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Preparation and Investigation of Antibacterial Protein-based Surfaces

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ABSTRACT

Surfaces bearing protein units (wool, silk) have been modified in a two step process to incorporate at the free side-chain hydroxyl groups functionalities (lipophilic with polycationic units) that bear antibacterial activity. The approach has involved tosylation of the hydroxyl groups followed by displacement with a tertiary amine bearing cationic and lipophilic components. The effectiveness of these modified surfaces for antibacterial action against a series of Gram + and Gram - bacteria is reported. Structural factors maximizing the activity against all species tested have been studied and appropriate surfaces have been generated. Preparative procedures along with methods of investigation of the antimicrobial activity are included along with a discussion of mode of activity.

Keywords: antibacterial, surfaces, antimicrobial

INTRODUCTION

It has for some time been recognized that cationic surfactants bear antibacterial activity.[1-5] Investigations of structure-activity relationships have demonstrated that in addition to a cationic site, a significant lipophilic component of the surfactant is involved in optimization of activity. With simple alkylbenzyldimethylammonium chlorides in their action against *Pseudomonas aeruginosa,* the optimal length of the alkyl chain has been noted to be twelve carbon atoms.[3] Optimal activity toward a variety of bacterial species for numerous structural variations of the water soluble cationic surfactants appears to occur when an alkyl chain of between ten and fourteen carbon atoms is present.[6-10]

The mechanism of action of such cationic surfactants on bacteria is understood to be one of electrostatic interaction and physical disruption, as opposed to interference with a metabolic pathway, as is commonly the situation with antibiotic species.[11] After the cationic site of the agent attached to a significant lipophilic component binds to anionic sites of the cell wall surface it is then able to diffuse through the cell wall and bind to the membrane. Acting as a surfactant, it is able to disrupt the membrane and permit the release of electrolytes and nucleic materials, leading to cell death.

While the construction of antibacterial agents that express their activity in such a manner has been well investigated, the remaining challenge has been to impart such activity to a surface from which the active agent is not released and will be able to maintain activity indefinitely. The binding of quaternary ammonium sites to glass surfaces through the use of silyl-ether linkages was found to impart antibacterial activity to such surfaces.[12] Several polymeric surfaces have also been investigated, including polystyrene[13,14] and poly(propylene imine).[15]

In this light, it appeared a reasonable possibility that other types of surfaces could be rendered antibacterial by the covalent attachment of polycationic units having lipophilic adjuncts. Prior efforts of our laboratory had demonstrated the facility with which such polyammonium units having lipophilic adjuncts could be prepared,[16,17] as well as the manner in which

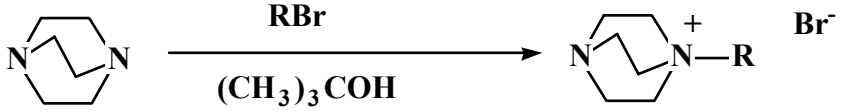
such materials could be attached to a variety of primary hydroxyl sites in complex structures.[18]

Following from this we previously have reported on the preparation and investigation of a variety of *carbohydrate* derived surface materials that were similarly functionalized.[18] These functionalized surfaces have been noted to be antibacterial with regard to a broad series of Gram + and Gram - bacteria. Success with such *carbohydrate*-derived surfaces, particularly cotton, has suggested that other types of fabric surfaces might also be rendered antibacterial. Using active cationic surfactant components as previously described, a similar approach and resultant activity might be anticipated for proteinaceous surfaces with a significant concentration of serine residues. The primary hydroxyl group of the amino acid appears to be an ideal site for incorporation of the active components. In this way, both wool and silk fabrics might be rendered antimicrobial as the serine content of each is significant (minimum values are for wool ~10%,[19,20] and for silk, 13-17%[19]). The current report is concerned with the functionalization of wool and silk surfaces in this manner and the investigation of their resultant antimicrobial activity.

RESULTS AND DISCUSSION

For the present effort parent polyammonium units having lipophilic adjuncts were prepared by a modification of previously noted techniques[16,17] using the bis-tertiaryamine 1,4-diazabicyclo[2.2.2]octane in reaction with appropriate haloalkanes in ethyl acetate solution (Equation 1). The resultant monocationic salts (**1** – **4**), which readily precipitated from solution, were isolated by suction filtration and dried under vacuum. All exhibited ^{1}H and ^{13}C NMR in accord with their proposed structures, as well as elemental analyses in accord with hydrated states of their elemental formulae, which have been reported previously.[16,17,21]

Protein-based surfaces (100% silk cloth, commercial grade; 100% wool cloth, commercial grade) were activated and functionalized (as noted in Equation 2), followed by washing and drying, to generate the modified surfaces.



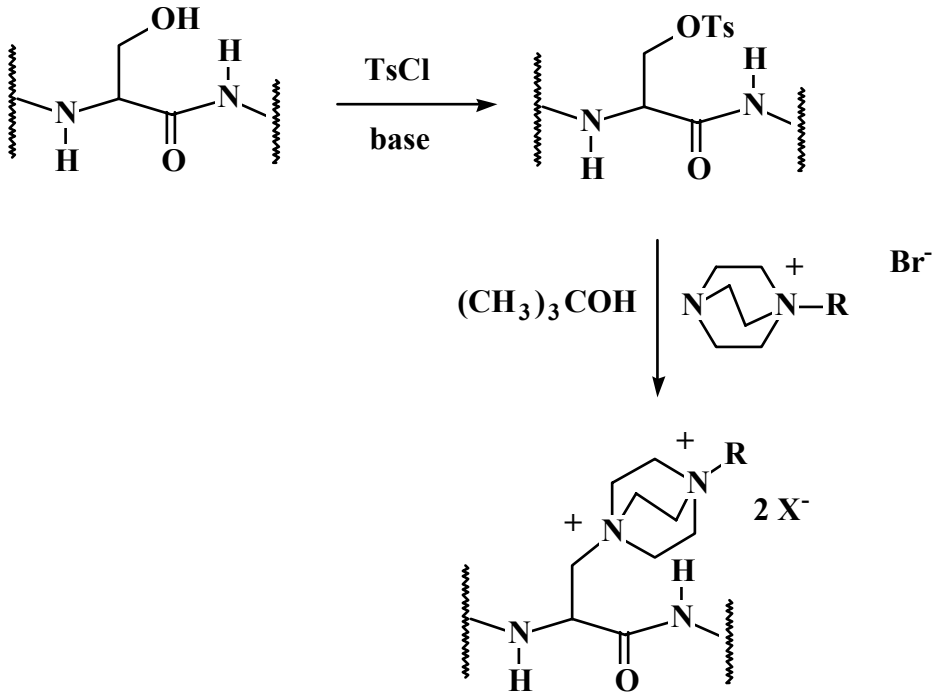
R = $-CH_2(CH_2)_{12}CH_3$ **(1)**

$-CH_2(CH_2)_{14}CH_3$ **(2)**

$-CH_2(CH_2)_{16}CH_3$ **(3)**

$-CH_2(CH_2)_{18}CH_3$ **(4)**

Equation 1



P-dabco-R

R =	**$-C_{12}H_{25}$**	**P-dabco-C12**
	$-C_{14}H_{29}$	**P-dabco-C14**
	$-C_{16}H_{33}$	**P-dabco-C16**
	$-C_{18}H_{37}$	**P-dabco-C18**

Equation 2

The structural components presumed for the protein surfaces are deduced from the corresponding results previously found for attachment of the cationic components to substrates bearing primary hydroxyl groups.[18,21] The reactivity of *p*-toluenesulfonyl chloride with alcohols is well known.[22] Primary hydroxyl sites can be functionalized quite specifically relative to secondary sites within the same molecule. Thus, tosylation (and subsequent displacement by the tertiary amine) of the available hydroxyls of the serine residues of the proteins is presumed to occur without reaction also occurring at any available secondary or aromatic hydroxyl sites. Very significantly, tosylation of these systems has been accomplished without the use of pyridine. Two alternative tosylation procedures have been accomplished using both wool and silk, the first using solid sodium bicarbonate present as an acid sink in acetonitrile medium. The second is most promising for applications, tosylation being performed in aqueous medium with sodium bicarbonate dissolved. This provides a

homogeneous reaction medium (except for the proteinaceous surface) minimizing the use of noxious organic reagents. The resultant modified proteinaceous materials exhibit the same activity as those produced using pyridine as a base in the tosylation step.

Four bacterial strains (two Gram - and two Gram +) were investigated, as noted below, with note of their American Type Culture Collection reference number:

Escherichia coli (ATCC #14948)
Proteus vulgaris (ATCC#13315)
Bacillus cereus (ATCC #14579)
Staphylococcus aureus (ATCC #6538)

Two types of experiments were performed to test the efficacy of the materials against these bacteria. First, ~10^5 bacteria were added to a test sample of the modified surface having an area of 0.25 in^2 placed on a rich medium and incubated, along with a control surface on the same plate. Control surfaces consist of the fabric of interest that have been washed with the same solvent system as the experimental surfaces, but have not been treated with the lipophilic adjunct.

Growth of bacteria off the edge of the surface was observed visually, following which the surfaces were placed in 4 mL of liquid growth medium and incubated for 16 hr. Growth of bacteria in these liquid media were measured turbidimetrically. (In all instances, involving each of the two types of protein-based surfaces, the control experiments exhibited full growth of the bacteria in all growth studies.) Growth of each of the bacteria in the liquid media experiments with modified silk is shown in Table 1. Corresponding results for modified wool surfaces are shown in Table 2.

Table 1
Growth of bacteria on modified silk surfaces as a percentage of control

	E. coli	*P. vulgaris*	*B. cereus*	*S. aureus*
P-dabco-C12	0	0	0	0
P-dabco-C14	44	4.6	0	0
P-dabco-C16	0	0	0	0
P-dabco-C18	70.9	24.6	0	0

Table 2
Growth of bacteria on modified wool surfaces as a percentage of control

E. coli	*P. vulgaris*	*B. cereus*	*S. aureus*

P-dabco-C12	17.8	21.4	0	0
P-dabco-C14	99.4	91.7	0	0
P-dabco-C16	50.7	19.8	0	0
P-dabco-C18	74.9	22.1	0	19.5

Fundamentally, this work demonstrates that broad antibacterial activity can be imparted to silk surfaces through the covalent attachment of cationic agents with lipophilic adjuncts. Both Gram + and Gram – bacteria were killed completely (*no* growth of bacteria in rich liquid medium after addition to treated surface on nutrient rich plate) with two of the four attached agents (**P-dabco-C12** and **P-dabco-C16**). Three of the cationic agents (**P-dabco-C12, P-dabco-C14** and **P-dabco-C16**) exhibited complete killing of Gram + bacteria but not (the more resistant) Gram – bacteria.

In a further type of experiment, 10^6 bacteria were spread on agar plates containing a rich medium, and modified surfaces were placed on top. Incubation was then performed overnight, after which the modified surfaces were discarded. Scrapings of agar from the region beneath the added surface were placed in 4 mL of liquid growth media and further incubated overnight. Growth of surviving bacteria in these liquid media was measured turbidimetrically. (Control surfaces were also included on each plate as noted before; all controls exhibited full growth of bacteria.) Full data for these growth experiments are shown in Table 3 for modified silk and Table 4 for modified wool surfaces. Again, with silk surfaces, **P-dabco-C12** and **P-dabco-C14** exhibited the greatest activity against both Gram + and Gram – bacteria, and **P-dabco-C16** exhibited significant activity against Gram + bacteria

Table 3
Growth of bacteria on agar under modified silk surfaces as a percentage of control

	E. coli	*P. vulgaris*	*B. cereus*	*S. aureus*
P-dabco-C12	0	26.5	0	0
P-dabco-C14	0	0	0	NA
P-dabco-C16	32.7	22.3	0	0
P-dabco-C18	42.4	NA	15.5	NA

Table 4
Growth of bacteria on agar under modified wool surfaces as a percentage of control

	E. coli	*P. vulgaris*	*B. cereus*	*S. aureus*
P-dabco-C12	31.7	0	0	0
P-dabco-C14	28	0	0	0
P-dabco-C16	82.5	63.7	0	0
P-dabco-C18	93.9	NA	0	NA

An optimal chain length of the lipophilic unit for antibacterial activity is observed for these protein-based surfaces as was previously with carbohydrate-based surfaces.[18] Maximal antibacterial activity can be observed toward particular types of bacteria using various chain lengths of lipophilic species. We are continuing investigation of variations in nature of the lipophilic chain in an effort to understand this structure/activity relationship.

The antibacterial activity may be understood as occurring in a stepwise manner. The lipophilic chains can be subsumed by the bacterial species to a stage where the cationic portion is brought into intimate contact with the cell surface, *and is subsumed sufficiently far that it is not easily expelled*. Detergent-like action then results in cell surface disruption initiating cell destruction. A particular advantage of such action is the lack of consumption of the antibacterial agent; it is not changed in the process and remains attached to the surface.

Clearly, modification of proteinaceous surfaces (silk and wool) by the covalent attachment of lipophilic/cationic adjuncts imbues those surfaces with an antibacterial character. However, the activity against both Gram + and Gram - bacteria is not as great as that previously reported using similarly activated carbohydrate-derived surfaces. We ascribe this characteristic to the necessarily decreased loading of the agent to the proteinaceous surfaces. Only 10-20% of the surface constituent units (serines) of the proteinaceous surfaces is capable of being modified, whereas virtually all of the surface units of a carbohydrate-based surface can be so modified. It is unlikely that antibacterial action results from a single active functionality interacting with the bacterial cell. Rather, with the large cell settling on the surface, the greater the number of active functionalities in a relatively small region that can interact with the cell the greater the likelihood that a sizable fissure of the cell envelope can result. This action is significantly less likely for a surface that is partially-serine than one that bears an active functionality throughout the exposed surface. Nonetheless, significant activity is observed for the modified proteinaceous surfaces.

Several other comparisons with the previously reported carbohydrate surface results may be noted. With proteinaceous surfaces (compared to carbohydrate-based surfaces) the adjunct with a saturated 16-carbon chain is no longer most active.[18] Shorter chains, particularly the 12-carbon chain, exhibit greater activity. This is in closer correspondence with prior work on other surfaces.[3,6-10] It would appear that the activity of such adjuncts is influenced by the nature of the surface to which they are attached. Further, Gram + bacteria are readily dispatched by all except the saturated 18-carbon chain adjuncts on proteinaceous surfaces, whereas Gram – species remain heartier.

We are continuing with efforts: 1) to broaden the range of surfaces that can be so modified; 2) to seek optimal structure for the antibacterial agent; and 3) expand the library of microorganisms susceptible to such activity. It would also be of interest to identify proteinaceous materials that are significantly higher in serine content than the silk and wool currently available commercially.

EXPERIMENTAL

General procedure for the preparation of modified surfaces:

A strip (2" x 10", 1.2 g, maximally 2.2 mmol of free serine hydroxyl for silk and 1.1 mmol for wool) of the raw surface material was activated by addition to a solution containing an excess of *p*-toluenesulfonyl chloride (10 g, 52.5 mmol) and sodium bicarbonate (5.0 g, 59.5 mmol) in water (100 mL). After standing in the stirred solution for 4 hr, the strip was removed and washed with water chilled with ice. Attachment of the polycationic ligand was then accomplished by placement in a stirred *t*-butyl alcohol solution (25 mL) containing the monocation unit (**1-4**) (an excess - *e.g.* with **4** to generate **P-dabco-C12**, 20 g, 44.7 mmol) and agitated for 24 hours. After this time the modified surface material was removed from the reaction medium and washed repeatedly with water, followed by a brine wash, and finally dried in air without heating.

General procedure for determination of the antibacterial characteristics of the modified surfaces:

Experiment 1:

The rich medium used for bacterial growth was prepared from Bacto-tryptone, Bacto-agar, yeast extract and sodium chloride. Each modified surface sample was investigated for its antibacterial effect with each of the bacteria studied in a two-part experiment. Specifically, on the same plate, bearing the growth medium, were placed two separate experimental runs, those being:

A - surface material that had been subjected to the solvent washing procedures of reaction but without addition of the reagent materials, to which the bacteria being investigated were added. In each instance μμL of the stock dispersion of bacteria in log growth phase were added using an Oxford Benchmate Pipetman, placing the entire load of bacteria at the center of the test swatch of material (square, 0.5 in to a side). For the strains investigated, this corresponded to addition of the following number of bacteria:

Escherichia coli 1.41×10^5

Proteus vulgaris 2.34×10^5

Bacillus cereus 1.54×10^5

Staphylococcus aureus 1.47×10^5

B – modified surface material, to which the bacteria being investigated were added in the same manner and amount as noted for **B** above.

The growth plate was incubated overnight at 35˚C. Growth was noted visually in the region around the material surface. Subsequently, the surface material was removed from the growth medium and placed in 4 mL of fresh growth medium and incubated at 35˚C for 16 hr. Growth of bacteria in this instance was measured turbidimetrically using a Beckman Model 25 UV/VIS spectrophotometer at 60 nm.

Experiment 2:

Approximately 10^6 bacteria (75 μL) were spread on an agar plate using a cell spreader. A test swatch of material (as in Experiment 1) was added to the top of the bacteria. Plates were incubated overnight at 35˚C. Material swatches were removed and discarded. Scrapings of agar located in the region under the placement of the material were collected and were placed in 4 mL of the growth medium and incubated at 35˚C for 16 hr. Growth was measured turbidimetrically as in Experiment 1.

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