IMPACT OF SILVER DRESSINGS ON *STAPHYLOCOCCUS AUREUS* BIOFILM FORMATION ON ROUGH AND SMOOTH SURFACES

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INTRODUCTION

Staphylococcus aureus is an important pathogen that frequently causes severe skin and soft tissue infections and indwelling medical device-associated infections. Biofilms, structured sessile communities of bacterial cells embedded in a polymeric matrix forming on biotic and abiotic surfaces, are a major factor in the pathogenesis on these infections [1] since they allow bacteria to persist at the infection site by protecting them from the host immune system and reducing antibiotic susceptibility [2]. An alternative approach in treating staphylococcal infections is silver products, which have been used for many centuries as effective antimicrobial and anti-inflammatory agents. Recently, a wide range of silver-containing dressings have been introduced to prevent and treat burn and wound infections. These formulations claim to have increased antimicrobial activity due to the controlled release of silver ions or other silver species. However, their impact on biofilm development is not fully elucidated, although reduced susceptibility to silver nitrate has been observed for *S.aureus* biofilm in comparison with planktonic populations [3].

Understanding the interaction between antimicrobial agents and the biofilm in realistic environments presents a challenging problem since the biofilm forming rate and susceptibility depend on surface physico-chemical properties such as hydrophilicity, surface free energy and surface topography. A direct correlation between surface roughness and biofilm development on enamel surface and orthodontic appliances, catheters and medical implants has been demonstrated [4]. Surface roughness can modify the local flow patterns and consequently the surface mass transfer rate, which may offer bacteria physical protection within crevices and allow them to survive under stress conditions [5]. Our recent research showed that staphylococci biofilms developed in crevices at vancomycin or tetracycline concentrations which exceeded 100 times the concentration levels that prevented biofilm formation on smooth surfaces. These observations strongly suggest that the testing of antimicrobial preparations for their ability to prevent or treat bacterial and fungi infections requires different and more suitable approaches that would allow predicting their behavior in realistic environments. In this study, topical silver-containing dressings were investigated for their ability to control biofilm development by *S.aureus* in crevices or on smooth surface in order to understand the response of bacteria, protected by surface roughness, to the aggressive effects of silver.

MATERIALS AND METHODS

Microorganisms and silver-containing dressings

Staphylococcus aureus ATCC 29213 and *S.aureus* MRSA UOC 18 were kindly provided by Biofilm Research Group of the University of Calgary. Nanocrystalline silver dressing Acticoat (Nucryst Pharmaceuticals Inc., CA), Antimicrobial wound contact dressing Silverlon (Argentum Medical, USA), Hydrofiber silver dressing Aquacel Ag^+ (ConvaTec, UK), Antimicrobial alginate dressing Silver Cel (Johnson&Johnson, USA) and Membrane wound dressing PolyMem Silver (FERRIS MFG Corp., USA) were used in experiments.

Biofilm formation

Biofilms were grown in MBEC-P&G devices (Innovotech, CA) as described previously [6]. Briefly, a sterile MBEC lid was inserted into wells with bacterial suspension of 10^7 CFU/ml in silver free medium or in the presence of dressing samples of 0.2 cm^2. After 1, 2, 5 or 7 days incubation at 37^0C and agitation at 135 rpm (with Gyratory Shaker, VWR International) pegs with biofilms were treated with 0.4% sodium thioglycolate for 15 min, rinsed with sterile distillated water, sonicated for 5 min at 40 kHz and, finally plate count was performed to assess viable cell numbers.

Scanning Electron Microscopy

After a desired period of incubation, pegs with biofilms and silver dressing samples were rinsed in sterile distillated water, fixed in 5 % glutaraldehyde for 24 hour at 4^oC, dehydrated in serial ethanol dilutions. Dehydrated samples were dried with a critical point dryer, covered with gold, and, finally, observed under a Scanning Electron Microscope FEI ESME XL30.

Silver release bioassay

Daily plate-to-plate transferring assay was performed to assess silver release. Briefly, a piece of dressing of 1 cm^2 was transferred to fresh confluent bacterial lawns daily.

Zones of bacterial growth inhibition (ZGI) were measured before each transfer, and when ZGI could no longer be observed, it was assumed that the dressing was no longer releasing sufficient concentration of silver. To investigate post-treatment effect, 'clean zone' after removal of dressing were daily shaded with bacterial culture until visible bacterial growth appeared.

RESULTS

Biofilm densities in silver-free medium or in the presence of silver dressings

Tested strains of *S.aureus* produced biofilms of approximately 10^7 CFU/peg in silver-free medium after two days of incubation and then kept the same population density levels until the 7th day (Fig. 1). In contrast, the dynamics of the biofilm formation in the presence of silver dressings lagged behind. Most of the tested dressings allowed a quick increase of bacterial population density during the first two days, after which the biofilm growth was significantly slow. PolyMem Silver™ allowed biofilm populations to grow up to 10^5 CFU/peg. In the presence of Silver Cel™ and Acticoat™, *S.aureus* developed the biofilms of approximately 10^4 and 10^2 CFU/peg, respectively.

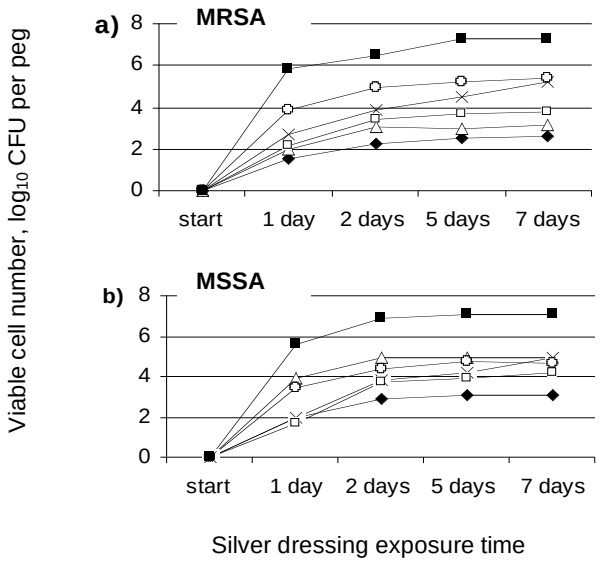


Figure 1: Formation of the biofilms by methicillin-resistant *S.aureus* UOC18 (*MRSA*) and methicillin-sensitive *S.aureus* ATCC 29213 (*MSSA*) in the presence Acticoat™ (*black diamonds*), Silverlon™ (*white triangles*), Aquacel Ag+™ (*crosses*), Silver Cel™ (*white squares*) and PolyMem Silver™ (*white circles*) versus longevity of silver dressing application in comparison with non-treated biofilm (*black squares*)

Silverlon™ demonstrated species-dependent activity allowing biofilm growth to 10^3 CFU/peg for *S.aureus* UOC 18 and 10^5 CFU/peg for *S.aureus* ATCC 29213. The number of bacterial cells within *S.aureus* biofilm growing in the presence of Aquacel Ag+™, increased continuously with exposure time and reached 10^5 CFU/peg after 7 days.

Scanning electron microscopy investigation of *S.aureus* ATCC 29213 biofilms developed in crevices and smooth surfaces

In silver-free medium, *S.aureus* ATCC 29213 formed well-developed biofilms, which covered 30 – 40% of smooth (Fig. 2a) and 100% of crevice surfaces (Fig. 3a). Despite the dehydration procedure used to prepare biofilm samples for SEM examination, the biofilm developed in crevices was observed to be embedded in abundant polymeric deposit.

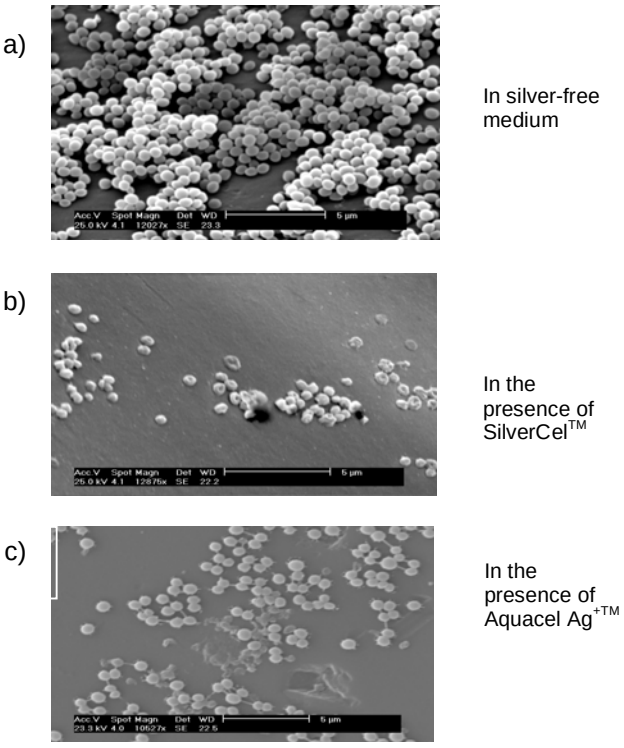
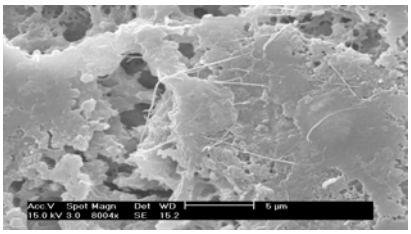


Figure 2: Sessile population of *S.aureus* ATCC 29213 developed on smooth surfaces in silver-free medium (*a*) or in the presence of in the presence Silver Cel™ (*b*) and Aquacel Ag+™. Magnification of 10000 – 12000.

The tested silver-containing dressings reduced or completely prevented staphylococcal biofilm formation on the smooth surfaces. After a 7 day incubation period, a single cell population of 38±12 cells per 100 μm² was observed on pegs exposed to PolyMem Silver™; however, the majority of the attached cells seemed to be damaged. The staphylococci exposed to Silver Cel™ were also damaged (Fig. 2b), although this population of 30±5cells per 100 μm² was gathered in small colonies. In contrast, Aquacel Ag+™ and

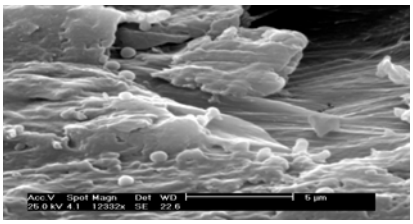
Silverlon™, which allowed development of sessile population of 40±6 and 16±2 cells per 100 μm^2, respectively, did not affect the integrity of the cell membranes. Moreover, in the presence of Aquacel Ag^{+}™, bacterial cells produced EPS which appears on the dehydrated samples as fiber-like bridges between the cells and the surface (Fig. 2c). Acticoat™ demonstrated the highest activity, completely preventing staphylococci adhesion to the smooth surface.

a)



In silver-free medium

b)



In the presence of Silverlon™

Figure 3: Sessile population of *S.aureus* ATCC 29213 developed in crevices in silver-free medium (*a*) or in the presence of Silverlon™. Magnification of 10000 – 12000.

Despite the high anti-biofilm activity of silver-containing preparations observed on smooth surface, *S.aureus* ATCC 29213 exposed to the dressings formed well-developed biofilms which covered 100% of the cracked surface. Figure 3b shows biofilm developed in crevices in the presence of Silverlon™ Bacterial populations were embedded in an abundant polymeric deposit, which seemed to be denser than that observed for biofilms developed in silver-free medium. The similar biofilms were detected for *S.aureus* grown in the presence of Aquacel Ag^{+}™, Silver Cel™ and PolyMem Silver™; whereas, Acticoat™ nanocrystalline silver dressing decreased coverage on cracked surface by 60%. Deposit was mostly localized on the walls of crevices leaving the bottom clean of microbes. A positive relation between crevice weights and biofilm deposition has been observed in both silver-free medium and in the presence of silver dressings. In particular, crevices narrower than 20 μm, were colonized in a manner similar to that seen for smooth surfaces, i.e. they were covered with single cells or small colonies without profuse polymeric deposition.

Time-dependent silver release bioassay

Bioassays were performed to estimate the release of sufficient dose of silver ions from tested dressings, which were detected by inhibition of *S.aureus* growth on solid media. In the first day, *S.aureus* strains were inhibited within zones of 2.5 – 5.5 mm by all tested dressings. Activity of most of the dressings markedly declined resulting in disappearance of inhibition zones after two – three days; whereas, Acticoat™ kept a level of silver sufficient to inhibit growth of *S.aureus* on solid media for 7 days. After removal of the silver dressings, sufficient levels of silver were present in the media to prevent bacterial growth. The sustained effect lasted by 5 days after Acticoat™ application and 2 – 4 days after removal of other dressings. Therefore, silver dressings might be able to inhibit *S.aureus* growth for 5 – 10 days due to a slow release and / or accumulation of silver in the medium.

DISCUSSION

In this paper we have studied the effects of topical silver-containing dressings on *S.aureus* biofilm formation on smooth surfaces and in crevices. Silver preparations such as silver-impregnated dressings, implants and catheters are widely used to treat burn and wound infections as well as post-surgery and medical device-associated complications due to bacterial contamination and dissemination. However, it is still an open question whether these preparations are effective against bacterial biofilms. Bacteria within biofilms express a distinct phenotype that makes them resistant to antimicrobial agents and the host immune system. A variety of environmental factors including substratum surface topography were reported to contribute in biofilm development and expression of the biofilm phenotype [5]. Consistent with earlier research, the present study showed a positive correlation between surface roughness and the biofilm-forming rate for methicillin-sensitive and methicillin resistant *S.aureus*, and a negative correlation between increased surface roughness and topical silver dressing efficacy to stave off staphylococcal biofilm formation. Tested dressings were observed to effectively prevent development of sessile populations on smooth surfaces, but, intensive biomass deposition was observed in crevices resulting in bacterial population of 10^2 – 10^5 CFU/peg. For comparison, biofilm populations in silver-free medium reached 10^7 CFU/peg.

The reduction in the biofilm-forming rate caused by silver dressings may be accounted for by the ability of silver ions to form insoluble compounds with essential bacterial cell macromolecules such as DNA and proteins, resulting in protein inactivation, alteration of cell proliferation, interference with the bacterial cell

membrane integrity, electrolyte transport and transmembranous energy metabolism. The silver release rate and, hence, the ability to maintain a sufficient concentration level in the environment are said to play an important role in topical dressing's efficacy. Commercially available topical silver dressings are claimed to continuously release sufficient amounts of silver ions into wound fluid to rapidly kill bacteria or prevent their growth [7]. The bioassays performed in this study demonstrated that all tested dressings were able to release and maintain sufficient levels of silver to kill staphylococci in suspension and prevent their deposition on the peg surfaces. However, despite maintaining a sufficient concentration of silver in media, tested dressings allowed biofilm development in crevices; although population densities of these biofilms were significantly lower that those observed in silver-free medium.

Numerous studies showed that the increasing surface roughness could increase bacterial adhesion and biofilm formation due to the increased surface available for attachment and favorable shear force distribution [5]. It has been reported that there are certain threshold surface roughness values which result in an increase in biofilm accumulation. For example, Scheuerman *et al.* [8] observed preferential biofilm deposition in crevices on silicon surface with crevice widths of 10 µm and more. Our data showed that biofilm accumulation in crevices narrower than 20 µm did not differ from that on smooth surfaces. A possible explanation for the preferential accumulation of the biofilms in large crevices is based on a hydrodynamic model which suggested that eddies generated at the edges of large crevices cause a localized hydrodynamic entrainment of the cells [8]. This assumption fits well with the fact that, in the presence of Acticoat™, biomass deposition was observed on the wall; whereas the bottom of cracks was clean of microbes. It was also reported that alteration of surface roughness might leads to changes in surface free energy and charges [9]. Regardless the mechanisms, the environment conditions in crevices favors bacterial adherence, which is governed by the interplay of hydrophilicity, surface charge and receptor-ligand binding [5]. More significantly, however, the conditions present in crevices seem to stimulate the expression of certain defense mechanisms within the bacterial community. In particular, it has been observed increased EPS production by *S.aureus* biofilms developed in crevices.

In summary, this study demonstrated that biofilm deposition in silver-free medium and in the presence of silver dressings was generally higher with increased roughness where bacteria meet favorable conditions for adherence and biofilm maturation. Despite the fact that tested silver-containing dressings inhibited *S.aureus* growth in suspension and on smooth surfaces, bacteria formed well-developed biofilms in the protected crevice environment. This observation indicates that traditional techniques used to study efficacy of antimicrobial preparations may not reflect their activity in realistic clinical conditions where bacteria might be protected by the specific environment in crevices found on the surfaces of medical devices. Additional research required to establish appropriate diagnostic techniques for antimicrobial agents used for treatment of biofilm-associated infections and to understand the mechanisms of increased biofilm-forming capacity and tolerances in crevices.

ACKNOWLEDGEMENTS

We would like to thank the Natural Sciences and Engineering Research Council of Canada and of Nucryst Pharmaceutical Inc. for their financial support through the Industrial Research Chair program.

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