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Linker-free covalent immobilization of heparin, SDF-1α, and CD47 on PTFE surface for antithrombogenicity, endothelialization and anti-inflammation

Ang Gao [a, b], Ruiqiang Hang [c], Wan Li [a], Wei Zhang [d], Penghui Li [b], Guomin Wang [a], Long Bai [c], Xue-Feng Yu [b], Huaiyu Wang [b, *], Liping Tong [b, **], Paul K. Chu [a, ***]

[a] Department of Physics and Materials Science, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong, China
[b] Institute of Biomedicine and Biotechnology, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, 518055, China
[c] Research Institute of Surface Engineering, Taiyuan University of Technology, Taiyuan, 030024, China
[d] Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, Beijing, 100190, China

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ABSTRACT

Small-diameter vascular grafts made of biomedical polytetrafluoroethylene (PTFE) suffer from the poor long-term patency rate originating from thrombosis and intimal hyperplasia, which can be ascribed to the insufficient endothelialization and chronic inflammation of the materials. Hence, bio-functionalization of PTFE grafts is highly desirable to circumvent these disadvantages. In this study, a versatile "implantation-incubation" approach in which the biomedical PTFE is initially modified by plasma immersion ion implantation (PIII) is described. After the N_2 PIII treatment, the surface of biomedical PTFE is roughened with nanostructures and more importantly, the abundant free radicals generated underneath the surface continuously migrate to the surface and react with environmental molecules. Taking advantage of this mechanism, various biomolecules with different functions can be steadily immobilized on the surface of PTFE by simple solution immersion. As examples, three typical biomolecules, heparin, SDF-1α, and CD47, are covalently grafted onto the PTFE. In addition to retaining the bioactivity, the surface-functionalized PTFE exhibits reduced thrombogenicity, facilitates the recruitment of endothelial progenitor cells, and even alleviates the inflammatory immune responses of monocytes-macrophages and is thus promising to the development of small-diameter prosthetic vascular grafts with good long-term patency.

1. Introduction

Prosthetic vascular grafts, particularly those made of expanded polytetrafluoroethylene (PTFE), have been utilized clinically for decades due to merits such as the excellent mechanical strength, tunable structure, and sufficient availability. The vascular prostheses are more acceptable by patients, as they can circumvent the additional surgical procedures and donor site morbidity by autologous vascular grafts [1,2]. Nevertheless, commercial PTFE grafts are only clinically successful as replacements of large-caliber vessels (>6 mm). In applications involving small-diameter vascular grafts, they generally suffer from the poor long-term patency rate and therefore fail to meet rigorous clinical requirements [1,3].

Luminal thrombosis and intimal hyperplasia are the main causes of poor long-term patency of prosthetic vascular grafts. Endothelialization of graft lumen is an effective strategy to prevent the luminal thrombosis and intimal hyperplasia of PTFE grafts [4–6] and the long-term patency can be significantly improved as a result. Since spontaneous endothelialization of pristine PTFE grafts is impossible *in vivo* [7], a tissue-engineered method by pre-seeding grafts with autologous endothelial cells has been proposed and is quite successful [8,9]. Nevertheless, its widespread clinical adoption is plagued by the high production cost, complicated procedures, and particularly the need for a lead time of up to

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: hy.wang1@siat.ac.cn (H. Wang), lp.tong@siat.ac.cn (L. Tong), paul.chu@cityu.edu.hk (P.K. Chu).

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several months [2,10]. In this respect, off-the-shelf grafts that facilitate rapid endothelialization *in situ* are highly desirable [11,12]. Circulating endothelial progenitor cells (EPCs), which can be mediated by various chemokines [13,14] including stromal cell-derived factor-1α (SDF-1α) [15,16], is essential to spontaneous endothelialization of prosthetic vascular grafts. By involving SDF-1α, prosthetic grafts can attain spontaneous endothelialization *via* recruitment of EPCs from the blood stream [17,18]. On the other hand, intimal hyperplasia, a result of the migration, over-proliferation, and matrix synthesis of native smooth muscle cells (SMCs), is provoked by mechanical stretching and chronic inflammatory response at the anastomotic site [19,20]. Immobilization of the transmembrane protein CD47 is another strategy to attenuate intimal hyperplasia [21,22], as the inflammatory response of prosthetic grafts can be effectively alleviated by the competence of CD47 as a "marker of self" [23]. In addition, luminal thrombosis can be ascribed to the intrinsic thrombogenicity of vascular prostheses and surface heparinization of prosthetic grafts has been proven successful in thrombus prevention [24,25].

Among the various techniques for surface modification of prosthetic grafts with biomolecules, covalent immobilization is a good choice on account of the long-term persistence of biomolecules after functionalization [26,27]. Nevertheless, owing to the prominent chemical inertness of PTFE, covalent modification of grafts by wet chemical methods is quite difficult. Alternatively, physical techniques such as plasma treatments have been demonstrated to alter the surface properties of biopolymers [28–31]. In particular, plasma immersion ion implantation (PIII) is a simple and effective technique to tailor the surface chemistry and topography of PTFE [32,33]. It is expected that covalent immobilization of biomolecules on PTFE is facilitated by PIII albeit in the absence of chemical cross-linkers [34,35].

In this study, PTFE which has undergone N_2 PIII is further functionalized by covalent immobilization of heparin, CD47, and/or SDF-1α, and the antithrombotic capability, endothelialization potential, as well as anti-inflammatory functions are investigated systematically. From the perspective of reduced thrombogenicity, rapid endothelialization, and low inflammatory immune response, PTFE after surface functionalization is promising enabling the development of small-diameter prosthetic vascular grafts with good long-term patency.

2. Materials and methods

2.1. Materials

The heparin sodium salt (from porcine intestinal mucosa, Grade I-A, ≥180 USP units/mg), toluidine blue O (TB), lipopolysaccharides (LPS, from *Escherichia coli*), AMD3100, thiazolyl blue tetrazolium bromide (MTT), horseradish peroxidase (HRP), paraformaldehyde (PFA), sodium dodecyl sulfate (SDS), Triton X-100, bovine serum albumin (BSA), and FITC labeled phalloidin were produced by Sigma-Aldrich, USA. The human TNF-α Valukine ELISA kit and human CXCL12/SDF-1α Quantikine ELISA kit were purchased from R&D systems, USA. The rabbit monoclonal vinculin antibody, Alexa Fluor 546 conjugated goat anti-rabbit IgG secondary antibody, fetal bovine serum (FBS), Dulbecco's modified eagle medium (DMEM), and RPMI 1640 medium were obtained from Life technologies, USA. The lactate dehydrogenase (LDH) assay kit, phorbol-12-myristate-13-acetate (PMA), 4′,6-diamidino-2-phenylindole dyes (DAPI) were obtained from Beyotime Biotechnology, China, and the recombinant human SDF-1α was obtained from Peprotech, USA. The recombinant human CD47 protein was acquired from Abcam, USA, and EGM-2 BulletKit was obtained from Lonza, Switzerland.

2.2. N_2 PIII treatment of PTFE

The PTFE sheets purchased from Goodfellow (0.25 mm in thickness) were cut into circular disks with a diameter of 15 mm. The substrates were ultrasonically cleaned in acetone, ethanol, and distilled water before inserting into the vacuum chamber of the gas PIII equipment. N_2 PIII was performed on the insulating PTFE samples according to procedures described previously [32]. Briefly, the PTFE samples were mounted on the sample stage which was connected to a negative high-voltage power supply. A stainless steel mesh placed 1 cm above the PTFE surface was electrically grounded to allow the plasma to diffuse through and be accelerated to the samples. High purity N_2 was introduced into the chamber to maintain a working pressure of 9.0×10^{-2} Pa. The N_2 plasma were generated by a radio frequency (RF) power of 1000 W and N_2 PIII was carried out for 3 h by applying a pulsed negative bias to the sample stage with the voltage of −15 kV, frequency of 500 Hz, and pulse duration of 20 μs.

2.3. Sample characterization

Scanning electron microscopy (SEM, JSM 7001F, JEOL, Japan) and atomic force microscopy (AFM, NanoScope V MultiMode system, Veeco, USA) were employed to evaluate the surface topography. The samples were dried and sputter-coated with gold prior to SEM observation and the surface roughness was determined by AFM. The static contact angles were measured with 5 μl of distilled water by the sessile drop method on the Rame'-Hart instrument (USA) under ambient conditions and X-ray photoelectron spectroscopy (XPS) was conducted on the Physical Electronics PHI 5802 (USA) equipped with a monochromatic Al K_α source.

2.4. Immobilization of biomolecules

The procedures for immobilization of heparin, CD47 and SDF-1α are schematically illustrated in Fig. 1. Briefly, the PTFE samples after undergoing N_2 PIII were degassed in the phosphate-buffered saline (PBS) solution and immersed in 500 μg/ml of the heparin solution or binary solution containing 500 μg/ml heparin and 1 μg/ml CD47 for 12 h at 4 °C. Afterwards, the samples were taken out, rinsed with PBS, and dipped into a 1 μg/ml SDF-1α solution for another 12 h at 4 °C. The above modified samples were designated as PTFE, N2, N2-Hep, N2-Hep-SDF, and N2-Hep-SDF-CD47, respectively.

2.5. Determination of heparin

The amount of immobilized heparin was determined by the modified TB method [36]. The TB solution was prepared by dissolving the TB reagent (0.005 w/v%) and NaCl (0.2 w/v%) in HCl (0.01 M). Using 24-well tissue culture plates as the holders, a series of 250 μl standard heparin solutions and various samples with 250 μl PBS solution were separately incubated with 250 μl of TB solution at room temperature for 30 min to allow the formation of TB-heparin complex. Subsequently, solutions were collected and mixed with 500 μl of n-hexane vigorously. The TB-heparin complex was extracted into the organic phase while the unreacted TB remained in the aqueous phase. 200 μl of the aqueous phase were transferred to a 96-well plate and the absorbance at 631 nm was monitored on a microplate spectrophotometer (Eon, Biotek, USA). According to the standard curve obtained with standard heparin solutions, the absolute amount of heparin immobilized on each sample was calculated.

To determine the shelf-life, the N2 samples were stored under ambient conditions for 72 and 198 days prior to heparin solution incubation and subsequent quantification of immobilized heparin.