Journal of Human Centered Technology Vol. 2: No. 2 (2023) 11 – 18
Journal of Human Centered Technology
E-ISSN: 2821-3467
https://humentech.utm.my

Antibacterial Analysis on Everolimus Grafted Surfaces using Polydopamine Intermediate Layer for Potential Use on Medical Devices

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Received 14 April 2023; Accepted 26 June 2023; Available online 06 August 2023
https://doi.org/10.11113/humentech.v2n2.52

Abstract:
Biomaterial-Centered Infection (BCI) is a significant issue in the implantation of medical devices, primarily caused by the formation of bacterial biofilms on the device surface. One potential solution to address this problem is the use of antibacterial coatings. This study examines the effectiveness of everolimus as an antibacterial coating agent with polydopamine (PDA) as an intermediate layer. The commonly used biomaterial for medical scaffolds, poly(lactic acid) (PLA), was coated with everolimus after being submerged in PDA for 24 hours. The coated PLA was then subjected to antibacterial analysis, including culturing Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* bacteria in Luria-Bertani broth, bacterial count tests, and disc inhibition tests. The results showed that everolimus has antibacterial properties, but its efficacy varies with different types of bacteria adhering to the biomaterial surfaces. Specifically, the everolimus coating was found to be more effective in killing Gram-positive *S. aureus*. The absence of inhibition zones and the lack of further growth of both Gram-positive *S. aureus* and Gram-negative *E. coli* on the samples demonstrated the controlled release of everolimus, indicating the potential of the PDA layer in holding the everolimus release to the surrounding. Therefore, the study concludes that the formation of everolimus coating on biomaterial surfaces aided by the PDA layer, have significant potential in retarding bacterial colonies on medical devices.

Keywords: Biomaterial-centered infection; Antibacterial coating; Everolimus; Polydopamine

1. Introduction

Biomaterials are increasingly being used in modern medicine to replace, support, or repair bodily functions, as well as for post-trauma treatment and oncological surgery. However, the use of biomaterials comes with the risk of biomaterials-centered infections (BCI). During perioperative procedures, bacteria can attach, colonize, and multiply on the surface of implants and biomedical equipment, which leads to the formation of biofilms and causes BCI. The presence of BCI is detrimental to both the implants and medical devices which will hinder the efficacy of the devices and endanger

the health of users [1–3]. Several medical devices that possess high risk of BCI include sutures, urinary catheters, IUDs, vascular grafts, and orthopedic devices [4].

Incorporating antibacterial agents onto biomaterial surfaces is an effective strategy for preventing bacterial colonization and infections associated with complications from biomedical devices [5, 6]. Everolimus, commonly used to prevent chronic rejection in organ transplants, has gained attention as a potential antibacterial coating due to its antiproliferative, antifungal, and immunosuppressive properties [7–9]. This compound exhibits strong binding to the FK506 binding protein-12 (FKBP-12), resulting in the suppression of the mammalian target of rapamycin (mTOR) pathway. The mTOR pathway plays a critical role in regulating DNA damage and repair checkpoints, which are crucial steps in the bacterial life cycle. By interfering with the late G1 phase of the bacterial life cycle, everolimus hinders cell progression into the S phase, where bacterial proliferation occurs, effectively inhibiting further bacterial growth [10–12].

The efficacy of antibacterial coatings and their biocompatibility with surfaces depend heavily on the coating technique used for the application of antibacterial agent [13]. One commonly used technique is immobilization, which involves the utilization of an intermediate layer to create chemical bonds between the coating and the biomaterial, and it is frequently employed in cell attachment and other biotechnological applications [14]. Polydopamine (PDA) has gained popularity as a material that can be cross-linked with other substances because of its ability to bind strongly to a variety of substrates, which was first observed in the invertebrate mussel [15–17]. Recent studies have demonstrated that PDA (polydopamine) exhibits antibacterial properties. The primary source of its antibacterial activity is attributed to the benzene ring of dopamine and the formation of active groups on the outer membrane of bacterial cells. These active groups induce local toxic effects, which in turn negatively impact the permeability of the cell membrane. As a result, essential components required for bacterial survival are obstructed [15, 18].

The primary aim of this study is to assess the potential of grafted everolimus in combination with PDA intermediate layer to eradicate bacterial colonies on biomaterial surfaces and retard bacterial growth around the coating. The study will contribute to the advancement of antibacterial coatings on biomaterial surfaces by exploring everolimus as a promising alternative antibacterial agent.

2. Methodology

2.1 Sample preparation

There are three main stages in preparing the sample for everolimus coating including the preparation of PLA filament, PDA grafting on the filaments, and everolimus coating on top of the PDA layer.

2.1.1 Preparation of PLA filament

The PLA filament with a diameter of 1.75 mm was cut into 1 cm length. Three PLA samples were prepared for this study including the control samples that were not coated with PDA and everolimus, the PLA-PDA samples that were coated only with PDA, and the PLA-PDA-everolimus samples that were grafted with both PDA and everolimus. Each sample was replicated three times.

2.1.2 Polydopamine coating

Polydopamine coating was developed by dissolving dopamine in an alkaline solution and allowing it to polymerize spontaneously. First, 200 mL of Tris buffer solution was prepared by dissolving 0.242 g of Tris powders in 200 mL of deionized (DI) water in a Schott bottle. Then, 20 mg of dopamine hydrochloride ($C_8H_{11}NO_2$.HCl) was dissolved in 10 mL of 10 mM Tris buffer solution. The pH level of the PDA solution was maintained at 8.5 by adding Tris buffer solution when the pH level is too low and adding dopamine hydrochloride when the pH level is too high. The solution was stirred until the color changed from transparent to light brown.

The prepared PLA filaments were then placed into a 24-well plate and 2 mL of PDA solution was pipetted into each well. Then, the well plate was wrapped with an aluminum foil to avoid ultraviolet (UV) exposure and kept for 24 hours. After 24 hours, the PLA-PDA samples were removed from the solution. A thin brown film was observed on the PLA, showing that the coating process was successful. The PLA-PDA samples were then rinsed with distilled water and dried.

2.1.3 Everolimus coating

Everolimus solution was prepared by diluting everolimus solution with dimethyl sulfoxide (DMSO). Equation 1 was used for calculating the amount of everolimus needed for the dilution, where *M* refers to the molarity of concentration and *V* refers to the volume of solution.

$$M_1V_1 = M_2V_2 \quad (1)$$

$$(1.0\ mM)\ V_1 = (0.05\ mM)(5\ mL)$$

$$V_1 = \frac{(0.05\ mM)(5\ mL)}{(1.0\ mM)}$$

$$V_1 = \ 0.25\ mL$$

$$V_{DMSO} = (5 - 0.25)\ mL = 4.75\ mL \quad (2)$$

For this study, 5 mL of 0.05 mM everolimus was needed from the available 1.0 mM everolimus solution. Hence, the volume of 1.0 mM everolimus which was required for the dilution to 0.05 mM everolimus was 0.25 mL. The volume of DMSO for the dilution was calculated, by subtracting the total volume needed for dilution with the everolimus volume, as shown in Equation 2. Based on Equation 2, the DMSO volume needed for the dilution was 4.75 mL. After the dilution was completed, each PLA-PDA sample was placed into another 24-well plate containing 1.25 mL of 0.5 mM everolimus solution to begin the immobilization coating process. After 24 hours, the PLA-PDA-everolimus samples were collected, rinsed, and dried. Jumat *et al*. [10] has reported the immobilization of 0.05 mM everolimus on PDA surfaces has resulted in 83.34 ± 0.03% immobilization.

2.2 Antibacterial analysis

For the antibacterial analyses, there are three stages involved which were bacterial culture, bacterial count test, and disc inhibition test. All analyses were conducted in triplicate.

2.2.1 Bacterial culture

The antibacterial analysis in this study used two strains of bacteria: Gram-positive *Staphylococcus aureus* (*S. Aureus*) and Gram-negative *Escherichia coli* (*E. coli*). *S. aureus* (ATCC 6538) and *E. coli* (ATCC 25922) were grown on nutrient agars and incubated at 37°C for 24 hours. Using a sterile inoculation loop, a single colony of bacteria was placed into 100 mL of Luria-Bertani (LB) broth. To obtain a concentrated suspension of bacteria, the bacteria were cultured for 16 hours in a shaking incubator at 200 rpm. After 16 hours, 10 mL of the concentrated bacterial suspension was pipetted into 100 mL of fresh LB broth and incubated for 1 hour at 200 rpm. The bacterial concentration was measured at an absorbance of 600 nm to attain a concentration of 1×10^6 cells/mL using UV-vis. Prior to the bactericidal testing, the surface of all samples was sterilized for 30 minutes on each side with UV light.

2.2.2 Bacterial count test

The bacterial count test was performed to evaluate the antibacterial capability on the sample surfaces by incubating the sterile samples in 2 mL of bacterial solution (1×10^6 cells/mL) for 3 hours at 200 rpm. The samples were removed after the prescribed incubation time, washed twice with phosphate buffer saline (PBS), and placed in 2 mL of new LB broth. The vortexing method was used to separate the adherent bacteria on the coated surfaces, and the suspension was diluted to six dilution series. Using a drop plate approach, the diluted bacteria were cultivated on nutrient agars. Each dilution was dropped over the agars in a total of 10 µL and incubated for 24 hours, in triplicate. The bacterial colonies formed on the agars were then manually counted and expressed as colony forming unit (CFU) that are counted with Equation 3. The CFU values were finally used to calculate the bactericidal percentage using Equation 4.

$$CFU = \frac{Number\ of\ colonies\ x\ Dilution\ factor}{Volume\ inoculated} \quad (3)$$

$$Bactericidal\ ratio = \left(\frac{CFU_{control} - CFU_{experiment}}{CFU_{control}}\right) \times 100\% \quad (4)$$

3. Results and Discussion

3.1 Bacterial count test

Three samples underwent the bacterial count test: PLA control sample, PLA- PDA sample, and PL-PDA-everolimus sample. In order to conduct the bacterial count test, a total of six dilutions were prepared. Following the incubation period of 24 hours, bacterial colonies were formed. The number of colonies that were formed declined significantly from the second to the sixth dilution, going from hundreds in the second dilution, to fewer than five in the sixth dilution. The bacterial colonies produced by the fourth dilution of the bacterial suspension for each sample were displayed in Figure 1 and Figure 2. For further analysis of the bacterial count test, the fourth dilution was chosen because it is easier to count and has sufficient colonies to compare with each sample.

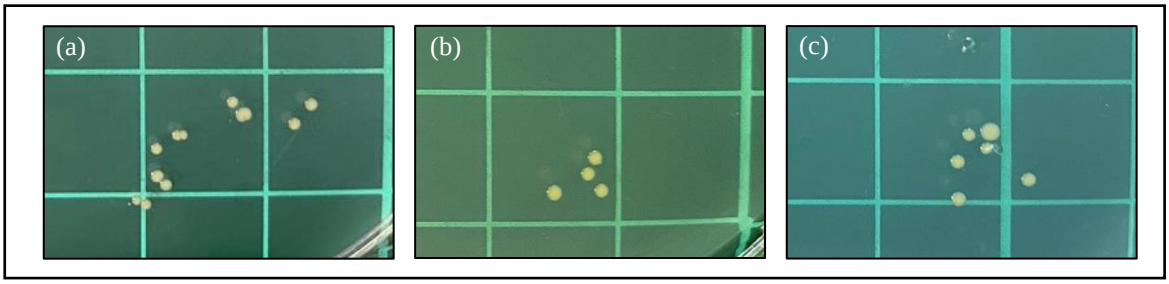

Figure 1. *S. aureus* colonies formed from each sample: (a) Control PLA (b) PLA-PDA and (c) PLA-PDA-everolimus

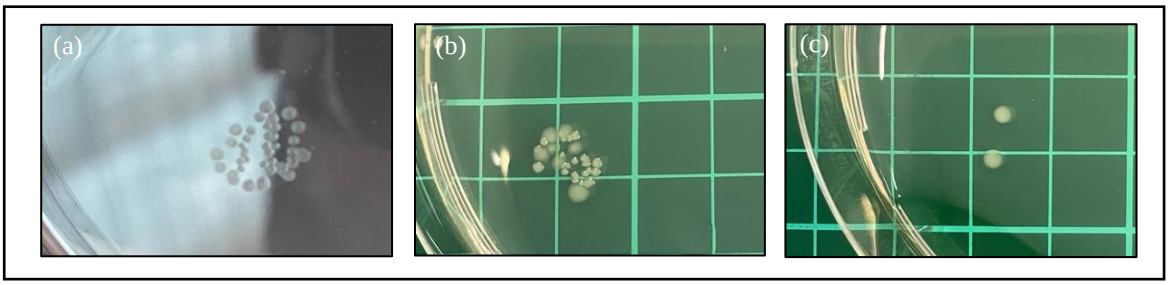

Figure 2. *E. coli* colonies formed from each sample: (a) Control PLA (b) PLA-PDA (c) PLA-PDA-everolimus

After manually counted the colonies, the CFU was calculated using Equation 3. CFU is defined as a single propagule that is viable and is capable of producing a single colony (a population of cells that can be seen with the naked eye) when grown on an appropriate semisolid growth medium [19]. The average CFU calculations from the three agar plates were later used for calculating the bactericidal ratio. The bactericidal ratio here refers to the number of bacteria killed compared to the bacteria in the control sample. Table 1 shows the bacterial count test results on the Gram-positive *S. aureus* and Gram-negative *E. coli*. Table 1 provides CFU average data and bactericidal percentages of PLA, PLA-PDA, and PLA-PDA-everolimus.

Table 1 - Bacterial count test results

Sample	Average of CFU ± SD	Bactericidal Percentage
	S. aureus	
PLA (Control)	$(8.33 \pm 2.31) \times 10^6$	0%
PLA-PDA	$(2.00 \pm 2.00) \times 10^6$	76.00%
PLA-PDA-everolimus	$(5.00 \pm 1.73) \times 10^6$	40.00%
	E. coli	
PLA (Control)	$(3.67 \pm 0.58) \times 10^6$	0%
PLA-PDA	$(7.67 \pm 1.53) \times 10^6$	0%
PLA-PDA-everolimus	$(2.67 \pm 1.15) \times 10^6$	27.27%

The bactericidal efficacy of different surfaces was evaluated against two types of bacteria: *S. aureus* (Gram-positive) and *E. coli* (Gram-negative). The surface coated solely with PDA exhibited a higher bactericidal percentage of 76% against *S. aureus*, whereas the PLA-PDA-everolimus surface achieved a bactericidal percentage of 40%. Conversely, the everolimus was proved more effective in killing *E. coli* than the surface grafted with PDA. Specifically, the PDA-coated surface failed to eliminate any attached *E. coli*, while the PDA and everolimus-coated surface achieved a bactericidal percentage of 27.27%. Several potential factors influence these results, including the structural differences between Gram-negative and Gram-positive bacteria, the binding mechanism between PDA and everolimus, and the antibacterial properties of PDA as the intermediate layer [19].

Gram-negative and Gram-positive bacteria are differed in their permeability towards antibacterial agents. Gram-negative bacteria possess a thin peptidoglycan layer, an outer membrane, and a cytoplasmic membrane. In contrast, Gram-positive bacteria lack an outer membrane and have a thick peptidoglycan layer surrounding the plasma membrane. This structural distinction requires antibacterial agents to traverse two lipidic membranes in Gram-negative bacteria, compared to just one membrane in Gram-positive bacteria. Consequently, Gram-negative bacteria exhibit higher resistance to antibacterial agents [20–22].

Moreover, the results were also influenced by the nature of everolimus and PDA as organic antibacterial agents. Organic antibacterial agents derived from organic material such as synthetic drugs or natural resources [4]. It is known that organic antibacterial agents do not strongly interact with the thin membrane layer of Gram-negative bacteria through ionization. Therefore, their primary antibacterial mechanisms do not involve electrostatic interactions between positively charged ions and negatively charged bacterial membranes. Instead, the action mechanisms of organic antibacterial agents are associated with organelle modification and disruption of bacterial intracellular biochemical pathways, which may explain their superior efficacy against Gram-positive bacteria compared to Gram-negative bacteria [4].

Another contributing factor to the observed results is the binding mechanism between the bacteria and antibacterial agents. The interaction between PDA and everolimus involved the formation of O–N bond between the hydroxyl (-OH) group of everolimus and the secondary amine of PDA. This interaction resulted in the release of hydrogen ions (H+) from the hydroxyl and secondary amine, leading to a decrease in the proportion of hydrogen (H) and an increase in the proportion of oxygen (O) on the surface [17]. Consequently, nucleation forms on the biomaterial surface but is not evenly distributed [17]. Bacteria can attach to the PDA-everolimus complex or the PDA film on the surface, leading to variations in the bacterial killing process and bactericidal ratios among the samples.

The characteristics of PDA as an antibacterial agent play a crucial role in the findings of this study. A critical property of PDA is, its contact-active antibacterial effect, primarily attributed to the presence of protic amine groups. When the positively charged functional groups of PDA came into contact with the negatively charged bacterial cell wall, bacterial lysis occurred, releasing cell contents [11]. It is important to note that Gram-negative and Gram-positive bacteria have different surface charges, with Gram-negative bacteria exhibiting higher negative surface charges. Consequently, the positive surface charge of PDA effectively killed Gram-positive bacteria but is insufficient to eliminate Gram-negative bacteria due to the disparity in surface charge. This disparity accounts for the observed outcomes in this study [20].

3.2 Disc inhibition test

A disc inhibition test was conducted to evaluate the release and efficacy of the antibacterial agent bound to the biomaterial surface. Nutrient agar plates were prepared with *S. aureus* and *E. coli* and then incubated for 24 hours. The presence of a clear area surrounding the sample, signifying reduced bacterial growth, and the formation of an inhibition zone confirm the successful release of antibacterial agents. Additionally, the absence of bacterial growth around the medium would further indicate the effectiveness of antibacterial agents [23].

The results of the disc inhibition test for the PLA, PLA-PDA, and PLA-PDA-everolimus samples are depicted in Figures 3 and 4. After 24 hours of incubation, no visible inhibitory zone was observed around the samples exposed to *S. aureus* and *E. coli*. Furthermore, no bacterial growth was detected beneath the samples. These findings suggest that everolimus was not released into the surrounding environment, demonstrating the ability of PDA as intermediate layer in controlling the release of the everolimus drug while pertaining everolimus capability as antibacterial agent.

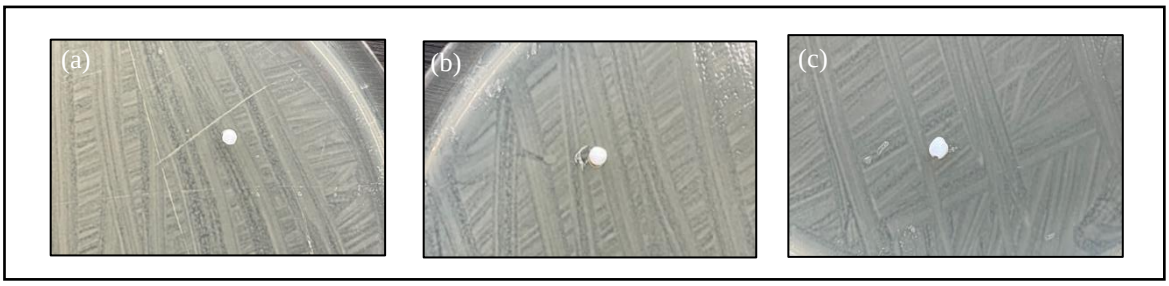

Figure 3. Results of *S. aureus* inhibition in each sample: (a) Control PLA (b) PLA-PDA and (c) PLA-PDA-everolimus

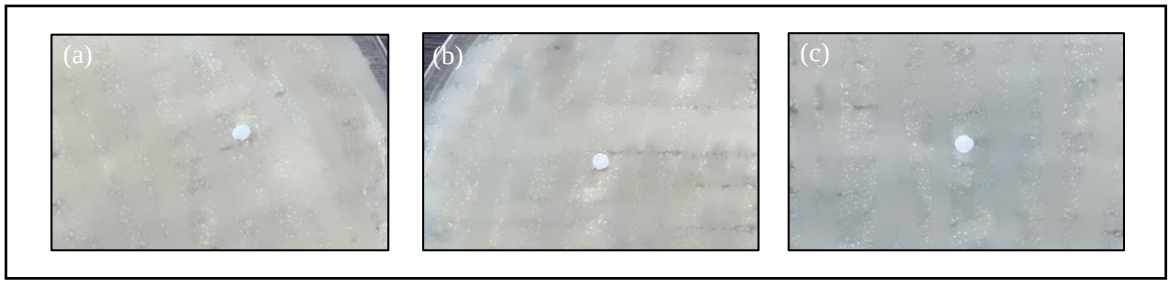

Figure 4. Results of *E. coli* inhibition for each sample: (a) Control PLA (b) PLA-PDA and (c) PLA-PDA-everolimus

Controlled release mechanisms can be categorized as both chemical and physical mechanisms. Physical mechanisms include drug diffusion through a polymer layer, polymer matrix dissolution or degradation determining the release rate, osmotic pressure-based release, ion exchange, and release of ionized pharmaceuticals. Chemical methods break covalent bonds between drug molecules and the delivery vehicle, such as polymer chains, through chemical or enzymatic processes [24].

Everolimus is commonly used in stent medications due to its immunosuppressive properties [10, 24]. However, the rapid release of everolimus can pose a risk by potentially delaying wound healing (endothelization) around the implantation site, leading to late-stage stent thrombosis [17]. To address this concern, immobilizing everolimus on stent surfaces offers a means of maintaining controlled drug release by forming cross-links for stable coating [10]. Polydopamine is highly favored for this purpose due to its exceptional capability to form robust chemical bonds with various base materials, making it versatile, cost-effective, and easily applicable. It contains two active functional groups, catechol and amine groups, which play a significant role in forming covalent bonds with a wide range of inorganic, organic, and metallic substrates. These covalent linkages are crucial in enabling PDA to stabilize and sustain the release of the immobilized molecules [10].

It has been demonstrated that a sample consisting of PLA, PDA, and everolimus can effectively control the release of the everolimus drug, assuming a sufficient drug concentration is utilized [10]. The successful disc inhibition test further supports releasing drugs as needed. Nevertheless, modifying the incubation time for the disc inhibition test can facilitate a more comprehensive investigation of the drug release mechanism to validate the hypotheses. These antibacterial surfaces are also well-suited for other medical applications, such as orthodontic braces, where they can eliminate bacteria while preventing chemical mixing with food.

4. Conclusion

This research demonstrates the potential antibacterial capabilities of PDA and Everolimus when applied to biomaterial surfaces. However, their effectiveness is influenced by various factors, including the characteristics of the bacteria, the specific antibacterial mechanisms of each agent, and the binding mechanism between PDA and everolimus. Notably, organic antibacterial agents like PDA and everolimus exhibit higher efficacy against Gram-positive bacteria compared to Gram-negative bacteria, which can be attributed to differences in cell wall structure and surface charge. The presence of nucleation on the surface also contributes to the variability observed in the results, suggesting the need for improvements in the coating process. Furthermore, PDA, serving as the intermediate layer, has successfully preserved both the release of everolimus from coated surfaces and the antibacterial capability of everolimus. To gain further insights into the antibacterial mechanisms of everolimus in different materials, future studies should explore its application on surfaces such as metals, ceramics, or other polymers, utilizing PDA as an intermediate layer.

Acknowledgment

This study was supported by UTMShine grant from Department of Deputy Vice Chancellor (Research & Innovation), Universiti Teknologi Malaysia, through the grant number Q.J130000.2451.09G93.

Conflict of Interest

The authors declare no conflict of interest.

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