New strategies to fight bacterial adhesion

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Initial adhesion of bacteria to biomaterials' surface is assumed to be an important stage in their colonization which may to lead to severe infections and loss of medical devices. Thus, the knowledge of how these microorganisms adhere and which factors affect this phenomenon proves to be of great importance in order to avoid their colonization. Hydrophobicity, charge, roughness and chemical composition of surfaces can rule the adhesion process. A better understanding of all these features is crucial for an effective microorganism's adhesion control. In this context, it is possible to modify the biomaterials' surfaces through coatings doped with an antimicrobial agent, in order to prevent biofilm formation. So, this mini-review gives a brief account of aspects involved in bacterial attachment to a surface, highlighting the interactions involved and presenting mechanisms that are being used to fight pathogens adhesion. For this intent, hydrophobicity, morphology and topography of doped silver coatings will be exploited as a new approach for the development of antibacterial surfaces.

Keywords bacterial adhesion; hydrophobicity; roughness; antimicrobial surface.

1. Introduction

Microorganisms play an important role in nature and in various technological processes [1]. Microbial adhesion to solid surfaces is a fundamental step in biofilm formation. Biofilms are a community of microorganisms strongly attached to each other and to a surface, embedded in an extracellular matrix. Moreover, due to their complex structure these communities are hardly eradicated by antibiotics. The medical devices are easily susceptible to microbial colonization, and are able to reduce or even eliminate this effect, so it is necessary to know the phase which leads to its formation - adhesion. Among the several microorganisms, bacteria are major group and are able to form biofilms, so it is important to known the mechanisms involved in bacterial adhesion. This is a complex process, involving physicochemical properties of bacteria and materials surface like hydrophobicity and roughness [2,3], microbiological factors of microorganisms, as the production of extracellular polymeric substances (EPS) [4] and also environmental factors [5]. The interactions involved in bacterial adhesion to materials' surface can be classified as nonspecific or specific [6]. The nonspecific interactions comprehend physicochemical interactions between bacterial cell wall and materials' surface. These interactions involve Van der Waals forces, electrostatic interactions and hydrophobic effects. This is the first stage where the adhesion is still reversible. The specific interactions are those in which the adhesion becomes irreversible. At this point, microorganisms have the ability to synthesize a variety of structural components, such as EPS that can fix to the surface of the materials. Moreover, bacteria can also bond to surface through appendages such as pili, fimbria, fibrils and flagella, when these are part of their structure [7]. One of the most commonly used materials in the development of medical devices is the stainless steel AISI 316L [8]. This material is the most popular in orthopaedics for its good mechanical properties and its low costs [8–10] . Additionally, in implant surgery it is common to use the cobalt–chromium alloys and titanium and its alloys [10], because they have good chemical and biological properties and excellent mechanical properties. However, stainless steel AISI 316L is well known like a material susceptible to corrosion in body fluids [9,11]. Ions released from the stainless steel can cause allergic reactions and infectious diseases [9,12]. So to overcome these drawbacks, different processes to modify the stainless steel surface should be assessed [10]. One of the most commonly used methods to improve and control the corrosion resistance and biocompatibility is the coating of its surface. It is possible to coat these alloys with transition metals [13,14], their nitrides [15–19], carbides [12,20], oxides [21,22] and carbonitrides [9,23–25]. This approach has been the subject of several studies, since these coatings display high hardness and resistance to corrosion, low wear, low friction and good biocompatibility. Nevertheless, the majority of these materials do not present any antimicrobial effect. Consequently, to confer them antimicrobial activity, it is necessary to develop strategies, as doping their surfaces with antimicrobial agents, as nanoparticles [26,27] and nanocomposites [28,29], or inorganic and organic-inorganic molecules [30,31]. Silver has been the most used metal to promote high antimicrobial activity, with relatively low cytotoxicity [32–35]. Studies have shown that silver ions, Ag^{+}, are able to penetrate the bacterial cell wall and to bond DNA, which inhibit bacterial replication [36,37] and binding to the sulfhydryl groups of metabolic enzymes inactivating the electron transport chain resulting in bacterial cell death [38]. In this sense, the aim of this mini-review is to briefly summarize the mechanisms of bacterial adhesion to surfaces and highlight the methods involved in the characterization of coatings doped with silver.

2. Surface parameters ruling bacterial adhesion

Hydrophobicity, roughness and chemical composition appear to play an important role in the microbial adhesion process.

2.1. Free energy of interaction

The initial phase of the adhesion process of bacteria to non-living surfaces is usually mediated by non-specific interactions. The approach of microorganisms to the substratum is ruled by diffusion due to Brownian motions in fluids, arbitrary movements of particles in a fluid as a result of collisions between all the molecules or atoms present in this fluid [39]. The initial adhesion of the microorganism to the surface occurs in the distances approximately 50 nm, in which the microorganisms and the surface can interact by short-and long-range forces leading to adhesion. The long-range forces involve Van der Waals and electrostatic forces. The former are the only operating at distances greater than approximately 50 nm. When distance is approximately 10 and 20 nm, the interactions occur as a result of electrostatic forces, these forces are originating by interaction of charges of microbial and material surface [7]. At the distance of approximately 0.5 and 2 nm, the interactions become stronger and are due to hydrophobic effects. These hydrophobic interactions between bacteria and material surfaces play an important role in initial adhesion of bacteria [40] by removing water films from the interacting surfaces. In this way, bacteria can attach to surfaces of material and simultaneously have the ability to segregate the EPS to set irreversibly on the surface of the material.

In the adhesion process, the interactions between microorganisms and the surfaces are carried out by a set of thermodynamic, physicochemical and microbiological phenomena. There are three physicochemical approaches that allow the quantification of the energies involved in the interactions between surfaces, the thermodynamic, the DLVO and xDLVO approaches.

2.1.1. Thermodynamic approach

The adhesion of a microorganism to a solid surface in aqueous solution can only be established if the film of water between both surfaces is removed. This approach describes the energy of interaction between material and bacteria surfaces and considers Van der Waals attractive forces and acid–base interactions. The acid–base interactions are based on electron-donor and electron-acceptor interactions or polar interactions. The influence of the acid–base interactions is enormous when compared with electrostatic and Van der Waals interactions, once that surrounding medium of the particles (bacteria, materials surface), is especially polar. However, the acid–base interactions are also relatively short ranged, and a close approach between the interacting surfaces (less than 5 nm) is required before these forces can become operative [41].

According to van Oss, the hydrogen-bonding energy of cohesion of water molecules originate hydrophobic interactions. As known hydrogen-bonding can be seen as a form of more general electron-donor, electron-acceptor, and thus the interactions sensed are due to acid-base interactions defined by Lewis interactions. The surface tension (γ) can be divided into a Lifshitz–van der Waals component (γ^{LW}) and an acid–base component (γ^{AB}) where γ^{LW} comprises dispersion, orientation and induction contributions to the van der Waals interactions. LW interactions when compared with the AB are tiniest, since these latest come to exceed 10 to 100 times [42,43]. Consequently, the sole origin of the hydrophobic interaction would be the strong hydrogen bonding of water causing the high internal cohesiveness of water. So, the hydrophobicity of a given material is defined in terms of the variation of the free energy of interaction (ΔG) between material's surface (s) immersed in water (w), $\Delta G_{sws,}$ and is given using the following equation:

$$\Delta G_{sws} = \Delta G_{sws}^{LW} + \Delta G_{sws}^{AB} \quad (1)$$

Where ΔG_{sws}^{LW} and ΔG_{sws}^{AB} are free apolar energy of Lifshitz–van der Waals and free polar energy of Lewis components, respectively. When ΔG_{sws} is negative, the free energy of interaction between molecules is attractive revealing a greater interaction with each other than with water, making the material surface hydrophobic. In opposition, a surface is hydrophilic when ΔG_{sws} is positive.

It is possible to determined ΔG_{sws}^{LW} and ΔG_{sws}^{AB} by the following equations:

$$\Delta G_{sws}^{LW} = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 \quad (2)$$

$$\Delta G_{sws}^{AB} = -4\times\left[\left(\sqrt{\gamma_s^{+}\times\gamma_s^{-}}\right)+\left(\sqrt{\gamma_w^{+}\times\gamma_w^{-}}\right)-\left(\sqrt{\gamma_s^{+}\times\gamma_w^{-}}\right)-\left(\sqrt{\gamma_w^{+}\times\gamma_s^{-}}\right)\right] \quad (3)$$

The surface tension components ($\gamma_s^{LW}, \gamma_s^{+}$ and γ_s^{-}) can be determined by measuring the contact angles formed by three different liquids on material or bacterial surface, one of them apolar and two polar liquids, which one should be water. Each component of surface tension is known and presented in table 1.

Table 1 Surface tension parameters (mJ/m^2) of the liquids commonly used in contact angle measurements.

Liquids	γ^{TOT}	γ^{LW}	γ^+	γ^-
Water	72.8	21.8	25.5	25.5
Glycerol	64.0	34.0	3.9	57.4
Formamide	58.0	39.0	2.3	39.6
Diiodomethane	50.8	50.8	0	0
n-Decane	23.8	23.8	0	0
α-Bromonaphthalene	44.4	44.4	0	0

Afterwards, using equation 4, resulting from Young's equation, for all de three liquids and solving the three unknowns system, it is possible to obtain each surface tension components (γ_s^{LW}, γ_s^+ and γ_s^-).

$$\gamma_l^{TOT}(1+cos\theta) = 2\sqrt{(\gamma_S^{LW}\gamma_l^{LW})} + 2\sqrt{(\gamma_S^-\gamma_l^+)} + 2\sqrt{(\gamma_S^+\gamma_l^-)} \quad (4)$$

Where, θ is the contact angle formed between the surface (of material or bacteria) and the liquid.
Surface hydrophobicity is one of the most important properties involved in the adhesion process [6]. As it has been previously mentioned, the hydrophobicity can be modified to increase the capacity of removing the film of water that interacts between two surfaces [44,45], but this definition is not sufficient since electrostatic interactions are not taken into account. Van Oss and Giese [46], defined hydrophobic interactions as the attraction among apolar, or slightly polar, cells or other molecules themselves, when immersed in an aqueous solution. As mentioned above, LW forces do not contribute much to the total interaction energy between apolar entities. The value of apolar surface tension component of water (γ_w^{LW}) is 21.8 mJ/m^2 at 20°C and as the majority of compounds have a γ_s^{LW} values proximally of γ_w^{LW} [46], by applying of the Eq. (2), ΔG_{sws}^{LW} have a very low value comparatively to ΔG_{sws}^{AB}. Thus, often the sole contribution driving force of hydrophobic attraction is the AB, or more precisely the hydrogen bonding component of the free energy of cohesion of water [47](van Oss1997). For completely apolar compounds the value of ΔG_{sws}^{AB} = -102 mJ/m^2, once the electron-acceptor surface tension parameter (γ_s^+) as well as the electron donor surface tension parameter (γ_s^-) are zero, substituting in the Eq. (3), one has: $\Delta G_{sws}^{AB} = -4\left[\left(\sqrt{\gamma_w^+ \times \gamma_w^-}\right)\right]$ which represents the hydrogen-bonding energy of cohesion of water. As $\gamma_w^+ = \gamma_w^-$ = 25.5 mJ/m^2 at 20 ºC, the ΔG_{sws}^{AB} = -102 mJ/m^2 for apolar compounds, immersed in water. The ΔG_{sws}^{LW} have a low contribution for the total free energy of hydrophobic attraction (ΔG_{sws}^{TOT}), according to van Oss [46], the contribution of ΔG_{sws}^{LW} is about 2% of the total ΔG_{sws}^{TOT}. When ΔG_{sws}^{TOT} is negative, the free energy of interaction between molecules of material surface is attractive revealing a poor or even no interaction with water, making the material surface hydrophobic. When ΔG_{sws}^{TOT} is positive, the material surface makes hydrophilic. In this sense the bacteria are more prone to attach the hydrophobic surfaces than hydrophilic. Bacteria and others microorganisms have developed several means to used hydrophobic effects, in order to adhere to material surfaces. According to Doyle [48], a microorganism may adhere to a material surface via the hydrophobic effect since that surface has seats where the density of apolar areas is high. Several studies have shown that bacterial adhesion is promoted by the hydrophobic effect [49,50].The hydrophobicity of the cell surface is important in adhesion since hydrophobic interactions tend to increase with an increasing of the apolar zones of one or both surfaces involved [50]. According to Drenkard and Ausubel [51], the ability of bacteria to attach to each other and to surfaces depends in part on the interaction of hydrophobic domains.

However, in this theory the electrostatic interactions are not accounted and these interactions are very important in aqueous systems, so this theory can only be applied if the energy barrier between the two surfaces is exceeded.

2.1.2. DLVO approach

The adhesion of microorganisms to surfaces can be interpreted according to the DLVO theory, developed by Derjaguin and Landau (1941), and Verwey and Overbeek (1948). This approach describes the energy of interaction between material and bacteria surfaces and considers only the interactions of long-range as Van der Waals attractive forces and electrostatic repulsive forces. The Van der Waals forces can be the London dispersion force, the Keesom dipole–dipole force, and the Debye dipole-induced dipole force, these forces are attractive and their intensity depends on the particles size involved. The electrostatic interactions are due to the formation, in aqueous solutions, of a charged layer, diffusely distributed around the bulk which is usually negative as a result of ionization of surface functional groups. The positive ions presents in the solution neutralizes the negative charge of the bacteria and material surfaces creating an electrical double layer. These interactions are due to overlapping of electrical double layer of bacteria surface with the double layer of the material surface, and as both surfaces have the same charge, the interaction between them is repulsive. This repulsive energy increases when the ionic strength of an aqueous solution decreases since the shield of the surface

charges due to the ions present in the electrical double layers decreases. At low ionic strengths, when a bacterial cell approaches a surface, there is an energy barrier which is difficult to overcome [52]. The total free energy of these interactions is the result of the sum of the free energies of each interaction forces, according to equation 5.

$$\Delta G_{adh}^{TOT} = \Delta G^{LW} + \Delta G^{DL} (5)$$

As mentioned above this theory contemplates only the interactions of long-range, thus allowing only the prediction that microorganisms approach up to a certain distance from the material surface.

Both approaches have proven merits for microbial adhesion. However, so far, they presented only partially interpretation of the phenomena. Consequently, Van Oss et al. [53] introduced an extended DLVO theory which integrated the thermodynamic aspects of adhesion to the DLVO theory.

2.1.3. Extended DLVO approach

This theory considers three fundamental interactions: Lifshitz–van der Waals (LW), electrostatic (DL) and Lewis acid–base (AB) forces. The total free energy of these interactions is the result of the sum of the free energies of each interaction forces, according to equation 6.

$$\Delta G_{adh}^{TOT} = \Delta G^{LW} + \Delta G^{AB} + \Delta G^{DL} (6)$$

2.2. Roughness

Biomaterial surface roughness is another relevant property for bacterial adhesion process. The roughness of these materials' surface usually promotes bacterial adhesion and consequently biofilm formation. According to some authors surface irregularities caused by the increased surface roughness provide shelter bacterial cells by inducing their attachment [54,55]. This is because shear forces are diminished, and surface area is higher on rougher surfaces [50,56]. Nevertheless, the growth of these microorganisms depends also on their sizes, cell morphology and their reproduction [57,58]. There are many different roughness parameters for measurement of surface roughness, such as Ra (arithmetical mean of surface roughness of every measurement within the total distance ½ roughness average), Rmax (maximum roughness within the distance measured), Rms (root mean square roughness), among others [59]. Ra is the most universally used roughness parameter since it is easy to define and to measure, providing a good general description of height variations [60]. However, this parameter alone is not enough to describe to whole topography of a surface, so, the other roughness parameters should always be used together. These parameters can be measured by atomic force microscopy, one of techniques for measurement of surface roughness. Several studies show that bacterial adhesion forces increase with increasing roughness of the substratum surfaces [61,62]. However, according to other authors, the influence of surface roughness on bacterial adhesion and biofilm formation is not that significant [61,63,64]. Some in vivo studies suggested a threshold surface roughness for bacterial retention (Ra = 0.2 μm) below which no further reduction in bacterial accumulation could be expected. An increase in surface roughness above this threshold roughness, however, resulted in a simultaneous increase in plaque accumulation [65]. In accordance to these studies, it was also reported that, when the surface roughness is lower than 0.2 μm, surface roughness has no significant effect on plaque accumulation and microbial composition on titanium abutments in vivo [66,67] nor on adhesion and colonization by *Staphylococcus epidermidis* of silicone surfaces in vitro [61,68]. Another study demonstrated that the attachment to and removal of microorganisms from stainless-steel surfaces did not depend on surface roughness when it varied between Ra values of 0.01 and 0.9 μm [64]. Some studies suggested that adhesion of different bacteria to surface substrata is not influenced by roughness. The adhesion of *S. epidermidis* and *Pseudomonas aeruginosa* are dependent on pyrolytic carbon surface roughness, although *Staphylococcus aureus* adhesion appears to be independent of these factors [69].
In the literature, the opinions vary in relation to the effect of bacterial attachment to surface features. Some researchers report that there is a positive correlation between adhesion and increase roughness [3,70], while other researchers do not show any correlation between the ability of bacteria to adhere and irregularities or roughness of surfaces [71]. This apparent conflict is related with the different roughness parameters determined, with the bacterial species studied, the physicochemical parameters of the surface and the method used to detect the bacteria on the surface.

2.3. Chemical composition

Another factor that influenced bacteria adherence to a biomaterial surface is the chemical composition of the latter. Material surfaces are composed by different functional groups. Hydrophobicity and charges which involved the biomaterial surface depend of those groups, influencing the bacterial adhesion. Tegoulia and Cooper [72] showed that *S. aureus* adhesion on self-assembled monolayers (SAMs), was lower on ethylene oxide-bearing surfaces followed by the hydroxyl surfaces and higher on carboxylic acid and methyl terminated SAMs. They measured the contact angle for all surfaces, and found that the ethylene oxide terminated SAM was more hydrophobic than the hydroxyl and carboxylic acid surface, which suggests that some methylene groups were exposed to water. In this study, it was demonstrated that surface hydrophobicity and the presence of specific functional groups such as ethylene oxide moieties can significantly affect the amount of cells that attach to the surface. Kiremitci-Gumustedereliooglou and Pesmen [73] studied the

microbial adhesion to polymeric biomaterials used in implants and showed that microbial adhesion was reduced on the negatively charged polymeric implants, while it was increased on the positively charged. Amanatides et al [74] investigated the effect of plasma surface treatment of polyethylene terephthalate (PET) films, based in He/O_2 rf discharges, on the *S. epidermidis* adhesion and showed that treatment increased the surface hydrophilicity leading to promote bacterial adhesion, and this might be due to the incorporation of oxygenated functional groups. Oliveira et al [75,76] studied the structural properties of Ti-Si-C-O-N coatings, including the biofilm formation of *S. epidermidis* on those surfaces and concluded that the biofilm formation could be related to the surface chemical composition. According to these authors coatings surfaces were less prone to be colonized with a decrease on the N/O atomic ratio since a decrease on the TiN structure promotes an increase on the Ti-O compounds. The change or modification in surface chemistry can hinder the bacterial adhesion to these surfaces.

3. Control of bacterial adhesion

3.1. Surface modification

The risk of microbial infection associated with the biomaterials used in implants happens more often at the time of implantation and revision surgeries [77]. Due to this fact, these materials are susceptible to colonization caused mainly by nosocomial pathogens. The most popular materials used in orthopaedic devices have already been mentioned earlier, being stainless steel the most appealing in terms of cost. However, this biomaterial has a serious problem with corrosion. In this sense, different types of physical vapour deposition, PVD, hard ceramic coatings are frequently considered to overcome this drawback. Titanium carbonitride (TiCN) for instance presents good mechanical and tribological properties [35]. The wear and fatigue behaviours of TiCN coatings are extensively studied [35,78,79]. In the literature, it is also reported the good corrosion resistance of TiCN [9]. Feng et al. [80] reported a significant decrease of the corrosion current density of TiCN-coated AISI 304 stainless steel. Besides, its non-cytotoxic character allied with mechanical and corrosion properties make it a very interesting material for biomedical applications. Concomitant to all these factors and has already referred, the risk of bacterial infection is also one of the causes of failure of these devices. Thus, surface modification by coatings doped with silver is one the most used approaches to control the bacterial adhesion and their colonization. Silver has been the most used metal by demonstrating high antimicrobial activity and a relatively low cytotoxicity [32–34,81,82]. It is important that addition of silver do not change the good mechanical and tribological properties of coatings. According to a previous study [35], the incorporation of silver into TiCN coatings for use in implant and medical devices must be limited up to 6 at.% to ensure a good balance between tribological and biocompatibility properties. Some studies revealed that the increase of silver contents leads to better antimicrobial effect, but also show an increase of the cytotoxicity [28,83]. Other authors have shown that the size of silver nanoparticles that can be associated to antimicrobial activity [84]. Studies done by Kelly et al. [29,85] with TiN/Ag coatings, showed that in addition to the quantity and size there is also a relationship between shape and distribution density of the silver particles and the nature of the surrounding matrix, which could also influence the antimicrobial activity. However, in our study the AgTiCN coated AISI 316 L stainless steel showed no antibacterial effect, even for relatively large quantities of silver (15 at.%), conflicting with other published results. In this study, the incorporation of Ag promoted an increase in (C+N)/Ti atomic ratio and consequently a segregation of an amorphous phase of a-CN_x The nanocomposite structure nc-TiCN/a-CN_x matrix where the silver nanoparticles are embedded can influence the Ag^+ ions release, making the release insufficient or even non-existent. The hydrophobicity of these materials was tested, and those with higher Ag content showed a more hydrophobic character that should favours microbial adhesion. The results also show that the coatings with Ag possess sites with high densities of apolar areas that can justify the bacterial adhesion to the surface via hydrophobic effect. Figure 1 confirms an increased in bacterial adhesion to coatings doped with silver.

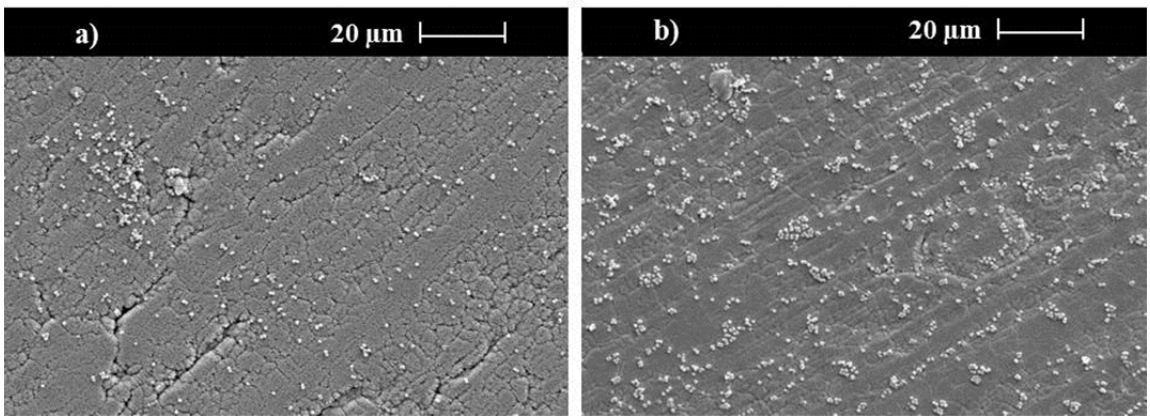

Fig. 1 SEM micrographs of *S. epidermidis* adhered to Ag-TiCN coatings, a) without Ag and b) with Ag.

The incorporation of silver in these coatings alters chemically the surface making it more hydrophobic and thus promoting bacterial adhesion.

Samples with and without silver were also subjected to measurement of surface roughness. In this study it is concluded that silver is preferably located in the grain boundary in the TiCN coatings becoming smoother. However, according to the studies mentioned above, the influence of the surface roughness was not significant since all coatings had a roughness less than 200 nm.

The modification of the material surfaces by the addition of an element like the case of silver alter the chemical composition and the structure/morphology and consequently the roughness and hydrophobicity, and as it has been described all these features can influence bacterial adhesion.

4. Conclusion

The phenomenon of bacterial adhesion depends on physicochemical interactions that occur between the cell and biomaterial surface. Application of surface thermodynamics, DLVO and xDLVO approaches may appear tempting and sometimes explain experimental observations, but it is not a priori obvious that the conditions required for application these methodologies are always fulfilled. The characteristics of the two interacting surfaces, such as hydrophobicity, charge, roughness and environmental conditions influence microbial adhesion process and subsequent biofilm formation. With the knowledge of physicochemical parameters of the surface it is possible to develop new strategies, which make the surface less attractive to microorganisms. One of them is the modification of biomaterial surface with an antibacterial agent, such as Ag. Silver ions realized have demonstrated a high antimicrobial activity even with low concentrations and have relatively low levels of cytotoxicity.

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