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Surface immobilization chemistry influences peptide-based detection of lipopolysaccharide and lipoteichoic acid

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Antimicrobial peptides (AMPs) have recently gained attention as potentially valuable diagnostic and therapeutic agents. The utilization of these peptides for diagnostic purposes relies on the ability to immobilize them on the surface of a detection platform in a predictable and reliable manner that facilitates target binding. The method for attachment of peptides to a solid support is guided by peptide length, amino acid composition, secondary structure, and the nature of the underlying substrate. While immobilization methods that target amine groups of amino acid sequences are widely used, they can result in heterogeneous conjugation at multiple sites on a peptide and have direct implications for peptide presentation and function. Using two types of commercial amine-reactive microtiter plates, we described the effects of analogous immobilization chemistries on the surface attachment of AMPs and their differential binding interaction with Gram-specific bacterial biomarkers, lipopolysaccharide and lipoteichoic acid. As might be expected, differences in overall binding affinities were noted when comparing AMPs immobilized on the two types of plates. However, the two-amine-targeted linking chemistries also affected the specificity of the attached peptides; lipopolysaccharide generally demonstrated a preference for peptides immobilized on one type of plate, while (when observed at all) lipoteichoic acid bound preferentially to AMPs immobilized on the other type of plate. These results demonstrate the potential for tuning not only the binding affinities but also the specificities of immobilized AMPs by simple alterations in linking strategy. Published 2012. This article is a U.S. Government work and is in the public domain in the USA.

Supporting information may be found in the online version of this article.

Keywords: antimicrobial peptides; lipopolysaccharide; lipoteichoic acid; surface immobilization; microtiter plate

Introduction

As part of the innate immune system of many organisms, antimicrobial peptides (AMPs) serve as the first line of defense against invading microbes. Almost 1000 naturally occurring AMPs have been isolated and characterized [1,2]. AMPs typically consist of 9–45 amino acids and can be categorized based on their secondary structures and mode of synthesis. Perhaps the most well-characterized group is composed of amphipathic α-helical peptides that most often possess between 2 and 9 positively charged residues provided by arginine and lysine. Studies with model membranes, liposomes, and live cells support the current dogma that many of these AMPs exert their antimicrobial activity by binding to invariant components of microbial surfaces, presumably through polyanions such as Gram-negative lipopolysaccharide (LPS), and causing membrane leakage/disruption either directly or through 'self-promoted uptake' [3–6]. Intriguing evidence for additional mechanisms of killing has been described, but it is difficult to confirm whether these other mechanisms arise as a result of membrane permeabilization [7–10]. A key feature of the interaction of AMPs with their target organisms is their broad selectivity. Interactions between AMPs and microbial targets may occur across different genera, but the strength of interaction varies according to the membrane composition, presence of different membrane components [e.g. LPS and Gram-positive lipoteichoic acid (LTA)], and the structure and amino acid sequence of the AMP (e.g. see Refs. [11–13]).

Numerous efforts have sought to utilize the semi-selective binding characteristics of AMPs, presented as free or surface-bound peptides, for various applications. Pawel, Welling, and coworkers have used radiolabeled AMPs for target delivery to and *in vivo* detection of localized bacterial and fungal infections [14–16]. For many years, polymyxins have been immobilized onto various substrates for neutralizing Gram-negative LPS-contaminated samples [17–19] and, more recently, for detoxifying clinical fluids [20,21]. As surface-sensitive techniques gain increasing importance in the field of biodetection, controlled immobilization of small biological molecules in a functionally active form on the sensor surface has become critical. Surface-bound AMPs have been utilized to enrich for and detect microbial cells [22–28] as well as markers for microbial infections such as LPS and LTA [17,29]. In our laboratory, AMP-based arrays have been used to detect and

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classify a wide range of microbial targets, including Gram-negative and Gram-positive bacteria, rickettsiae, and certain viruses [24,25,28,30].

In our effort to transition low-density peptide-based detection assays to high-throughput screening (HTS) applications, we integrated a set of AMPs previously demonstrated in planar arrays to an HTS microtiter plate platform. It has been shown by some researchers that immobilization parameters, such as linker length and peptide density, can affect the antimicrobial activity of surface-bound AMPs without affecting the target specificity and activity profile [31–35]. However, we have observed that the choice of immobilization method significantly affects AMP–target interactions and binding affinities in rapid detection assays [23,28,30]. The current study was undertaken to quantify the effects of two analogous amine-reactive immobilization to commercial pre-activated microtiter plates on the presentation and binding activity of AMPs of varied length, charge, and structure. The density of immobilized peptides was quantified; the number of free amines on the immobilized peptides was assessed; and finally, the functionality of the immobilized peptides was characterized with respect to binding of the bacterial biomarkers LPS and LTA.

Materials and Methods

Materials

The following AMPs were purchased from American Peptide Company, Inc. (Sunnyvale, CA): cecropin B (cecB), cecropin P1 (cecP1), cecropin A–melittin hybrid [CA(1-8)ME(1-18)NH_2] (ceme), melittin (mel), and indolicidin (indol); their amino acid sequences and domain structures are shown in Table 1. LPS from *Salmonella typhimurium* and LTA from *Streptococcus pyogenes*, phosphate buffered saline (PBS; pH 7.4), Tween 20, bovine serum albumin (BSA), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Cy5 monofunctional *N*-hydroxysuccinimidyl ester was purchased from Amersham Pharmacia (Piscataway, NJ). Alexa Fluor 555 *N*-hydroxysuccinimidyl ester (AF555-NHS), AF555-cadaverine, and AF555-maleimide were purchased from Invitrogen (Carlsbad, CA). Two analogous amine-reactive microtiter plate surfaces were used in this study (Figure 1): maleic anhydride-activated (MA) plates (Pierce Scientific, Rockford, IL) and NUNC Immobilizer Amino® (IA) (Thermo Fisher Scientific, Waltham, MA). Microfluor I® untreated polystyrene plates were used as controls and were also purchased from Thermo Fisher.

Preparation of Fluorescently Labeled LPS and LTA

Cy5-labeled LPS and LTA were used as labeled targets in peptide-target binding assays. One milligram of LPS or LTA was incubated in 1 ml of 50 mM sodium borate (pH 8.5) with one packet of Cy5 monoreactive dye dissolved in 25 μl of anhydrous DMSO. After 1-h incubation at room temperature, the labeled LPS or LTA was purified from unincorporated dye by gel filtration on BioGel P-2 (Bio-Rad, Hercules, CA). The labeled biomolecules were stored in the dark at 4 °C until use. The molar ratios of dye to labeled species ranged from 1.1 to 1.4 for both LPS and LTA. The concentrations of labeled LPS and LTA were determined from absorbance readings at 259 and 650 nm, using a calibration curve generated with unlabeled LPS or LTA standards and corrected for Cy5 background absorbance at 259 nm.

Peptide Immobilization

Lyophilized peptides were resuspended at 200 μg/ml in 100 mM carbonate coating buffer (30 mM Na_2CO_3, 70 mM $NaHCO_3$, pH 9.6) and sonicated for 5 min. Sonicated solutions were then added to IA, MA, or control polystyrene plates (100 μl/well) and incubated for 2 h at room temperature with gentle agitation; each peptide was patterned in quadruplicate. Following peptide immobilization, plates were washed three times with PBS + 0.005% Tween 20 (PBST) and incubated with 125 μl of blocking buffer (10 mg/ml BSA in 0.1 M Tris, pH 8.5) for 1 h.

For the assessment of the total number of peptides immobilized on IA and MA plates, cysteine-terminated peptides were used in place of the standard peptides during immobilization, and thiol groups were labeled by incubating with AF555-maleimide in PBS (pH 6.5) for 2 h at room temperature with gentle agitation. The number of free amines on immobilized peptides was measured by incubating the coated plates for 1 h at room temperature with AF555-NHS monoreactive dye [in 50 mM sodium borate (pH 8.5), 100 μl/well]. After labeling of the immobilized peptides with AF555-NHS (amine-specific) or AF555-maleimide (thiol-specific), the plates were washed with PBST, wells were filled with 100 μl of PBS, and AF555 fluorescence was read on a SAFIRE microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

LPS/LTA Binding Assay

For determination of peptide binding activity, peptide-coated plates were washed three times and each well received 100 μl

Table 1. Peptide sequences

Peptide	Sequence*	Structure upon interaction with membrane/LPS/LTA	No. of amino acids
CecB	Lys-Trp-Lys-Val-Phe-Lys-Lys-Ile-Glu-Lys-Met-Gly-Arg-Asn-Ile-Arg-Asn-Gly-Ile-Val-Lys-Ala-Gly-Pro-Ala-Ile-Ala-Val-Leu-Gly-Glu-Ala-Lys-Ala-Leu-NH_2	Helix-hinge-helix	35
Ceme	Lys-Trp-Lys-Leu-Phe-Lys-Lys-Ile-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-NH_2	Helix-hinge-helix	26
CecP1	Ser-Trp-Leu-Ser-Lys-Thr-Ala-Lys-Lys-Leu-Glu-Asn-Ser-Ala-Lys-Lys-Arg-Ile-Ser-Glu-Gly-Ile-Ala-Ile-Ala-Ile-Gln-Gly-Gly-Pro-Arg	Uninterrupted helix	31
Mel	Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH_2	Helix-hinge-helix	26
Indol	Ile-Leu-Pro-Trp-Lys-Trp-Pro-Trp-Trp-Pro-Trp-Arg-Arg-NH_2	Unstructured	13

* Sequence of native peptide; custom peptides with C-terminal cysteine were also used.

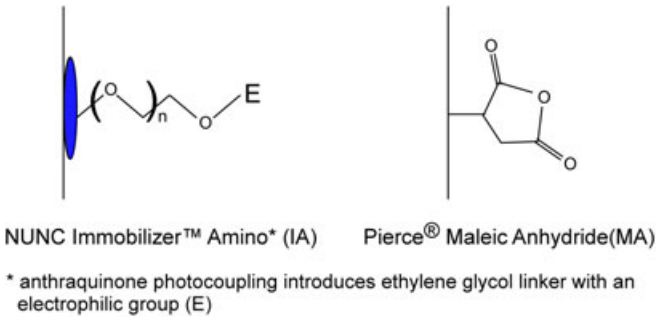

Figure 1. Schematic representations of commercial, amine-reactive microtiter plate surfaces used for AMP immobilization.

of Cy5-conjugated LPS or LTA. Plates were then incubated for an additional 2 h at room temperature with gentle agitation. After being rinsed three times with PBST, the wells were filled with 100 μl of PBS, and fluorescence was read on a SAFIRE microplate reader.

Statistical Analyses

Data are presented as mean ± standard error, with a minimum of four replicates for each experiment. Each experiment was performed at least twice. Analyses were conducted using InStat 3 (GraphPad Software, San Diego, CA). Statistical significance was determined using Student's *t*-test. Limit of detection (LOD) is defined as the lowest analyte concentration that produces a fluorescence signal equal to or greater than 3 SD above the background.

Results

Quantification of Reactive Sites on Commercial Microtiter Plates

Immobilizer Amino® and maleic anhydride-activated plates possess chemically activated surfaces to which peptides can be covalently coupled via primary amines. To quantify the number of reactive groups on each plate type that could potentially mediate peptide immobilization, we incubated fluorescently labeled cadaverine (cad-AF555) with the amine-reactive plates. As cad-AF555 possesses a single -NH_2 end group, the level of AF555 fluorescence detected should be directly proportional to the number of reactive sites on the plate surface. Statistical analysis indicated significant differences in the surface density of reactive sites between IA and MA plates ($p < 0.001$; Figure 2). Control polystyrene plates, which have no chemically reactive groups, showed cad-AF555 labeling equivalent to background ($p > 0.5$). The total fluorescence intensity signal on the MA plates was approximately 1.5-fold higher than that of the IA plates.

Quantification of Packing Density of Immobilized Peptides on IA and MA Plates

A series of peptides incorporating C-terminal cysteines were custom synthesized as analogs to the native peptides used throughout this study (New England Peptides, Gardner, MA; cys-cecB, cys-ceme, cys-cecP, cys-mel, and cys-indol). For quantification of the density of immobilized peptides, cysteine-terminated peptides were immobilized according to standard protocols onto MA and IA plates in place of native peptides using amine-directed chemistry. After immobilization and rinsing, the C-terminal cysteines were then targeted for labeling with AF555-maleimide. As each custom peptide possessed only a single maleimide-targeted moiety (cysteine), this labeling strategy was expected to result in the incorporation of a single fluorophore per peptide.

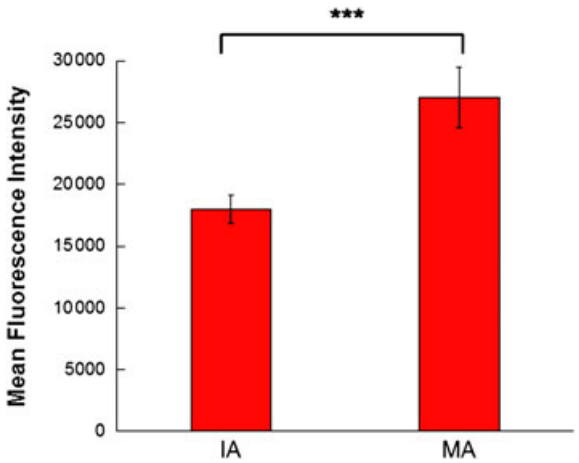

Figure 2. Quantitation of surface reactive groups for commercial microtiter plates. Fluorescence intensities of cad-AF555 bound to amine-reactive IA or MA microtiter plates. ***$p < 0.001$.

On the basis of the fluorescence labeling of the bound cysteinyl peptides, the surface densities of all peptides tested were significantly higher on MA plates than on IA plates ($p < 0.001$; Figure 3). Differences ranged from 3- to 48-fold higher fluorescence intensities

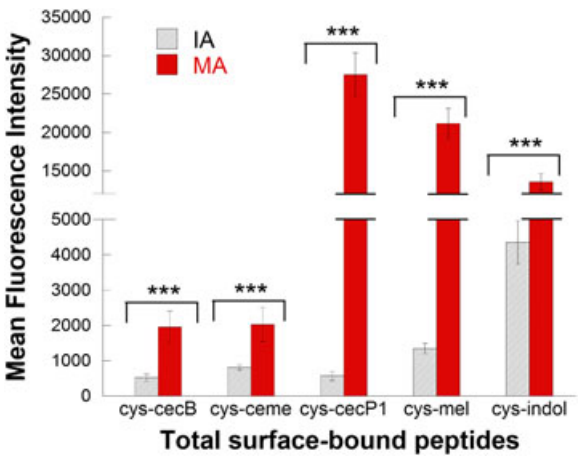

Figure 3. Differential immobilization efficiencies of AMPs attached to IA or MA plates. Fluorescence intensities of AF555-maleimide-labeled, cysteine-terminated AMPs (cys-cecB, cys-ceme, cys-cecP, cys-mel, and cys-indol) immobilized to IA or MA microtiter plates.

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on the MA plates. The densities of immobilized cecP1 and mel on MA plates were the highest.

Presentation of Peptides after Immobilization

For characterization of the orientation of covalently immobilized AMPs on the plate surface, native peptides were immobilized onto IA and MA plates and were subsequently treated with AF555-NHS to determine the levels of free (unlinked) amines (Figure 4). Significant differences in labeling between plates were observed for all peptides except cecB. All peptides immobilized onto IA plates exhibited high levels of amine-targeted labeling, with fluorescence for all five peptides within a twofold range of values. In contrast, peptides immobilized on MA plates demonstrated a wider range of labeling efficiencies, ranging from no modification [cecP1, not significantly above background levels ($p > 0.05$)] to levels higher than those observed on IA plates (ceme, mel). However, a trend was observed on both plates for peptides possessing a helix-turn-helix motif (cecB, ceme, and mel), wherein fluorescence values for the three peptides increased in the same order (cecB < ceme < mel).

The fluorescence values presented in Figure 4 are related to both the number of free amines and the number of peptides present on the surface (i.e. peptide density). To account for peptide density, we determined the number of free amines per peptide

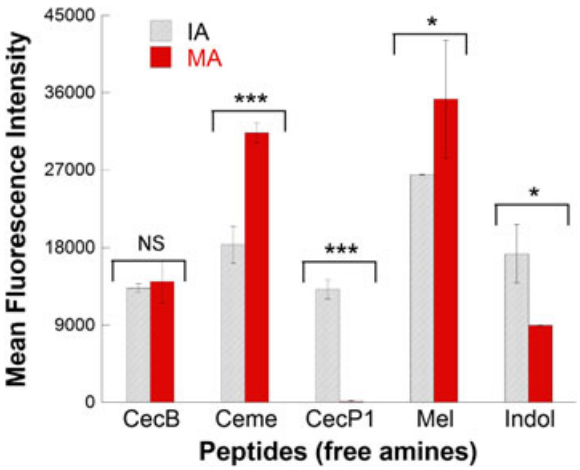


Figure 4. Effects of immobilization chemistry on the orientation/presentation of AMPs on microtiter plate surfaces. Fluorescence intensities of AF555-NHS-labeled amines of AMPs immobilized to IA or MA microtiter plates. NS, statistically not significant. *$p < 0.05$; ***$p < 0.001$.

by normalizing the free amine fluorescence (AF555-NHS) with respect to the total number of amines immobilized on each plate (AF555-maleimide); the results are presented in Table 2. On IA plates, the number of labeled amines per peptide (Table 2, 4th column) mirrored the total number of primary amines available for labeling (Table 2, 2nd column) based on their amino acid sequences, with indol having the fewest and cecB having the highest number of primary amines. Perhaps more importantly, the IA normalized values roughly approximated the expected numbers of free amines if peptides were immobilized on the plates by a single linkage (Table 2, 3rd column). Only cecB showed a significantly lower value from the expected single-point linkage number; this difference may reflect either multipoint linkage to the plate surface or steric effects after immobilization, leading to inaccessibility of the primary amines to the labeling solution.

Interestingly, trends observed with the IA-immobilized peptides were not observed on MA plates. Levels of amine labeling did not reflect the expected number of amines available based on sequence: Ceme labeled to a higher degree than cecB, which possesses a larger number of lysines, and results with cecP indicated that all primary amines were either linked to the plate or inaccessible for modification after immobilization.

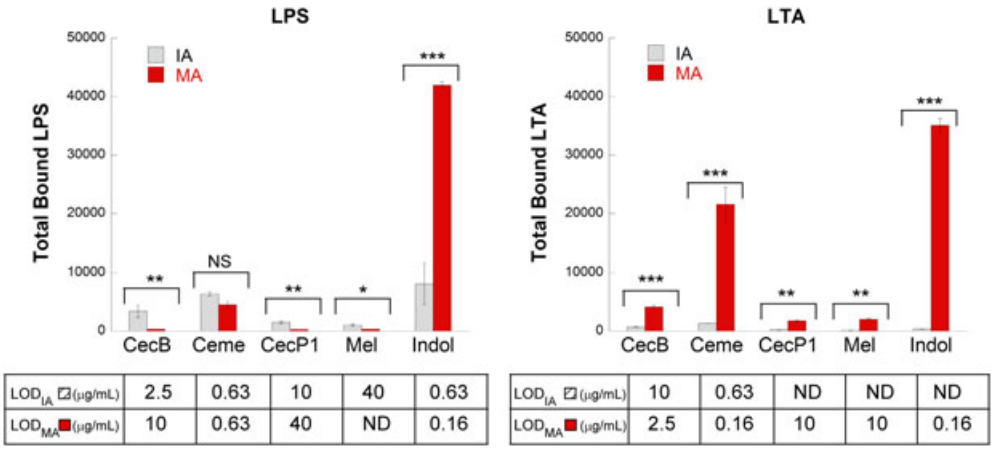
Binding Activity of Immobilized Peptides

We examined the effect of immobilization on the peptides' ability to bind fluorescently labeled LPS and LTA, two bacterial markers released by Gram-negative and Gram-positive bacteria, respectively, into the circulation of infected individuals. The LPS and LTA dose–response curves of immobilized peptides were determined (see Supporting Information), and the LODs, defined as the LPS/LTA concentrations that produce a fluorescence signal equal to or above 3 SD of the background signals, are indicated (tables shown at the bottom of Figure 5). The binding activity of LPS or LTA at the highest concentration tested (40 μg/ml) is shown both as a function of total fluorescence signal (Figure 5) and after normalization to account for the density of surface-bound peptides (Figure 6). The fluorescence signal intensities in the target binding assays varied considerably from plate to plate and from peptide to peptide, yet we identified some general trends in LPS and LTA binding.

Overall, with the exception of indol, LPS binding to IA-immobilized peptides resulted in significantly higher signals than the corresponding MA-immobilized peptides (Figure 5, left panel; $p < 0.05$). The higher signals on IA plates for cecB, cecP1, and mel translated into improved LODs. In spite of the higher signals with IA-immobilized ceme, there was, however, no difference in

Table 2. Number of free amines per peptide

Peptide	Total no. of primary amines	Primary amines available for modification after single-point immobilization (column 2 minus 1)	No. of labeled amines per peptide	
			IA plates	MA plates
CecB	7 Lys + N-terminus = 8	7	4.5	1.2
Ceme	5 Lys + N-terminus = 6	5	4.0	2.6
CecP	5 Lys + N-terminus = 5	5	4.0	0.0
Mel	3 Lys + N-terminus = 4	3	3.5	0.3
Indol	1 Lys + N-terminus = 2	1	0.7	0.2



LOD_{IA} (μg/mL)	2.5	0.63	10	40	0.63
LOD_{MA} (μg/mL)	10	0.63	40	ND	0.16

LOD_{IA} (μg/mL)	10	0.63	ND	ND	ND
LOD_{MA} (μg/mL)	2.5	0.16	10	10	0.16

Figure 5. Binding of Cy5-labeled LPS and LTA to MA- and IA-immobilized peptides. The values shown were obtained at 40 μg/ml of each target; complete dose–response curves are available as supporting information. ND, not detected; NS, statistically not significant. *$p < 0.05$; **$p < 0.005$; ***$p < 0.001$.

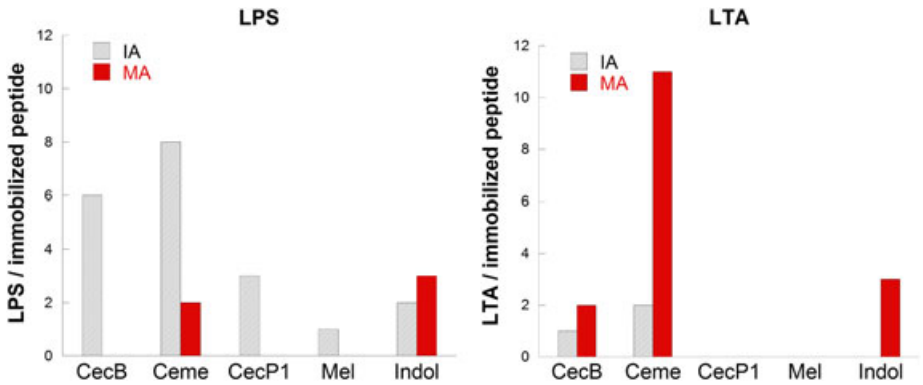


Figure 6. Binding of Cy5-labeled LPS and LTA to MA- and IA-immobilized peptides after normalization for peptide density. The values shown were obtained at 40 μg/ml of each target.

LOD for LPS binding to IA-immobilized versus MA-immobilized ceme. In contrast, fluorescent signals in LTA binding assays were much higher on MA plates than on IA plates (Figure 5, right panel). Signals for LTA binding to three of the peptides attached to IA plates were indistinguishable from background, and therefore, LODs could be determined only for two of the IA-immobilized AMPs; when comparing the LODs for LTA binding to cecB and ceme, lower values (i.e. higher sensitivity) were obtained with the MA-immobilized AMPs. When normalized to account for the peptide densities on each plate type, other trends emerged (Figure 6). On a per-peptide basis, all peptides immobilized on IA plates were observed to be functionally active and capable of binding either LPS or LTA. Interestingly, IA-bound peptides demonstrated a greater binding specificity to LPS than to LTA, with cecB and ceme demonstrating the highest fluorescence signal intensities. In contrast, on MA plates, only cecB, ceme, and indol demonstrated any significant binding to LPS or LTA when normalized for density. MA-bound cecP1 and mel did not bind LPS or LTA above background levels.

Discussion

There are many papers describing surface immobilization of AMPs to provide materials for decontamination or implantation (for an excellent review, see Ref. [33]). However, the design requirements for implantable materials differ from those applied to biosensing and biodetection, in that the latter group seeks to promote binding of target microbes, rather than prevent it. Several studies have recently suggested that surface attachment chemistries in detection-intended systems influence the conformation [36,37] and activity of surface attached peptides [30] and will therefore affect their performance in sensors. The purpose of the present work was to characterize the effects of covalent AMP immobilization onto two presumably analogous, commercially available HTS platforms on the activity and specificity of several AMPs of varied length and structure. Five candidate peptides were studied: three peptides with helix-hinge-helix structures (cecB, ceme, mel; Refs. [38,39]), one peptide comprising an uninterrupted α-helix (cecP1; Ref. [40]), and a short peptide whose principal structural motif is type VI turns (indol; Ref. [41]). These peptides were immobilized onto two plates designed for covalent attachment of biomolecules via primary amines: maleic anhydride-activated (MA) and Immobilizer Amino® (IA) plates. The commercial plates were evaluated for the number of reactive sites, the density and presentation of the surface-bound peptides, and finally, the ability to bind Gram-negative LPS or Gram-positive LTA.

The first parameters measured were the density of reactive groups on IA and MA plate surfaces and the number of peptides

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attached, following the manufacturers' standard immobilization procedures. Surprisingly, the higher number of reactive groups on the MA plates did not translate to proportional levels of peptide packing. Indeed, the surface densities of covalently immobilized peptides varied considerably with each peptide. The MA plate exhibited ~1.5-fold more reactive sites than the IA plate (Figure 2), yet surface densities of cecP1 and mel were 48- and 15-fold greater, respectively (Figure 3). The results suggest that (1) the maleic anhydride moieties on MA plates bind preferentially to certain peptides, or (2) peptides immobilized on IA plates take up more surface area per peptide than those immobilized to MA plates.

Each of the four α-helical peptides possesses multiple lysines in its sequence (Table 1), which potentially provide multiple points for attachment of the peptide to the plate. As these charged residues also play an important role in the peptide's interaction with the polyanionic LPS/LTA [13], we sought to determine the availability of these amine residues for binding to LPS and LTA after the peptides were immobilized. Although the peptide packing density on the IA plates was low (Figure 3), the surface-bound α-helical peptides exhibited high levels of free amines (Figure 4) as well as increased biomarker binding activity, with greater specificity for LPS than LTA (Figure 6).

On the MA plates, the levels of peptide activity, measured on a per-peptide basis (Figure 6), appeared to mirror the trend of free amines per peptide (Table 2, 5th column) for the four α-helical peptides: α-Helical peptides with higher numbers of free amines per peptide (cecB, ceme) had higher per-peptide functionality in LPS and LTA assays. More interesting were the results that despite extremely high levels of MA-bound cecP1 and mel (Figure 3), the number of free amines per peptide was extremely low for both of these peptides (Table 2, 5th column). These results suggested that all reactive amines were involved in covalent bonds to the plate surface or were otherwise sterically prevented from modification by AF555-NHS and also, presumably, from interacting with LPS or LTA. This hypothesis was supported by the low binding of MA-immobilized cecP1 and mel to either of the LPS or LTA species tested. The only non-helical peptide tested, indol (random-coiled structure), was also present in high concentrations on MA plates but had a low amine/peptide ratio. This peptide is postulated to bind LPS via both hydrophobic interactions between its tryptophans and salt bridges between its two arginines [42]; therefore, loss of its lysine (not involved in salt bridges) or amino terminus through covalent attachment to the MA was not expected to have much effect on its ability to bind LPS and presumably LTA.

In summary, these results suggest that the target binding activities of surface-bound AMPs are highly sensitive to the choice of immobilization chemistry used to covalently attach peptides to microtiter plates. Although IA and MA microtiter plates should display similar results due to their presumptively analogous amine-directed immobilization chemistries, significant differences were observed in peptide-specific immobilization efficiency, as well as the presentation/orientation of surface-bound peptides. These qualities translated into significant and potentially useful differences in the ability of the immobilized peptides to recognize and bind to LPS and LTA. While the density and total number of recognition molecules present on a surface may be important for target binding in many detection platforms (e.g. immunosensors, ELISAs), clearly, they are not the only factors affecting the peptide-based assays described here. Specifically, if the number of free amines per immobilized peptide is sufficiently high, we have seen that peptide density is less important for target binding. These results serve as an important illustration that experiments utilizing small molecules, such as peptides, immobilized onto HTS platforms such as microtiter plates through presumably predictable attachment chemistries, may result in highly unpredictable and unexpected observations. Researchers using such technologies should therefore be aware that presumptively analogous methods of immobilization (i.e. amine-directed covalent attachment on IA and MA plates) may not give equivalent results; assays incorporating small biomolecules such as peptides on HTS platforms may well require significant optimization for the most favorable results.

Perhaps more importantly, the differences in peptide density and presentation on the two plates translated into changes in binding specificity. A large number of studies have been published assessing the effects of linkage on binding/killing activity (see Refs. [33,35] for excellent reviews); as many of these studies aimed to develop antimicrobial coatings and materials, the research groups typically strove to retain broad-spectrum antimicrobial activity. Of the few studies with direct, quantitative comparisons of different linking strategies on specificity (versus overall affinity or antimicrobial/killing activity)[31,32,34], only Chen *et al.* [43] described any significant alterations in peptide selectivity; such a change – while undesirable for antimicrobial coatings – is advantageous when peptide-binding patterns might be used for classification. In our study, the preferences of LPS for some IA-immobilized AMPs and of LTA for the same AMPs immobilized on MA plates can potentially be used for discrimination, although clearly, additional sources of LPS and LTA should be tested. Overall, our results clearly demonstrate the potential for fine-tuning not just the affinities but also the binding specificities of immobilized AMPs by simple alterations in linking strategies.

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