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A study on the long term effect of biofilm produced by biosurfactant producing microbe on medical implant

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ABSTRACT

Low density polyethylene (LDPE) is used as a long term medical implant. Biofilm forming ability of two pathogenic microorganisms, namely, *Bacillus subtilis* (*B. subtilis*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) on this polymer and the differences in the properties of these matrices are studied for a year. There are very few long term studies on biofilms formed on medical implants. After three months, colonies of *B. subtilis* were two times higher when compared to those of *P. aeruginosa*. And at the end of one year, they were two orders of magnitude higher than the later. The exopolysaccharide (EPS) and biosurfactant recovered from the polymer surface after three months were 21 and 10.4 μg/cm^2 for *B. subtilis* and 13 and 8.6 μg/cm^2 for *P. aeruginosa*. After one year, these were higher in *B. subtilis* (50 and 37.1 μg/cm^2, respectively) than in *P. aeruginosa* (34.1 and 31.8 μg/cm^2, respectively). *B. subtilis* consisted of protein controlling the community and sporulation development, while *P. aeruginosa* had either housekeeping or metabolic proteins. The EPS in the respective biofilm consisted of biosurfactants produced by *B. subtilis* (surfactins, m/z = 1029 to 1134) and *P. aeruginosa* (rhamnolipids, m/z = 568 to 705). Thermogravimetric analysis indicated that LDPE incubated with these organisms underwent a weight loss of 4 and 3% after three months and 11.1 and 9.2% after one year, respectively at 435 °C. Laccase and manganese peroxidase were detected in the biofilm which could be involved in the degradation. The biosurfactant of these microorganisms altered the hydrophobicity of the surface, favoring their attachment and proliferation.

1. Introduction

The global biomaterial market is estimated to reach $88.4 billion by 2017, from $44.0 billion in 2012, while orthopedic implants is projected to reach US$ 46.5 billion by 2017 from an estimated US$ 21.1 billion in 2007 [1]. Polymers including polypropylene (PP), polyethylene (PE) and polytetra-fluoroethylene (PTFE) are high molecular weight materials for medical use. They are also used as negative control materials for ISO 10993-6 International Standard Biological Evaluation of Medical Devices [2]. Among the above polymers, LDPE is commonly used as a medical implant [3,4]. Its unique properties which include high abrasion resistance, low friction and high impact strength, excellent toughness, low density, ease of fabrication, biocompatibility and biostability make it an ideal candidate [5]. So it accounts for approximately one-tenth of disposable medical devices and a higher percentage in non-disposable surgical implants [6]. However, its surface is often colonized by bacterial biofilm leading to infection, inflammation and rejection [7,8]. Although hypoxic environment may prevail in the area of the infected implant located distant from the arterial blood flow, 7 to 10% of O_2 is sufficient for significant biofilm formation [9,10]. A variety of microorganisms may be involved as pathogens and their ability to adhere to materials and to promote formation of a biofilm depend on several factors [7].

Pseudomonas aeruginosa (Gram −ve) and *Bacillus* sp. (Gram +ve) are two well known pathogens which cause infections. The former is one of the notorious biofilm former that widely causes infections on medical implants [11,12], responsible for 4 to 6% of infected orthopedic devices [13]. *P. aeruginosa* represents 10% of all microorganisms involved in hip prosthesis infections [13]. *Bacillus subtilis* is a typically studied model organism in research [14], since Gram-positive bacteria are responsible for 50% of infections in the United States, and 60% of overall nosocomial infections [15,16]. Intra vascular implants cannot be removed for 3 months to 10 years in most of the cases [17]; hence, treatment of implant-associated infections without the removal of the device remains an only option. Once a biofilm has been formed on a surface, the bacteria inside the matrix are less exposed to the immune response of the host and are less susceptible to antibiotics, leading to long term infections.

In this paper the biofilm formed by two microorganisms, namely, *B. subtilis* and *P. aeruginosa* on LDPE surface and their subsequent effect on the polymer are studied over a period of twelve months. Both

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organisms produce biosurfactants, the former a mixture of cyclic lipopeptides and the latter, rhamnolipids [18]. Arutchelvi and Doble [19] have shown that the formation of biofilm requires the production of biosurfactant which serves specific developmental functions, including favoring the expression of adhesins on the outer surface thereby promoting both cell–cell and cell–surface adherence [20]. There is very little study reported on biofilm formed on medical devices in patients where the surgical device cannot be removed as a means of cure. Furthermore, there exist differences in antibiotic susceptibility between young, recently formed biofilm, and mature and old chronic biofilm [21]. Hence, long-term studies are helpful in determining the composition of the biofilms formed by different organisms and the mechanism of this process. Such knowledge can lead to treatment strategies or prevention techniques which are specific to the nature and type of biofilm. Polyethylene is chosen for this study, since implants made up of this polymer remain in the body for very long periods of time.

2. Materials and methods

2.1. Low density polyethylene (LDPE)

LDPE pieces (gifted by Reliance Industries Ltd., Mumbai, India) of size 1.15 ± 0.03 cm^2 and weighing 30 ± 3 mg were used for this study. All the chemicals used here were procured from HiMedia Laboratories, India.

2.2. Bacterial culture and incubation studies

B. subtilis YB7 and *P. aeruginosa* CPCL were isolated from polymer dump yard and petroleum contaminated soil, Chennai, India, respectively [18,22]. The composition of the minimal medium (1 l) used for culturing both the strains is KH_2PO_4 — 3 g, NaH_2PO_4 — 6 g, NH_4Cl — 2 g, NaCl — 5 g, glucose — 8 g, and $MgSO_4 \cdot 7H_2O$ — 0.1 g at a pH of 7.0 ± 0.2. 20 ml of the sterilized minimal medium and pre-weighed LDPE (weighing approximately 30 mg) were taken in a 60 ml boiling tube. 1% of 24 h grown pure bacterial culture (one of the above two species) was then inoculated (the CFU/ml count of this was brought to 10^7 by either dilution or concentration as required after overnight growth) [23]. The study was carried out for twelve months at neutral pH and at 35–37 °C temperature, under aerobic conditions at 180 rpm on an Orbitrek shaker (Scigenics Biotech, Chennai, India). Fresh sterilized minimal medium was replaced in the tubes once in every 30 days. All the reactions were performed simultaneously in triplicate. Tubes with polymers but without the culture were also run as control. The composition and morphology of the biofilms were determined at the end of one year as described below.

2.3. Biofilm morphology

The number of live bacteria in the biofilm was determined by serial dilution and spread plating technique and the results were interpreted in the form of colony forming units/cm^2 of LDPE (CFU/cm^2) [24]. The live and dead cells present on the polymer were visually observed using a Live/Dead® BacLight™ Bacterial Viability Kit (Invitrogen, Germany), which consisted of SYTO 9 and propidium iodide dyes. The former stained the live as well as the dead cells and the latter stained only the dead cells [18]. The polymer was stained with this dye mixture, incubated for 10–15 min in the dark and then the images were captured under a fluorescence microscope (Leica DM5000, Germany) with a blue filter at an excitation of 475 nm [24]. BATH assay was performed to determine the hydrophobicity of the bacterial cell surface [24]. LDPE with the biofilm was washed mildly in sterile distilled water and incubated with 3% glutaraldehyde for 1 h and again washed with various concentrations of ethanol (50, 60, 70, 80, 90 and 100%) for 30 min. Image of this surface was captured using a scanning electron microscope (Hitachi 3400N) after gold sputtering at 10 kV [25].

2.4. Biofilm studies

Polymer samples were removed from the media using sterile forceps and carefully washed with distilled water to remove loosely adhered bacteria [24]. Then the biofilm was removed from the surface by subjecting it to a mild water bath sonication in 1 ml of 0.85% saline, for a total of 4 min at 1 min intervals [26,27]. The carbohydrate and protein in this solution were estimated using phenol-sulfuric and Bradford methods respectively [28].

B. subtilis biofilm was acidified with 6 N of HCl to bring the pH to 2.0, [29] followed by extraction with equal volume of dichloromethane. The lower phase was collected and evaporated to concentrate the biosurfactant, surfactin. This was dissolved in 50 mM of sodium bicarbonate. In 100 μl of extract, pyrene was added (final concentration of pyrene as 1 μM) and the fluorescence was measured with a Jasco FP-6500 Spectrofluorometer. The biofilm solution produced by *P. aeruginosa* was adjusted to a pH of 2.0 with 6 M of H_2SO_4, followed by extraction with a mixture of chloroform and methanol (2:1) [30]. This extract was then concentrated by rotoevaporation, and the amount of biosurfactant, rhamnolipid, here was quantified using orcinol/sulfuric acid method [18]. Amount of laccase in the medium was determined as per a reported method with ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) as the substrate [31]. Amount of enzyme, manganese peroxidase produced by the microorganisms was determined according to a reported method with phenol red as the substrate [32]. One activity unit was defined as the amount of enzyme necessary to oxidize 1 μmol of substrate per minute. Thermo gravimetric weight loss of the polymer at the end of one year, which was a measure of the change in its crystallinity, was determined using a thermogravimetric analyzer (TGA), TA-Q 500 (Thermal Analyzer, model 204, Netzsch, Germany).

2.5. Statistical analysis

Data reported here were expressed as mean ± standard error (SE) of three samples.

3. Results and discussion

Medical implant related infection indicates that microorganisms are capable of adhering to foreign materials and survive by forming biofilm thereby causing pathogenicity [33–35]. Hence they escape the antibiotic treatment and host defense mechanisms including antibodies and phagocytes [36]. The communication between the Gram-positive and Gram-negative bacteria occurs through diffusible signal molecules. This results in the expression of genes associated with the production of specific enzymes, virulence factors and metabolites and leads to the development of microbial communities known as the biofilm [37]. The composition of the biofilm is greatly different from species to species and its analysis enables the control and the adhesion of the bacteria in different environments.

In this study, two bacterial species, namely *B. subtilis* and *P. aeruginosa* belonging to Gram-positive and Gram-negative, respectively, which are capable of producing biosurfactants are examined for their ability to form a biofilm on LDPE and their subsequent effect on the polymer. After three months, colonies of *B. subtilis* are two times higher when compared to those of *P. aeruginosa*. At the end of one year, colonies of *B. subtilis* are two orders of magnitude higher than those of *P. aeruginosa* (Table 1), probably because the former is a sporulating bacteria while the latter is a non-spore former. Sporulating bacteria are resistant to environmental stresses, enabling their proliferation during favorable conditions and remain dormant and alive during nutrient depleted conditions.