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Biochemical and electrochemical characterization of biofilms formed on everolimus-eluting coronary stents

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ABSTRACT

Drug-eluting stents (DES) are mostly used in percutaneous coronary intervention, which is the main treatment for coronary artery occlusion. This procedure aims to restore the natural lumen, while minimizing the risk of restenosis. However, stent insertion increases the risk for infections, due to contamination of the device or insertion hub with normal skin flora. While coronary stent infection is a rare complication, it can be fatal. Currently, there is little information on biofilm formation on everolimus-eluting stents. Although everolimus is not designed as an antimicrobial agent, its antimicrobial activity should be investigated. In this study, biofilm formation on everolimus-eluting and bare metal stents (BMS) is characterized through biochemical and electrochemical methods. DES and BMS are inoculated with *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*, both independently and in co-culture. Biofilms formed on DES were 49.6 %, 12.9 % and 47.5 % higher than on BMS for *P. aeruginosa*, *S. epidermidis* and their co-culture, respectively. Further, the charge output for DES was 18.9 % and 59.7 % higher than BMS for *P. aeruginosa* and its co-culture with *S. epidermidis,* respectively. This observation is most likely due to higher surface roughness of DES, which favors biofilm formation. This work shows that bioelectrochemical methods can be used for rapid detection of biofilms on drug-eluting and bare metal stents, which may find application in quality assessment of stents and in characterization of stents removed after polymicrobial infections.

1. Introduction

Coronary artery disease (CAD) is the leading cause of mortality worldwide, resulting in approximately 8.9 million deaths globally every year [1]. CAD is a chronic inflammatory disease of the coronary arteries, leading to the accumulation of lipid-laden plaque within the tunica intima of the coronary artery, which limits the perfusion of blood throughout the heart. Rupture of the atherosclerotic plaque results in dangerous thrombosis, leading to complete arterial occlusion. A severe shortage of blood flow results in hypoxic conditions within the heart, leading to cardiomyocyte necrosis and myocardial infarction, which eventually results in heart failure.

To restore normal blood flow within the coronary artery of CAD patients, the artery is expanded using a balloon-tipped catheter and a drug-eluting wire mesh stent is placed at the disease site to prevent re-occlusion in a process known as restenosis. As with the majority of cardiovascular diseases, vascular endothelial injury underlies restenosis [2]. Stent implantation induces ischemia-reperfusion injury (IRI), in which the sudden return of blood flow to ischemic tissues causes further vascular damage, inducing endothelial cell apoptosis. Endothelial cell apoptosis induces vascular inflammation and the proliferation of medial vascular smooth muscle cells, which narrows the lumen.

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Restenosis is a serious problem for patients and health care providers, as reoccluded stents must be either replaced or the blood flow redirected by coronary bypass surgery. Coronary stents are expensive and the surgical procedure required for their removal carries a significant risk [3] of post-operative complications, which include wound infections, pneumonia, thromboembolic phenomena, graft failure, atrial fibrillation, pulmonary hypertension, pericardial effusion, strokes, renal injury, and hemodynamic instability. A possible solution to minimize restenosis is to use drug-eluting coronary stents (DES) like everolimus-eluting stents, which in 2014 comprised 82 % of the stents implanted in the USA [4]. DES are coated in a bio-compatible polymer that is then used to bind and slowly release antiproliferative drugs. For example, Everolimus released from the wall surface of the coronary stent inhibits smooth muscle cell proliferation and vascular endothelial cell migration. However, despite the widespread use of DES, as many as 25–50 % of implanted stents still become reoccluded due to restenosis [5,6].

DES are the most effective option in percutaneous coronary intervention while reducing restenosis [7,8]. However, stent implantation may cause other complications such as infection and thrombosis. Among infections, biofilms are of particular concern. Bacterial biofilms are microstructured microbial communities that grow on solid surfaces, in which bacterial cells are encased in a self-produced matrix, comprising exopolysaccharides, extracellular DNA (eDNA), and proteins [9]. As biofilms show high antibiotic and antimicrobial resistance, the majority of microbial and chronic infections (65 % and 80 %, respectively) are associated with biofilm formation. The formation of biofilms on biomedical implants can lead to surface and structural degradation, which in turn affects their functionality. With biofilm formation, there is the potential for the persistence of antibiotic-resistant phenotypes that may be solved only by reimplantation [9].

The main bacterial species found in biomedical implants are *Enterococcus faecalis*, *S. aureus*, *S. epidermidis*, *Streptococcus viridans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *P. aeruginosa* [10]. *P. aeruginosa* is a model biofilm-forming microorganism due to its ease of culture and shows rapid adaption and high resistance to antibiotics [11]. *S. epidermidis* is an ubiquitous component of skin flora in a healthy individual. Biofilms can form on the surface of virtually every biomedical device, including catheters, endotracheal tubes, prosthetic joints, mechanical heart valves, intrauterine devices, pacemakers, and contact lenses. The longer the device is in use, the higher the risk of biofilm formation and growth. The number and type of cells in the surrounding liquid, temperature, and availability of nutrients affect the rate of biofilm formation [12].

Commensal microorganisms within the body collectively referred to as the microbiome, assist the host in various processes of synthesis, digestion, protection, and production of energy. However, overgrowth of opportunistic pathogens proceeding a trauma, a lesion, or concurrent pathology, may result in inflammation and dissemination of bacterial cells into the bloodstream, a condition known as bacteremia [11]. Bacteremia can be caused by Gram-negative and Gram-positive bacteria. The source of infection can be large foci of pneumonia, meningitis, tissue infections (e.g., periodontitis), as well as bacteria introduced during surgical interventions, followed by implantation of a biomedical device. The progress of bacteremia depends on the bacterial species, the immune status of the patient, and other factors such as the location of the implant. The entry of bacteria into the bloodstream, both from infected organs and from the implant itself, increases the likelihood of biofilm formation since most implants have an uneven or microporous surface, which favors bacterial attachment [13].

Coronary stent infection is a rare complication of percutaneous coronary intervention. However, it is often fatal because it can lead to purulent pericarditis, myocardial infarction, myocardial rupture, and coronary aneurysm rupture[14]. Moreover, this complication does not have a well-defined treatment protocol, with a significant difference among patients [15]. Surgical intervention may not be an option for many patients, due to their underlying diseases, which increases the risk of death. Therefore, early detection of stent-related bacteremia and infection may help improve the safety of stent implantation and re-implantation. Through early identification of biofilm formation, the stent can be replaced, and the patient can be put on a prophylactic antibiotic treatment regime to limit the spread and pathological effects of dispersed bacteria cells from the biofilm.

In this work, we used for the first time a bioelectrochemical method for the determination of early biofilm formation on everolimus-eluting cobalt-chromium (Drug Eluting Stents - DES in the following) coronary stents and bare cobalt-chromium stents (Bare Metal Stents - BMS in the following) to analyze *P. aeruginosa* and *S. epidermidis* as co-culture models on stents. Results show that bioelectrochemical characterization of biofilms is consistent with biochemical characterization and can be used for quality assessment of stents in vitro and potentially in vivo.

2. Experimental

2.1. Materials

Nutrient agar, cetrimide agar, mannitol salt agar, crystal violet, and acetic acid were obtained from Sigma Aldrich (Germany) and prepared according to the manufacturer's instructions. Coronary stents used were provided by Professor Paul Cahill, Dublin City University, Ireland. *S. epidermidis* ATCC 12228 (SE) and *P. aeruginosa* ATCC 10145 (PA) were purchased from a local microorganism repository. Nutrient broth (NB) (beef extract 3 g L^{-1}, peptone 5 g L^{-1}) was used because it is a general nutrient medium for the growth of most culturable bacterial strains, thus its suitability for co-culture. Screen-printed Carbon Electrodes (SPE Ref. C110) obtained from Metrohm DropSens, Spain, with graphite working electrode (WE) of 4 mm diameter and 0.126 cm^2 surface area, graphite counter electrode (CE), and silver reference electrode (RE) were used in all electrochemistry experiments. Electrochemical cells of 10 mL capacity were used (with 8 mL working volume). For bioelectrochemical experiments involving stents, cobalt-chromium stents (surface area 0.47 cm^2) were used as the working electrode. The RE and CE were Ag/AgCl and platinum wire, respectively. Electrochemical cells of 25 mL capacity (with a working volume of 18 mL) were used. In both SPE and stents electrochemical experiments, no external redox mediator was added. The current output observed in *P. aeruginosa* and co-culture bioelectrochemical experiments is due to microbially-produced phenazines [16,17].

2.2. Pretreatment of stents

Stents were cut using a pair of pre-sterilized scissors under a controlled environment devoid of dust, oil or other extraneous materials and pre-treated as follows: the stents with a diameter of 0.3 cm and length of 1.5 cm were cut into three parts, with approximately 0.47 cm^2 area each. All the stents were surfaced sterilized in 70 % v/v ethanol; washed twice in sterile distilled water, allowed to dry in the hood and finally autoclaved for 15 min at 121 °C and 101 kPa and kept under sterile conditions until used.

2.3. Planktonic cell growth in microtiter plates

Overnight and independent cultures of *S. epidermidis* and *P. aeruginosa* grown in 30 mL nutrient broth in an incubator at 37 °C for 18–24 h were used as inoculum, after which each well of a 48 wells microtiter plate was inoculated with 0.1 optical density (OD_{600}) of *S. epidermidis*, *P. aeruginosa*, and their co-culture. The co-culture was prepared as 0.1 OD_{600} of *P. aeruginosa* in 0.5 mL + 0.1 OD_{600} of *S. epidermidis* in 0.5 mL of nutrient broth. The plates were incubated in the plate reader at 37 °C for 48 h, under static conditions. The OD_{600} was recorded every 20 min using Gen5 TM Microplate Reader and Imager Software (BioTek Instruments).

2.4. Colony forming unit (CFU) assay

S. epidermidis and *P. aeruginosa* cultures were grown in 30 mL nutrient broth separately in an incubator under static conditions for 24 h at 37 °C. After dilution with nutrient broth to a final OD_{600} of 0.5, serial dilutions were carried out. Then, 100 μL of the final diluted solution was plated on a Petri dish with nutrient agar and another 100 μL on a cetrimide agar. The number of colonies from the nutrient agar Petri dish was calculated according to standard laboratory protocols.

2.5. Biofilm formation assay in microtiter plates

S. epidermidis and *P. aeruginosa* cultures were grown in 30 mL nutrient broth in an incubator for 24 h at 37 °C. Then, 0.1 OD_{600} of separate and mixed bacteria were inoculated on 48 wells microtiter plate. Co-culture was prepared as 0.1 OD_{600} of *P. aeruginosa* in 0.5 mL + 0.1 OD_{600} of *S. epidermidis* in 0.5 mL. The plate was covered with sealing films to prevent sample evaporation. Since early infections can occur in the first 10 days after stent implantation, the plate was kept in the incubator without shaking for 7 days [15,18]. After that the planktonic cells and their media were discarded, then the plate was gently washed with sterile distilled water twice. Biofilms on the well walls were fixed by 1 mL of crystal violet that has already been prepared with ethanol. After 20 min, the wells were gently washed with sterile distilled water twice, air-dried for 10 min, then 33 % v/v acetic acid was added and OD_{600} was measured. The same procedure was done for stents which surface area was area 0.47 cm^2. Each stent was added to well with 1 mL of media prepared as described above. After 7 days of incubation, the stents were moved into separate empty wells. 1 mL of crystal violet prepared with ethanol was added into each well, after 20 min the stents were placed in another empty well and 33 % v/v acetic acid was added. After 10 min, the stents were removed and the OD_{600} of the residual medium was measured. In these 7 days-experiments, the OD_{600} of the planktonic cells was determined as well.

2.6. Electrochemical assay

Fresh inocula were prepared the same way as previously described. The initial OD_{600} of *S. epidermidis* and *P. aeruginosa* was 0.5 OD_{600} in 18 mL of media. Mixed culture was prepared as 0.5 OD_{600} of *P. aeruginosa* in 9 mL + 0.5 OD_{600} of *S. epidermidis* in 9 mL. The assay was carried out with a three-electrode system comprising Ag/ACl RE, platinum coil CE, and a coronary stent with a 0.47 cm^2 surface area as a WE. The WE was maintained at E = 0.4 V vs. Ag/AgCl for 7 days in chronoamperometry (CA) and differential pulse voltammetry (DPV) was carried out immediately after inoculation and every 24 h. The DPV parameters were set as follows: $E_i = -0.4$ V and $E_f = 0.4$ V, pulse height 50 mV, and pulse time 200 ms. The electrical charge output (mC) for each CA experiment was also calculated using EC Lab® software (Bio-Logic, France). The same parameters were used for the 48 h experiments using graphite SPE. In this case, the inocula were prepared as described above and the working volume was 8 mL in a 10 mL container.

2.7. Pyocyanin concentration measurement

P. aeruginosa PA and the co-culture of *S. epidermidis* and *P. aeruginosa* were cultivated in 30 mL nutrient broth each for 48 h at 37 °C, and then centrifuged at 4200 rpm for 30 min. The supernatant was mixed with 3 mL of chloroform. After 2 min, 1 mL of 0.2 M HCl was added, then the absorbance was measured at 520 nm. The result was multiplied by 17.072 to obtain the pyocyanin concentration in μg mL^{-1} [19].

2.8. Scanning Electron Microscopic (SEM) imaging

At the end of bioelectrochemical experiments, selected SPE and stent samples were fixed overnight in 1 mL of 1 % w/w glutaraldehyde. Then, the samples were washed with phosphate buffer saline (PBS) twice for 10 min each time. Further, the samples were fixed with 1 mL of 1 % osmium for 2 h was performed. The samples were then washed again with PBS twice for 10 min. After dehydration in alcohols of ascending concentration: 50 % of ethanol - twice for 10 min each time, 70 % of ethanol - twice for 10 min each time, 96 % of ethanol - twice for 10 min each time, and absolute alcohol 100 % - twice for 20 min each time, the samples were covered with 5 nm thick gold film and examined under SEM with the following parameters: EHT = 5.00 kV, Mag = 5000x and 10000x, I probe = 92 pA, Signal A = InLens.

3. Results and discussion

3.1. Planktonic cell growth

In full strength nutrient broth (NB) medium, the generation times of *S. epidermidis* and *P. aeruginosa* at 37 °C are approximately 17–38 and 44 min, respectively [20]. However, the planktonic growth curves of *S. epidermidis*, *P. aeruginosa,* and their co-culture (Fig. 1) show that *P. aeruginosa* grows faster than *S. epidermidis* and their co-culture grows approximately the same as *P. aeruginosa*. To our knowledge, there are no reports on in vitro cultivation and biofilm formation by co-culture of *S. epidermidis* and *P. aeruginosa*. However, there are several studies on *S. aureus* and *P. aeruginosa* co-culture. Tognon et al. found that planktonic *P. aeruginosa* grows like the *S. aureus* - *P. aeruginosa* co-culture. Transcriptomic data indicate that early responses between *P. aeruginosa* and *S. aureus* involve competition for resources and metabolic adaptations, rather than the expression of bacteria or host-directed virulence factors [21].

In another study, it was observed that established *S. aureus* biofilm was not outcompeted by the introduction of *P. aeruginosa*, however, the actual ratio of *S. aureus* / *P. aeruginosa* cells and biofilm depends on several co-culture parameters, like inoculation time, initial number of cells, etc. [22]. With these caveats in mind, the results reported here should be considered as a first step in the simulation of *S. epidermidis* - *P. aeruginosa* co-culture. The planktonic growth (Fig. 1) shows that the maximum OD_{600} of *P. aeruginosa* is approximately three times that of *S. epidermidis* and occurs between 10 and 12 h, then decrease due to nutrient exhaustion. In co-culture, the growth pattern is similar to *P. aeruginosa*, indicating that *P. aeruginosa* either outcompetes *S. epidermidis* or release antimicrobial toxins that inhibit the proliferation of *S. epidermidis* (e.g., phenazine) or a combination of both effects. It

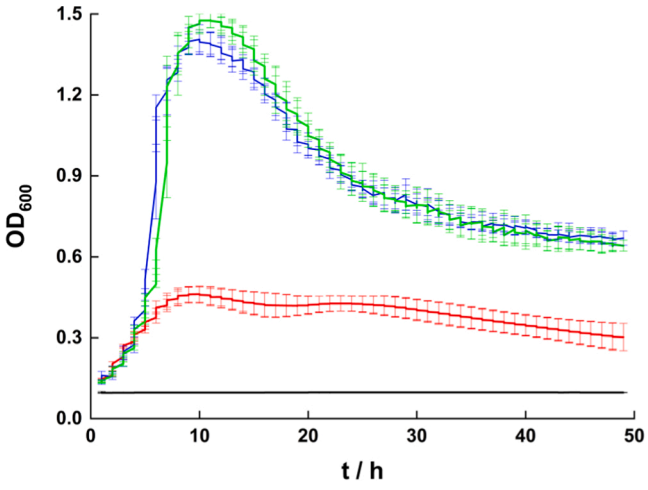

Fig. 1. Optical density of *S. epidermidis* (red curve), *P. aeruginosa* (blue curve), and co-culture of (green curve) planktonic cells during 48 h incubation at 37 °C in nutrient broth in a microtiter plate. Each curve is the average of 6 independent biological replicates. The control experiment with sterile nutrient broth is also shown (black line).

should be noted that in situ co-culture (e.g., sputum) might behave differently, as previously shown [23], due to nutrient limitation, which results in different generation times. When comparing the results to those of previous studies, it must be pointed out that *P. aeruginosa* has an antibacterial effect in co-culture with *Staphylococcus* sp., as *P. aeruginosa* -*S. aureus* co-culture resulted in a decrease in the number of *S. aureus* cells after 18 h of mixed culture growth [24].

3.2. Microtiter plate assay

S. epidermidis formed a substantially smaller biofilm on DES than *P. aeruginosa* or mixed culture on DES (Fig. 2A). A similar behavior was observed in BMS, however more biofilm formed on DES than on BMS. The planktonic growth on DES was not affected by the antimicrobial activity of everolimus, which has been reported effective against mycobacteria [25]. This is possibly due to the low eluted concentration of everolimus in the bulk fluid. The planktonic growth in presence of the stents shows similar trend, with higher *P. aeruginosa* growth than *S. epidermidis* (Fig. 2B). As expected, the planktonic growth on BMS is similar to that on DES, indicating the stent surface affects only biofilm cells, and there is no long-distance effect. Both co-culture biofilm and planktonic growth are not significantly different from *P. aeruginosa*, possibly because the microbially produced phenazines inhibit *S. epidermidis* growth. The difference between biofilm and planktonic growth between DES and BMS can be explained by a biofilm-promoting effect of DES. However, it is also possible that the everolimus-containing coating protects cells from the cobalt-chromium antimicrobial effect or that the coating roughness promotes biofilm formation (see Section 3.4).

3.3. Electrochemical assay

The chronoamperometric characterization as carried out using the DES, showed that the current output of *P. aeruginosa* and co-culture in DES is higher than *S. epidermidis* (Fig. 3). This was expected based on the fact that the current output at oxidative potential is determined by the presence of microbially-produced redox mediators phenazines. Interestingly, the current output in co-culture was higher than *P. aeruginosa*, indicating that *S. epidermidis* cells can use phenazines to transfer electrons extracellularly, despite its microbial toxicity. This effect was first observed in mixed consortia MFC [26].

Further experiments comparing current produced in DES and BMS test conditions for the bacteria were carried out but expressed as charge output. In fact, due to the inherent difficulty of measuring small currents

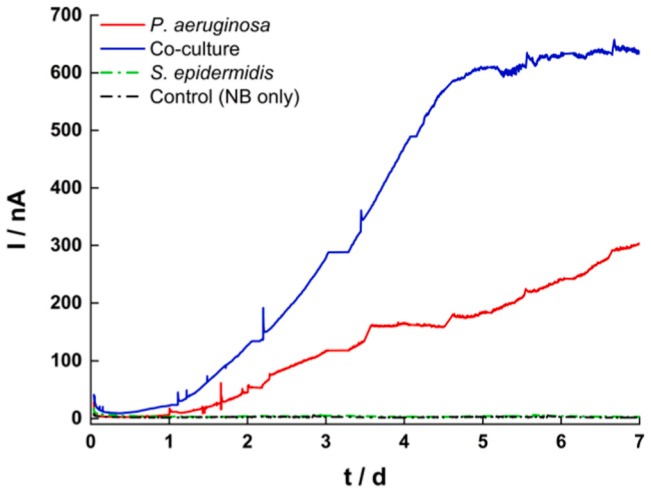

Fig. 3. Representative chronoamperometric trace of *S. epidermidis* (green dashed line), *P. aeruginosa* (red line), co-culture *S. epidermidis* and *P. aeruginosa* (blue line), and sterile control (black dashed line) on DES represented on a current versus time graph after incubation for 7 days. The OD_{600} of each inoculum was 0.5.

of biological origin, the overall charge output over a given time is often preferred as the integration process compensate for the noise of the measurement, particularly for long-term experiments (> 2–3 day). Fig. 4 shows the charge output for both *P. aeruginosa* and co-culture on DES and BMS. The charge output for *S. epidermidis* and sterile control was approximately zero (similar to the chronoamperometric trace shown in Fig. 3 above) and was not included in Fig. 4. The output for the co-culture on BMS was different from all other conditions and was significantly lower. This raises questions about specific interactions between *S. epidermidis* and *P. aeruginosa* in the biofilm with the stent surface. In this case, biofilm behavior as captured by biofilm quantification and electrochemical measurements showed differences linked to the composition of the stent surface.

This result can be explained by the difference in surface texture and roughness of the DES and BMS (see Section 3.4), which might affect cell adherence and biofilm formation. It could also be that the bare metal was toxic to the co-culture or elicited a specific interaction within the co-culture that reduced biofilm formation and the charge produced. Quantification of biofilm formation on the DES and BMS (Fig. 2) clearly

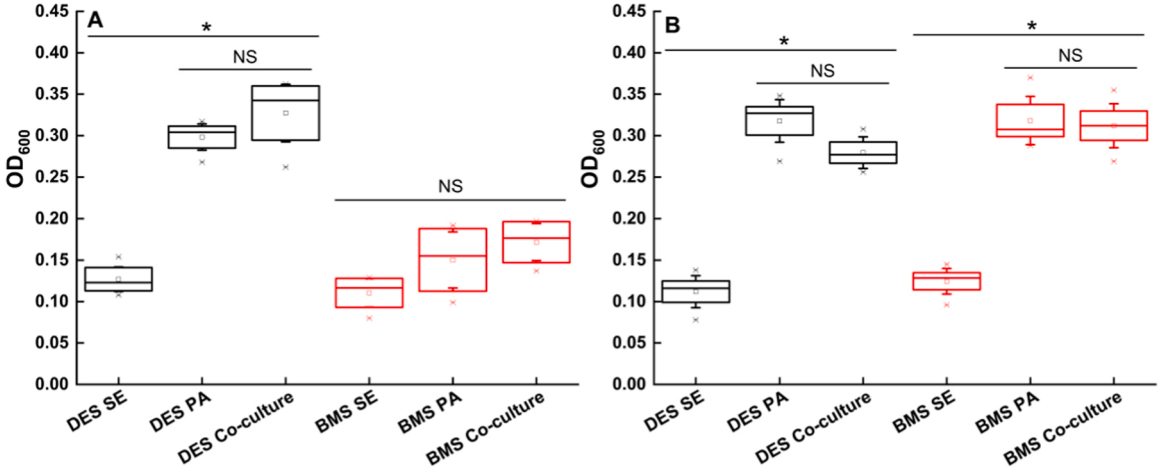

Fig. 2. (A) Optical density (OD_{600}) of *S. epidermidis* and *P. aeruginosa* (individual and co-culture) biofilm in nutrient broth on DES (black) and BMS (red) in microtiter plate after 7 days. (B) Optical density of *S. epidermidis* and *P. aeruginosa* (individual and co-culture) planktonic cells in nutrient broth after removal of DES (black) and BMS (red) in microtiter plate after 7 days. Four independent biological replicate experiments were carried out. Asterisk (*) indicates significant difference between the experimental conditions ($p < 0.05$) (Table S1) following Tukey's test. NS means not significant.

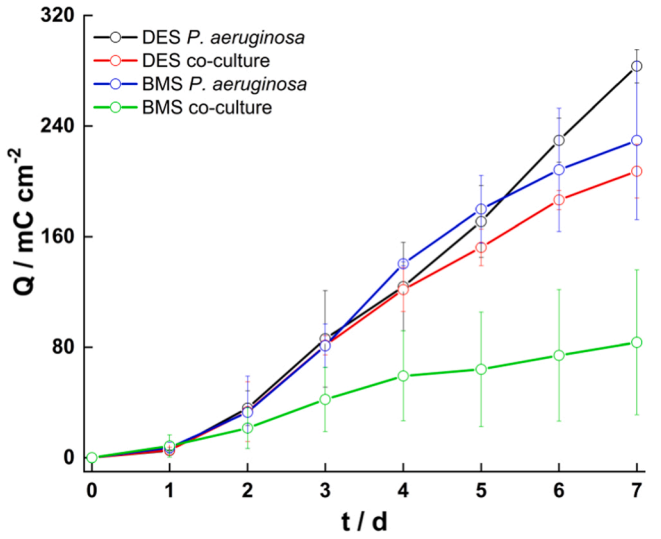

Fig. 4. Total charge per unit of surface (Q) for *P. aeruginosa*, and *S. epidermidis* and *P. aeruginosa* co-cultures on BMS and DES after incubation for seven days. Three independent biological replicates for each condition were carried out.

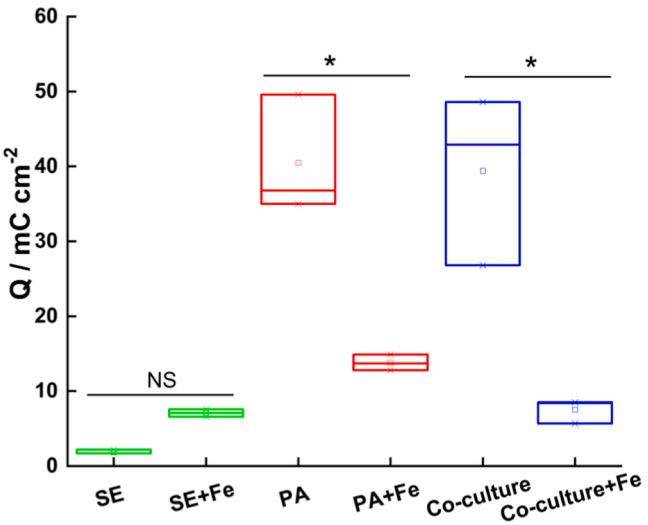

Fig. 5. Total charge per unit of surface (Q) for *S. epidermidis* (SE), *P. aeruginosa* (PA), and their co-culture after 48 h experiments with SPE in nutrient broth and in nutrient broth with Iron ($FeSO_4{\cdot}7H_2O$). 30 μM was taken as the normal concentration of iron in the blood. Three independent biological replicate experiments were carried out. Asterisk (*) indicates significant difference between the experimental conditions ($p < 0.05$) (Table S2) following Tukey's test. NS means not significant.

showed that lower biofilm was produced in BMS compared to DES for co-culture. However, these results only partially agreed with the biofilm formation shown in Fig. 2A. In fact, the bare metal surface seems to have no effect on *P. aeruginosa* biofilm charge output even though there was an observed reduced biofilm concentration for *P. aeruginosa* in comparison with the drug-eluting stents. The specific effects and mechanisms of interactions of everolimus on planktonic and biofilm cells could be investigated in further work.

To verify if the trend in charge output was due to the electrode material or the inherent characteristics of the co-culture, experiments on screen printed carbon electrodes (SPEs), which are chemically inert, were carried out. In these experiments, Iron (Fe) was added as $FeSO_4$ to simulate a normal condition in blood, in which soluble iron enhances biofilm formation and charge output. Interestingly, Fe addition decreased the overall charge output over 48 h. This could be due to the low bioavailability of Fe as inorganic $FeSO_4$. Approximately three-fourths of the available iron in the blood exists as heme iron, an Fe^{2+} chelation into a complex organic compound known as the 'heme structure', which is usually present in hemoglobin, myoglobin and important redox active human body enzymes. Further experiments using heme iron are important to specifically decipher their role in biofilm formation on blood-based implants.

In presence of Fe, the charge output was lower for both *P. aeruginosa* and co-culture than for *S. epidermidis*, suggesting that Fe alone did not serve as a redox mediator across the two species. There is a possible interference between Fe and phenazines produced in both co-culture and *P. aeruginosa* cultures. After 48 h of cultivation of *P. aeruginosa* and co-culture of *S. epidermidis* and *P. aeruginosa* in NB, the pyocyanin concentration in *P. aeruginosa* (7.47 ± 0.1 μg/mL) was lower than in the co-culture of (12.58 ± 0.5 μg/mL). This result is interesting, as it shows a complex ecological role for pyocyanin, which can be an antimicrobial or a culture-enhancer depending on the species co-cultured. Previous studies show that pyocyanin concentration increases in a mixed culture of *P. aeruginosa* and *E. coli* [27]. In contrast to our previous results where *S. epidermidis* does not show current/charge response, the addition of Fe to the medium increased the charge output in SPE experiments (Fig. 5). The results of this experiment support the hypothesis that iron supplementation can promote the electrochemical activity of *S. epidermidis* biofilms [28].

The DPV of the co-culture showed a higher peak than observed with *P. aeruginosa* at 24 and 48 h. After 48 h, a negative shift in the potential of co-culture can be observed. Thus, it appears that the presence of other bacteria co-cultured with *P. aeruginosa* can induce increased pyocyanin production and potentially other changes in the bioelectrochemistry of *P. aeruginosa*. (Fig. 6).

3.4. Scanning Electron Microscopic (SEM) Imaging

SEM was performed to visualize bacterial biofilms formed on the coronary stent at different magnification from x5000 and above. Single species and dual species biofilms were grown on both DES and BMS stents for 7 days. Prior to that, SEM imaging of un-inoculated BMS and DES were performed (Fig. 7).

There are observable roughness and undulations on the surface of the DES in comparison with the smooth surface of the BMS. This could be a result of the everolimus coating applied to the DES in the manufacturing process. It is also plausible that the difference in topography and chemical composition (i.e., the coating masks the microbial toxicity of chromium and cobalt) could affect the rate of cellular adherence to the surface and subsequent formation of biofilms, hence the differences in biofilm formation and charge output when comparing the two stents.

SEM analyses of the biofilms formed on the DES/BMS suggest a correlation between the abundance of biofilm formation on the stent surface and the electrochemical activity of the biofilm can be observed. For *S. epidermidis* experiments, BMS harbored far fewer biofilms in comparison with the DES, as shown in Fig. 8A and B. The biofilm formations of *S. epidermidis* as shown on the DES were characteristic of the biofilm proliferation pattern of *S. epidermidis* as proven by infection model studies of *S. epidermidis* on orthopedic implants [16]. The reduced biofilm formation on BMS however needs to be further investigated to elucidate individual effects on species. Despite poor biofilm colonization of BMS by *S. epidermidis*, it was observed that a good biofilm growth of *P. aeruginosa* formed on both BMS and DES (Fig. 8C and D). However, SEM is not an ideal technique for accurate quantification of biofilm microstructure. Earlier studies of biofilm infection models of *P. aeruginosa* [29] had proved that SEM images could show *P. aeruginosa* biofilms with reduced production of exopolymeric substances (EPS) within their biofilms. This was evidenced by the appearance of the biofilm showing scanty individual cells of the bacteria not clumped together in a matrix. In our work, it was however observed that on both BMS and DES, *P. aeruginosa* exhibited strong biofilm formation with well

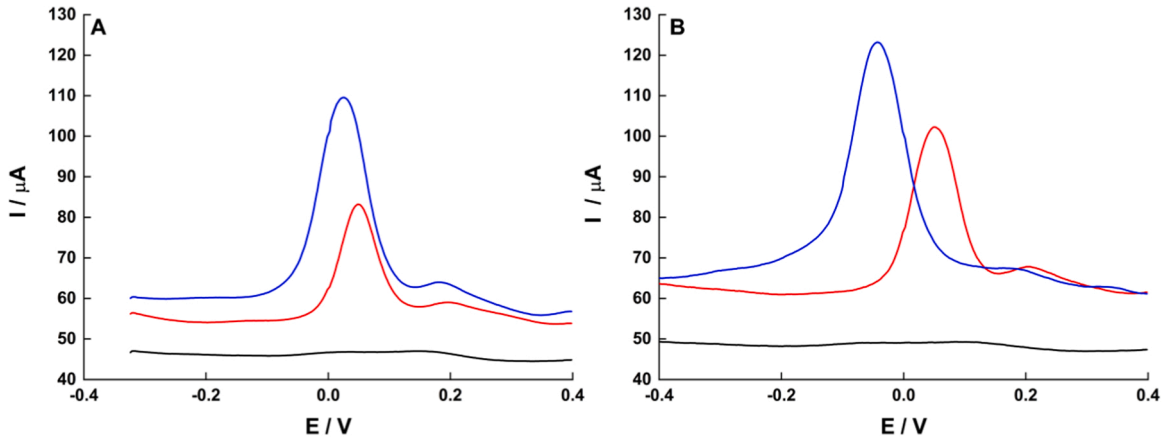

Fig. 6. DPV of SPE with *P. aeruginosa* (A) and co-culture (B) at 0 h (black line), 24 h (red line), 48 h (blue line).

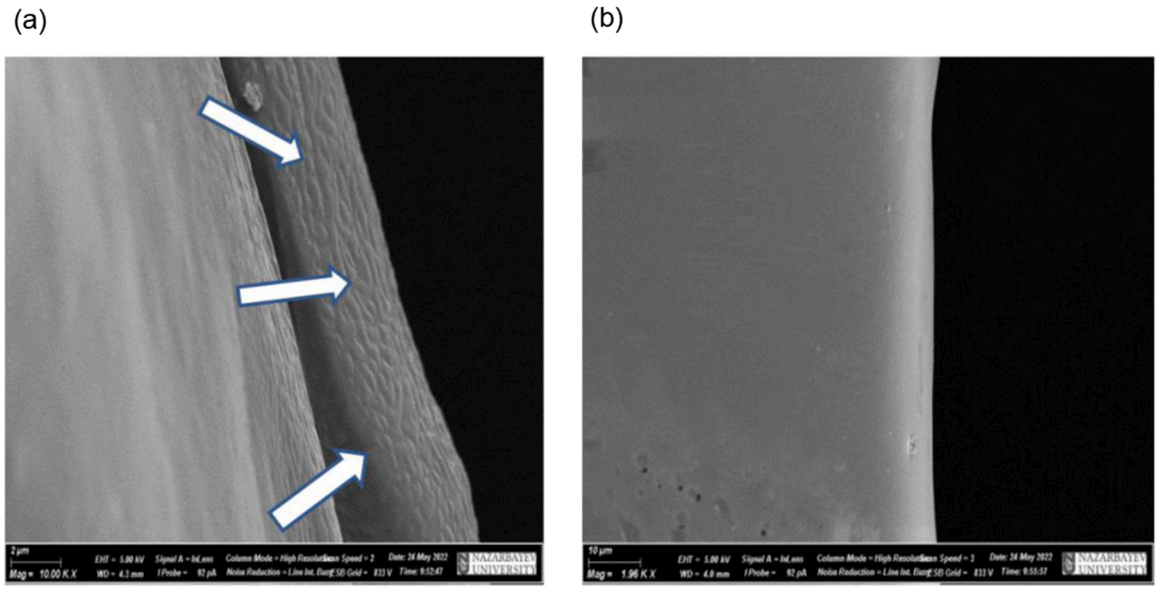

Fig. 7. Representative SEM images of two different surfaces of sterile stents; (A) rough undulating surface of DES, white arrows pointing to rough areas (B) smooth surface of BMS.

clumped cells and good formation of exopolymeric matrices that effectively covered the individual cells (Fig. 8C and D). Co-culture SEM experiments showed two distinct cell types, cocci and rods, present on the stent surface albeit in grossly different quantities. Based on morphological appearances, co-culture biofilms formed on both DES and BMS were similar. Major clumps of *P. aeruginosa* biofilms with good EPS formation can be seen (Fig. 8E and F), however few cells of *S. epidermidis* dispersed and exposed without any EPS protection can also be seen dotted on the surface of the luxuriant lawn of *P. aeruginosa* biofilms.

This loose interaction between *P. aeruginosa* and *S. epidermidis* based on observed biofilm morphology is pointing towards a *P. aeruginosa* driven co-habitation system of the two organisms within a potential infection. The observable presence of cells of *S. epidermidis* on the *P. aeruginosa* biofilms in the co-culture, shows that it is not exactly clear to what extent *P. aeruginosa* can act as an antagonist to *S. epidermidis*. This is also particularly valid given the fact that the secretion of pyocyanin (the main antimicrobial and redox active agent) by *P. aeruginosa* is controlled by several quorum sensing and genetic factors [19]. Cells of *S. epidermidis* could also be embedded within the thick covering of *P. aeruginosa* biofilms formed on both DES and BMS in the co-culture. Previously reported co-culture experiments between Staphylococci and *P. aeruginosa* show interactions between the two organisms and that *P. aeruginosa* biofilms could protect Staphylococci cells from phagocytosis during infection conditions [30,31]. It was also observed that Staphylococci cells were clustered onto well-formed biofilm of *P. aeruginosa* in a possibly dependent manner. This was similar to cellular interactions observed in our experiments with *S. epidermidis* and *P. aeruginosa*.

SEM analyses show how bacteria form biofilm on stent separately and combined with *P. aeruginosa,* exhibiting a thicker layer of biofilm than *S. epidermidis*. The adhesion potential and biofilm formation of bacteria on the two different stent surfaces could also differ according to the nutrient concentration in the medium used. Experimenting with different media, Wijesinghe and colleagues reported that brain heart infusion media induced higher biofilm formation of the co-culture of *P. aeruginosa* and staphylococcal biofilms as it caused improved biofilm concentrations in comparison with nutrient broth, phosphate buffered saline and Roswell Park Memorial Institute (RPMI) Medium [30]. Regarding our work on *P. aeruginosa* and *S. epidermidis* on BMS and DES, it is plausible that some interactions between the nutrients in the medium and the elemental composition of the stent might lead to an extended effect on the cell adherence and colonization potentials of *S. epidermidis* and *P. aeruginosa*. Further biomechanistic insights would be needed to fully elucidate this.

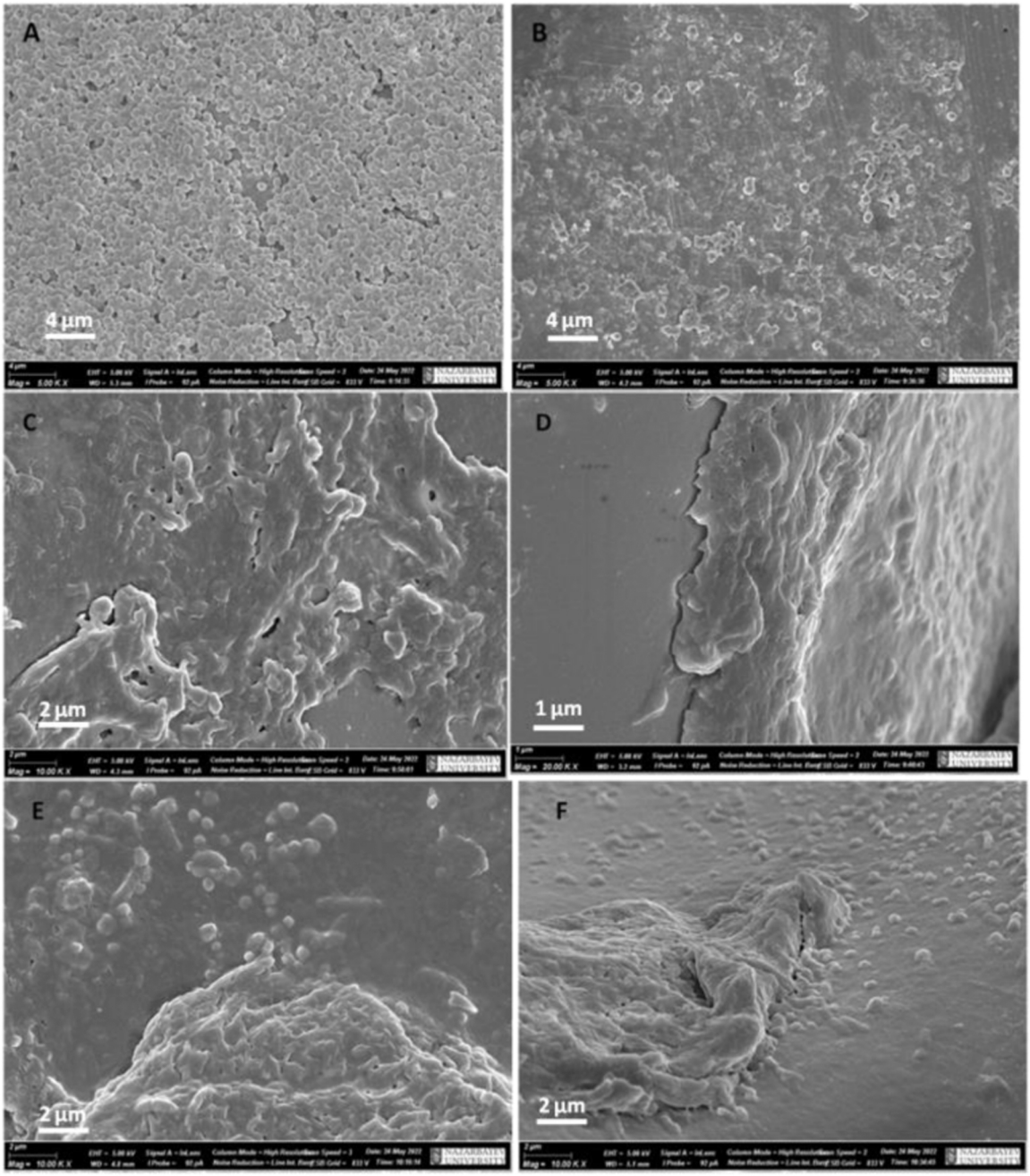

Fig. 8. Representative SEM images of bacteria on the coronary stent after 7 days of incubation in microtiter plate. (A) DES *S. epidermidis* (B) BMS *S. epidermidis* (C) DES *P. aeruginosa* (D) BMS *P. aeruginosa* (E) DES co-culture (F) BMS co-culture.

4. Conclusions

Comparisons between bare metal coronary stents and everolimus-eluting coronary stents showed that the surface coating and/or the elemental composition of the stent surface affected cell adhesion and biofilm formation on the stents in a species-dependent fashion. Surface topography was also observed to be different for both the DES and BMS, and this might have also influenced the quantities of biofilms formed. Bioelectrochemical analyses indicated that pyocyanin could act as an electron shuttle for *S. epidermidis* in the co-culture with *P. aeruginosa*, with higher pyocyanin levels being produced during co-cultures than in *P. aeruginosa* single cultures. Elevated levels of pyocyanin produced by *P. aeruginosa* in co-culture suggests an antagonistic response to the presence of another microbial species. This is plausible as pyocyanin has been identified as an antibacterial agent. No antibacterial effect was observed with everolimus under the reported experimental conditions. The functionality of everolimus as a drug coating for cardiovascular stents needs to be further investigated as it might lead to higher biofilm formation on stents in the possible scenario of contamination during the stent insertion.

Ethical statement

This article does not contain any studies with human participants or animals.

CRediT authorship contribution statement

Gauhar Akhmetzhan: Investigation, Data curation. **Kayode Olaifa:** Data curation, Writing – review & editing. **Michael Kitching:**

Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Paul A. Cahill:** Conceptualization, Writing – review & editing. **Tri T. Pham:** Writing – review & editing. **Obinna M. Ajunwa:** Methodology, Data curation, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Enrico Marsili:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data Availability

Data will be made available on request.

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Author Agreement

All authors have seen and approved the final manuscript before its submission. We declare that this manuscript is original, has not been published previously and is not currently under consideration for publication elsewhere.

Data Availability

All data generated or analysed during this study are included in this published article and its supplementary information files. Raw data from the EC-lab software as well as the microtiter equipment can be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.enzmictec.2022.110156.

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