ($p \leq 0.05$).

Influence of surface energy components

Figure 6 shows that the live, dead and total *E. coli* cells on the Ti-DLC coatings decreased with the γ^- surface energy of the Ti-DLC coatings increasing. Figure 7 demonstrates that the bacterial attachment had strong correlation with the γ_2^{LW}/γ_2^- ratio, which varied in a wide range between 4.52 and 10.44. The live, dead and total *E. coli* cells on the polished stainless steel, DLC coating and Ti-DLC coatings decreased with the γ_2^{LW}/γ_2^- ratio decreasing with the correlation coefficient R^2 values 0.921, 0.903 and 0.914, respectively. Figure 8 shows that the bacterial removal percentage also had strong correlation with the γ_2^{LW}/γ_2^- ratio. The removal percentage of *E. coli* cells (live, dead and total *E. coli* cells) increased with the γ_2^{LW}/γ_2^- ratio decreasing with the correlation coefficient R^2 values 0.972, 0.956 and 0.968, respectively.

Influence of surface roughness

The effect of surface roughness on bacterial adhesion has also been investigated. These studies clearly demonstrate that substrate topography at the micro- and nano-scale had significant influence on bacterial attachment ($p \leq 0.05$). However, no universal relationship between the surface roughness and the number of attached cells was observed (Hsu et al. 2013). Some studies showed that increased surface roughness at micro-scale leads to an increased bacterial adhesion, as roughening of the

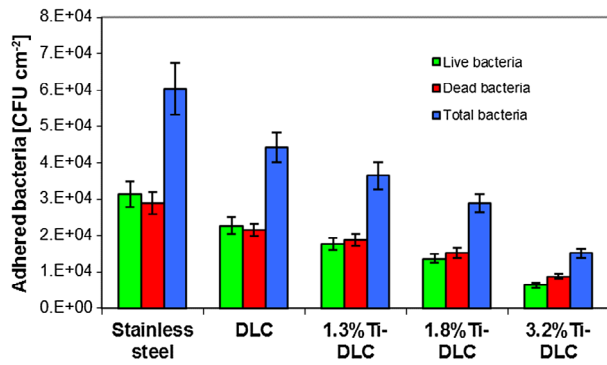


Figure 2. Attachment of *E. coli* cells on stainless steel, DLC coating and Ti-DLC coatings ($N = 36$, error bars are standard error).

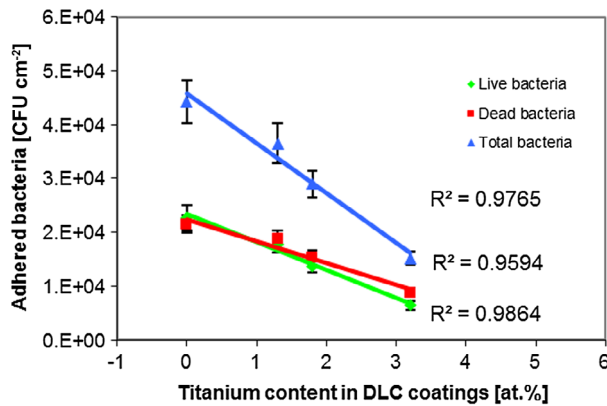


Figure 3. Effect of Ti contents of Ti-DLC coatings on the attachment of *E. coli* cells ($N = 36$, error bars are standard error).

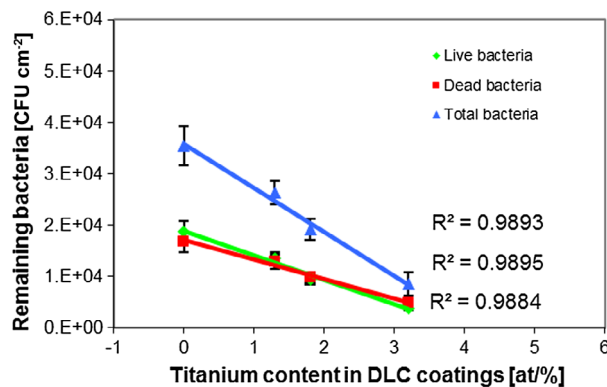


Figure 4. Effect of Ti content of Ti-DLC coatings on the remaining *E. coli* cells ($N = 36$, error bars are standard error).

surface increases the area available for bacterial adhesion (Gharechahi et al. 2012). Yoda et al. (2014) demonstrated that surface roughness (R_a) below 30 nm can also promote bacterial adhesion. In this study, the R_a values of the Ti-DLC coatings were in the range of 4.7–17.3 nm (Table 1). Figure 9 shows that the number of attached bacteria decreased with increasing surface roughness

($p \leq 0.05$). These findings are in accordance with the studies of Yoda et al. (2014).

Influence of bacterial species

According to the extended DLVO theory, the surface properties of bacteria, including surface charge and surface energy, have significant influence on bacterial adhesion (van Oss 1994; Liu and Zhao 2011). In order to validate the antibacterial properties of the Ti-DLC coatings, the assays of bacterial adhesion and removal were also performed with *P. aeruginosa*. Figure 10 shows the attachment of *P. aeruginosa* cells on stainless steel, DLC coating and Ti-DLC coatings; similar results to Figure 2 with *E. coli* were obtained. However it was observed that the total number of adhered *P. aeruginosa* cells was significant higher than that of adhered *E. coli* ($p \leq 0.05$). Figure 11 shows the removal percentage of *P. aeruginosa* cells from stainless steel, the DLC coating and the Ti-DLC coatings. Again, similar results to Figure 5 with *E. coli* were obtained.

Discussion

The influence of total surface energy on bacterial adhesion has been investigated intensively, with the frequent conclusion that bacterial adhesion is less on low energy surfaces and easier to clean because of weaker binding at the surface interface (Ahn et al. 2009; Liu and Zhao 2011; Brajkovic et al. 2014). Brajkovic et al. (2014) demonstrated that the surfaces with lower values of surface energy were less prone to biofilm adhesion and the number of adhered bacteria increased with increasing surface energy. However, there are also a number of contrary findings that suggest high energy surfaces have a smaller biofouling tendency and higher removal rate than low energy surfaces (Brink et al. 1993; Pasmore et al. 2001). Hahnel et al. (2009) reported that there was no relationship between total surface energy and bacterial adhesion. The above research indicated that there is no consistent conclusion on the effect of total surface energy of substrates on bacterial attachment and removal. Little research work has been reported on the effect of surface energy components of substrates on bacterial attachment and removal.

It was reported that the larger the electron donor component γ^- of a surface, the more negatively charged the surface (Chibowski et al. 1994; Liu and Zhao 2011). Therefore, bacterial adhesion should decrease with increasing electron donor γ^- values of the coatings if other parameters that affect bacterial adhesion are identical. The γ^- surface energy component of Ti-DLC coatings increased with Ti content increasing, meaning that the incorporation of Ti element increased the negative charge

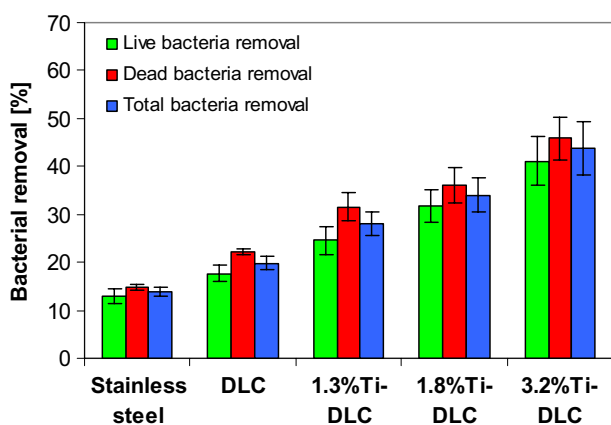


Figure 5. Removal percentage of *E. coli* cells from stainless steel, pure DLC coating and Ti-DLC coatings after dipping process ($N = 36$, error bars are standard error).

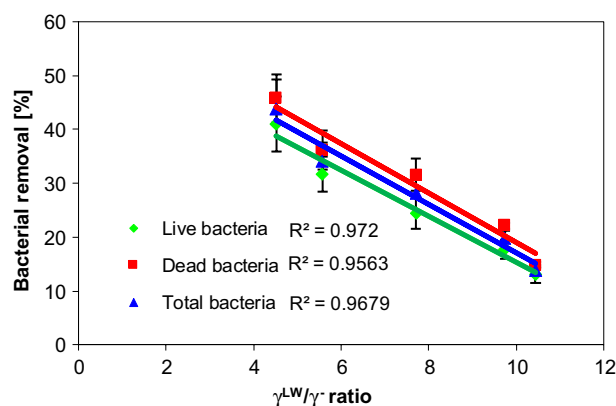


Figure 8. Effect of γ_2^{LW}/γ_2^- ratio on the removal percentage of *E. coli* cells from stainless steel, DLC coating and Ti-DLC coatings after dipping process ($N = 36$, error bars are standard error).

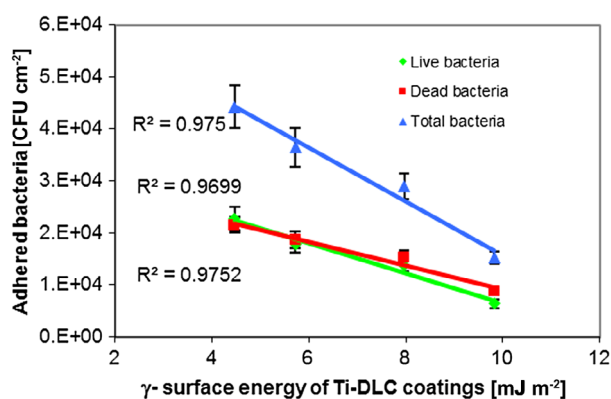


Figure 6. Effect of γ^- surface energy of Ti-DLC coatings on the attachment of *E. coli* cells ($N = 36$, error bars are standard error).

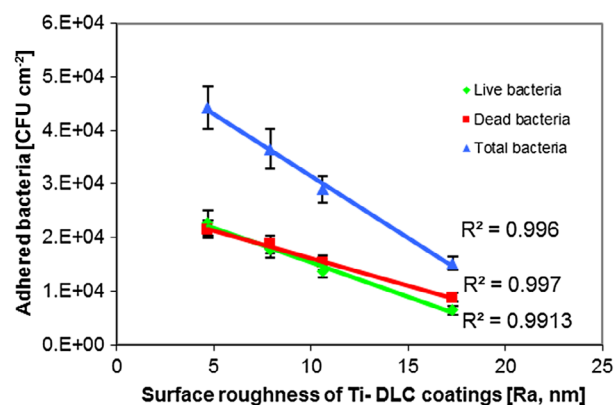


Figure 9. Effect of surface roughness R_a of Ti-DLC coatings on the attachment of *E. coli* cells ($N = 36$, error bars are standard error).

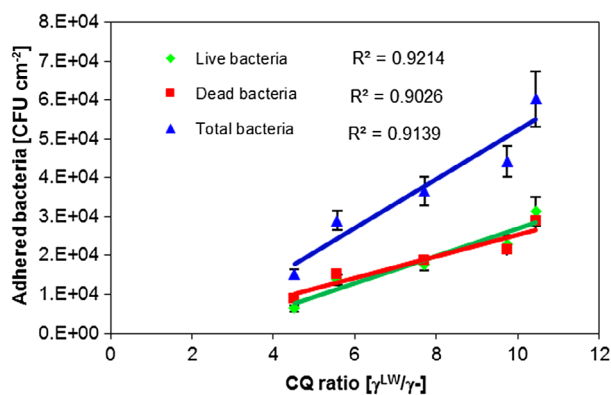


Figure 7. Effect of γ_2^{LW}/γ_2^- ratio of stainless steel, DLC coating and Ti-DLC coatings on the attachment of *E. coli* cells ($N = 36$, error bars are standard error).

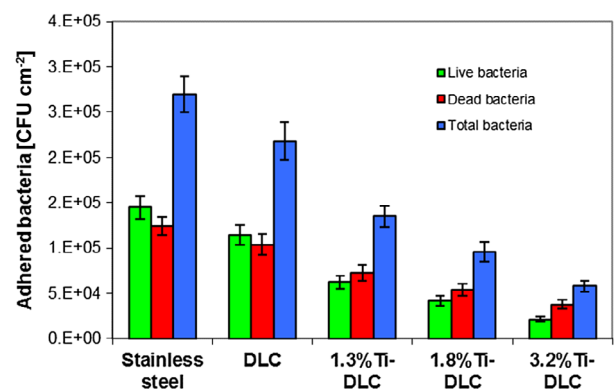


Figure 10. Attachment of *P. aeruginosa* cells on stainless steel, DLC coating and Ti-DLC coatings ($N = 36$, error bars are standard error).

of the Ti-DLC coatings. As *E. coli* and *P. aeruginosa* are negatively charged, this explains why the number of the adhered bacteria significantly decreased with increasing the electron donor γ^- values of the coatings and why the

Ti-DLC coatings performed much better than stainless steel or pure DLC coating against bacterial adhesion. The higher electron donor surface energy approach may lead to a way of controlling biofilm formation.

The comparison of the numbers of adhered bacteria shows that the numbers of *E. coli* was much lower than

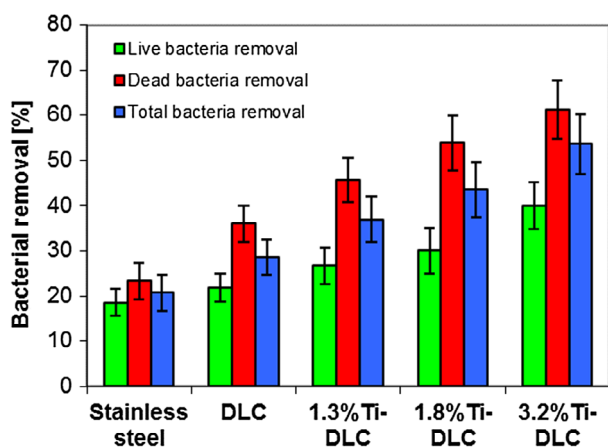


Figure 11. Removal percentage of *P. aeruginosa* cells from Stainless steel, pure DLC coating and Ti-DLC coatings after dipping process ($N = 36$, error bars are standard error).

P. aeruginosa. The γ_2^- value of *E. coli* (70.01 mJ m^{-2}) is much higher than that of *P. aeruginosa* (7.43 mJ m^{-2}). This helps to explain why *E. coli* is relative more repellent to the coatings with high γ_2^- values.

Conclusions

A range of Ti-DLC coatings with different Ti contents (1.3, 1.8 and 3.2 at.%) on stainless steel substrates were prepared using a plasma-enhanced chemical vapour deposition technique. The incorporation of Ti into the DLC coatings increased both the electron donor surface energy γ_2^- and surface roughness of the Ti-DLC coatings, which lead to the reduction of bacterial adhesion and the increase of bacterial removal rate. The 3.2% Ti-DLC coating performed best, which reduced bacterial attachment by 75% and increased bacterial detachment from 15% to 45%, compared with stainless steel. There were strong correlation between bacterial attachment (or removal) and the γ_2^{LW}/γ_2^- ratio. The γ_2^{LW}/γ_2^- ratio gives us a clear direction for the design of anti-biofouling and biofouling-release coatings through surface modification.

Disclosure statement

No potential conflict of interest was reported by the authors.

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