Colloids and Surfaces B: Biointerfaces 156 (2017) 186–193

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Protocols

Elaboration of antibacterial plastic surfaces by a combination of antiadhesive and biocidal coatings of natural products

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ARTICLE INFO

Article history:
Received 7 November 2016
Received in revised form 2 May 2017
Accepted 10 May 2017
Available online 13 May 2017

Keywords:
Antimicrobial peptides
Hyaluronic acid
Antibacterial surface
Biocidal coating
Antiadhesive coating
Xps

ABSTRACT

Antibacterial polyolefins surfaces, combining biocidal and antiadhesive properties, were elaborated by a covalent grafting of antimicrobial peptides (AMPs), able to kill adherent bacteria, on a pre-immobilized hyaluronic acid (HA) layer, able to repel the micro-organisms. Different HA activation rate for its immobilization, were used to change HA layer morphology and number of residual free carboxylic acid functions for AMPs grafting. Based on adhesion tests on *Staphylococcus epidermidis* and microscopy fluorescent observations, the presence of the two combined properties was shown to be depended on the HA activation rate. Thus, the best addition effect was observed for an AMP grafting on a surface based on a high HA activation, data pointing out a decrease of the bacterial adhesion up to 99.8% and a perturbation of the bacterial membrane integrity of adhered bacteria. On the contrary, a decrease of the antibacterial activity was observed for an AMP grafting on a surface based on a low HA activation.

1. Introduction

Polyolefins, in particular polyethylene, are used for various applications, such as medical devices or food and cosmetic packaging. In all of these applications, bacterial contamination is a major problem. This contamination often occurs by an initial bacterial adhesion on the polyolefin surface, leading to a biofilm formation [1]. This phenomenon is thus one of the main cause of nosocomial infections [2,3], food and cosmetics products [4] degradation, food intoxication [5] or cutaneous problems.

To overcome this surface contamination, two main strategies to elaborate antibacterial surfaces have been yet developed. The first one consists to avoid bacterial adhesion by designing antiadhesive surfaces [6,7] while the second approach is to kill or to inhibit growth of adhered bacteria by used of biocidal coatings [8,9].

Antiadhesive surfaces can be elaborated by using low surface energy compounds, which are generally fluorinated polymers. Thus, Everaert et al. [10]. developed silicon prostheses with covalent grafted perfluoroalkylsilanes; they observed that the antiadhesive properties were proportional to the length of the fluorinated chain. Nevertheless, this strategy is essentially adapted to surfaces submitted to a liquid flow [11]. Another approach to avoid the bacterial adhesion is to design hydrophilic surfaces by immobilization of polar polymers [2,7] which can be highly flexible [11] and form a wide exclusion volume [12] e.g., polyethylene glycol (PEG) [13,14], acrylic or methacrylic acid based polymers [15,16], or anionic polysaccharides [17]. This last compound family is naturally secreted by various plants, animals or bacterial species. Some studies have investigated the immobilization of heparin [18,19], ulvan [20], dextran [21], or hyaluronic acid (HA) [22,23]. They pointed out a decrease of the bacterial adhesion as compared with unmodified surfaces.

Antibacterial surfaces can also be designed by biocidal coatings through entrapment of biocides in a layer-by-layer assembly [24] or by their covalent grafting on a functionalized surface [25]. The latter strategy avoids release of active molecules into the product or the environment. Immobilization of antibiotics [26,27] or quaternary ammonium salts [28,29] have been extensively studied, but the emergence of bacterial resistance towards these compounds limits their use [30].

For this reason, natural compounds such cationic antimicrobial peptides (AMPs) are promising candidates [31,32]. AMPs are secreted by a lot of organisms (micro-organisms, vegetables, insects, fishes, amphibians and mammalians) to protect themselves against invading micro-organisms by, in most cases, permeabilizing the cellular membranes [33].

Nowadays a strategy to enhance antibacterial activity of modified surfaces is to combine antiadhesive and biocidal properties,

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http://dx.doi.org/10.1016/j.colsurfb.2017.05.025
0927-7765/

by grafting inhibitors on antifouling layers, in order to kill the few bacteria able to adhere despite the antiadhesive treatment [34]. Furthermore, the polymer layer acts as a spacer and confers mobility to the biocidal compounds, allowing them a better interaction with the microbial cells [35,36]. For example, Muszanska et al. [37] used a first brush layer of triblock copolymer, based on poly(oxyethylene) (PEO) and poly(oxypropylene) (PPO), named Pluronic PF-127, to immobilize a short AMP on silicone rubber. Microbiological results on *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* showed an increase of the bacterial adhesion in the presence of the AMP, in comparison with Pluronic PF-127. Confocal microscopy investigations demonstrated that, after 20 h of contact, the antibacterial activity of the modified surfaces seemed to depend on a balance between antiadhesive and biocidal properties. This study also showed that the combination of antiadhesive and biocidal coatings is governed by antagonist mechanisms (i.e., bacterial repelling and bacterial attraction), leading to a complex control of the antibacterial activity of such surfaces. But currently there is no structure/activity relationship between the antiadhesive coating (usually a polymer layer) and the biocidal coating.

The aim of the present study was to design highly antibacterial plastic surfaces based on natural compounds, but also to find some rules to elaborate combined antibacterial surfaces. To create such surfaces an antifouling HA polysaccharide was associated with an antimicrobial peptide (i.e., the nisin Z). HA is a non-toxic and biocompatible compound [38] which is interesting to use in an active packaging or on a prosthetic implant. Nisin Z was chosen because this is the only AMP authorized by the FDA and the WHO [39], and it is used as a preservative in food. Surface modifications were characterized by contact angle measurements and XPS analyses. The antibacterial activity of the modified surfaces was evaluated against *S. epidermidis,* through bacterial enumerations and fluorescent microscopy observations, to analyze the role of both compounds on the final surface activity.

We also used various anchoring points to immobilize hyaluronic acid and investigated the impact of this parameter on (i) the antiadhesive properties of the polysaccharide layer and (ii) the combination of antiadhesive and biocidal properties by playing on the nisin immobilization (i.e., density, accessibility, orientation) on HA layer.

2. Experimental

2.1. Materials

3-aminopropyltrimethoxysilane (APTMS), acetic acid, ethanol, N-hydrosuccinimide (NHS), 2-(N-morpholino)ethanesulfonic acid (MES) and toluidine blue O (TBO), fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich.1-éthyl-3-(3-diméthylaminopropyl) carbodiimide hydrochloride (EDC).Hyaluronic acid (HA) were purchased from Acros organics and propidium iodine, syto9® and mowiol from molecular probes® (Life technologies). Phosphate buffer saline (PBS) was purchased from Fischer bioreagents. Brain and heart infusion (BHI) and BHI-agar was purchased from BD Difco.

Nisin Z was purchased from Anhui mimetals development, China. Nisin stock solutions were prepared at 10 mg/ml in HCl 0,2N.

High density polyethylene (HDPE) was provided by Albea company.

2.2. Surface modification

The three different strategies used to elaborate the antibacterial surfaces are described on Fig. 1.

2.2.1. Surface treatment

HDPE surfaces were cut (coupons of 10 mm × 10 mm) and were successively sonicated during 10 min in MilliQ water, acetone and ethanol, and finally dried under nitrogen flow.

These surfaces were then treated by UV/ozone (Jelight Model 42) during 5 and 15 min to hydroxylate and carboxylate them, respectively. The optimal UV/ozone irradiation times were determined by XPS analyses (not shown).

2.2.2. Direct nisin immobilization (Fig. 1A)

Carboxylated surfaces were treated with a 150 μl drop of NHS (30 mM) and EDC (150 mM) in MES solution at 50 mM (pH 4.5) for 2 h, rinsed in MilliQ water and dried under nitrogen flow.

Then, the immobilization of nisin (1 and 0.1 mg/ml in PBS) on plastic surfaces was carried out by depositing a 150 μl drop of peptide solution on the activated surfaces, at room temperature for 3 h. After the immobilization step, the surfaces were vigorously rinsed in MilliQ water and dried under nitrogen flow. Surfaces were named Nis1 and Nis0.1 according the peptide concentration used.

2.2.3. Nisin immobilization on hyaluronic acid layer (Fig. 1 B and C)

Immediately after UV/ozone treatment, hydroxylated surfaces were immersed in a methanol solution containing 4% (v/v) of APTMS for 18 h. Samples were rinsed in methanol and dried under a nitrogen flow. The surface thus obtained was named APTMS.

A solution of HA was prepared at a final concentration of 1 mg/ml in PBS buffer (pH 7.4). An EDC/NHS (molar ratio of 5/1) solution was added to the HA solution in order to convert 10%, 50% or 100% of HA carboxylic acid functions into NHS esters (i.e. respectively 0.26 mM of EDC and 0.052 mM of NHS, 1.3 mM and 0.26 mM, and 2.6 mM and 0.52 mM). After 5 min of stirring, a 150 μl drop of the activated HA solution was deposited on APTMS functionalized surfaces. After 18 h of contact, the surfaces were rinsed twice in MilliQ water and dried under nitrogen flow. Surfaces were named HA10, HA50 and HA100 according to the percentage of carboxylic functions conversion.

Inactivated carboxylic acid functions of immobilized HA were then activated by a deposition of a 150 μl drop of EDC/NHS (respectively at 150 and 30 mM) solution on HA modified surfaces for 2 h, excepted for HA100 surfaces which have been directly used for the next step. Surfaces were rinsed twice in MilliQ water and dried under nitrogen flow.

Immobilization of nisin (1 and 0.1 mg/ml in PBS) on activated HA surfaces was carried out by depositing a 150 μl drop of nisin solution on the surfaces, at room temperature for 3 h. After the immobilization step, the surfaces were vigorously rinsed in MilliQ water and dried under nitrogen flow. Surfaces were named HA10-Nis1, HA50-Nis1, HA100-Nis1, and HA100-Nis0.1.

2.3. Surface characterization

2.3.1. Contact angle measurements

Static contact angle measurements with MilliQ water (2.5 μl drops) were carried out with a Multiskop goniometer (Optrel-Gmbh) equipped with a syringe with a micrometric screw-type piston. Water angle values were expressed as the mean value of five measurements (on three different samples each) ± SD (n = 15)

2.3.2. XPS analyses

XPS measurements were carried out with a Thermo Electron K-Alpha Spectrometer using a monochromatic Al-K-Alpha X-ray source (1486.6 eV) with a spot size of 400 μm. The survey spectra were collected over a range of −10 to 1350 eV with pass energy of 200 eV. The high resolution spectra over the C 1s, O 1s, S 2p, N 1s,