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Antibacterial and non-cytotoxic ultra-thin polyethylenimine film

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ABSTRACT

In recent years, a common strategy, to obtain more uniform and controlled synthesis of polyelectrolytes multilayers (PEMs), relies on a previous polyethylenimine (PEI) coating of the substrate surface. PEI is a synthetic cationic polymer which provides a positive charge distribution on the materials to be covered with PEMs. Despite being an important step, this pre-layer deposition is frequently overlooked and no comprehensive characterizations or deep discussions are reported in literature. In that sense, this work reports on the synthesis of a typical PEI film that works as a precursor for PEMs, and its detailed physicochemical characterization. As many PEMs are produced for antibacterial and biomedical applications, the cytotoxicity of the film was also tested using fibroblasts, and its antibacterial activity was studied using *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Our results present the formation of an ultra-thin film of PEI with a thickness around 3.5 nm, and with a significant percent of NH_3^+ (35% of the total amount of N) in its chemical structure; NH_3^+ is a key chemical group because it is considered an important bacterial killer agent. The film was stable and did not present important cytotoxic effect for fibroblasts up to 7 days, contrary to other reports. Finally, the PEI film showed high antibacterial activity against the *S. aureus* strain: reductions in cell density were higher than 95% up to 24 h.

1. Introduction

Polyethylenimine (PEI) is a synthetic, weakly basic aliphatic polymer, which is polycationic due to the presence of primary, secondary and tertiary amino groups [1]. PEI can be linear, which is insoluble, and branched, which is freely miscible with water at room temperature [2]. Both are available in different molecular weights. PEI has been used in a wide range of industrial applications [3], such as wet-strength agent in the paper-making process, chelator of heavy metal salts, flocculant with silica sols, and even in cosmetics as clear antidandruff hair products and antiperspirants. However, most specialized applications are in the field of biotechnology, such as immobilization of enzymes [2], flocculation of cellular contaminants [4], cell-attachment promoter [5,6], bactericide as nanoparticles in composites [7,8], gene and DNA vaccine delivery reagent [9,10], and others.

In the last decade, PEI has been widely used as a pre-layer to build polyelectrolyte multilayers (PEMs) [11–16]. PEMs are thin films assembled using oppositely charged polyelectrolytes, mainly by the layer-by-layer technique (LbL) [17]. In these systems, an initial layer of PEI provides a homogeneous electrical charge distribution onto the surface of the substrate to be coated [14]. Then, subsequent PEMs are deposited, in a more uniformly and better controlled way. However, authors overlook the pre-layer of PEI and no further characterizations and descriptions are usually included about this precursor film.

Most of the studies that have used PEI as pre-layer focused on PEMs synthesis as antibacterial surfaces and obtained satisfactory results [11–13,15,16]. But PEMs tend to exhibit degradation [18]; this process may expose the underlying PEI layer and lead to a possible loss of the sample antibacterial activity. Therefore, it is important to determine if a typical deposition of PEI film, commonly obtained by dip coating, can also work as antibacterial surface. Hence, our work contributes to a better understanding of the ultra-thin PEI film, widely used as pre-layer for PEMs, by a detailed surface characterization. Besides, PEI cytotoxicity was tested using fibroblasts cultured up to 7 days, and its antibacterial activity

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was studied using *Staphylococcus aureus* and *Pseudomonas aeruginosa*, gram-positive and gram-negative bacteria, respectively. These two bacteria strains represent some of the most important cause of nosocomial infections in recent years [19] and thus were chosen for this study.

2. Materials and methods

2.1. Materials

Polyethylenimine (PEI, 50 wt.% solution in water, $M_W \approx 7.5 \times 10^5$ g/mol), rose Bengal (RB, $M_W \approx 1017.64$ g/mol), methylene blue (MB, $M_W \approx 373.90$ g/mol) and phosphate buffer solution (PBS) 0.01 M (0.138 M NaCl, 0.0027 M KCl, pH = 7.4 at 25 °C) were purchased from Sigma-Aldrich, USA. Acetone (C_3H_6O), isopropanol (C_3H_7OH), sodium hydroxide (NaOH), sodium chloride (NaCl), and hydrochloric acid (HCl) were purchased from Synth, Brazil. All chemicals were used without further purification, and solutions were prepared using Milli-Q water with resistivity of 18.2 MΩcm (pH ~ 7.6, otherwise mentioned). Silicon (Si) wafers with ⟨100⟩ orientation were used as substrates and purchased from University Wafer, USA.

2.2. Samples preparation

Si substrates (1×1 cm^2) were cleaned by ultra-sonication in acetone, isopropanol and distilled water, for a period of 15 min in each solvent. Acetone removes greasy and oily substances; isopropanol is necessary to rinse acetone off, and distilled water removes any isopropanol residues. Afterwards, Si wafers were dried under N_2 flow and subsequently treated with O_2 plasma at 100 mTorr for 15 min (720 V DC, 25 mA DC, 18 W; Harrick Plasma Cleaner, PDC-32G).

A PEI solution was prepared by dissolving the polymer at a concentration of 1 mg/mL in a 0.5 M NaCl solution and its pH was adjusted to 4 with a 0.1 M HCl and/or NaOH solution. PEI was deposited onto the substrates using an automatic dipping procedure (LbL Nanostructure Pro, ECSIA NanoScience, Brazil) with constant stirring under room conditions. Si substrates were immersed in the PEI solution for 15 min, followed by three consecutive Milli-Q water rinse steps of 2, 1 and 1 min, respectively. The pH of water rinse was previously set at 4 units.

2.3. Physicochemical characterization

The PEI film deposited on the surface of Si substrates was detected measuring the water contact angle and evaluating the presence of free amino and carboxylic groups on the surface of samples. The water contact angle was determined using a contact angle goniometer Easy DropDSA-150 Krüss (Germany) in the static sessile drop mode. The free amino and carboxylic groups in the samples were determined by immersion of the tested samples in RB (0.001 M, pH 5.5) and MB (0.001 M, pH 7.0) solutions for 15 min, followed by three consecutive Milli-Q water rinse steps of 2, 1 and 1 min, respectively. The pH of the water rinse was previously adjusted at 5.5 for the RB solution and 7 for the MB solution. Subsequently, stains from both sides of the slides (2 cm^2 of total area) were removed for UV–Vis spectroscopy measurements on absorbance mode. Samples with RB were immersed in a solution of 1 M NaOH and samples with MB were immersed in a solution of glacial acetic acid at 5% (*v*/v). RB and MB absorbance values were detected at 545 and 663 nm, respectively, using a UV–Vis VarianCary 1G spectrophotometer (Agilent Technologies, USA). Measurements were normalized by the sample total area.

The topography of the samples was acquired by atomic force microscopy (AFM) using a Keysight equipment Model 5500 (Keysight Technologies, Chandler, AZ, USA). Images were acquired in tapping mode in air using conical Si tips with a typical tip radius of 10 nm and tip length of ~20 μm (MPP-21120-10, Veeco, NY, USA). The spring constant and resonance frequency were typically 3 Nm^{-1} and 75–95 kHz, respectively. The root-mean-squared roughness (R_{RMS}) of the surface was determined from imaging over 4×4 μm^2 areas for each sample. AFM images, thickness measurement and roughness calculations were processed using freely available software (Gwyddion V. 2.37). Furthermore, the morphology of the coating was observed by field-emission scanning electron microscopy (FESEM; model F50, FEI Inspect) operated at 30 keV.

Surface chemical composition was determined by X-ray photoelectron spectroscopy (XPS). A surface analysis system (SPECS, Germany) equipped with a Phoibos 150 electron analyzer was used for the measurements. Monochromatized aluminum radiation (1486.6 eV) with an output power set at 380 W was used for the analyses of all samples. The C1s signal (284.6 eV) was employed as the reference to calibrate the binding energies (BE) of different elements in order to correct the charge effect. CasaXPS software was used to analyze all XPS data. Surface atomic concentrations in the samples were estimated using the instrument sensitivity factors to scale for the calculated photoelectron peak areas.

2.4. Cytotoxicity assays

To verify the toxic effect of the PEI film, cytotoxicity assays were performed using the BALB/c 3 T3 cell line, which was purchased from the National Institute of Health Baltimore, USA (NIH). The cells were routinely grown in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% fetal bovine serum, antibiotics (100 U/mL penicillin, 10 μg/mL streptomycin) and L-glutamine (2 mM) in a humidified incubator with 5% carbon dioxide, at 37 °C. Aliquots of 10^4 cells/well were incubated in 24-well plates until reaching semiconfluence, the RPMI medium was then removed and substituted by fresh supplemented medium to culture the cells on Si control and PEI samples (1×1 cm^2) for 1, 3, 5 and 7 days. Cell viability was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. After the respective culture time, the samples were rinsed with PBS solution and 100 μL of MTT solution (0.5 mg/mL of culture medium) was added to each sample. After incubation for 4 h at 37 °C, the medium was removed and the formed crystals solubilized in 100 μL of ethanol. The samples were shaken for 1 min on a plate shaker and the absorbance was measured at 570 nm in a microplate reader (ELx800, BioTek Instruments, Inc., Winooski, VT) [20].

Cell viability data were expressed as the means ± SD of experiments. A percentage of viability compared with control Si (the mean optical density of cells was set to 100% viability) was calculated from the concentration-response curves by linear regression analysis. Cell cultures were performed in triplicate and data from each assay were analyzed statistically by analysis of variance (ANOVA) with subsequent Tukey post-hoc test using the Statistica 12.0 software; *p*-values of 0.05 or less were considered statistically significant.

2.5. Stability of the PEI film

PBS was used to study the stability of the PEI film [21]. Samples were placed in PBS solution under stirring (100 rpm) in an orbital shaker at 37 °C (Novatecnica, Ltda. Piracicaba, Sao Paulo, Brazil) for 1, 3, 5 and 7 d. Afterwards, samples were washed for 1 min in distilled water to remove residual salt traces and dried under a N_2 flow. The stability of the PEI film was evaluated by detecting the nitrogenated groups by UV–Vis spectroscopy using the RB stain as indicated in the protocol of Section 2.3. Experiments were carried out in triplicates.

2.6. Bacterial adhesion assays

In order to test the antibacterial activity of the PEI film, *S. aureus* (ATCC 25933) and *P. aeruginosa* (ATCC 27853) were purchased from Thermo Fisher Scientific, USA. Bacterial cultures were performed in the laboratories of IPEL Itibanyl Products Special Ltda. Aliquots of 1×10^6 CFU/mL were inoculated in tryptic soy broth (TSB) medium