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A Novel Strategy for Control of Microbial Biofilms through Generation of Biocide at the Biofilm-Surface Interface

PAUL WOOD,[1] MARTIN JONES,[2] MOHAN BHAKOO,[2] AND PETER GILBERT[1]*

Department of Pharmacy, University of Manchester, Manchester M13 9PL,[1] and Unilever Research, Port Sunlight Laboratory, Wirral L63 3JW,[2] United Kingdom

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Biofilms of a mucoid clinical isolate of *Pseudomonas aeruginosa* (24 h; ca. 10^6 CFU/cm^2) were established by immersion of polymer discs in nutrient broth cultures at 37°C. Biofilms exposed for 30 min to various concentrations (0 to 3 mg/ml) of hydrogen peroxide or potassium monopersulfate were rinsed and shaken vigorously in sterile saline to detach loosely associated cells, and the residual viable attached population was quantified by a blot succession method on agar plates. Incorporation of copper and cobalt phthalocyanine catalysts within the polymers significantly enhanced the activity of these oxidizing biocides towards biofilm bacteria by several orders of magnitude. Biofilms established on the control discs resisted treatment with concentrations of either agent of up to 3 mg/ml. Enhancement through incorporation of a catalyst was such that concentrations of potassium monopersulfate of as low as 20 μg/ml gave no recoverable survivors either on the discs or within the washings. Catalysts such as these will promote the formation of active oxygen species from a number of oxidizing agents such as peroxides and persulfates, and it is thought that generation of these at the surface-biofilm interface concentrates the antimicrobial effect to the interfacial cells and generates a diffusion pump which further provides active species to the biofilm matrix. The survivors of low-concentration treatments with these agents were more readily removed from the catalyst-containing discs than from the control discs. This indicated advantages gained in hygienic cleansing of such modified surfaces.

Microbial biofilms are not only widely distributed in nature (11) but also problematic in many industrial environments where relatively high nutrient fluxes and large areas of submerged surface provide choice niches for the formation of extensive microbial biofilms (20, 23). This may not only cause increased fluid frictional resistance in pipes (10), increased heat transfer resistance (6, 10), biocorrosion (25), and reduced water quality (13, 21) but also provide foci for the contamination of hygienic products (20, 23). Implanted biomaterials and medical devices also favor microbial colonization, such that their use is associated with increased infection rates (14, 15).

While suspended populations of bacteria (planktonic) and device-related bacteremias respond readily to antimicrobial treatment, the bacteria within biofilms are protected from even the most aggressive of treatment regimens (11, 12, 18). Device-related infections therefore recur unless the device is removed before treatment. Conventional methods of microbial control and eradication within domestic and industrial settings also often prove inadequate with respect to adherent microorganisms associated with biofilms (9). It has been suggested (1, 5, 19, 22) that one of the primary mechanisms associated with the resistance of biofilms to antimicrobial agents relates to the presence of highly hydrated, polyanionic matrices of extracellular polymeric substances. These encase the cells growing at a surface and provide protection from external aggression by reacting chemically with, or binding, biocides and providing a diffusion barrier (7, 8, 24, 27, 28). These factors reduce access to and availability of the biocide at the colonized surface and within the depths of the biofilm.

The precise manner by which extracellular polymeric substances protect the cells is unclear, but the presence of bound extracellular enzymes, such as β-lactamases, within the glycocalyx may reinforce its action as a diffusion barrier (5) with respect to some antibiotics, and its molecular sieving properties are enhanced through binding of divalent cations, such as calcium, from the environment (22). It has also been proposed that the glycocalyx provides intrinsic protective effects against antimicrobial agents which are additional to those associated with its diffusion and charge-related properties (19).

If the glycocalyx confers resistance through exclusion of the treatment agent from the underlying cells, biocide would become concentrated at the colonized surface by the same processes if biocide could be generated at this site. Provision of biocides at the colonized surface has been reported by other workers to reduce the viability of attached organisms. In such studies, the biocides have been impregnated into biomaterials which then serve as antimicrobial reservoirs (3, 4). In such instances, biocide must be applied to the surface before growth of the biofilm, and this will become depleted with time.

The present article describes a novel approach to hygienic control and eradication of microbial biofilms from surfaces through the generation of biocide at the surface by catalysis from an extrinsically applied treatment agent.

MATERIALS AND METHODS

Strains and culture maintenance. A stable mucoid clinical isolate of *Pseudomonas aeruginosa* PaWH was used in the study. Cultures were maintained on tryptone soy agar (TSA; Oxoid CM131) slants, in the dark at 4°C, after overnight incubation at 37°C. Overnight cultures were prepared from the slopes by inoculating 100-ml volumes of tryptone soy broth (TSB; Oxoid CM129) in 250-ml Erlenmeyer flasks and incubating the flasks at 37°C for 16 h in an orbital incubator (200 rpm).

Media and chemicals. Dehydrated culture media were obtained from Oxoid (Basingstoke, United Kingdom). All other reagents were of the purest available grade and were obtained from BDH Ltd. (London, United Kingdom).

Copper sulfonated phthalocyanine (CuPC), cobalt sulfonated phthalocyanine

* Corresponding author. Mailing address: Microbiology Research Group, Department of Pharmacy, University of Manchester, Oxford Rd., Manchester M13 9PL, England. Phone: 44 161 275 2361. Fax: 44 161 2396. Electronic mail address: PGilbert@fs1.pa.man.ac.uk.

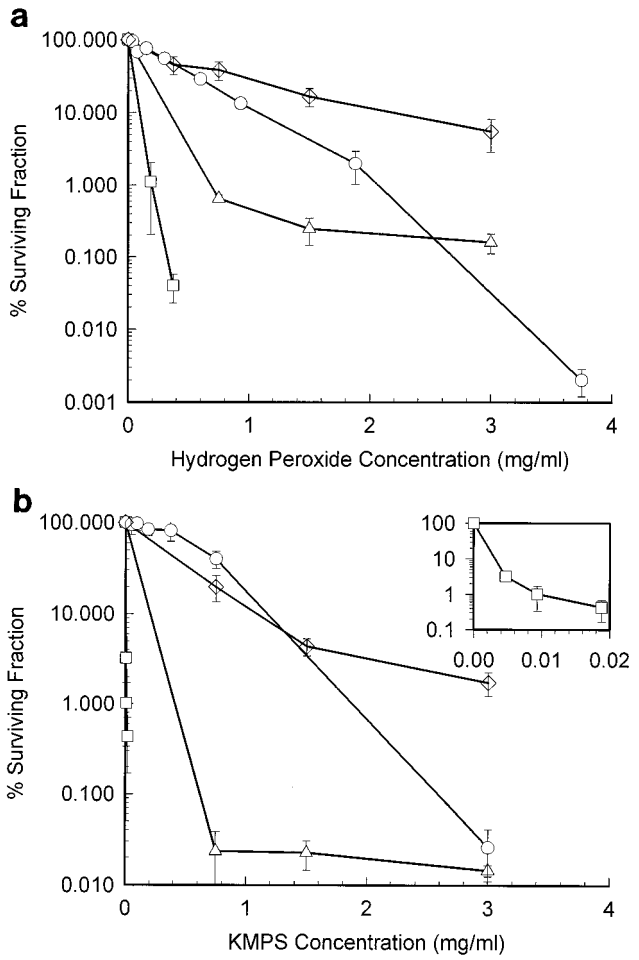

FIG. 1. Effects of exposure for 30 min at room temperature to various concentrations of H_2O_2 (a) or KMPS (b) on the survival of *P. aeruginosa* grown planktonically (○) and as biofilms on Trylon discs without (◇) and with incorporation of a CuPC (△) or CoPC (□) catalyst. Survivors of treated biofilms were estimated from the numbers of CFU removed to saline during rinsing and vigorous shaking. Bars indicate standard errors.

(CoPC), and potassium monopersulfate (KMPS) were provided by Unilever Research, Port Sunlight Laboratories.

Preparation of control materials and surfaces. Trylon resin (polyester resin in styrene monomer, 49.5 ml; Trylon Ltd., Wollaston, Northamptonshire, United Kingdom) was mixed with hardener (methyl ethyl ketone peroxide, 0.5 ml; Trylon) to a concentration of 1%, poured into 20-mm-diameter boiling tubes, and allowed to cure overnight at room temperature. Cylinders of Trylon resin were removed from the tubes, machine-cut into discs (thickness, 1.5 mm; diameter, 20 mm), and used without further polishing.

Preparation of catalyst-containing materials and surfaces. CuPC (5 mg) or CoPC (5 mg) was mixed with unhardened Trylon resin (1 ml). The volume of Trylon resin was made up to 49.5 ml. Hardener (0.5 ml) was added, and the mixture was poured into a 20-mm-diameter boiling tube. Once cured, cylinders of resin containing catalyst (100 μg/ml) were removed from the tubes and cut into discs as described above. The discs were stored dry in a darkened cupboard at room temperature. Prior to use, the discs were soaked in ethanol (70% [wt/wt]) for 1 h or autoclaved at 121°C for 15 min.

Susceptibility testing of planktonic cultures. The susceptibility of planktonically grown *P. aeruginosa* towards H_2O_2 and KMPS was assessed by inoculating volumes (20 ml) of sterile saline containing biocide (0 to 3.75 mg/ml) with aliquots (1 ml) taken from an overnight TSB culture. This gave initial cell concentrations of ca. 10^8 CFU/ml. Test suspensions were held at room temperature for 30 min, after which the biocide was quenched by performing a 1:10 dilution in sodium thiosulfate (9 mg/ml). The quenched suspensions were further diluted in sterile saline, and aliquots (0.1 ml) were spread onto the surfaces of quintuplicate predried nutrient agar plates. The plates were incubated for 16 h at 37°C, and colony counts were made. Results were expressed as percentage reductions in viability relative to that of controls which had been exposed only to saline and sodium thiosulfate.

Culture of biofilms. Catalyst-containing and control Trylon discs were clamped vertically within a Teflon cassette (17). The cassette holds up to 16 discs in a radial arrangement which allows the free circulation of liquid when immersed in culture medium. Beakers (500 ml) containing TSB (200 ml) and a filled cassette were inoculated from a TSA plate and incubated in an orbital incubator (37°C, 200 rpm) for 24 h. Biofilms (ca. 10^6 to 10^7 CFU/cm^2) formed on the surfaces of the discs.

Viable count determinations for biofilms. Four replicate biofilms together with their Trylon-resin supports were removed from the cassettes, and each one was rinsed in two successive volumes (20 ml) of sterile saline to remove loosely associated cells and carryover. Discs were each shaken vigorously for 10 min in saline (process A; 10 ml) with a flask shaker (Griffin and George Scientific, London, United Kingdom). This removed the bulk of the attached cells. The discs were removed and rinsed in successive volumes (three times, 20 ml) of sterile saline (process B). Viable counts were performed on bulked suspensions obtained from processes A and B and related to the total surface area of the colonized discs (7.2 cm^2). These easily removed cells corresponded to ca. 99% of the total biofilm community.

The residual attached population after shaking and rinsing was assessed by the plate succession method of Eginton et al. (17). Each of the discs was transferred to the surface of an individual, predried, TSA plate, left for 2 min, removed with forceps, and placed on a fresh plate. The initial plate was spread with a sterile rod and incubated overnight at 37°C. This procedure was repeated for a succession of 15 plates. In all cases, the mean CFU from the four replicate discs decreased as an exponential function of the plate succession number. Regression analyses were performed by use of the Marquardt-Levenberg algorithm to determine the coefficients of the independent variables that gave the best fit between equation and data. This is an iterative process carried out with Sigma Plot software (Jandel Scientific, Erkrath, Germany). Data were fitted to the following equation: $\text{CFU} = A \cdot 10^{-KN}$, where A is a constant, K is the reduction exponent of the best-fit line and gives a measure of the ease of removal of the attached cells, and N is the plate succession number.

K values for the lines of best fit reflect the ease of removal of the residual attached biofilm cells from the test surfaces (16, 17). From the equation described above, and the best-fit values of A and K, the size of the residual attached population could be calculated. This was done by an iterative procedure based on integer values of N from 1 to that calculated to give only one transferred colony per plate (17). Numbers of residual attached cells were expressed as the means of the four biofilm samples relative to the surface area of one side of the test piece (3.143 cm^2). Total biofilm populations could be estimated from the sum of the CFU obtained in the rinse solutions and estimates from the plate succession experiment.

Susceptibility testing of biofilms. Biofilms were cultured, as described above, on catalyst-containing and control Trylon discs. After a preliminary rinsing in saline (twice, 20 ml each) to remove loosely attached cells, each disc was placed in saline containing various concentrations (0 to 3 mg/ml) of either H_2O_2 or KMPS (20 ml). After 30 min at room temperature, the KMPS-treated discs were transferred by forceps to sodium thiosulfate neutralizer (20 ml, 9 mg/ml) and peroxide-treated discs were transferred to sterile saline (20 ml). This was followed after 5 min by successive rinses in sterile saline (twice, 20 ml each). Each disc was then transferred to a sample tube containing sterile saline (10 ml) and shaken vigorously for 10 min in a flask shaker (Griffin and George Scientific). After further rinsing in sterile saline (three times, 20 ml each), the residual viable attached population of cells was assessed by the plate succession method (described above). All data were expressed relative to those of untreated control biofilms generated on appropriate resin discs. The effects of biocide treatment were related, for the mean results from four replicate discs, to (i) the residual, attached-biofilm population, after shaking and rinsing, determined from the results of the plate succession experiments, and (ii) the population of the biofilm which had become dispersed during and after treatment. The latter was estimated from the summation of the viable counts performed on the treatment solution and the various washings. Reduction exponents (K), reflecting the ease of removal of the residual attached populations, were determined in each case.

RESULTS

Effect of catalysts on biofilm development. Biofilms of *P. aeruginosa* were established on Trylon resin discs by immersion of the discs into TSB batch cultures. Estimates made of the size of the biofilm communities established at 24 h showed total viable, attached populations of 4.5×10^6, 3.0×10^5, and 3.8×10^5 CFU/cm^2 on the control, CuPC-containing, and CoPC-containing discs, respectively. This indicated a lower level of colonization of the catalyst-containing discs. Growth of the planktonic cultures was unaffected by the presence of the catalyst-containing discs. This indicated that any antimicrobial effect associated with the catalyst was confined to the disc

TABLE 1. Concentrations of biocide required to reduce populations of *P. aeruginosa* by 90% within 30 min at room temperature when grown planktonically or as biofilms on Trylon discs with or without incorporation of a CuPC or CoPC catalyst

Nature of target cells	H_2O_2 concn (mg/ml)		KMPS concn (mg/ml)	
	Attached[a]	Total biofilm[b]	Attached[a]	Total biofilm[b]
Planktonic cells	ND[c]	1.120	ND	1.200
Biofilms on Trylon discs	ND	2.200	ND	1.100
Biofilms on CuPC discs	2.200	0.375	0.375	0.200
Biofilms on CoPC discs	0.150	0.125	0.004	0.003

[a] Determined by the agar succession method (23).
[b] Attached plus those cells removed by processes A and B.
[c] ND, not defined.

surface. Vigorous shaking of the cultured discs in a Griffin flask shaker was sufficient to remove >99% of the biofilm population. The residual attached cells, determined by the plate succession method, in these instances, corresponded to 8×10^4, 7.6×10^3, and 7.7×10^3 CFU/cm^2 for the control, CuPC-containing, and CoPC-containing discs, respectively.

Biocidal susceptibilities. The extent of killing of suspended cells and of biofilms associated with the test and control discs, caused through exposure to either H_2O_2 or KMPS, was assessed, and the results are presented in Fig. 1 and 2. The survivors of biocide-treated biofilms were considered separately as those cells which were removed from the biofilm during and after treatment (Fig. 1) and as the residual attached population (Fig. 2). The latter cells had resisted the actions both of the biocide and the posttreatment removal either by rinsing or vigorous shaking in saline. Concentrations of KMPS and H_2O_2 of 3.0 mg/ml gave ca. 4-log reductions in the numbers of exposed planktonic cells. Control biofilms, established on Trylon discs, were significantly less sensitive to concentrations of these biocides of >1.5 mg/ml (ca. 1.5-log reductions at 3 mg/ml). This was indicated not only through a greater survival of the biofilm-treated cells recovered in the washings (Fig. 1) but also through failure of the higher concentrations to affect the numbers of residual attached cells (Fig. 2). This residual population, however, constituted less than 1% of the total biofilm.

The activities of KMPS and H_2O_2 against the control biofilms and planktonic cells were similar. Significant increases in the effectiveness of the two biocides were, however, observed when either the CuPC or CoPC catalyst was incorporated into the test material. This increase in activity was greater for the CoPC-containing discs than for the CuPC-containing discs and was more marked for combinations which included KMPS rather than H_2O_2 as the biocide. The extent of killing, particularly at the lower concentrations of biocides, was greater than that obtained when planktonic suspensions of cells had been exposed. The concentrations of biocide required to reduce the numbers of viable cells by 90% are given in Table 1. These values indicate resistance of biofilms towards KMPS and H_2O_2 biocides and order-of-magnitude improvements in activity toward biofilm achieved through the inclusion of catalyst at the colonized surface.

Ease of removal of biofilm-derived cells from treated surfaces. The plate succession method (17) not only gives a measure of the numbers of viable cells which remain attached to a test surface after rinsing and vigorous shaking in saline but also indicates their ease of transfer from that surface to an agar plate. In the biocide susceptibility experiments described in this article, the removal exponent (K), obtained from the equation in Materials and Methods, is a measure of the ease of removal of the residual attached population (Fig. 3) from the treated test pieces. The ease of removal of the cells from the test surface increases with K. The effects of H_2O_2 and KMPS treatments on K for the residual attached cells are illustrated in Fig. 3. The nature of the catalyst had little or no effect on the magnitude of K for untreated biofilms but markedly increased the ease of removal of the residual survivors following biocide treatment. As for the catalyst-promoted killing (see above), this effect was more marked for the CoPC- than for the CuPC-containing discs and greater for KMPS than for H_2O_2 treatments.

DISCUSSION

Biofilms are generally less sensitive to antimicrobial treatments than are the equivalent cells growing planktonically (1, 5, 22). Much of this resistance has been attributed to the exclusion properties of the glycocalyx. These are thought to provide a relatively biocide-free environment for the underlying cells (19). If biocides could be released from the colonized

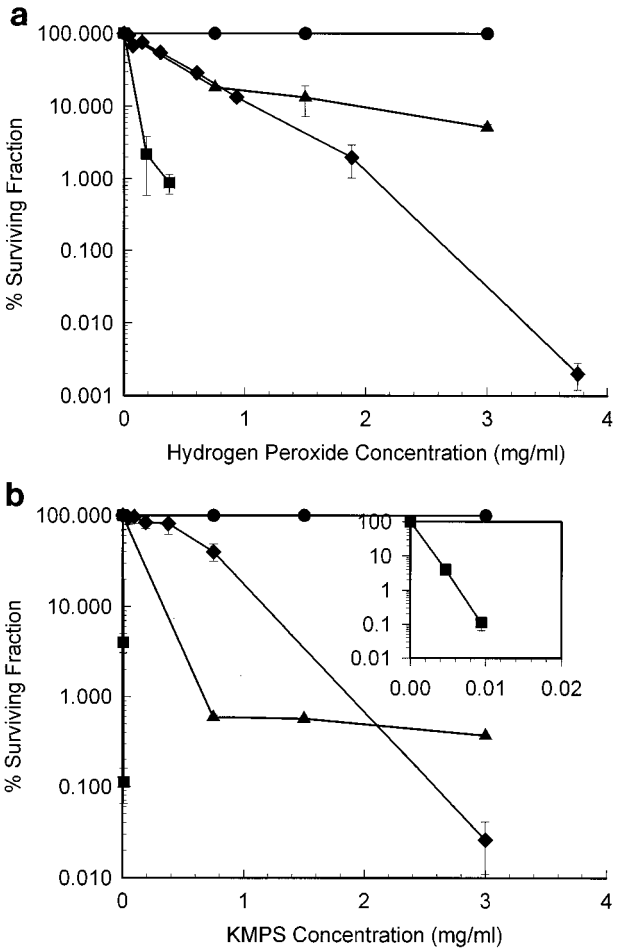

FIG. 2. Effects of exposure for 30 min at room temperature to various concentrations of H_2O_2 (a) or KMPS (b) on the survival of *P. aeruginosa* grown planktonically (◆) and as biofilms on Trylon discs without (●) and with incorporation of a CuPC (▲) or CoPC (■) catalyst. Survivors of treated biofilms were estimated as the residual attached population which remained on the test surfaces after rinsing and vigorous shaking. This was determined by the agar succession method (23). Bars indicate standard errors.

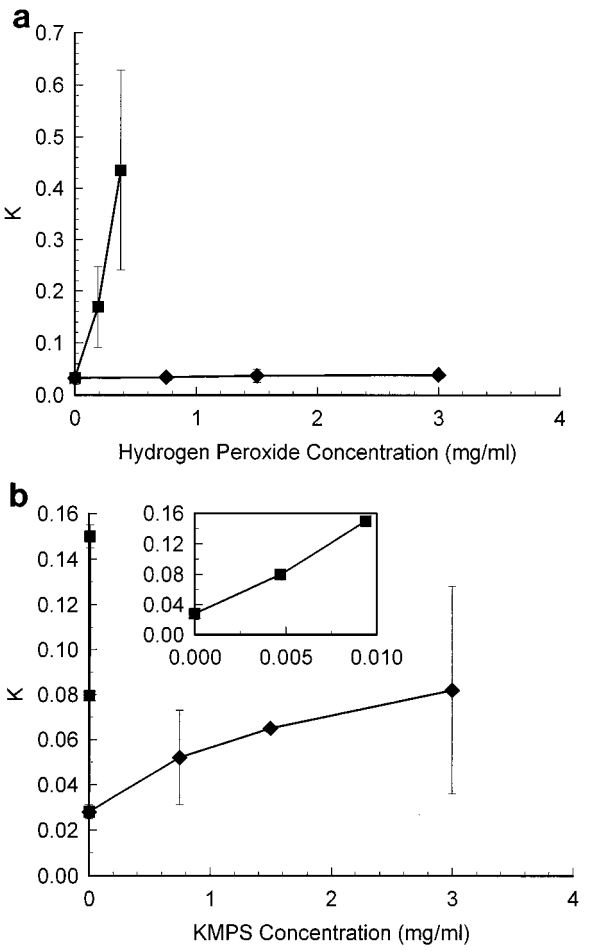

FIG. 3. Effects of exposure of *P. aeruginosa* biofilms for 30 min at room temperature to various concentrations of H_2O_2 (a) or KMPS (b) on the ease of removal (*K*) of the residual attached survivors. Biofilms were cultured on Trylon discs containing either a CuPC (◆) or CoPC (■) catalyst. *K* was determined by analysis of the results of plate successions conducted after washing and shaking the treated biofilms in saline (23). Bars indicate standard errors.

surface, rather than applied extrinsically, then cells adjacent to the surface would no longer be protected. This could be achieved if biocide was deposited on or within the material before exposure to a colonizing population of microorganisms (3, 4) or it might be generated in situ, after biofilm formation, from an extrinsically applied biocide precursor. This latter effect might be achieved if the colonized surface incorporated a catalyst which was able to generate or activate biocide from a treatment agent. In such an instance, catalysis would deplete levels of the treatment agent both within the biofilm matrix and adjacent to the surface-biofilm interface. Such depletion might power a diffusion pump and deliver more treatment agent to the interface. Those same factors which normally protect the underlying cells in a biofilm against extrinsic biocide would now accumulate the active agent at the colonized surface. Concentrated action at the interface might have additional effects on the adhesion properties of the biofilm.

The feasibility of this approach, for the control of biofilms on surfaces, was examined in the current study by incorporating transition metal catalysts into polymer surfaces. These catalysts promote the formation of active oxygen species from peroxides and persulfates (2). Biofilms were allowed to form before being treated with hydrogen peroxide and KMPS. The killing actions of these compounds relate not only to their oxidizing properties per se but also to their slow breakdown to water, releasing various oxygen species and free radicals (2, 26). These compounds are more reactive chemically than the parent compounds but are very short-lived. As a consequence of their short-lived nature, only those radicals which are generated in the immediate vicinity of a cell are likely to have antimicrobial effects. The presence of catalysts which promote the breakdown of peroxides, within peroxide-based disinfectant formulations, would therefore reduce killing capacity when directed against planktonic cells. If, however, the catalyst was localized at sites critical to the target cells (such as the cell membrane or adhesion interface of a biofilm), then the generated free radicals would act predominantly at these locations and diffusion would replenish the peroxide.

The results of the present study indicate that such an approach dramatically enhances the killing properties of both hydrogen peroxide and KMPS against bacterial biofilm (2- to 3-log improvement). At biocide concentrations of less than 3 mg/ml, the killing of biofilms formed on catalyst-containing surfaces occurred to a greater extent than even that of planktonic populations. As a catalyst of the breakdown of peroxides, cobalt salts are well known to be significantly more effective than copper salts, and it is therefore not surprising that incorporation of a CoPC catalyst into the test surface gave a far greater enhancement of biofilm killing than incorporation of CuPC (Fig. 1). Residual attached populations were unaffected by the biocide treatments unless a catalyst was included in the test surface. The enhancement effects of the catalysts were most marked when examined for these populations (Fig. 2). When the strength of bacterial attachment to the test surfaces was examined (Fig. 3), it was noted that inclusion of the catalysts had markedly loosened the survivors. Having resisted the actions of the biocide, they were now only weakly associated with the surface. Presumably, such loosening is related to action of breakdown products of the biocides with matrix polymers rather than with the cell membranes or envelopes. In hygienic cleaning, and in antifouling treatments, this implies that the survivors of biocide treatment might be more readily removed by normal cleaning regimens when on catalyst-containing surfaces rather than conventional surfaces.

Immediate applications of these findings might be the inclusion of transition metal catalysts such as CoPC and CuPC within plastic moldings and extrusions intended for hygienic uses or applications (pipework, laminated work surfaces, contact-lens cases, and toothbrushes, etc.). This would not only facilitate their disinfection but also reduce the levels of treatment agent required to sanitize. The latter is particularly relevant to possible applications in the biomedical field (urinary catheters and contact lenses, etc.). The catalyst need not necessarily be included within the materials at manufacture but might presumably be applied to surfaces during or after cleaning. The results also indicate that other forms of catalysis, i.e., enzyme mediated, at colonized surfaces might also promote antibiofilm activity and that the treatment agent need not be antimicrobial in its own right. In this respect, it is amusing to note that the inclusion of urease in urinary catheters or glucose-oxidase on tooth surfaces might provide for urea or glucose to become the treatment agents which could then be applied by urination and sweet-eating, respectively.

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