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Detection of antimicrobial efficacy in silver-coated medical devices

Summary

Background: There is an increasing interest in using silver- and nanosilver-coated materials for medical devices. These materials can prevent microbial colonisation and/or they can protect sensitive surfaces from microbial contamination. The silver compounds used for these surfaces vary and may range from elemental silver to complex silver coatings. In order to prove their antimicrobial activity, a number of different test methods have been employed. However, as a result of varying test protocols, the comparability of their findings is limited.
Method: Test specimens from commercially available medical devices were used to test antimicrobial efficacy against *Enterococcus faecium* with comparable test protocols and experimental conditions (same investigator, same laboratory conditions). The test methods employed included were the agar diffusion test, the suspension test and modified Dow Shake methods.
Results: The inhibition zones in silver-coated materials increased in correlation with the silver compound and contents. In contrast, no inhibition zones were detectable on any of the uncoated specimens. The most accurate results were obtained with the suspension test with germ recovery. Similarly, the modified Dow Shake Methods were also able to demonstrate different extents of germ reduction and, thus, antimicrobial activity on the silver-coated specimens. The individual values of the two Dow Shake methods correlated well.
Conclusion: In conclusion, all silver-coated materials tested exhibited a high degree of antimicrobial activity against *Enterococcus faecium* and the methods employed proved to be suitable for demonstrating the antimicrobial effects.
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Introduction

Today, insertion of temporary catheters and permanent implants is becoming an increasingly more widespread practice. Against a background of biomaterial-associated infections, increasingly more stringent requirements are being addressed to medical devices. As far as possible, the surface materials of the latter should prevent adhesion and, in particular, proliferation of microroganisms [1]. Antimicrobial surface coatings can prevent or reduce colonisation by microroganisms, thus constituting an important element of infection prophylaxis.

There are various methods for testing the antimicrobial properties, including in particular the agar diffusion test, inhibition zone test, or Kirby-Bauer test, microbial count test, suspension test based on the American method AATCC 100, ASTM E-2149-01 (Dow 0923, Shake Flask) and tests based on the Japanese standard JIS Z 2801, JIS L 1902 [2–5]. In North America other tests are common for investigating the antimicrobial properties of medical devices and industrial products, but in general there are no precise protocols specifying how these are conducted [3–6].

All tests have their own pros and cons, and there are also marked differences in the reproducibility of the measurement results [2–5]. The literature does not advocate any standard method for evaluation of the antimicrobial properties of medical devices and industrial products. Many of the test methods have been modified by the test institutes or enterprises so as to tailor them to the respective test product. This applies in terms of the test organisms used, exposure times, incubation time and nutrient media.

The aim of the present study was to investigate the various antimicrobial silver coatings of medical devices while using uniform methods (agar diffusion test, suspension test and modified Dow Shake methods).

Materials and Methods

For the experiments a variety of commercially available silver-coated medical devices, of identical dimensions (12 × 5 mm), were employed as process challenge devices (PCDs) (Table 1). The PCDs measured 2 mm in thickness. In all cases, silver-coated materials and uncoated reference samples made of the same basic material were used. The test materials were classified as follows in accordance with the type of silver used: silver sodium hydrogen zirconium phosphate 1 (silver Na-HZP-1), silver sodium hydrogen zirconium phosphate 2 (silver Na HZP-2), nanosilver 1, nanosilver 2, elemental silver and silver zeolith.

Silver-Na-HZP-1 is a highly woven material combination of calcium alginate and carboxymethylcellulose to which silver is applied in the form of the inorganic complex silver sodium hydrogen zirconium phosphate (trade name: Alphas an®, Milliken Chemicals). In the material silver Na-HZP -2 the silver complex was embedded into a polyurethane matrix. In both materials the active component was ionised silver in the form of silver sodium hydrogen zirconium phosphate. Nanosilver 1 was applied to a polyethylene net using sputter technology in the form of nanocrystalline silver particles (diameter 10–20 nm, trade name: SILCRYSTTM, NUCRYST Pharmaceuticals). Nanosilver 2 was a suspension with nanoscale silver particles applied to glass PCDs as a layer (layer thickness = 30 µm). For the elemental silver coating, an ion beam deposition process was used, as PVD process, to apply elemental silver as a continuous silver coating (layer thickness of around. 1200 Å (Ångström) = 0.12 µm) on polyester (polyethylene terephthalate). The silver-ion-releasing component of the silver zeolith material is a patented silver compound AgION™, which was homogenously embedded into the silicone catheter material. The AgION™ antimicrobial continuously releases silver ions through exchange of electrolytes, such as e.g. sodium ions. The ionised silver is ionically bound in crystal grids, known as zeoliths. The AgION™ clusters measure 1–2 µm.

Table 1: Overview of the samples used for the tests.

Material	Type of silver	Silver content [mg/cm²]	Silver content in PCD [mg]
Silber-Na-HZP-1	Silver sodium hydrogen zirconium phosphate	0,8	4,80
Silber-Na-HZP-2	Silver sodium hydrogen zirconium phosphate	10,0	60,0
NanoSilber 1	Nanocrystalline silver (SILCRYSTTM nanosilver)	12,0	72,00
NanoSilber 2	Nanocrystalline silver in suspension	Not known	Not known
Elemental silver	Elemental silver	0,07–0,16	0,42–0,96
Silver zeolith	Zeolith AgIONTM	ca. 3,0	18,00

The samples of silver-coated materials und uncoated reference samples were distributed among glass petri dishes and sterilised in autoclaves using a sterilisation programme at 121 °C for solid materials.

Agar diffusion test

For the agar diffusion test, from a cultured *Enterococcus faecium* (DSM 2146, ATCC 6057) culture in trypticase broth a fresh working culture was prepared with 100 µl in trypticase soybean broth and incubated at 36±1 °C.

The tests were performed with trypticase soybean and Kanamycin Aesculin Azide (KAA) agar plates.

From the bacterial suspension (microbial count: 10^8 cfu/ml) aliquots of 0.1 ml were pipetted onto trypticase soybean and KAA agar plates and evenly spread using sterilised glass spatulas. After applying these samples to the plates, the PCDs and reference controls, which had first been immersed in NaCl, were placed on the plates using sterile tweezers. The plates were then incubated for 48 hours at 36±1 °C. For evaluation, the inhibition zone diameters around the outer margin of the PCDs was measured using a ruler (reading 1/10 (0.1 mm) precision, as per DIN 862). To check the measurement results, the tests were repeated 10 times for each material sample as well as for the associated reference on trypticase soybean and KAA agar plates.

Suspension test, recovery test

From the bacterial suspension (10^8 cfu ml) 10 µl aliquots were applied in each case to the PCDs and these contaminated PCDs were incubated at 36±1 °C. At intervals of 10 minutes, 1 hour, 2 hours, 4 hours and 24 hours the PCD was withdrawn, transferred to a test tube with 10 ml stripping fluid and shaken. Using a 10^{-1} and 10^{-7} dilution series, the microbial count was determined by means of a surface culture on trypticase and KAA agar. To that effect, 0.1 ml aliquots were plated out twice in each case per dilution series onto agar plates and spread evenly using sterilised glass spatulas. The agar plates were incubated for 48 hours at 36±1 °C, followed by counting of the agar plates and calculation of the colony forming units (cfu/ml).

Modified Dow Shake method 1

The PCD and reference were each contaminated with 10 µl of the bacterial suspension (10^8 cfu/ ml) and inoculated in 10 ml NaCl 0.85%. At intervals of 1 hour, 3 hours, 6 hours and 24 hours, 1 ml aliquots of the sample were taken, a decadic dilution series was pipetted and 0.1 ml aliquots were plated out twice in each case per dilution series onto trypticase soybean agar plates. The agar plates were incubated for 48 hours at 36±1 °C and counted. All tests were conducted in duplicate.

Modified Dow Shake method 2

The PCD and reference sample were each contaminated with 10 µl of the bacterial suspension (108 cfu/ ml) and inoculated in 10 ml NaCl 0.85%. At defined intervals (of 1 hour, 3 hours, 6 hours and 24 hours). 0.1 ml aliquots of the solution were plated out twice onto trypticase soybean agar plates. After incubation for 48 hours at 36±1 °C the microbial count was determined using a 0 to 5 scoring system (0=no colonies, 1=isolated colonies, 2=lit-

tle growth, 3=moderate growth, 4=heavy growth and 5=extremely heavy growth).

Evaluation of the results

For the inhibition zone values obtained in the agar diffusion test the mean values and standard deviations were calculated in each case. Each test was conducted in duplicate. The mean value was calculated for the results of individual tests. The results of the modified Dow Shake method 1 (MDS-1) and modified Dow Shake method 2 (MDS-2) were statistically compared. The colony forming unit was then calculated on the basis of the mean values (cfu/ml) obtained. The data were recorded and entered into a table using the program Excel (Microsoft, USA) and evaluated. Statistical calculations were carried out using the program SPSS (SPSS Inc., Chicago, IL, USA). A value of $p<0.05$ was set to determine significance of the results.

Results

Agar diffusion test, inhibition zone test

After incubating for 48 hours, inhibition zones were observed for all samples (Figure 1), whereas no inhibition zones were seen for the silver-free controls. The size of the inhibition zones varied in accordance with the initial silver content and the concentration of silver ions released. The biggest inhibition zones were found in the silver Na-HZP 2 and nanosilver groups (Figure 1). The smallest inhibition zones were noted for elemental silver, where mean values of 0.95 mm (trypticase soybean) and 0.97 mm (KAA) were measured (Figure 1). In all the samples of the elemental silver group a narrow inhibition zone border could be clearly seen around the sample. In none of the measurements was there any significant difference noted between the measured values obtained for trypticase soybean and KAA agar.

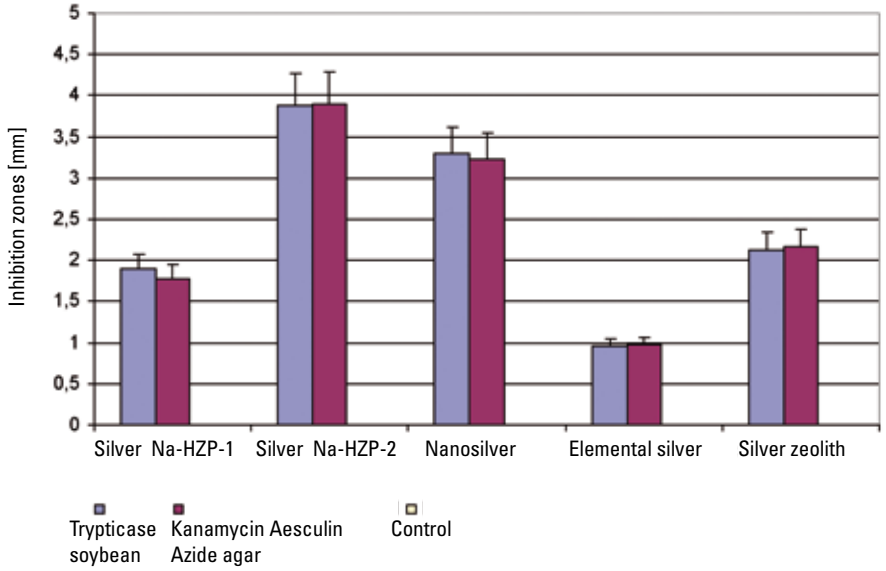

Figure 1: Inhibition zones measured around the process challenge device (MV±SD).

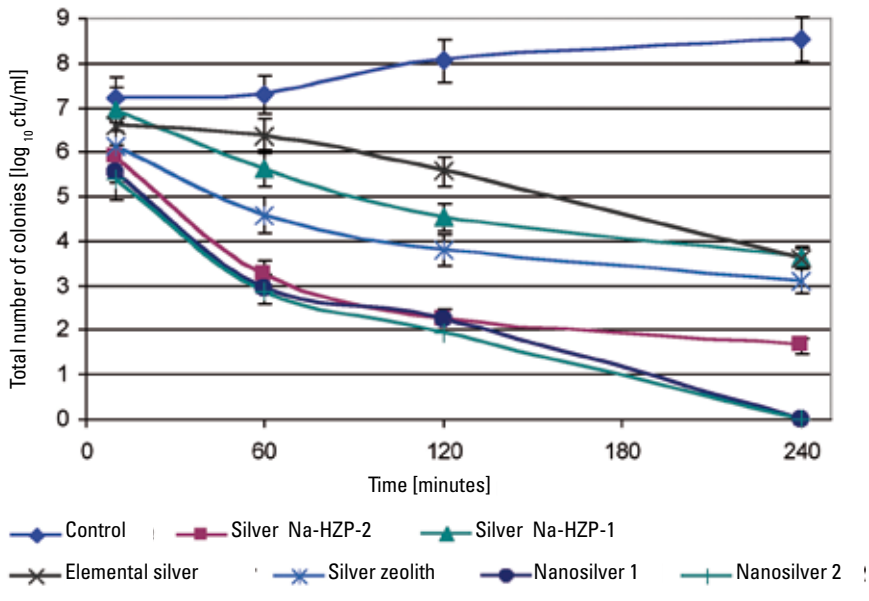

Figure 2: Trends in the total number of colonies on materials up to 4 hours (MV±SD).

Suspension test, recovery test

To enhance transparency, the control values have been shown as mean values. While the entire number of colonies continued to rise in the control samples, this continued to decline for the silver-coated materials (Figures 2 and 3). The magnitude of this decline depended essentially on the silver content and on the type of silver. The biggest decline was noted for silver Na-HZP2 and nanosilver, whereas the slowest action was seen in the case of elemental silver (Figures 2 and 3). Already after one hour the PCDs silver zeolith, silver Na-HZP 2 and nanosilver had achieved a $\log_{10}$ reduction of three orders of magnitude. After four hours a reduction by more than four log levels was noted for all materials (Figure 2). After 24 hours, no bacteria could be detected anymore on the agar plates in the case of all silver-coated materials (Figure 3).

Modified Dow Shake method 1

The trends noted in the number of colonies on using the Dow Shake method 1 (MDS-1) were similar to those observed in the suspension test. Here, too, after three hours the greatest log 10 reductions were achieved for the PCDs silver Na-HZP 2, nanosilver and silver zeolith.

Modified Dow Shake method 2

With this modification of the Dow Shake method 2 (MDS-2) bacterial growth on the agar plates was defined using a scoring system defined by the authors ((5=extremely heavy, 4=heavy, 3=moderate, 2=little,=isolated and 0=no growth. On us-

ing this scoring system, with its attendant subjectivity, trends very similar to those of the MDS-1 were detected..

Comparison of the modified Dow Shake methods

The values obtained for the total number of colonies for the modified Dow Shake method 1 (MDS- 1) were closely correlated with the values scored for the modified Dow Shake method 2 (MDS-2) (94 value pairs, r=0.9806; Pearson) (Figure 4).

Discussion

Against a background of burgeoning antibiotic-resistant bacteria, including even vancomycin-resistant bacteria, the interest elicited in the clinical setting by silver-coated medical devices is becoming more widespread [7,8]. For example, various strains of *E. faecium*, which cause nosocomial (hospital-acquired) infections (endocarditis, sepsis), have in the meantime become resistant to several antibiotics [9].

The term "antimicrobial efficacy" defines all principles of action that stop growth of microorganisms, prevent microbial colonisation or kill microorganisms [5,6]. The efficacy of an antimicrobial surface is defined as the microbial reduction effected during the contact time. This is expressed in log levels, whereby one log level corresponds to a reduction in the microbial count by one order of magnitude ($\log_{10}$).

To demonstrate bacteridical efficacy, a reduction of three or more orders of magnitude ($\log_{10}$ levels) in the number of colony forming units (cfus) is needed [3–5]. Just as the different types of silver coatings used for the medical devices are not standardised, so too is there a lack of standardisation of the methods used by manufacturers to investigate their antimicrobial efficacy. For example, there is no standardised definition for test methods or for interpretation of results to permit comparison [3]. The present study was therefore aimed at using uniform test methods to investigate various medical devices in respect of their antimicrobial activity. The microorganism employed in our study, *E. faecium*, is used as per DIN 58949 Part 4 as a biological indicator for investigation of thermal disinfection in the 75 °C programme and is endowed with a high level of resistance to temperature and pH [10, 11]. Furthermore, enterococci are a common cause of nosocomial infections, in particular of wound and urinary tract infections [12]. Therefore *E. faecium* appeared to be a particularly suitable candidate for our tests.

A commonly used test is the agar diffusion test – also known as the inhibition zone test – where the antimicrobial activity is determined on the basis of the appearance, and size, of inhibition zones around the PCDs.

In our investigations an inhibition zone, of varying sizes, was noted around each PCD belonging to the silver test groups compared to the controls, thus attesting to the antimicrobial activity of the material. Whereas when using circular / round PCDs for antibiotic testing it is the diameter of the inhibition zones which are measured, in our experiments we measured the inhibition zones around the PCD starting from the edge of the PCD . That method has been used by other study groups when using rectangular PCDs [13,14]. The smallest inhibition zones were observed for elemental silver, which gave rise to a narrow inhibition zone around the PCD. The biggest inhibition zones were observed for the silver Na-HZP

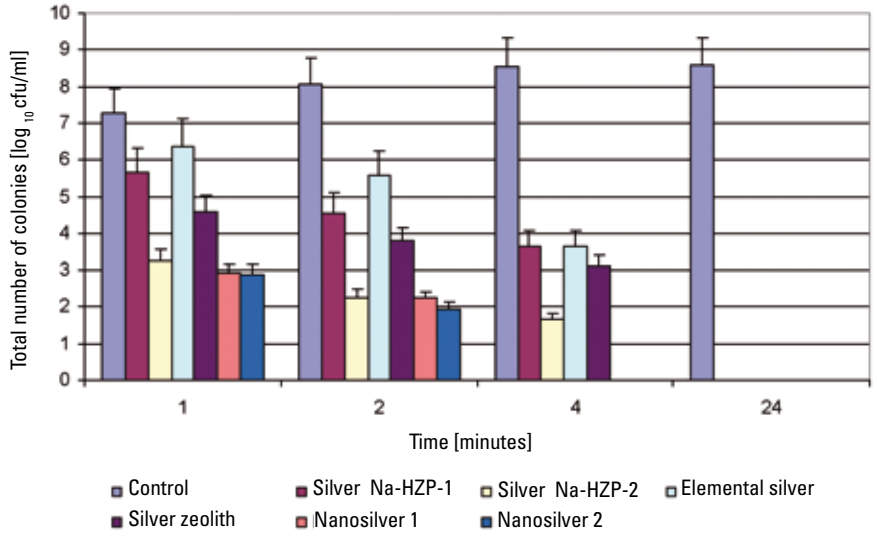

Figure 3: Total number of colonies on materials (MV±SD).

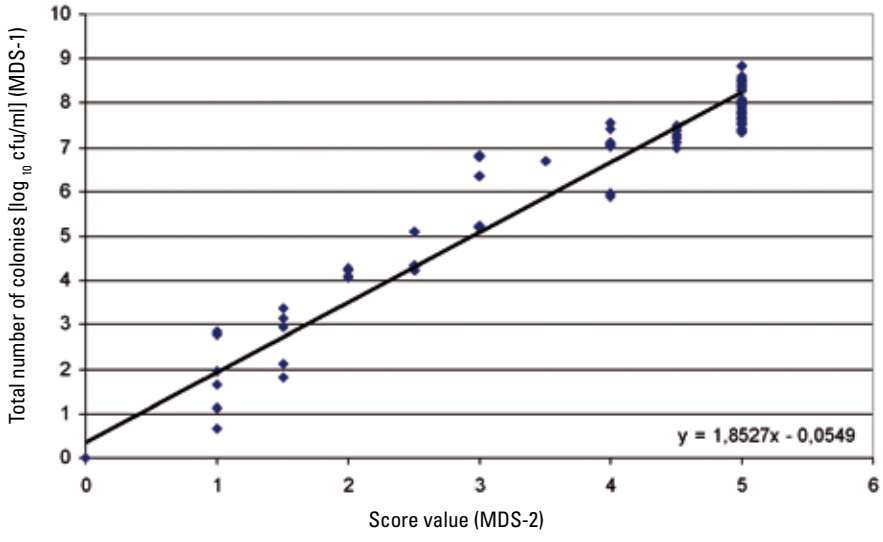

Figure 4: Correlation between total number of colonies for MDS-1 and scored values in MDS-2.

2 and nanosilver groups, which contained the highest silver content and in which the release of silver ions was greatest. The size of the inhibition zones was related to the silver concentration in the material as well as to the silver-ion release kinetics measured.

In similar experiments, inhibition zones were observed in wound dressings, but with marked differences seen depending on the different microorganisms investigated (*S. aureus, E. coli, C. albicans*) [14]. Conversely, in experiments conducted within the framework of the Kirby-Bauer test, which is a modified version of the agar diffusion test, with multi-layers of nanosilver, Lee et al. noted that the inhibition zone values measured were a function of the silver-containing layer (thickness, diffusion) [15]. Starting with the mechanism of action of silver, i.e. release of silver ions and diffusion to the surface of the material, it should be possible to detect such inhibition zones for all active materials. The results, which are quickly and easily obtained with the agar diffusion test, provide insights into the antimicrobial action of the materials investigated, however, they should be supplemented with microbiology tests. Whereas the agar diffusion test is firmly established in research into antibiotics, no consensus has been reached on its role in providing information on surface-active materials [3].

Another type of antimicrobial test is based on contamination of PCDs with a microbial solution and incubation for various periods of time, followed by determination of the microbial count on agar plates. The reduction in the microbial count is compared with that of the control. In the literature various designations are used for this, such as the suspension test or recovery test. There are several variants and modifications of these tests, e.g. Japanese Industry Standard (JIS Z 2801:2000) and the Standard of the American Association of Textile Chemists and Colorists (AATCC-100), which are tailored by the manufacturers to the respective product [2,3,4,6].

Based on the methods for testing disinfectants, a quantitative suspension test is a test that provides proof of the disinfectant's efficacy which is determinted by means of the reduction in the baseline microbial count, calculated as $\log_{10}$ levels. In the germ carrier test the disinfectant action is determined on contaminated materials, i.e. defined quantities of a microbial suspension are applied to PCDs (germ carriers) of a defined size, exposed to the disinfectant and after defined periods of time the germ carriers are examined for surviving microorganisms. Based on these definitions, the tests conducted by us were quantitative suspension tests involving the use of germ carriers.

Evaluation of the suspension test revealed that all materials investigated were able to reduce *E. faecium* counts. In particular, the nanosilver materials evidenced a reduction by almost 2 $\log_{10}$ levels already after 10 minutes. For elemental silver, containing the lowest silver content, a reduction in the microbial count was also noted after a two-hour exposure time. In the 24 hour samples, no bacteria were found any longer on the agar plates in any of the materials investigated.

To evaluate the antimicrobial efficacy the Dow Shake test was also conducted. In the description given for the test [7] the antimicrobial activity of the free silver ions eluted from the surface was measured. For this method catheter pieces with 10^9 bacteria (*S. epidermidis*) were inoculated in 1 ml NaCl phys and at six-hour intervals aliquots were withdrawn with a calibrated eyelet (0.02 ml) and plated onto an agar plate [7]. The cultures were incubated for 24 hours. The culture results were evaluated using a scoring system [7].

Since preliminary tests revealed it was not possible to assure valid counting of controls purely on the basis of bacterial growth,, and hence these could only be estimated, we changed the method to permit two practicable series of tests. In the modified Dow Shake-Test 1, 1 ml aliquots of the inoculation solution were withdrawn and counted at intervals of 1, 3, 6 and 24 hours. In the modified Dow Shake test 2, 0,1 ml aliquots of the inoculation solution were withdrawn and plated onto trypticase soybean agar. Visual assessment was carried out using a scoring system based on the literature [16]. The findings show that both methods were able to show reductions as seen in the quantitative microbiology tests with the suspension test and germ carrier test, but the corresponding values showed deviations. That highlights the importance of a high recovery rate, as achieved thanks to the recovery buffer in the suspension test. Statistical evaluation showed that both Dow Shake methods were closely correlated, hence even the simplified test involving direct plating out and evaluation on the basis of a scoring system is able to produce valid results.

Conflict of Interest

The authors declare that there is no conflict of interest as understood by the International Committee of Medical Journal Editors.

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