New Approach Towards m-PEG Grafting Onto Commercially Available Nylon 6 To Resist Bacterial Adhesion On Surface

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Abstract: Biofilm formation, caused particularly by pathogenic bacteria like methicillin-resistant Staphylococcus aureus (MRSA) on medical devices, is imposing threat to public health. There is thus an ever growing demand for designing materials that are both cytocompatible and resistant to biofilm formation as well as bacterial infections. Surface functionalized polyamides, such as Nylon 6, are widely used as biomaterial due to its strength, flexibility, toughness and cytocompatibility. The undertaken study is focused on the surface functionalization of Nylon 6 by reducing the surface with borane-tetrahydrofuran complex (BH₃-THF), followed by grafting with poly(ethylene glycol) methyl ether tosylate (mPEG-OTs) via a novel lithiation approach. The modified Nylon 6 surfaces were characterized by various techniques such as water contact angle (WCA) analysis, atomic force microscopy (AFM), scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR) to confirm the modification of Nylon 6 surface. Evaluation of bacterial adhesion on the pure and modified surface against biofilm active pathogenic bacterial strain: Staphylococcus aureus (S.A.) CCM 3953 was accomplished. The functionalized Nylon 6 surfaces showed significant resistance towards bacterial adhesion compared to pure Nylon 6.

Keywords — grafting, lithiation, Nylon 6, pathogenic bacteria, surface functionalization.

I. INTRODUCTION

THE successes in modern biomedical industry is closely related to the increasing field of polymeric biomedical devices [1]. Despite the considerable success in biomedical field, one of the key challenges is the device associated infections due to microbial attachment to the polymeric surface leading to biofilm formation [2]. Amongst various pathogenic bacteria, methicillin-resistant Staphylococcus aureus (MRSA) has been a great concern in health sectors [3]. There are various approaches to deal with this problem by controlling hydrophobicity, surface roughness, surface functional groups and electrostatic interactions [4]. One of the key strategies is to prevent bacterial adhesion by immobilizing biocompatible hydrophilic polymer poly(ethylene glycol) (PEG) and its derivatives such as poly(ethylene glycol) methyl ether (mPEG) [5,6]. The research studies have reported that higher molecular weight PEG and longer brushes can resist bacterial adhesion more strongly [7].

The newly developed synthetic polymers like polyesters and polyamides were introduced in medical application during World War II [8]. Nylon 6 is widely used in catheters, wound dressing materials, suture material, dialysis membrane etc. but without surface modification, it may cause other complications like thrombogenesis [9]. Therefore, surface functionalization with specific functional groups is necessary to improve the performance as biomaterial. Both the physical (UV radiation or plasma) and chemical methods can be used for surface functionalization whereas, both the methods have their own advantages and disadvantages [10,11].

The present research is focused on amide group reduction on Nylon 6 surface with borane complex (BH₃-THF) [12], followed by a novel method of grafting poly(ethylene glycol) methyl ether (mPEG) onto reduced Nylon 6 surface using poly(ethylene glycol) methyl ether tosylate (mPEG-OTs) and tert-butyllithium (t-BuLi). The changes in WCAs of modified Nylon 6 surface indicated the surface morphology alteration that was further
confirmed by atomic force microscopy (AFM), scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). The bacterial adhesion assessment was performed using standard protocol CSN EN ISO 6222.

II. PROCEDURES

A. MATERIALS

Nylon 6 film, with the thickness of 0.015 mm, was supplied by Goodfellow Cambridge Ltd., (Huntingdon, England). Borane-Tetrahydrofuran complex (1 M, BH3-THF), poly(ethylene glycol) methyl ether tosylate (Mn = 2000 g/mol, mPEG-OTs), tert-butyl lithium (1.7 M in hexane, t-BuLi) and THF (anhydrous, ≥99.9%, inhibitor-free) were obtained from Sigma-Aldrich Co. Solvents tetrahydrofuran (99.8% THF), dimethyl sulfoxide (99% DMSO), acetone (99.5%), ethanol (99%), 2-propanol (99.8%), hexane (99.9%) and hydrochloric acid (35% HCl) were purchased from Lach: Ner, s.r.o., CZ. Sodium hydroxide (NaOH) was supplied by PENTA Ltd. CZ. All washings were performed by deionized water.

Staphylococcus aureus (S.A.), CCM 3953 strain was used for bacterial adhesion tests. Soybean Casein Digest Medium: HIMEDIA® REF, Plate Count Agar without Dextrose (BIORAD a.s., CZ) and sterile physiological solution (6% NaCl) were used for bacterial inoculum preparation. Globular protein Bovine Serum Albumin (BSA, Sigma-Aldrich, CZ) was used as a support for better S.A proliferation.

B. METHODS

Sample preparation

Nylon 6 samples (size 2 cm × 2 cm), were thoroughly rinsed with water, ethanol, 2-propanol, acetone, THF and hexane in sonicating bath for 3 mins per solvent. The washed samples were dried at 50°C/3 h in vacuum and stored for further use.

Nylon 6 surface reduction and grafting

The amide functional groups present in Nylon 6 were reduced to secondary amine by BH3-THF according to procedure described by Herrera-Alonso et al. [12]. The reduced samples have been referred as Nylon 6-NH. The modified samples were washed with THF, 1 M HCl, deionized water, 1 M NaOH, deionized water, THF, ethanol, acetone and hexane, each by sonication for 3 minute. The reduced Nylon 6 films were dried at 50°C overnight in vacuum and used for grafting. Dry THF (50 ml) was added into the Schlenk flask containing Nylon 6-NH samples. Then t-BuLi (0.6 ml, 1 ml and 2 ml) was introduced into it for 2 h lithiation at r.t./250 rpm in inert atmosphere. After lithiation, the solution of remaining t-BuLi was removed carefully by syringe, maintaining the inert condition. 2g (1 mmol) mPEG-OTs, dissolved in dry THF (50 ml), was added into the same flask and allowed to react overnight. The grafted samples were washed with THF, ethanol, acetone and hexane; dried in the vacuum. Modified samples have been referred as Nylon 6-N-mPEG.

Surface characterization

The WCAs was measured using a portable computer-based instrument with special purpose software following ISO 27448:2009 test method (See System E, Advex Instruments, CZ). The topography of the unmodified and modified Nylon 6 films was studied in the air at atmospheric pressure with NanoWizard® 3 NanoScience AFM (JPK Instruments, Germany). The surface morphology was determined as secondary electron images (1 kV) by SEM (ZEISS, Sigma Family, Germany). The Nicolet™ iS™10 FT-IR Spectrometer (Thermo Scientific™, USA) was used to examine the changes in surface composition after the modifications of Nylon 6.

Bacterial adhesion test

UV A sterilized pure Nylon 6, Nylon 6-NH and Nylon 6-N-mPEG samples were added into 50 ml of bacterial inoculum homogenous solution (3.0 x 10^7 CFU/ml). Total 5 sets were prepared with S. aureus (S.A.). The bacterial solutions, as well as control, were incubated for 48 h at 37° C. Each sample was taken out from the inoculum and transferred to 100 ml Fisher bottle containing 20 ml sterile physiological solution (6% NaCl). The bottles were shook vigorously for an hour to remove surface adhered bacteria in solution. 1 ml each of the solution at different concentrations (10^0, 10^-1, 10^-2, 10^-3 and 10^-4) was directly used for bacterial proliferation in agar medium and subsequently stored in the incubator 48 h/37° C. In the case of control, the concentration line was as follows (10^0
\(10^6 = 3.0 \times 10^7\) CFU/ml. The results are mentioned in TABLE III.

### III. RESULTS

The reduction of amide functional groups on Nylon 6 surface to secondary amine (Nylon 6-NH), followed by grafting onto Nylon 6-NH forming Nylon 6-N-mPEG are shown in schematic representation (Fig. 1).

![Fig. 1. Schematic presentation of Nylon 6 surface modification to Nylon 6-N-mPEG.](image)

The WCAs measurements with standard deviations (± SD) related to the unmodified and modified Nylon 6 films are presented in TABLE I. The significant increase in WCAs was noticed after Nylon 6 film modification into Nylon 6-NH. On the contrary, the mean WCAs have been decreased significantly, as expected due to the mPEG chain immobilization, after grafting with mPEG-OTs via lithiation. The \(t\)-BuLi concentration played an important role in grafting intensity, thus differed the WCAs of three different grafted samples. Nylon 6-NH activated by 0.6 ml \(t\)-BuLi is more hydrophilic after grafting compared to the other two grafted samples activated with higher concentrations of \(t\)-BuLi.

| Sample                      | Mean WCA (°) ± SD |
|-----------------------------|-------------------|
| Nylon 6                     | 71.4 ± 4.3        |
| Nylon 6-NH                  | 82 ± 4.8          |
| Nylon 6-N-mPEG (0.6 ml \(t\)-BuLi) | 44.5 ± 3.8 |
| Nylon 6-N-mPEG (1 ml \(t\)-BuLi)    | 46 ± 4.2         |
| Nylon 6-N-mPEG (2 ml \(t\)-BuLi)    | 60.1 ± 4.8       |

The AFM analyses showed significant changes in surface topography which were reflected in surface roughness (Ra) values for measured areas (1 μm x 1 μm and 10 μm x 10 μm) in case of the pure Nylon 6, modified Nylon 6-NH and Nylon 6-N-mPEG. The obtained Ra values are presented in the TABLE II. The Nylon 6 surface roughness decreased noticeably after reduction, whereas increased after grafting. The lithiation concentration did not show any major effect on Ra values for all the grafted samples.

| Surface area | Nylon 6 | Nylon 6-NH | Nylon 6-N-mPEG (2 ml \(t\)-BuLi) |
|--------------|---------|------------|----------------------------------|
| (1 × 1) μm²  | 5.1 nm  | 1.3 nm     | 1.3 nm                           |
| (10 × 10) μm²| 34.8 nm | 7.2 nm     | 12.8 nm                          |

Surface morphologies were examined by SEM images before and after Nylon 6 surface modification (Fig. 2a – 2d). SEM images clearly showed a successful grafting on reduced Nylon 6 surface. The grafting intensity difference was visible between Nylon 6-N-mPEG (0.6 ml \(t\)-BuLi) and Nylon 6-N-mPEG (2 ml \(t\)-BuLi). This explained the difference in WCAs among the various grafted samples. The higher concentration of \(t\)-BuLi (2ml) during Nylon 6-NH lithiation caused denser grafting layer than lower \(t\)-BuLi concentration (0.6 ml). This was further supported by FT-IR spectra shown in Fig. 3.
Fig. 2. SEM images of a) Nylon 6; b) Nylon 6-NH; c) Nylon 6-N-mPEG (0.6 ml t-BuLi); d) Nylon 6-N-mPEG (2 ml t-BuLi).

Fig. 3 shows the comparison between the FT-IR spectra of pure Nylon 6 and functionalized Nylon 6-NH and Nylon 6-N-mPEG films. In FT-IR spectra, the characteristic stretching vibrations of amide group (1660 cm\(^{-1}\) and 1541 cm\(^{-1}\)), the aliphatic groups (2858 cm\(^{-1}\) and 2931 cm\(^{-1}\)) and secondary amine groups (3293 cm\(^{-1}\)) for Nylon 6 were also visible in case of both reduced and grafted samples. This occurred due to 1 µm penetration depth of FT-IR. A new band appeared at the range of 2420 - 2250 cm\(^{-1}\) corresponded to the imine groups introduced on the surface due to chemical treatment with borane. This indicated the step towards amide reduction to amine. FT-IR spectra of all three Nylon 6-N-mPEG showed that the imine stretching band disappeared after grafting. The characteristic PEG stretching band (1087 cm\(^{-1}\)) for Nylon 6-N-mPEG was significant for the successful grafting. The stretching band intensity at 1087 cm\(^{-1}\) in spectra correlated the grafting intensity of Nylon 6-N-mPEG samples.

Fig. 3. FT-IR spectra showing pure, reduced and grafted Nylon 6.
The bacterial adhesion resistance to the surface of the pure Nylon 6, Nylon 6-NH and grafted Nylon 6-N-mPEG samples against \textit{S. aureus} was evaluated by counting the number of bacterial colonies formed in agar media incubated for 48 h at 37° C. The results are summarized in TABLE III. The grafted Nylon 6-N-mPEG (0.6 ml \textit{t-BuLi}) sample showed significant resistance towards \textit{S. aureus} adhesion compared to other two grafted ones. Nylon 6-NH also exhibited efficiency to resist bacterial adhesion, but pure Nylon 6 was very prone to bacterial adhesion as it showed high number of bacterial colony formation similar to the control.

| Number of bacterial colonies formed at various bacterial inoculum concentrations | | | | | |
|---|---|---|---|---|
| Sample | $10^0$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ |
| Control * | * | * | * | 975 | |
| Nylon 6 | * | * | * | 904 | 100 |
| Nylon 6-NH | 13 | 11 | 7 | 0 | 0 |
| Nylon 6-N-mPEG (0.6 ml \textit{t-BuLi}) | 5 | 0 | 0 | 0 | 0 |
| Nylon 6-N-mPEG (1 ml \textit{t-BuLi}) | 370 | 59 | 7 | 3 | 0 |
| Nylon 6-N-mPEG (2 ml \textit{t-BuLi}) * | 672 | 78 | 8 | 0 | |

* implies uncountable bacterial colonies (> 1500)

IV. CONCLUSION

The Nylon 6 surface reduction and grafting were confirmed by the microscopic as well as spectroscopic analyses. The novel method of grafting mPEG on reduced Nylon 6 surface via lithiation has shown a new, simple yet efficient approach to functionalize Nylon 6 surface for providing effective resistance against \textit{S. aureus} bacterial adhesion. The grafting intensity could be tuned by various concentrations of \textit{t-}butyllithium to obtain desirable efficacy. Even Nylon 6 surface reduction forming Nylon 6-NH proved to be efficient to resist the bacterial adhesion. Thus, our new approach has great potential for other biomedical polyamide surface functionalization to resist bacterial adhesion significantly.

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