Colloids and Surfaces B: Biointerfaces 164 (2018) 262–271

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Two-step biocompatible surface functionalization for two-pathway antimicrobial action against Gram-positive bacteria

Diego E. Pissinis, Guillermo A. Benítez, Patricia L. Schilardi*

Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas (INIFTA), CONICET- Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Casilla de Correo 16, Sucursal 4, 1900, La Plata, Argentina

ARTICLE INFO

Article history:
Received 24 October 2017
Received in revised form 14 January 2018
Accepted 28 January 2018
Available online 31 January 2018

Keywords:
Antibacterial
Surface functionalization
Silver nanoparticles
Ampicillin

ABSTRACT

The use of indwelling devices has emerged as a frequent and often life-saving medical procedure. However, infection in prosthetic surgery is one of the most important and devastating complications. Once the biofilm has been formed, its eradication is extremely difficult, due to an increased resistance to host defense and conventional antimicrobials. Thus, the design of novel strategies for inhibiting the bacterial adhesion on implantable devices is a key point for successful surgical procedures. In this work, the development of a simple two-step protocol to prepare surfaces able to prevent the bacterial growth was successfully achieved. The surface-modification design includes a combined approach involving the multi-functionalization of Ti surfaces with silver nanoparticles (AgNPs) and/or ampicillin (AMP). The surface chemistry involved in AMP adsorption on titanium and silver surfaces was elucidated for the first time, thus establishing the basis for the further anchoring of other antibacterial compounds having similar functional groups. Our results show that the antibiotic binds to the titanium surface through covalent interactions between the —COOH groups in AMP and the —OH groups of the native TiO_2 on the surface, although electrostatic interactions between protonated AMP and negatively charged TiO_2 can also contribute to the antibiotic anchoring to the surface. The AMP immobilization on the AgNPs is carried out by thiolate-like bonds. The β-lactam ring functionality is preserved after the adsorption process, since the Ti-AgNPs-AMP surface was able to decrease the bacterial viability in more than 80%. Moreover, the antimicrobial capacity is maintained over time due to a two-pathway antibacterial mechanism: death by contact (AMP) and death by release (AgNPs). The effect of AMP prevails on AgNPs at early stages of bacterial adhesion, while AgNPs are responsible for sustaining the relatively low but steady release of Ag(I), preserving the bacteriostatic activity of the surface over time. This effect would contribute to prevent infections due to sessile cells on indwelling devices, powering the action of the immune system and the conventional antibiotics usually dosed in implanted patients.

1. Introduction

The use of indwelling devices has emerged as a frequent and often life-saving medical procedure. However, infection in prosthetic surgery is one of the most important and devastating complications [1]. Its incidence is about 2–3% in primary prosthesis and twice in revision surgeries, reaching percentages above 15% in constrained or hinged implants [2]. The infection may be originated from bacteria already present in the individual or from those that entered by contamination of the material or improper handling during the surgical procedure. Once the biofilm has been formed, its eradication is extremely difficult due to an increased resistance to host defense and conventional antimicrobials. This resistive mechanism comprises: (i) protection of internal cells from aggressive environments and, in the case of prostheses, from the host immune system; (ii) creation of a diffusional barrier to large molecules or the entrapment of antibacterial substances and (iii) reduction in metabolic rate and induction of oxygen gradients across the biofilm, which contribute to the phenotypic heterogeneity within the bacterial population [3]. It is also important to highlight that, in conventional treatments, the antimicrobial agent acts effectively on the outer part of the biofilm (rapidly growing cells) and hardly on the region adjacent to the surface of the biomaterial (stressed cells or persisters). Bacterial strategies also involve a high cell density, which minimizes both the area exposed

* Corresponding author.
E-mail addresses: dpissinis@inifta.unlp.edu.ar (D.E. Pissinis), gbenitez@inifta.unlp.edu.ar (G.A. Benítez), pls@inifta.unlp.edu.ar, patricia.schilardi@gmail.com (P.L. Schilardi).

https://doi.org/10.1016/j.colsurfb.2018.01.057
0927-7765/

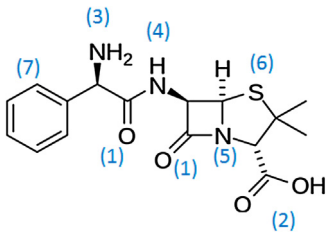

Fig. 1. Chemical structure of ampicillin. The functional groups are labeled: (1) carbonyl; (2) carboxyl; (3) primary amine; (4) amide; (5) lactam; (6) sulfide; (7) aromatic.

to the aggressive environment and the sacrifice of microbes that are directly exposed to the antimicrobial agent [4]. Therefore, an effective strategy to enhance the antimicrobial action and to inhibit the development of biofilms is the prevention of the formation of two- or three-dimensional compact layers of bacteria, which can be done by the proper modification of the surface chemistry. For instance, for this aim, the antimicrobials adsorption on polymeric surfaces has already been explored: polytetrafluoroethylene (PTFE) modified by penicillin [5,6] and ampicillin (AMP) [7], amoxicillin on poly(dimethylsiloxane) (PDMS) surfaces [8], etc. These antibiotics belong to the β-lactam family, which binds to penicillin-binding proteins (PBPs) which are responsible for the cross-linking of peptidoglycan in the cell wall synthesis. In this process, the β-lactam ring (Fig. 1) reacts with these enzymes to form stable covalent complexes, leading to inactivation of the PBPs and ultimately to cell death. These covalent complexes are formed as a result of the covalent binding of —OH group in the enzyme and the carbonyl group from the β-lactam ring after the cleavage of the N—CO bond [9].

The molecular structure of ampicillin (Fig. 1) shows several functional groups which are potentially adequate for the anchoring of AMP to surfaces. Self-assembled monolayers (SAMs) of $R\text{-}COO^-$/RCOOH type compounds can be easily formed on oxidized surfaces such as $\alpha\text{-}Al_2O_3$, Fe_xO_y and TiO_2, while organosulfur compounds, including alkanethiols, dialkylsulfides and thiophenes are suitable for SAMs formation on varied metallic surfaces (Au, Ag, Cu, etc.) [10]. Thus, AMP seems to be a good candidate for the chemical modification of several surfaces in order to confer them antimicrobial characteristics, since, *a priori*, the β-lactam ring is not involved in the surface reaction and, thus, would keep its antimicrobial function.

On the other hand, the increasing resistance of microorganisms to antibiotic is a matter of great concern worldwide [11]. Therefore, the search for new strategies for the inhibition of the bacterial proliferation is in continuous progress. In view of the fact that microbes have not been able to develop resistance to silver yet, the use of silver nanoparticles (AgNPs), as relatively new antibacterial agents, has been widely studied [12–15]. The mechanism involving the antimicrobial activity of AgNPs is controversial [16] and it is still under discussion: some studies suggest that the nanoparticles interact directly with the cell wall, whereas some others attribute the antibacterial effect to Ag(I) ions release from the nanoparticles. There are authors who also postulate the combined action of both effects [17–26]. What is certainly involved is Ag(I) ions release from the AgNPs. These Ag(I) ions either interact with bacterial proteins through thiol groups [27] or affect the respiratory chain [19], and even interfere in DNA replication [24,28]. Furthermore, Xiu et al. [25] have reported that AgNPs *per se* did not significantly represent a direct particle-specific toxicity to bacteria, but they may only act as carriers for silver ions that then will conduct the toxic action on the bacteria.

The aim of this work is the design of surfaces having highly inhibiting efficiency for the adhesion and proliferation of bacteria through the immobilization of conventional antimicrobials (AMP) and/or nanomaterials (AgNPs). These agents have different mechanisms of action, which, when combined in a surface can, in turn, operate at different times. These characteristics would allow keeping the antimicrobial effect for longer periods when compared to AMP or AgNPs as single factors. We have chosen Ti as substrate because it is one of the most widely used in dental and orthopedic implants due to its high strength-to-weight ratio, corrosion resistance and mechanical wear, inert nature, ability for adsorbing proteins on their surface and biocompatibility, which allows the osseointegration [29], among other properties. On the other hand, on this metal, native oxide film (dense and stable anatase TiO_2) is naturally formed on the surface when it is exposed to air, leading to a spontaneous passivation of the metal [30,31]. To the best of our knowledge, there is not previous information about the performance of AMP and/or AgNPs immobilized on titanium surfaces with an exhaustive analysis of the surface chemistry involved. The mentioned surface chemistry analysis would help in the subsequent smart design of new antimicrobial surfaces with applications in medical devices.

2. Material and methods

2.1. Reagents

All solutions were prepared using ultrapure MilliQ® water. All reagents were analytical grade and used as received, without further purification: silver nitrate (Sigma Aldrich), sodium citrate (J.T. Baker), hydrogen peroxide 30% (Merck), sodium borohydride (Fluka), ampicillin (Fabra laboratory), nutrient broth and nutrient agar (Britania), disodium hydrogen phosphate (Fluka), potassium dihydrogen phosphate (Fluka), sodium chloride (Sigma Aldrich), phosphoric acid (J.T. Baker).

2.2. Silver nanoparticles preparation

AgNPs in aqueous solution were prepared following the methodology described by Frank et al. [32]. This synthesis leads to silver nanoprisms with controlled size. Briefly, the synthesis was carried out by adding these solutions in the following order: 2.0 mL of 1.25×10^{-2} M sodium citrate, 5.0 mL of 3.75×10^{-4} M silver nitrate, and 5.0 mL of 5.0×10^{-2} M hydrogen peroxide. The silver reduction was achieved by adding 2.5 mL of freshly prepared 5.0×10^{-3}M sodium borohydride under vigorously magnetic stirring. After approximately 3 min, a stable color is reached, indicating the end of the synthesis. The colloidal dispersion was then dialyzed for 2 h to eliminate the excess of reactives. The final Ag concentration in the nanoparticles dispersion is 18.38 μg/mL.

2.3. Silver nanoparticles functionalization with ampicillin

AgNPs functionalized with ampicillin (AMP) were obtained by adding AMP to the AgNPs dispersion until the AMP concentration reached 2 mM [33]. After 24 h, the resulting dispersion was centrifuged twice at 14.000 rpm for 30 min in order to remove the AMP-containing supernatant. Then, the pellet with ampicillin functionalized AgNPs (AgNPs/AMP) was re-suspended in ultrapure water and preserved in the dark at 25 °C.

2.4. Titanium surface preparation

The substrates were titanium discs (Johnson-Mathey, 99.7%) 1 cm in diameter and 0.25 mm in thickness. The substrates were first polished with abrasive paper, sonicated for 15 min, and then polished at mirror grade with 1 μm diamond paste. After that, the