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Penicillin impregnation on oxygen plasma surface functionalized chitosan/*Antheraea assama* silk fibroin: Studies of antibacterial activity and antithrombogenic property

Arup Jyoti Choudhury [a,*], Dolly Gogoi [b,1], Raghuram Kandimalla [c,1], Sanjeeb Kalita [c], Yogesh B. Chaudhari [d], Mojibur R. Khan [d], Jibon Kotoky [c], Joyanti Chutia [b]

[a] Department of Physics, Tezpur University, Napaam, Sonitpur 784028, Assam, India
[b] Physical Sciences Division, Institute of Advanced Study in Science and Technology, Paschim Boragaon, Garchuk, Guwahati 781035, Assam, India
[c] Drug Discovery Lab, Institute of Advanced Study in Science and Technology, Guwahati 781035, Assam, India
[d] Molecular Biology and Microbial Biotechnology Lab, Institute of Advanced Study in Science and Technology, Guwahati 781035, Assam, India

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ABSTRACT

Low temperature plasma can effectively tailor the surface properties of natural polymeric biomaterials according to the need for various biomedical applications. Non-mulberry silk, *Antheraea assama* silk fibroin (AASF) is a natural polymer having excellent biocompatibility and mechanical strength yet unlike mulberry silk, *Bombyx mori* silk fibroin, has drawn less interest in biomedical research. In the quest for developing as potential biomaterial, surface functionalization of plasma induced chitosan (Cs) grafted AASF ($(AASF/O_2–Cs)_g/O_2$) yarn is carried out using oxygen (O_2) plasma. The $(AASF/O_2–Cs)_g/O_2$ yarn exhibits enhanced antithrombogenic property as well as antimicrobial activity against Gram positive (*Bacillus subtilis*) and Gram negative (*Escherichia coli*) bacteria as compared to AASF yarn. Moreover, impregnation of antibiotic drug (penicillin G sodium salt, PEN) on $(AASF/O_2–Cs)_g/O_2$ yarn further improves the observed properties. *In-vitro* hemolysis assay reveals that O_2 plasma treatment and subsequent impregnation of PEN do not affect the hemocompatibility of AASF yarn. The present research findings demonstrate that plasma induced grafting of Cs followed by penicillin impregnation could significantly improve the potential applicability of AASF in the field of surgical research.

1. Introduction

Low temperature plasma technology for surface modification of polymers has drawn a great deal of interest in the field of biomedical research [1–3]. The proper choice of polymers in various biomedical applications is very crucial as these polymers very often do not possess the surface properties that satisfactorily meet their biological responses including biocompatibility, bioactivity, non-cytotoxicity, hemocompatibility and antibacterial activity [2,3]. It therefore requires modifying the specific surface properties like hydrophobicity/hydrophilicity, surface morphology, chemical structure, cross-linking density, conductivity, etc. of polymers to fulfill certain specific requirements for successful utilization in biomedical applications. Through plasma technology it is possible to achieve surface modification of polymers without the use of any solvent and chemicals [1]. In this regard, plasma technology can be a better alternative to chemical treatment in terms of more environmentally friendly nature, low processing temperature (<100 °C) and less consumption of chemical, energy and time [4]. Another advantage of plasma technology is that it allows the selective treatment of a polymer surface and that the surface modification takes place up to few nanometer depths without influencing the bulk properties of the polymer [4].

Natural polymers like silk fibroin (SF) and chitosan (Cs) are considered as promising biomaterials due to their favorable properties such as biocompatibility, biodegradability and mechanical and physical properties [5,6]. SF, the natural fibrous protein produced by silkworm is broadly classified into two main types, (a) mulberry produced by bombycid species, *Bombyx mori* (*B. mori*), and (b) non-mulberry having several varieties including *Antheraea assama* (*A. assama*) [7]. *B. mori* SF (BMSF) has been widely characterized and used as a suture for centuries whereas the potential applicability of *A. assama* SF (AASF) as biomaterial is yet to be fully explored [8,9]. AASF is well known for some of its distinctive properties such as highest tensile strength among all natural silk, hydrophobicity, acid resistivity, UV resistance, low dye uptake behavior, high durability, etc. and is superior to BMSF in terms of physico-chemical, thermal and biological properties [9]. All such properties make AASF an interesting biomaterial for extensive research work. The effective applicability of AASF as biomaterial can be further achieved by improving its certain properties such as tensile strength, antithrombogenicity and antimicrobial activity by means of surface modification process through plasma treatment and/or grafting of a polymer [9]. For improving

* Corresponding author.
E-mail address: arupjchoudhury@gmail.com (A.J. Choudhury).
[1] Dolly Gogoi and Raghuram Kandimalla contributed equally to this work.

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desired properties through plasma polymer grafting, Cs can be a proper choice of a polymer as it is biocompatible and has active sites capable of interacting with SF macromolecules [10]. Cs has impressive roles in wound healing process and generates a minimal foreign body response with accelerated angiogenesis [11]. By combining the properties of Cs and AASF, development of a new advanced functionalized biomaterial can practically be realized. Besides, the surface properties including hydrophobicity of Cs grafted AASF can further be modified through plasma treatment for improving impregnation of proteins, enzymes, biomolecules, antibiotic drugs, etc. in order to meet specific biomedical application depending on an end user requirement.

In this present work surface functionalization of Cs grafted AASF ($(AASF/O_2–Cs)_g/O_2$) yarn is carried out using low temperature oxygen (O_2) plasma followed by impregnation of antibiotic drug with an aim to improve its antithrombogenic property, biocompatibility and antimicrobial activity. In this work penicillin G sodium salt (PEN) is chosen as an antibiotic drug as it is a natural β-lactam antibiotic and is commonly used for treatment of skin, ENT (ear, nose and throat) and respiratory infections caused by certain bacteria such as *Staphylococcus aureus* (*S. aureus*), *Bacillus subtilis* (*B. subtilis*), *Escherichia coli* (*E. coli*), etc. Besides, PEN has advantages over semisynthetic penicillin in terms of low cost and low toxicity [12]. The results obtained from various characterization techniques are further discussed in this work and compared with antithrombogenic property and antimicrobial activity of AASF before and after plasma induced Cs grafting followed by impregnation of PEN.

2. Experimental

2.1. Materials

A. assama silk cocoons are provided by Central Silk Board, Assam, India. Before O_2 plasma treatment the cocoons are degummed twice with sodium carbonate (Na_2CO_3, Merck, Germany) solution at 100 °C for 45 min and washed thoroughly with deionized water. The AASF fibers are extracted from the degummed silk cocoons and reeled together to form yarns. The AASF yarns are then rinsed three times in deionized water, dried in ambient environment (temperature: 27 °C; relative humidity (RH): 65%) and stored in a desiccator prior to use. O_2 gas (purity: 99.995%, Assam Air Products, Assam, India) is used for plasma generation and surface functionalization of Cs grafted AASF yarn.

Chitosan (Cs, 85% deacetylated, molecular weight: 4.0×10^4) and penicillin G sodium salt (PEN) are purchased from Sigma-Aldrich, Germany. Water used in experiments is purified by a Millipore (Elix 1/Milli-Q) purification system.

2.2. Methods

2.2.1. O_2 plasma treatment of AASF yarn

Surface functionalization of AASF yarn using O_2 plasma treatment is carried out in a stainless steel horizontal cylindrical chamber of 30 cm diameter and 100 cm length. Prior to O_2 plasma treatment the chamber is evacuated to a base pressure of 1×10^{-3} mbar using a rotary pump (pumping speed: 21 $m^3\ h^{-1}$). The pressure inside the vacuum chamber is measured by a capacitance manometer (MKS, PDR2000, Singapore) while the flow rate of O_2 is monitored by mass flow controller (MFC, Aalborg, USA). Uniform and stable plasma discharge is obtained using an RF generator (Seren, 0–300 W, USA) that is connected to a water cooled RF electrode through an L-type matching network. The AASF yarn is placed on the surface of the RF electrode and O_2 is introduced into the chamber through a gas shower plate that is placed 5 cm above the electrode. In the present wok, the plasma parameters are optimized for imparting hydrophilicity to AASF yarn through formation of nano-structured surface and accordingly the surface treatment of AASF yarn is carried out for 45 s at RF power value of 5 W and working pressure of 2×10^{-1} mbar. After O_2 plasma treatment, the sample ($AASF/O_2$ yarn) is transferred to a vacuum desiccator for further characterization.

2.2.2. Preparation of Cs solution

Cs solution with concentration of 2% (*w*/*v*) is prepared by dissolution of Cs in 0.2 M acetic acid. The mixture is stirred at 50 °C for 2 h by a magnetic stirrer (500 rpm) to obtain a homogeneous polymer solution followed by filtration through a filter paper (Whatman, grade 1) to remove air bubbles trapped in the viscous liquid. For various characterization purposes, Cs film is prepared by casting the solution onto 50 mm glass petri dish (Borosil, India). The film so obtained is then air-dried for 24 h in ambient environment (temperature: 25 °C, RH: 50–55%). The dried film is neutralized with 0.5 N NaOH solution, washed with deionized water, and then dried at ambient environment.

2.2.3. Plasma induced grafting of Cs on $AASF/O_2$ and impregnation of PEN

The procedures of O_2 plasma induced grafting of Cs on $AASF/O_2$ yarn followed by impregnation of PEN involve three steps as shown in the schematic (Fig. 1). In step 1, $AASF/O_2$ yarn of 90 cm in length is placed in Cs solution for 24 h under static condition. After taking out from Cs solution, the Cs attached $AASF/O_2$ ($AASF/O_2–Cs$) yarn is washed with deionized water to remove loosely attached Cs from the surface and allowed to dry in a vacuum at 40 °C for 24 h and then subjected to O_2 plasma treatment (step 2) which results in simultaneous grafting of Cs on $AASF/O_2$ ($(AASF/O_2–Cs)_g/O_2$) as well as surface functionalization of $(AASF/O_2–Cs)_g/O_2$ yarn. The plasma treatment conditions for processing $(AASF/O_2–Cs)_g/O_2$ yarn remain the same as that described in plasma treatment of AASF yarn. The amount of Cs grafted is calculated from the weight difference (dry) between $(AASF/O_2–Cs)_g/O_2$ and $AASF/O_2$ yarn using an analytical balance (Shimadzu, ATX, resolution: 0.01 mg) at ambient environment. In this work, five equal lengths (90 cm) of $AASF/O_2$ yarns are placed in different Cs solutions of same concentration (2% *w*/*v*) and the amount of Cs grafted on the surface of each $AASF/O_2$ yarn after O_2 plasma treatment is calculated to be 763 μg, 765 μg, 752 μg, 766 μg and 755 μg respectively. From these data, the mean of the amount of Cs grafted on $AASF/O_2$ yarn is evaluated and expressed as mean ± SD (standard deviation). Accordingly, the amount of Cs grafted on $AASF/O_2$ yarn is determined as (760 ± 6) μg. In step 3, the $(AASF/O_2–Cs)_g/O_2$ yarn is then placed in PEN solution for 24 h at ambient condition (temperature: 27 °C, RH: 65%) with constant shaking (50 rpm). The PEN solution is prepared by dissolving 1 mg of PEN in 10 ml of deionized water. After taking out from the solution, the PEN impregnated $(AASF/O_2–Cs)_g/O_2$/PEN yarn is dried under a vacuum for 24 h and then transferred to a desiccator for further characterization. The amount of PEN impregnated on $(AASF/O_2–Cs)_g/O_2$ yarn is calculated using the following formula [13]:

$$\text{Drug impregnation}(\mu g) = W_1 - W_2 \quad (1)$$

where W_1 is the total weight of PEN dissolved in DD water and W_2 is the total weight of remaining PEN in DD water after removal of $(AASF/O_2–Cs)_g/O_2$ yarn. W_2 is calculated from concentration values obtained from the calibration curve on UV spectrophotometric analysis of the samples at the absorbance peak with a wavelength of 264 nm corresponding to PEN. The amount of PEN impregnation calculated from Eq. (1) is found to be 845 μg, 850 μg, 855 μg, 859 μg and 844 μg respectively for each of the $(AASF/O_2–Cs)_g/O_2$ yarn. From these data, the mean of the amount of PEN impregnated on $(AASF/O_2–Cs)_g/O_2$ yarn is evaluated and expressed as mean ± SD. Accordingly, the amount of PEN impregnated on $(AASF/O_2–Cs)_g/O_2$ yarn is determined as (850 ± 7) μg. In this work, no impregnation of PEN on AASF and $AASF/O_2–Cs$ yarns is observed after the yarns are placed in PEN solution for aforementioned period and studied using UV spectrophotometric analysis (Eq. (1)).