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Evaluation of antibacterial properties of polyelectrolyte multilayer coatings by norm tests

Abstract: Medical implants play a central role in modern medicine and both, naturally derived and synthetic materials have been explored as biomaterials for such devices. However, when implanted into living tissue, most materials initiate a host response. In addition, implants often cause bacterial infections leading to complications. Polyelectrolyte multilayer (PEM) coatings can be used for functionalization of medical implants improving the implant integration and reducing foreign body reactions. Some PEMs are also known to show antibacterial properties. We developed a PEM coating suggesting that it can decrease the risk of bacterial infections occurring after implantation while being highly biocompatible. We applied two different standard tests for evaluating the PEM's antibacterial properties, the ISO norm (ISO 22196) and one ASTM norm (ASTM E2180) test. We found a reduction of bacterial growth on the PEM but to a different degree depending on the testing method. This result demonstrates the need for defining proper method to evaluate antibacterial properties of surface coatings.

Keywords: polyelectrolyte multilayer coatings, implants, antibacterial coating, norm tests

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1 Introduction

Medical implants are crucial devices allowing modern therapeutics or diagnostics and improving the lives of many

patients. Both naturally derived and synthetic materials have been successfully used as biomaterials in the recent decades. However, when in long-term contact with living tissue most materials initiate a host response such as a foreign body response. Polyelectrolyte multilayer (PEM) coatings have been used to functionalize the surfaces of medical devices in order to improve the implant integration. Such PEM coatings are only a few nanometers thick, but may dramatically modulate the interaction between biomaterial surface and biological systems. In addition, PEM productions methods are in principle simple.

Next to issues of the biocompatibility of the implant materials, bacterial infections and inflammatory processes caused by contaminations and subsequent local or systemic defence reactions of the body are a major challenge for using implants, often requiring clinical treatment or the revision of the implant. Modern sterilization procedures and the use of antibiotics are common strategies to reduce infections caused by implants. However, not only the increasing resistance to antibiotics requires the development of novel material surface modifications and coatings with antibacterial properties. PEM coatings have also been suggested to have antibacterial properties depending on their composition and properties [1, 2].

For evaluation of the potential antibacterial effect of such modified implant surfaces appropriate testing is required, either on the lab scale for early research or standardized for R&D close to applications or transfer to market. Two protocols commonly used in industry are the ISO 22196 test "Plastics–Measurement of antibacterial activity on plastics surfaces" [3] and the ASTM E2180 test "Determining the activity of incorporated antimicrobial agent(s) in polymeric or hydrophobic materials" [4]. In these protocols, surfaces are tested using a liquid bacterial culture, covered with a plastic film (ISO) or bacteria are added to an agar slurry (ASTM), and grown for a certain time in contact with the test samples. After incubation, surfaces are rinsed and after further incubation, bacteria colonies are counted. Although such standard tests are widely used, it is suggested that they are limited in reflecting the complex practical condition and are thus limited in their

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usability [5]. In this work we developed a PEM coating suitable for implants and tested its antibacterial activity applying the ISO 22196 and ASTM E2180 standard methods. With both tests, the PEM coating was found to generally reduce the bacterial growth compared to the positive control (an uncoated sample). However, the two tests result in different quantitative degrees of bacterial reduction.

2 Materials and Methods

2.1 Preparation of surface coatings

Materials. Polyethylenimine (PEI) (MW 750 kDa) and poly(acrylic acid) (PAA) (MW 100 kDa), both from Sigma-Aldrich (Steinheim, Germany), and poly(allylamine hydrochloride) (PAH) (MW 120-200 kDa) from Alfa Aesar (Massachusetts, USA) were all used as received. PAA and PAH were dissolved in 0.5 M NaCl solution to a concentration of 2 mg/ml, pH 7.0. PEI (2 mg/ml, pH 7.0) was dissolved in ultrapure water and deposited as a first layer, acting as an anchoring layer for the adsorption of consecutive layers. The whole coating procedure was conducted in a sterile environment under a laminar flow bench.

Preparation of PEM coatings. PEI(PAA/PAH)₅ coatings were prepared by layer-by-layer (LbL) technique using the hand dipping method [7] on microscope slides 2×2.5 cm (Thermo Scientific™ Polysine Adhäsionsobjektträger). The film build-up was pursued at 25°C by alternating dipping of the glass slides into PAA and PAH solutions (for 10 min) followed by three washing steps (2 min each) in water. After the last deposition step, the coatings were dried in a nitrogen stream. The same microscope glass slides, but uncoated were used in the antibacterial tests as a negative control, and thin copper foil 2×2.5 cm (Kupferfolie, unbeschichtet, 99.8 %, Alfa Aesar™) was used as a positive control.

2.2 Characterization of surface coatings

Ellipsometry. The thickness and the refractive index of the PEM films were monitored at five different locations on each sample (prepared in triplicate) by spectroscopic ellipsometer Sentech SE800 (Sentech Instruments GmbH, Germany) with wavelength range from 280 to 850 nm, at an angle of incidence 70 deg. The raw data were fitted by four-layer model considering the contribution from air, PEM, SiO₂, and Si.

Static water contact angles were measured by the sessile drop method using an optical contact angle measuring and contour

analysis system (OCA15EC DataPhysics Instruments, Germany) equipped with video capture. 3 µL of ultrapure water were placed on the leveled surface of the sample by microsyringe forming a single sessile drop. An image was taken 5 seconds post droplet formation. The left and right angles were determined at 5 different spots for each specimen (prepared in triplicate) applying the Young-Laplace-fitting of the profile.

2.3 Bacteria cultivation and tests

A Gram-negative bacterial strain was used for both of the testing methods (*Escherichia coli*, DSMZ 10199). The liquid culture was prepared in Tryptic Soy Broth (TSB) (Sigma-Aldrich), the cultivation occurred on Tryptic Soy Agar plates (TSA) (Sigma-Aldrich).

International Organization for Standardization (ISO) 22196: Measurement of Antibacterial Activity on Plastics and other non-porous Surfaces.

A modified protocol, according to ISO 22196 was applied. Before inoculation, the *E. coli* were incubated in TSB for 24 h at 37°C and bacteria number was determined with a photometer. Thereafter a cell suspension of *E. coli* was prepared and a 150 µL drop was placed onto the surfaces of the coated samples and the controls. The bacterial inoculum on the test samples was covered with a plastic film (Bemis™ Curwood Parafilm™). The samples were then incubated in the dark for 24 h at 37°C. After incubation, the *E. coli* on the surface were removed by 5 mL of sterile washing solution (0.85% Saline, 0.2% Tween 80). Then serial dilutions were made from the washing solution and afterwards incubated on TSA plates for 24 h at 37°C. The number of colony forming units (CFU) was counted. Thereafter the number of viable bacteria was determined according to the ISO norm [3]. Numbers are given in log₁₀ reduction relative to the negative control sample.

Figure 1: General procedure of the standard test methods and main difference: (a) a cover film protects the inoculum according to ISO 2296; (b) a agar-slurry contains the inoculum according to ASTM E2180.

American Society for Testing and Materials (ASTM) E2180: Determining the Activity of Incorporated Antimicrobial Agent(s) In Polymeric or Hydrophobic Materials.

An adapted protocol was used. The bacterial culture was prepared in TSB and incubated for 24 h at 37°C, then bacteria were counted with a spectrometer. The culture was then diluted to an end concentration of 10^6 cells/mL into a semi-solid agar-slurry cooled down to 45°C. 500 μ L of the prepared suspension was spread on the samples. Subsequent the samples were incubated 24 h at 37°C in the dark. Afterwards the samples and controls were placed in a neutralizing medium, first treated with ultrasound, then vortexed for transferring the inoculum into the solution. The solutions were then diluted and spread on TSA, followed by incubation for 24 h at 37°C. Afterwards the CFU could be counted according to the ASTM norm [4]. Numbers are given in percent reductions relative to the negative control sample.

3 Results and Discussion

PEM offer a simple, versatile platform for controlling the interaction between materials and cells and can have antibacterial properties. We used PEM as a well-defined model system to compare the both test procedures described in ISO 22196 and ASTM E2180.

The coatings comprise five bilayers of the weak synthetic polyelectrolytes PAH and PAA - PEI(PAA/PAH)₅. PEI was applied as an adhering layer. Ellipsometry measurements showed that PEI(PAA/PAH)₅ PEM have thickness of about 15.0 nm. The contact angle of the coating was 72°.

Weak polyelectrolytes are charged only in a small pH range, hence, their polymeric chains conformations, surface and bulk properties can be easily modulated upon changing their net charge by adjusting the pH of the deposition solutions. At our deposition conditions the amine groups of PAH are protonated and carry positive charge. The degree of ionization of PAH at that conditions is about 85% [7]. The coatings are overlaid with the positively charged PAH and have in total a net positive charge.

The antibacterial activity of the coated samples against *E. coli* evaluated using the two different standard norms is presented in Figure 2. The results show that both test methods demonstrate a reduction in bacteria number compared to the negative control (uncoated sample). ISO 22196 results are given in log₁₀ reductions (figure 2 (A)). The positive control (Cu foil) yield in a reduction of 8.0-Log demonstrating the high antibacterial properties. The PEM coating performs

slightly antibacterial by showing a 1.4 Log-reduction in comparison to the positive control (copper foil). The measured Log-reduction means that the reduction of living bacterial cells as result of the incubation on the coating is nearly over 90%.

The results of the measured antibacterial activity applying the protocol according to ASTM E2180 is shown in Figure 2 (C). The antibacterial activity was calculated in percentage reduction in compliance with the norm. With this test method the PEM coating shows only a moderate antibacterial property with a reduction of approximately 20.6%. The positive control (Cu-foil) led to a reduction of nearly 100% as expected.

To compare the results of the two tests methods we calculated the log₁₀-reduction according to the ISO norm for the experimental results, the CFU, for the results of the ASTM measurements. The Log-reduction for the ASTM experimental data is significantly smaller than the one determined by the ISO standard method.

The moderate antibacterial action of the PAH-finished PEM could be attributed to the positive surface charge and high charge density that may yield strong electrostatic interaction between PAH-chains and bacterial surface. PAH has been identified to bind to phosphate moieties present in the lipopolysaccharides on the outer membrane of Gram-negative bacteria. A similar mechanism of antibacterial action was found for other cationic polymers [8].

Figure 2: Results for the antibacterial properties of the PEM coating and Cu-foil (positive control) according to (A) ISO22196, (B) ASTM measurement according to ISO calculation yielding a Log-reduction value and (C) the ASTM measurement evaluated according to the ASTM standard in %-reduction, all compared to the uncoated control.

In summary, the PEM coating induced a reduction in living bacteria incubated in contact with the samples, however the measured Log-reductions are moderate compared to the positive control of Cu-foil. Nonetheless, the quantitative degree of the antibacterial properties highly depends on the test method demonstrating the need for careful consideration of the test methods even if relying on norm standards.

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