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Immobilization of Antibacterial Dihydropyrrol-2-ones on Functional Polymer Supports To Prevent Bacterial Infections *In Vivo*

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Antibiotic-resistant *Staphylococcus aureus* is of great concern, as it causes a wide range of life-threatening infections. The current study demonstrates that dihydropyrrolone (DHP)-coated polyacrylamide substrates are effective in reducing the number of culturable clinical isolates of *S. aureus in vitro* in a dose-dependent manner and are able to reduce the pathogenic potential of staphylococcal infection in a subcutaneous infection model. Covalently bound DHPs therefore show great potential for use as an antimicrobial strategy in device-related applications.

The rapid emergence of antibiotic-resistant *Staphylococcus aureus* is of great concern in medicine due to its ability to cause a wide range of life-threatening infections and to easily gain resistance to antibiotic therapies (13, 17). For this reason, alternatives to treat or prevent staphylococcal infections without inducing drug resistance are needed. The use of dihydropyrrolones (DHPs) for control of *S. aureus* infection is attractive, as they exhibit good antimicrobial properties against several bacterial species, are of low cytotoxicity toward mammalian cells (10, 11), and are also likely to have a low propensity to induce resistance (1, 3).

Recent studies on surface attachment of a range of DHPs covalently attached to glass surfaces showed excellent prevention of biofilm formation and bacterial adhesion (4). In particular, DHP-1 demonstrated excellent broad-spectrum activity. In this study, a polymeric substrate was modified by covalent attachment of DHP-1, and the resulting material was investigated for its *in vitro* and *in vivo* antimicrobial activity.

DHP-coated polyacrylamide beads were prepared by mixing UltraLink biosupport medium (azlactone-functional beads; Pierce Biotechnology, Inc., Rockford, IL) with 1,2-ethylenediamine solution (0.5 M in absolute ethanol). The resultant suspension was vortexed and agitated at room temperature overnight, filtered, and washed successively with absolute ethanol to remove unreacted ethylenediamine. The amine-functional beads were then reacted with DHP (0.03 M in ethanol), followed by use of the same procedure described above and air drying. A schematic for the reaction is shown in Fig. 1. Attachment of the DHP to the surface was analyzed using X-ray photoelectron spectroscopy (XPS) as described previously (4). Finally, the beads were sterilized by exposure to 70% (wt/vol) ethanol before suspension in 500 μl sterile phosphate-buffered saline (PBS).

Successful surface modification of polyacrylamide beads was indicated by the changes in the carbon/nitrogen (C/N) ratio and oxygen/nitrogen (O/N) ratio, measured by XPS (Table 1). The amine-treated beads showed a small reduction in the C/N ratio (4.0 to 3.9) in comparison to the untreated beads, which is in agreement with the addition of nitrogen and carbon from ethylenediamine. The subsequent attachment of DHP was demonstrated by a significant increase in the C/N ratio (3.9 to 4.6) and a slight increase in the O/N ratio (1.1 to 1.3), signifying a higher proportion of carbon and a small amount of oxygen from the DHP were added to the amine-functionalized polyacrylamide support. In addition, a fluorinated DHP (DHP-F) was also coupled to the polyacrylamide support, in which the fluorine was used as a marker for XPS. Detection of fluorine on the DHP-F-coated bead (1.5% F) and the consistent increase in the C/N and O/N ratios further ascertain the covalent attachment of DHP on the polyacrylamide bead.

In order to verify that DHP was not released from the material, DHP-coated beads were suspended in PBS (10 mg dry beads/ml), with agitation for 24 h. Evaluation of the PBS solution by UV spectrometry did not show any free DHP (data not shown).

Bacterial cultures of a clinical isolate, *S. aureus* strain 38 (6), were prepared by a previously described method (4) to give a final bacterial suspension of 10^6 CFU/ml. This was confirmed by a retrospective plate count. Equal volumes of this bacterial suspension were added to different proportions of dry beads in PBS to final concentrations of 0.5, 5, and 10 mg dry beads/ml, resulting in a final bacterial suspension of 5×10^5 CFU/ml. The bead-bacteria suspension was incubated on a laboratory rotator (Stuart Scientific, Great Britain) at 37°C for 24 h. After this time, the beads were allowed to settle for 20 min. The culturable bacteria in the supernatant were quantified by plating triplicate aliquots of serial dilutions onto nutrient agar plates. Plates were incubated for 24 h at 37°C, and the number of CFU was counted. Each experiment was repeated a minimum of three times in duplicate. Statistical differences were determined by a one-way analysis of variance (ANOVA), followed by a Games-Howell *post hoc* test. Results with P values of <0.05 were considered significant.

The growth of *S. aureus* in the presence of different proportions of polyacrylamide beads was evaluated, and the results are presented in Table 2. There was no effect of the untreated and

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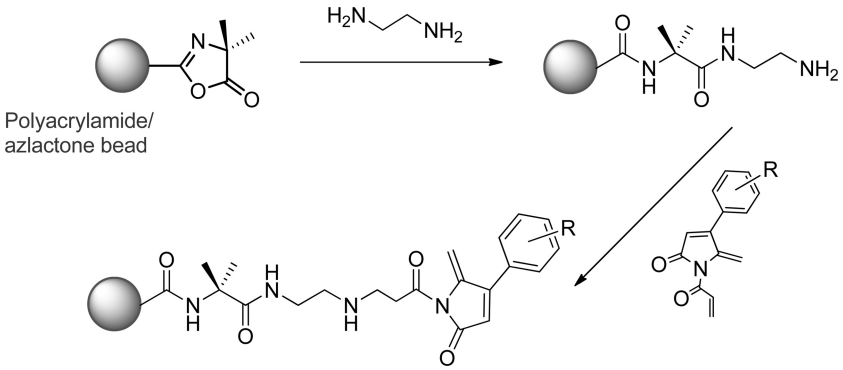

FIG 1 Schematic of the attachment chemistry. Azlactone groups on the polyacrylamide beads were first ring opened with ethylenediamine, followed by reaction with DHP.

amine beads, at any concentration tested, on the number of *S. aureus* CFU recovered (Table 2).

For DHP-coated beads, no significant difference in the number of bacteria recovered from the control beads was observed at a bead concentration of 0.5 mg/ml. However, in the presence of 5 or 10 mg/ml DHP beads, a dose response was observed, with reductions in culturable bacterial counts of 4 logs (99.99%; $P = 0.037$ and $P = 0.000$) and 5 logs (99.999%; $P = 0.006$ and $P = 0.002$), respectively, compared to those of the blank and amine-functionalized controls.

Polyacrylamide beads retained after the *in vitro* growth studies were spread onto nutrient agar plates without being washed. After 24 h of incubation, *S. aureus* growth on the untreated and amine-treated beads was too numerous to count. In contrast, sparse bacterial growth and variation in colony sizes associated with the DHP-coated beads were observed (data not shown), suggesting that bacterial culturability was affected once the bacteria had interacted with the DHP. Moreover, the supernatant suspension and the beads were observed under fluorescence microscopy (Olympus FV1000 confocal inverted microscope) with the aid of LIVE/DEAD staining (data not shown). The number of bacteria in the suspensions correlates to the number of bacteria obtained from retrospective counting. Very few bacteria were observed around the DHP beads as opposed to the control beads, indicating that the bacteria were not adhered to the DHP beads.

The mechanisms of action of DHP are believed to be similar to those reported for fimbrolides and furanones, acting as bacterial quorum-sensing (QS) inhibitors by interacting with the membrane-associated surface proteins of bacterial cells (9, 12, 16). In addition, *N*-acylhomoserine lactones (AHLs) and furanones have been shown to affect the biofilm formation and growth of *S. aureus*, and it has been suggested that this is mediated by interference with the QS systems *sarA* and *agr* (15). Previous *in vivo* study has demonstrated activity for covalently attached furanones (5); however, the precise mechanism has not yet been confirmed. Together, these findings imply that the interaction between DHP and bacteria occurs on the surface of the cells, which indirectly affects the cell replication of the bacterium or may have rendered the cells viable but nonculturable (8, 14). Similar behavior of *S. aureus* was observed when the bacterium was grown in the presence of silver nanoparticles, in which the growth is affected due to a prolonged bacterial lag phase (7).

To examine the effects of DHP surface coating *in vivo*, *S. aureus* strain 38 prepared as described above was adjusted to 10^6 CFU/ml in PBS and then mixed with polyacrylamide beads (DHP-treated beads or controls) to a final bead concentration of 5 mg dry beads/ml. The mixture of bacteria and beads in a total volume of 200 μl was injected subcutaneously into the shaved flanks of five 8-week-old male BALB/c mice per treatment. The mice were monitored for 4 days. Clinical parameters were scored on a scale of 0 to 4, for which 0 meant no pathology and 4 meant severe redness and swelling. All protocols for animal use were approved by the institutional animal ethics committee. Experiments were performed in triplicate. Statistical differences were determined by a nonparametric Mann-Whitney test. Results with P values of <0.05 were considered significant.

For the groups injected with untreated or amine-functionalized beads, the lesions showed a significant increase of 2- to 3-fold over those injected with the DHP beads ($P = 0.036$ and 0.001, respectively) in the average clinical score by day 2. Further increases in gross pathology in all control mice were observed over the subsequent days (days 3 and 4) (Fig. 2A and B). In contrast, the group injected with DHP-coated beads showed no significant change in gross clinical observations throughout the monitoring period (Fig. 2C).

At day 4, mice were euthanized, and the beads and tissue asso-

TABLE 1 Elemental surface concentration ratios as determined by XPS[a]

Treatment of beads	Elemental composition (%)				C/N ratio	O/N ratio
	C	N	O	F		
Untreated	65.3	16.2	18.5		4.0	1.1
Amine	64.5	16.6	18.9		3.9	1.1
DHP	66.9	14.6	18.6		4.6	1.3
DHP-F	65.3	14.3	18.9	1.5	4.6	1.3

[a] C, carbon; O, oxygen; N, nitrogen; F, fluorine.

TABLE 2 Numbers of culturable *S. aureus* CFU/ml after 24 h of growth in medium containing different proportions of functionalized polyacrylamide beads

Treatment of beads	No. of CFU/ml with indicated proportions (mg/ml) of beads[a]		
	0.5	5	10
Untreated	$4.8 \times 10^8 \pm 7.8 \times 10^7$	$4.2 \times 10^8 \pm 5.6 \times 10^7$	$4.3 \times 10^8 \pm 1.5 \times 10^7$
Amine	$4.9 \times 10^8 \pm 2.5 \times 10^7$	$5.1 \times 10^8 \pm 3.1 \times 10^7$	$5.0 \times 10^8 \pm 3.7 \times 10^7$
DHP	$4.0 \times 10^8 \pm 4.6 \times 10^7$	$1.7 \times 10^4 \pm 1.4 \times 10^3$[b]	$3.0 \times 10^3 \pm 6.6 \times 10^2$[b]

[a] Results reexpressed as the numbers of CFU/ml ± standard derivations of the means.
[b] Significant differences to the same quantity of untreated beads ($P < 0.05$).

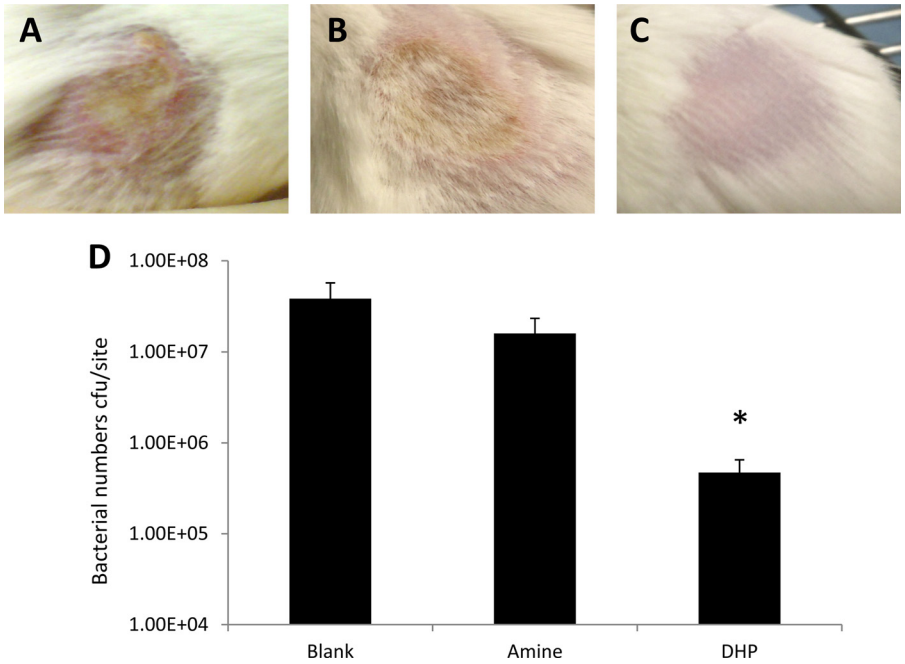

FIG 2 (A to C) Photographic images of a representative mouse from each group injected subcutaneously with untreated (A), amine (B), and DHP (C) polyacrylamide beads; 5 days after injection. (D) Average numbers of bacteria recovered from explanted biomaterials and associated tissue. Errors are standard errors of the means, and "*" indicates a P value of <0.05 compared to blank and amine surfaces.

ciated with them at the injection site were collected and homogenized in 1 ml of PBS. Culturable bacteria were quantified by serial dilution and plate counting, as described above. Bacterial numbers recovered in the presence of untreated or amine-treated beads were not significantly different ($P = 0.284$) (Fig. 2D) and were consistent with findings in other models (2). However, bacterial numbers recovered from the DHP-coated bead sites showed a significant, approximately 2-log reduction in bacterial numbers compared to those from both the blank and amine-functionalized surfaces ($P = 0.03$ and 0.048, respectively) (Fig. 2D), with no morphotypic variants in the bacterial colonies.

There was an apparent reduction in inhibitory activity of the DHP in the animal model compared to the *in vitro* data. This could be due to effects of deposition of host biomolecules such as proteins and lipids onto the surface of the beads, which may partly block the ability of the DHP to interact with the bacteria.

In vivo results suggest that DHP-coated polyacrylamide beads are able to reduce the gross pathology and reduce the bacterial load in a subcutaneous *S. aureus* infection model. Importantly, the DHP-coated beads did not cause any apparent adverse effect on the mice and therefore appear to be biocompatible. The reduced virulence may be an effect of quorum-sensing inhibition, as the quorum-sensing signal *N*-3-oxo-dode-anoyl-L-homoserine lactone (3-oxo-C_{12}-HSL) was found to inhibit exotoxin production in *S. aureus* (15), and DHP, having effects similar to those of 3-oxo-C_{12}-HSL on the growth of *S. aureus*, is believed to interfere with the same pathway.

This study is the first to demonstrate the efficacy of surface-attached antibacterial DHP *in vivo*. The present study shows that DHP-coated polyacrylamide beads are effective against staphylococcal infections *in vivo* and shows great potential for prevention of infection in topical and device-related applications.

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