J Ind Microbiol Biotechnol (2003) 30: 102–106
DOI 10.1007/s10295-002-0022-0

ORIGINAL PAPER

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Antimicrobial efficacy of a silver-zeolite matrix coating on stainless steel

Received: 22 July 2002 / Accepted: 18 November 2002 / Published online: 14 January 2003

Abstract A silver- and zinc-containing zeolite matrix (AgION) used as a coating for stainless steel was tested for antimicrobial efficacy against *Escherichia coli* 25922, *Staphylococcus aureus* 25923, *Pseudomonas aeruginosa* 27853, and *Listeria monocytogenes* 7644. Assays were performed on flat coupon surfaces and in formed steel cups. AgION reduced microbial colony-forming units when compared to uncoated steel surfaces under all conditions tested. Percent reductions ranged from 84.536 to 99.999 after 4 h exposure, and from 99.992 to 100 after 24 h in all cases. The durability of the coatings declined most markedly when the coating had been applied with a wet process and scrubbed between uses with a test tube brush. Powder-coated surfaces cleaned with a towel retained a high degree of activity after five cycles of use.

Keywords Silver · Antimicrobial · Stainless steel

Introduction

There is increasing interest in materials that possess antimicrobial properties. In medicine and in dentistry, biomaterials impregnated with various types of antimicrobials have been in use for many years [8, 9, 12, 18]. The antimicrobial compounds used include traditional antibiotics as well as organic antimicrobials such as triclosan [5] and benzalkonium chloride [14], and inorganic compounds such as silver [6, 23, 24] and other heavy metals. These same antimicrobials have also been incorporated into materials intended for non-medical applications, such as carpets [15], hand lotions, wallpaper adhesives [11], gloves [10], pavement-marking materials [3] and window cleaners [7]. Some experts estimate that there are between 600 and 700 different types of consumer items containing some form of antimicrobial [7, 21].

The food industry is particularly interested in surfaces that can reduce microbial loads [16, 26] since an estimated 76 million people contract a foodborne illness in the United States each year [4]. *Escherichia coli* and *Listeria monocytogenes* are two of the most common foodborne pathogens and are commonly targeted by antimicrobial strategies. Another nonmedical industry with great interest in antimicrobial materials is the construction industry. Builders and users of residential and commercial properties are looking for ways to prevent growth of molds in building materials and in ventilation systems, as there has been an increase in concern over their possible health effects [22] and in indoor air quality, which can be adversely affected by the growth of microorganisms [19].

Perhaps the most common antimicrobial being incorporated into solid materials is silver, one of the oldest antimicrobial agents on record. Silver ions are thought to inhibit bacterial enzymes, interfere with electron transport, and bind to DNA [25]. Silver in the form of silver sulfadiazine is also one of the primary antimicrobials used in the treatment of burn patients [20]. This paper describes the antimicrobial efficacy against *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa* and *L. monocytogenes* of a new compound that can be coated onto solid surfaces and which uses ion exchange to release active silver particles. The compound material is a zeolite containing 2.5% (w/w) silver (Ag) and 14% zinc (Zn) ions within alumino-silicate matrices.

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Materials and methods

Bacteria and growth conditions

E. coli ATCC 25922, *P. aeruginosa* 27853 and *S. aureus* ATCC 25923 were streaked on tryptic soy agar (TSA) (Difco, Detroit, Mich.) from −70°C stocks. Overnight agar cultures were

transferred to tryptic soy broth (TSB; Difco) and incubated at 37°C statically for 18–24 h. *L. monocytogenes* ATCC 7644 was cultivated similarly on brain heart infusion agar and broth (Difco) at 37°C.

Bacteria were harvested by centrifugation at 8,000 *g* at 4°C for 10 min, and resuspended to $A_{540} = 0.1$ or to McFarland standard 1 in Butterfield's buffer (International Bioproducts, Bothwell, Wash.). This suspension was further diluted by a factor of 10 in Butterfield's before use in antimicrobial assays.

Coating of steel coupons and cups

Stainless steel coupons (2 inches × 2 inches), either bare or coated with AgION, the zeolite preparation containing 2.5% silver and 14% zinc, were supplied by AK Steel Corporation (Middletown, Ohio). Steel products were coated with an epoxy containing the silver-zeolite additive by two different methods. In the wet-process method the epoxy was dissolved in solvent, applied with rollers to a moving steel strip and heated to remove the solvent. The powder-coating method involved applying an electrical charge to the epoxy-zeolite mixture in a dry form. The electrical charge caused the powder to adhere to the surface of the steel, which was then heated so that the powder melted, flowed and cured to form a continuous film.

Antimicrobial efficacy assays

Minimum bactericidal concentration of AgION powder

Zeolite (sodium aluminosilicate) powders were provided by AgION Technologies (Wakefield, Mass.). Powders were amended with 2.5% Ag and 14% Zn ions. These concentrations of Ag and Zn were chosen after extensive testing, which demonstrated that 14% Zn stabilizes the product by slowing the release of Ag ions, enabling the use of relatively low concentrations of Ag. The powder was prepared in serial dilutions in TSB or Luria-Bertani (LB) broth. A 3-h broth culture of *E. coli*, *S. aureus* or *P. aeruginosa* was each made into a powder suspension. The test suspensions were incubated statically at 37°C and samples were removed and placed into neutralizing D/E broth (Remel, Lenexa, Kan.). Dilutions of the surviving bacteria were made in sterile saline and enumerated using the spread plate method on TSA.

Incubation on steel coupons

Coupons were cleaned by gentle rubbing with 70% isopropyl alcohol and allowed to air dry prior to analysis. Single coupons were placed on sterile supports inside sterile Petri dishes so that Butterfield's buffer (6 ml) could be added under the coupon without touching it to maintain ambient humidity. Bacterial suspension (0.5 ml) was pipetted on to the coupon and the Petri dish was closed and incubated statically at either 37°C or 23°C. Humidity was maintained by placing the coupons inside a closed container with a beaker containing 750 ml hot water. Three coupons of each type were inoculated with bacteria and incubated for 2 h, 6 h and 24 h. One coupon of each type was inoculated with bacteria and immediately processed to determine the input number of bacteria.

At the designated time, coupon supports were aseptically removed from under the coupons. The coupon was swirled in the 6 ml Butterfield's buffer in the bottom of the Petri dish, which was supplemented with fresh buffer to reach a volume of 10 ml. Sterile disposable plastic inoculating loops (International Bioproducts) were used to scrape the surface of each coupon into the buffer. The scraping was performed in an overlapping manner from right to left so as to cover the entire surface and then the coupon was turned 90° and scraped again. The coupon was then removed and discarded. Removal of bacteria was confirmed by periodic staining of coupons with BacLight LiveDead reagent (Molecular Probes, Eugene, Ore.) followed by fluorescent microscopy.

Incubation in steel cups

Stainless steel, either bare or coated with AgION as above, was formed into small cups (approximately 4.5 cm in diameter). Cleaning was performed as with the coupons above. Cups were then filled with 15 ml bacterial suspension and incubated at 37°C with gentle rotation (55 rpm). Fluid samples were withdrawn at designated time points and bacteria were enumerated (see below).

Enumeration of bacteria

The buffer in the Petri dish or in the steel cups was serially diluted in triplicate in Butterfield's solution. Aliquots (100 μl) from these dilution tubes were plated on the appropriate agar medium and incubated at 37°C for 24–36 h. Colonies were counted visually and colony-forming units (cfu) were calculated. Percent reduction was calculated with the formula: $[1-(\text{mean cfu}_{\text{treated coupon at time } t})/\text{mean cfu}_{\text{untreated coupon at time } t})]\times 100$. Negative values were designated as zero percent reduction. All experiments were performed in triplicate.

Results

Minimum bactericidal activity of silver/zinc zeolite powder

The minimal bactericidal concentration (MBC) of the silver-zeolite powder was 3.13 mg/ml for *E. coli* (grown in LB), *S. aureus*, and *P. aeruginosa*. *E. coli* grown in TSB was killed at a powder concentration of 1.56 mg/ml. Minimum inhibitory concentrations were not determined since the zeolite powder made broth suspensions highly turbid.

Bactericidal activity of coated steel coupons against broth cultures

Cultures of *S. aureus* and *E. coli* were both effectively killed by exposure to AgION-coated stainless steel (Table 1). Both bacteria were placed on the coupons at a concentration of $>1\times10^6$ cfu. *S. aureus* experienced a 5-log reduction in viability after only 6 h of exposure to the surface, while *E. coli* was reduced by 3.6 logs in the same period of time. Both bacteria were virtually eliminated by the 24 h time point.

Bactericidal activity of coated formed steel cups against buffer suspensions of bacteria

Tables 2 and 3 present the results from experiments testing the bactericidal activity of stainless steel cups treated with the AgION formulation using two different processes: "wet" coating and a powder coating. Bacteria were suspended in buffer inside the cups. Cups coated using both processes were highly effective at reducing bacterial counts, though the wet-coated cups resulted in less than a 2.5-log reduction for all bacteria after a 4 h incubation. After a 24 h incubation, these cups reduced

Table 1 Recovery of bacterial from stainless steel coupons

	Bare stainless steel		Wet-process coated stainless steel		
	Average log cfu (SD)	Log change from time 0	Average log cfu (SD)	Log change from time 0	Percent reduction
Staphylococcus aureus					
0 h	6.340 (0.012)	–	6.340 (0.012)	–	–
2 h	5.987 (0.080)	–0.348	2.260 (0.449)	–3.915	99.973
6 h	4.442 (2.728)	–0.462	1.301 (0.000)	–5.040	99.997
24 h	6.347 (0.185)	+0.033	1.301 (0.000)	–5.040	99.999
Escherichia coli					
0 h	6.579 (0.023)	–	6.579 (0.023)	–	–
2 h	6.737 (0.174)	+0.181	5.826 (0.160)	–0.712	87.225
6 h	8.284 (0.022)	+1.705	2.923 (0.185)	–3.632	99.999
24 h	8.428 (0.037)	+1.850	1.301 (0.000)	–5.279	100.000

Table 2 Bactericidal activity of wet-process-coated AgION stainless steel cups

	Bare stainless steel		Wet-process-coated stainless steel		
	Average log cfu (SD)	Log change from time 0	Average log cfu (SD)	Log change from time 0	Percent reduction
S. aureus					
0 h	6.380 (0.018)	–	6.380 (0.018)	–	–
4 h	5.962 (0.043)	–0.417	5.000 (0.063)	–1.378	89.058
24 h	4.982 (0.334)	–0.311	1.000 (0.000)	–5.380	99.992
E. coli					
0 h	6.176 (0.055)	–	6.176 (0.055)	–	–
4 h	6.806 (0.080)	+0.191	5.990 (0.109)	–0.620	84.536
24 h	6.998 (0.101)	+0.386	2.000 (0.000)	–4.620	99.999
Pseudomonas aeruginosa					
0 h	6.782 (0.042)	–	6.873 (0.003)	–	
4 h	6.594 (0.104)	–0.272	5.186 (0.192)	–1.657	95.885
24 h	7.119 (0.232)	+0.282	0.301 (0.000)	–6.572	100.000
Listeria monocytogenes					
0 h	6.743 (0.059)	–	6.743 (0.059)	–	
4 h	6.250 (0.188)	–0.493	4.374 (0.092)	–2.369	98.726
24 h	5.714 (0.289)	–1.029	0.301 (0.000)	–6.442	100.000

Table 3 Bactericidal activity of powder-coated AgION stainless steel cups

	Bare stainless steel		Powder-coated stainless steel		
	Average log cfu (SD)	Log change from time 0	Average log cfu (SD)	Log change from time 0	Percent reduction
E. coli					
0 h	6.418 (0.052)	–	6.418 (0.052)	–	–
4 h	6.131 (0.142)	–0.288	0.816 (0.891)	–5.603	99.998
24 h	6.018 (0.182)	–0.400	0.301 (0.000)	–6.117	100.000
L. monocytogenes					
0 h	6.796 (0.134)	–	6.796 (0.134)	–	
4 h	6.260 (0.155)	–0.536	1.286 (0.302)	–5.511	99.999
24 h	5.810 (0.168)	–0.987	0.301 (0.000)	–6.495	100.000

the numbers of all four bacterial species by greater than 99.99%.

The powder-coated cups were more rapidly bactericidal to the two bacteria tested (*E. coli* and *L. monocytogenes*). Both bacteria were reduced by greater than 99.998% (5.5 log reduction) as early as 4 h post-inoculation.

Retention of antimicrobial efficacy

Steel cups coated with both processes were tested sequentially for maintenance of antimicrobial efficacy against *E. coli*. Cups coated via the wet process that were scrubbed with test tube brushes between tests showed the lowest durability of efficacy (Table 4). The 4 h

Table 4 Durability of antimicrobial efficacy against *E. coli* after repeated testing and washing

Coating/washing method	Percent reduction on trial 1		Percent reduction on trial 3		Percent reduction on trial 5	
	4 h exposure	24 h exposure	4 h exposure	24 h exposure	4 h exposure	24 h exposure
Wet process/test tubebrush	97.170[a]	99.999	69.608	99.907	54.506	99.851
Wet process/towel	92.536	99.999	92.077	99.999	15.933	99.997
Powder coat/towel	99.998	100	99.999	100	86.372	100

[a]Percent reduction was calculated from the mean of three cfu counts (see Materials and methods)

antimicrobial efficacy decreased significantly over the course of five tests, although the 24 h efficacy showed a lower diminution with use. These coupons were continuously tested a total of 11 times, and while the 4 h efficacy decreased to 20% reduction on the 11th trial, the 24 h efficacy remained relatively high: >90% reduction on the 11th trial (Fig. 1). Cleaning these cups using paper towels and 70% ethyl alcohol did not alleviate the minor diminution in efficacy. In the fifth sequential test of cups cleaned in this way, the 4 h percent reduction in cfu was 15.933, while the 24 h percent reduction was 99.997.

Five sequential tests were also performed on powder-coated AgION cups, using the paper-towel washing method. These cups retained their efficacy to the highest degree after five trials, displaying a 4 h reduction of 86.372% and a 24 h reduction of 100% is (Table 4).

Discussion

Silver ions captured in a ceramic alumino-silicate matrix form the basis for a wide variety of coatings applied to many different types of substrata. This study investigates such a zeolite coating on stainless steel surfaces and investigates the degree of antimicrobial efficacy it confers. Zinc, present in the mixture at a concentration of 14%, provides additional antimicrobial activity. It is thought to work by inhibiting nutrient uptake and interfering with proton transfer [2, 13].

When bacteria in low concentrations of broth were placed on flat coupons coated with the silver zeolite (AgION) matrix, there was a 99.997% reduction in viable counts as compared to uncoated stainless steel within 6 h. After 24 h both *S. aureus* and *E. coli* were virtually eliminated from AgION-coated surfaces, while uncoated surfaces supported a modest increase in numbers of bacteria (Table 1). These experiments were conducted in high humidity; bacterial "puddles" did not evaporate over the course of the experiment. Under conditions in which the bacterial suspensions are exposed to ambient humidity and allowed to dry, the killing effects are amplified (data not shown).

A relatively high concentration of the zeolite powder was required for killing activity in minimal bactericidal concentration assays. Either 3.13 or 1.56 mg/ml zeolite was required for complete killing of the three species tested. This represents a silver ion concentration of 78 or 39 μg/ml. In addition, previous studies have shown that silver can form insoluble AgCl or Ag_2S complexes in media containing chloride or sulfur anions [17], a phenomenon also reflected in the differential susceptibility of *E. coli* to the silver-zeolite mixture in media of differing composition. It is of interest that *P. aeruginosa*, a bacterial species with a high degree of natural resistance to many antimicrobials, was as sensitive to this mixture as *E. coli* and *S. aureus* in both the soluble powder assay and the solid-surface assay (Table 2).

The use of formed cups coated with the silver-zeolite matrix represents a new, simple and reproducible method for testing solid-surface antimicrobials. In this assay, coatings generated by both methods (the wet-process and powder-coating process) had high antimicrobial efficacy against all four bacterial species tested,

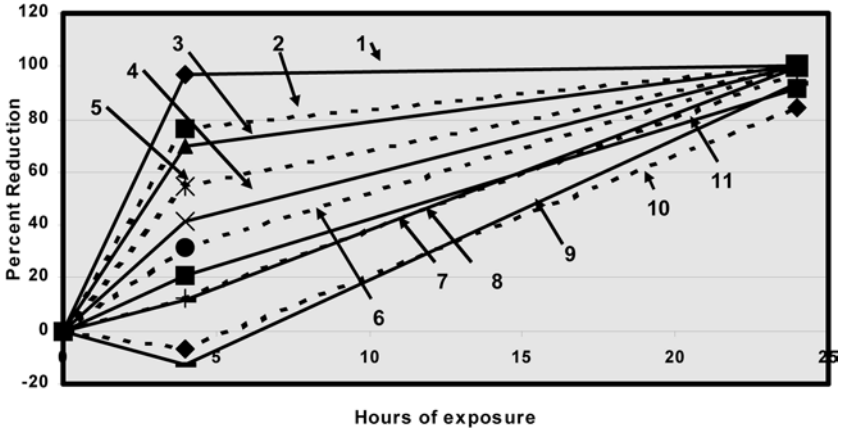

Fig. 1 Retention of antimicrobial efficacy under the worst-performing conditions. Wet-process coated coupons were scrubbed with test tube brushes after each of 11 trials. Antimicrobial efficacy at 4 h and 24 h is shown. Percent reduction was calculated from the mean of three cfu counts

including *P. aeruginosa* and *L. monocytogenes*, with the powder-coated cups displaying superior killing abilities (Table 2). The powder-coated cups also had increased durability compared to the wet-process-coated cups, an observation attributed to the increased thickness of the coating and the higher viscosity of the material maintained during application (AK Steel Corporation, personal communication).

In summary, a silver- and zinc-containing zeolite matrix applied to stainless steel greatly enhances the antimicrobial properties of the stainless steel and could prove useful in settings where microbial contamination is undesirable. The powder-coating process results in higher activity and durability than the wet-coating process. Akiyama et al. [1] have also shown that silver (in the form of silver sulphadiazine and silver nitrate) is highly effective at preventing biofilm formation by *S. aureus*. Future studies will investigate the efficacy of this silver-zeolite mixture on biofilms produced by various microorganisms on steel surfaces.

Acknowledgements The technical assistance of Jim Lewellyn and Matt Renners is gratefully acknowledged. This work was supported by funding from AK Steel Corporation, Middletown Ohio, and by the Miami University Office for the Advancement of Scholarship and Teaching.

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