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Covalent immobilization of lysozyme onto woven and knitted crimped polyethylene terephthalate grafts to minimize the adhesion of broad spectrum pathogens

Bassam M. Al Meslmani [a], Gihan F. Mahmoud [a,b], Thomas Leichtweiß [c], Boris Strehlow [a], Frank O. Sommer [d], Michael D. Lohoff [d], Udo Bakowsky [a,*]

[a] Department of Pharmaceutical Technology and Biopharmaceutics, Marburg University, Ketzerbach 63, 35037 Marburg, Germany
[b] Department of Pharmaceutics and Industrial Pharmacy, Helwan University, Ain Helwan, 11795 Cairo, Egypt
[c] Institute of Physical Chemistry, Justus-Liebig-University Giessen, Heinrich-Buff-Ring 58, 35392 Giessen, Germany
[d] Institute for Medical Microbiology and Hospital Hygiene, Marburg University, Hans Meerwein Str 2, 35032 Marburg, Germany

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ABSTRACT

Graft-associated infections entirely determine the short-term patency of polyethylene terephthalate PET cardiovascular graft. We attempted to enzymatically inhibit the initial bacterial adhesion to PET grafts using lysozyme. Lysozyme was covalently immobilized onto woven and knitted forms of crimped PET grafts by the end-point method. Our figures of merit revealed lysozyme immobilization yield of 15.7 μg/cm^2, as determined by the Bradford assay. The activity of immobilized lysozyme on woven and knitted PET manifested 58.4% and 55.87% using *Micrococcus lysodeikticus* cells, respectively. Noteworthy, the adhesion of vein catheter-isolated *Staphylococcus epidermidis* decreased by 6- to 8-folds and of *Staphylococcus aureus* by 11- to 12-folds, while the Gram-negative *Escherichia coli* showed only a decrease by 3- to 4-folds. The anti-adhesion efficiency was specific for bacterial cells and no significant effect was observed on adhesion and growth of L929 cells. In conclusion, immobilization of lysozyme onto PET grafts can inhibit the graft-associated infection.

1. Introduction

The utilization of polyethylene terephthalate, (PET) (Dacron®) in cardiovascular prosthesis has given rise to critical infections, mainly prosthetic valve endocarditis (PVE) and prosthetic vascular graft infection (PVGI) [1–3]. The development of infection starts primarily by bacterial adhesion onto graft surface *via* weak, reversible, physical forces followed by an adhesion cascade to secure successful attachment. The adhered bacteria proliferate and develop an irreversible attachment forming dense microbial communities called biofilm [4]. The formed biofilm exhibits a great ability to evade host defenses and to resist antimicrobial therapy and the human immune system [4,5]. Studies on pathogenic organisms responsible for PET graft infections have shown that *Staphylococcus aureus* is the infecting organism in ~70% of cases, and *Staphylococcus epidermidis* encounters ~30% of the cases, resulting in prolonged hospitalization, graft failure, and patient death [6,7].

Different strategies have been developed to prevent bacterial colonization on cardiovascular prostheses after implantation. The most commonly used approaches are focused primarily on bonding of anti-infective agents onto-graft surface, mainly silver [8], quaternary ammonium [9] or antibiotics [10], in addition to systemic antibiotics as prophylaxis [11]. Alternatively, recent approaches to prevent bacterial adhesion and to eradicate the bacteria upon contact with the biomaterial surface were applied. For instance, strategies were used to block bacterial adhesion including; changing surface morphology [12], modifying surface features using either bio surfactants [13], or plasma [14]. In addition, soluble agents toxic to bacteria were used [15]. Nevertheless, previous strategies were limited by difficulties related to short active duration, high cost, and high host-cytotoxic potential even at low concentrations [16]. Furthermore, contradictory results were also reported by several studies concerning biofilm eradication [17,18].

Unlike previous studies, we aimed to inhibit the bacterial adhesion enzymatically using an enzyme with lytic activity against bacteria. Accordingly, an antibacterial graft resisting broad-spectrum pathogens was developed. Lysozyme, or 1,4-β-N-acetylmuramidase, is an enzyme

Abbreviations: PET, polyethylene terephthalate; PET-Enz, lysozyme immobilized polyethylene terephthalate; PET-COOH, polyethylene terephthalate functionalized with carboxyl group; PET-NH_2, polyethylene terephthalate grafted with ethylenediamine.

* Corresponding author.
E-mail addresses: almeslmanib@yahoo.com (B.M. Al Meslmani), mahmoudg@staff.uni-marburg.de (G.F. Mahmoud), Thomas.Leichtweiss@phys.Chemie.uni-giessen.de (T. Leichtweiß), strehlo4@staff.uni-marburg.de (B. Strehlow), sommerf@med.uni-marburg.de (F.O. Sommer), lohoff@med.uni-marburg.de (M.D. Lohoff), ubakowsky@aol.com (U. Bakowsky).

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that displays lytic activity especially for Gram-positive bacteria such as *S. aureus* and *S. epidermidis* [19,20]. Lysozyme damages the bacterial cell wall by catalyzing the β(1–4) glucosidic bonds of murein resulting in an effective protection against bacterial adhesion and biofilm formation. This strategy provides many advantages compared to antibiotic or other agents including long-term duration, nontoxicity, local application without adverse effect on normal flora and inability of bacteria to develop a resistance against the enzyme.

To the best of our knowledge, the covalent immobilization of lysozyme enzyme on woven and knitted forms of crimped PET cardiovascular grafts (Fig. 1) is rarely addressed. In the current study, a hen egg white lysozyme was immobilized by the covalent end-point attachment technique to maintain its chemical stability and bioactivity after the immobilization process.

The woven and knitted forms were surface-modified as schematically shown in Fig. 2. Denier reduction was carried out to selectively cleave the ester linkage within the PET surface, resulting in a free carboxyl group that was used as an anchor site for covalent immobilization of lysozyme. The surface-modified PET was characterized by FTIR and XPS. The immobilization efficiency of the enzyme was determined by the Bradford assay and its activity compared to free enzyme using *Micrococcus lysodeikticus* cells was detected. The bacterial anti-adhesion efficiency of the enzyme-immobilized grafts was studied against two Gram-positive (*S. aureus*, *S. epidermidis*) and one Gram-negative pathogen (*Escherichia coli*). The host cell behavior on the modified grafts was evaluated using mouse L929 fibroblasts as a model.

2. Materials and methods

2.1. Materials

Knitted and woven forms of crimped PET grafts (14 mm internal diameter, 30 cm length, 0.45 mm wall thickness, and 12 filaments per yarn bundle) were kindly provided by Vascutek GmbH, Germany. The bacterial strains *S. aureus* (ATCC 29213), *S. epidermidis* (isolated from a vein catheter of a patient), and *E. coli* (ATCC 25922) were supplied from the Institute of Medical Microbiology and Hospital Epidemiology, Marburg University, Germany. Lyophilized *Micrococcus lysodeikticus* (ATCC 4698) cells were purchased from Sigma Aldrich, Germany. Lyophilized powder lysozyme (EC 3.2.1.17) obtained from hen egg white (activity ≥40,000 units/mg protein), N-hydroxysulfosuccinimide (Sulfo-NHS), 1-Ethyl-3-(3-dimethyl amidopropyl) carbodiimide hydrochloride (EDC), glutaraldehyde, and ethylenediamine were purchased from Sigma Aldrich, Germany. Sodium hydroxide was supplied by Carl Roth, Germany. Paraformaldehyde, Tris buffer, methylene blue, and Giemsa stain were purchased from Merck, Germany. 2-(N-morpholino) ethanesulfonic acid (MES) was acquired from Serva, Germany. The mouse L929 fibroblast cell line was from DSMZ, Braunschweig, Germany. Dulbecco's modified Eagle's medium (DMEM), Earle's Balanced Salt Solution (EBSS), Fetal Bovine Serum (FBS), trypsin, streptomycin, penicillin, and amphotericin B were purchased from PAA Laboratories GmbH, Germany. L-glutamine was purchased from VWR, Germany. All other used chemicals were of analytical reagent grade.

2.2. Methods

2.2.1. Surface modification of crimped PET grafts

In the present study, two different forms of crimped PET cardiovascular grafts woven and knitted were used for surface modification (Fig. 1). All steps in the subsequent experiments were performed for each form of crimped PET graft.

To introduce a functional carboxyl group on the surface of the crimped PET grafts, modified Denier reduction was utilized as previously reported [21], and schematically illustrated in Fig. 2. Briefly, slices (2×2 cm^2) from woven and knitted forms of crimped PET grafts were immersed in 20 ml of 50% ethanol for 15 min in an ultrasonic water bath (Sonorex RK 100H, Bandelin, Germany) to clean the surface. Subsequently, the grafts were rinsed several times with distilled water under stirring and then oven dried at 55 °C for 2 h. The cleaned grafts were dipped in 20 ml of 1%NaOH solution and incubated in a boiling water

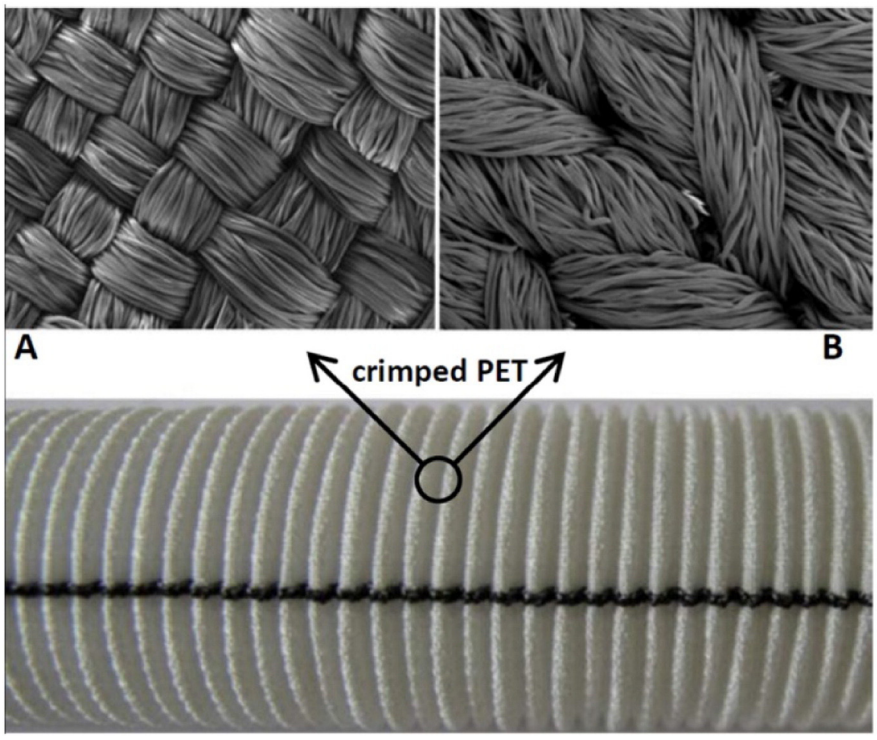

Fig. 1. SEM micrographs of woven (A) and knitted (B) crimped PET grafts. The multifilament PET threads in woven grafts are fabricated in an over-and-under pattern, while in knitted grafts are looped. The crimped technique, shown in the photographic picture, is utilized to increase the flexibility, distensibility, and kink-resistance of grafts.