ANTIMICROBIAL EFFICACY OF A SILVER-ZEOLITE MATRIX COATING ON STAINLESS STEEL

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Summary

A silver and zinc-containing zeolite matrix (AgION®) used as a coating for stainless steel was tested for antimicrobial efficacy against *E. coli* 25922, *Staphylococcus aureus* 25923, *Pseudomonas aeruginosa* xxxxx, and *Listeria monocytogenes* 7644. Assays were performed on flat coupon surfaces and in formed steel cups. The silver-zeolite mixture reduced microbial colony-forming units from 84.536 – 99.999% after 4 h exposure, and from 99.992-100% 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 in materials intended for non-medical applications, such as in carpets [15], hand lotions, wallpaper adhesives [11], gloves [10], pavement marking materials [3] and window cleaners [7]. Some experts estimate that there are from 600-700 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 U.S. 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 the 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. Silver is one of the oldest antimicrobial agents of 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*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Listeria monocytogenes* of a new compound which 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% (wt/wt) silver (Ag) and 14% zinc (Zn) ions within alumino-silicate matrices (AK Steel Corporation, Middletown, OH).

Materials and methods

Bacteria and growth conditions

Escherichia coli ATCC 25922, *Pseudomonas aeruginosa xxxx* and *Staphylococcus aureus* ATCC 25923 were streaked on tryptic soy agar (TSA) (Difco, Detroit, MI) from -70° C stocks. Overnight agar cultures were transferred to tryptic soy broth (Difco) and incubated at 37° C statically for 18-24 h. *Listeria monocytogenes* ATCC 7644 was cultivated similarly on brain heart infusion agar and broth (Difco) at 37° C.

Bacteria were harvested by centrifugation at 8000 *x g* at 4° C for 10 minutes, and resuspended to A_{540} = 0.1 or to McFarland standard 1 in Butterfield's buffer (International Bioproducts, Bothell, WA). 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 in x 2 in), either bare or coated with AgION®, the zeolite preparation containing 2.5% silver and 14% zinc, were supplied by AK Steel Corporation, Middletown OH. 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, MA). Powders were amended with 2.5% Ag and 14% Zn ions. The powder was prepared in serial dilutions in tryptic soy broth or Luria broth in triplicate. A 1:100 dilution of 3-h broth culture of *E. coli*, *S. aureus* or *P. aeruginosa* was made into each powder suspension. The test suspensions were incubated statically at 37° C and samples were removed and placed into neutralizing D/E broth (Remel, Lenexa, KS). Dilutions of the surviving bacteria were made in sterile saline and enumerated using the spread plate method on tryptic soy agar.

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. One-half ml of bacterial suspension was pipeted on to the coupon and the petri dish was closed and incubated at either 37° C or 23° C statically. Humidity was maintained by placing the coupons inside a closed container with a beaker containing 750 ml of hot water. Three coupons of each type were inoculated with bacteria and incubated for 2h, 6h and 24 h. One coupon of each type was inoculated with bacteria and immediately processed to determine 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 OR) followed by fluorescent microscopy.

Incubation in steel cups: Stainless steel, either bare or coated with AgION® as above, was formed into small cups (approximately 3 in diameter). Cleaning was performed as with the coupons above. Cups were then filled with 15 ml of 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. 100 µl aliquots 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 CFUs (colony forming units) 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.

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 Luria broth), *S. aureus*, and *P. aeruginosa*. *E. coli* grown in tryptic soy broth was killed at a powder concentration of 1.56 mg/ml. Minimum inhibitory concentrations (MIC) 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 x 10^6 colony-forming-units (CFUs). *S. aureus* experienced a 5-log reduction 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

Table 2 presents the results from experiments testing the bactericidal activity of stainless steel pans 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-coat cups resulted in less than a 2.5-log reduction for all bacteria after a 4 h incubation. After 24 h of incubation these cups reduced the numbers of all 4 bacterial species by greater than 99.99%.

The powder-coated cups were more quickly 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 which were scrubbed with test tube brushes between tests showed the lowest durability of efficacy (Table 3). The 4 h antimicrobial efficacy decreased significantly over the course of 5 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 approx. 20% reduction on the 11^{th} trial, the 24 h efficacy remained relatively high - 90% reduction on the 11^{th} trial. Cleaning these cups using paper towels and 70% ethyl alcohol did not alleviate the diminution in efficacy. In the 5^{th} sequential test of cups cleaned in this way, the 4h percent reduction in CFUs was 15.933, while the 24h 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 4h reduction of 86.372% and a 24 h reduction of 100%.

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 micrograms/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 to note that *Pseudomonas 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 2a).

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) were shown to have high antimicrobial efficacy against all four bacterial species tested, including *P. aeruginosa* and *L. monocytogenes*, with the powder-coated cups displaying superior killing abilities (Table 2). The powder-coated cups also were shown to have increased durability when 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 (personal communication, AK Steel).

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.

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Table 1 Bacterial recovery from stainless steel coupons

	Bare stainless steel		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	6.340 (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
E. 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 2a 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
P. 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
L. 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 2b 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. mono-cytogenes					
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

Table 3 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	
	4h exposure	24h exposure	4h exposure	24h exposure	4h exposure	24h exposure
Wet process / test tube brush	97.170	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

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