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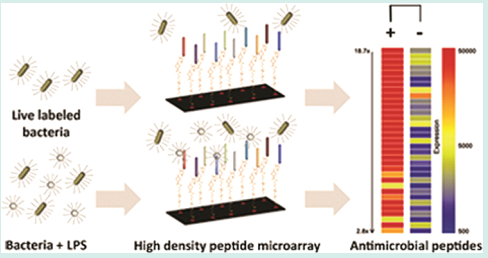
High-Throughput Platform for Rapid Deployment of Antimicrobial Agents

Sergei A. Svarovsky*,† and Maria J. Gonzalez-Moa†

Center for Innovations in Medicine at the Biodesign Institute, Arizona State University, 1001 South McAllister Avenue, Tempe, Arizona 85287-5901, United States

Supporting Information

ABSTRACT: A new approach to conducting bacterial binding assays by using an addressable high density random sequence peptide microarray is described. When bacterial binding is carried out in the presence of a competing excess of corresponding bacterial lipopolysaccharide (LPS), most of the observed bacterial binding is inhibited, suggesting that LPS is the major target of the bacterial binding peptides. Importantly, the amino acid composition of the selected peptides closely resembles the composition of natural antimicrobial peptides. Conjugation of selected peptides to polyvalent nanoparticle scaffold yields constructs that show potent antibacterial agglutination activities. The system is general enough to potentially create antimicrobial agents to virtually any pathogen.



KEYWORDS: antimicrobial, peptide, microarray, high-throughput screening

The increasing development of bacterial resistance to traditional antibiotics has reached unprecedented levels. As a result, there is a strong need to develop new antimicrobial agents with novel modes of action or different cellular targets. Many pathogens expose large quantities of glycans at their cell surface in the form of lipopolysaccharides, capsular polysaccharides, peptidoglycans, and other species-specific glycans. These bacterial glycans are not only very distinct from host glycans but also are essential for pathogen survival and therefore constitute perfect targets for new antibiotic development.[1]

While many antibiotics act on enzymes involved in the cell wall and protein biosynthesis or on DNA replication and repair, antibiotics that target bacterial cell membranes directly have not been fully explored in the clinical settings, with the main difficulty involving finding molecules with selective specificity for bacterial glycans. Two prominent examples of direct bacterial cell wall targeting are represented by vancomycin and teicoplanin, glycopeptides that bind specifically to the outer peptidoglycan backbone in Gram-positive bacteria, inhibiting proper cell wall biosynthesis.[2] Other examples include gene-encoded antimicrobial peptides (AmPs) that are ubiquitous components of innate host defense systems in animals and plants.[3] Although the exact mode of action of these peptides is poorly understood, it is believed that many of them act by first binding to the pathogenic glycans, for example, lipopolysaccharides (LPS) in Gram-negative bacteria, with subsequent permeabilization of bacterial cell membranes eventually leading to irreversible structural damage.[4−6] One of the major strengths of AmPs includes their ability to kill multidrug-resistant bacteria, which along with extremely rare cases of developed resistance helped rekindle interest in this class of compounds in recent years and many of them are now in clinical trials.[7]

To maintain a status quo in the fight against rapidly evolving pathogens, it is important to develop technologies that do not involve protracted research and development stages traditionally associated with drug discovery. Attempts to rapidly select peptides that are specific to the components of outer bacterial membranes by using biocombinatorial strategies, such as ribosome-displayed peptide libraries[8] or aptamer libraries,[9] have been attempted in the past with promising results. Arrays of peptides were recently used to identify antimicrobial peptides while tethered to the surface[10] and to detect pathogenic bacteria in a multiplex fashion.[11] To advance this area further, we have recently introduced a purely combinatorial "shot-gun" strategy where high density random sequence peptide microarrays were used as a powerful glyco-recognition platform to discover peptide sequences that not only specifically bound to LPS, but incidentally inhibited bacterial cell growth.[12] One of the distinct advantages offered by the microarray approach is the immediate visual assessment of all binding events on the array, which unlike biocombinatorial approach, enables parallel analysis of all binding sequences at once. This can be useful for selection of orthogonal functional sequences that have different binding targets or mechanisms of action. A distinct disadvantage, however, is the limited number of potential binding ligands that generally does not allow meaningful selection of consensus sequences or binding motifs, a goal commonly attained by biocombinatorial approaches. This drawback is partially offset by the potentially unrestricted structural and chemical diversity

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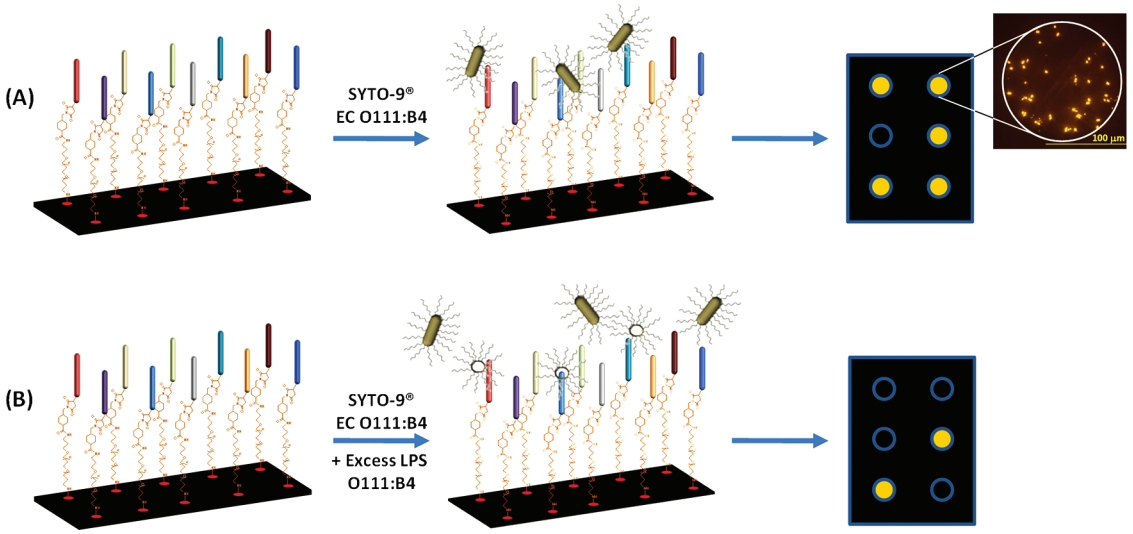

Figure 1. Schematic representation of the bacterial binding assay. PEG3400 linker modified random sequence peptide microarrays were incubated (A) with fluorescently labeled bacteria and (B) with bacteria in presence of excess free LPS. Peptide spots that were quenched or significantly diminished by added LPS were selected as LPS-binding peptides. Insert shows a microscope image of fluorescently labeled bacteria binding to one of the array spots.

of the synthetic ligands and by the flexibility to conduct binding/inhibition assays under more diverse conditions.

Our previous system was based on screening peptide microarrays using novel quantum dot labeled LPS micelles[13] with a main disadvantage being the need for laborious extraction and labeling of bacterial membrane components. Here we report a new improved system to select peptides that bind to specific bacterial membrane components by using live fluorescently labeled bacteria. At the core of this system is the high-density peptide microarray constructed by spotting with a robotic spotter 10 410 presynthesized 20-mer random sequence peptides[14] onto a glass microscope slide functionalized with a polymeric (3400 Da, ~66 EG units) PEG linker terminated with thiol reactive maleimide (Supporting Inforamtion Scheme 1). This extended linker separates the peptides from the slide surface to allow efficient binding to the live bacteria cell surface, which reduces nonspecific binding of bacteria to the array surface presumably by making it cytophobic.[15,16] Noteworthy, when peptides were printed directly onto two-dimensional SMCC-modified aminosilane sildes,[12,14] epoxysilane slides, commercially available three-dimensional hydrogel slides (Schott H-slide) or poly-L-lysine slides the bacterial binding occurred nonspecifically all over the slide surface. These PEGylated arrays were probed directly with fluorescently labeled *E. coli* O111:B4 as schematically shown in Figure 1A and each experiment was done in triplicates.

Since more than 90% of Gram-negative bacterial surface is composed of LPS, it is reasonable to assume that bacteria would bind to the peptide microarray mostly through LPS. This feature was successfully explored in the past for whole bacteria glycoprofiling by using lectin microarrays.[17] To demonstrate that peptides on the microarray indeed bind bacteria through the LPS and to eliminate possible dye binding, we conducted a competition assay with free LPS. Same number of *E. coli* O111:B4 cells stained with SYTO-9 was used to probe the microarrays with 100x excess of unlabeled LPS derived from the O111:B4 strain (Figure 1B). In this case decreased signal in the presence of LPS is indicative of specific LPS mediated bacterial binding. We found that 54 peptides (0.5% of total 10 410, Supporting Information Table 1) showed a decrease in raw signal of more than 2-fold in the presence of LPS. The heat map in Figure 2 shows raw signal intensities (I) for the top 32 peptides that

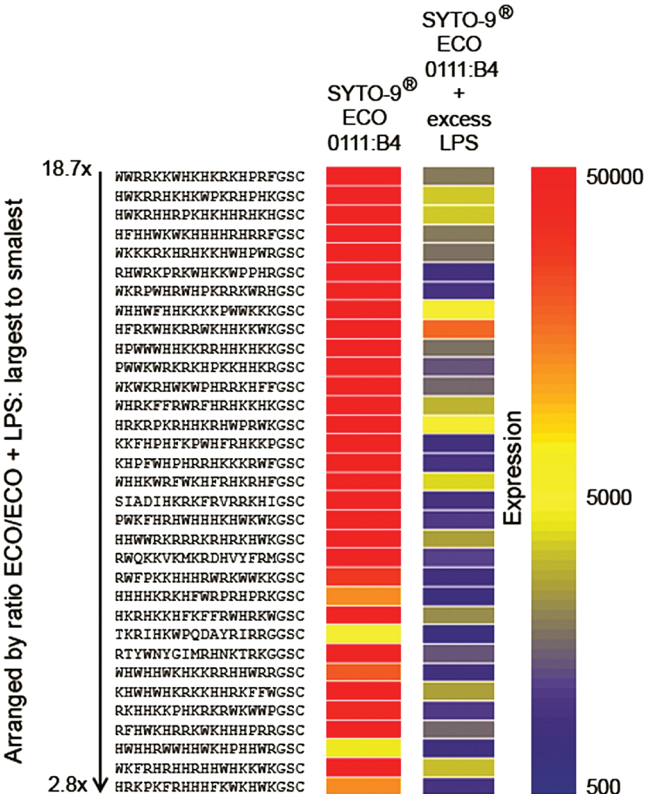

Figure 2. Heat map compares the levels of raw luminescent intensity (log 2) between direct *E. coli* O111:B4 (ECO) binding and binding of ECO in presence of excess LPS (ECO + LPS). In this image, the range of fluorescent intensity on the microarray is represented by red (highest) to blue (lowest). Only top 32 peptides with 2.8−18.7 ECO/(ECO + LPS) ratios are shown. Full list and physical properties of 54 peptides with ECO/(ECO + LPS) > 2 is available in Supporting Information Table 1.

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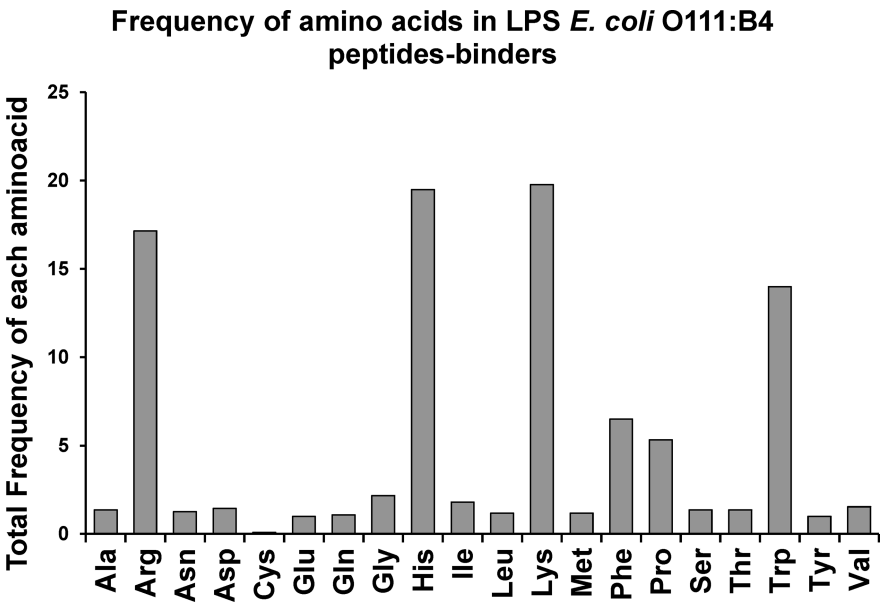

Figure 3. Chart of frequencies of amino acids in the sequences of peptide-candidates for LPS *E. coli* O111:B4 binders.

bound *E. coli* with and without excess LPS with 2.8 to 18.7-fold inhibition ratio $I(E.\ coli)/I(E.\ coli$ + LPS).

To further validate that the fluorescent signal resulted from the bacterial cell adhesion to the peptides on microarray and not from a nonspecific dye binding, we attempted direct visualization of bound bacteria with fluorescent microscopy. Five sequences (Seq. 29−33, Supporting Information Table 1) that appeared in the middle of the list of LPS binding peptide were resynthesized and printed on a custom array on the slides prepared according to procedure for 10K microarray, but using wider spatial separation. All the sequences have shown similar ability to bind stained bacteria on the array. For example, insert in Figure 1A shows ∼30 stained bacterial cells adhering to 100 μm spot modified with QF8 (Seq. 33, Supporting Information Table 1) peptide. This serves as an additional proof for the applicability of our surface chemistry and cell binding techniques for selection of peptides interacting specifically with bacteria.

The physical properties of the selected 54 LPS binding peptides are very similar. All selected sequences appear to be cationic amphipathic peptides, which is also a major characteristic of naturally occurring AmPs generally characterized by an overall positive charge imparted by the presence of multiple lysine and arginine residues and a substantial portion of hydrophobic residues.[3,7] On the other hand, the high prevalence of phenylalanine, histidine, lysine, arginine and tryptophan in the selected bacteria binding peptides (Figure 3) agrees with our previous data obtained by direct LPS binding assays.[11] The strong sequence homology between microarray selected and natural AmPs was discussed in more detail in our previous publication.

Despite the prevalence of LPS on the membrane of Gram-negative bacteria, there are other membrane components, such as peptidoglycans, porins, fimbrial proteins, etc., that could potentially serve as peptide binding targets. Indeed, 21 peptides bound *E. coli* O111:B4 independently of LPS addition, although this binding may in principle be attributed to the cationic SYTO-9 dye that could potentially be released by the stained bacteria. Inspection of the physical properties of these non-LPS binders reveals a dramatically different amino acid distribution and their mostly negative charge, as illustrated in Supporting Information Figure 1. Thus, while the mean pI of LPS binding peptides was almost 12, the mean pI of non-LPS binding peptides was 6.76 ($p < 0.001$). These data and our ongoing work with Gram-positive bacteria indicate that screening whole bacteria may yield peptide candidates that bind bacterial membrane components other than LPS.

Since the selected peptides are positively charged and the bacteria typically carry negative charge, it can be argued that LPS binding sequences were selected due to nonspecific electrostatic interactions alone. Although electrostatic interactions certainly play a role, it must be kept in mind that nearly half (∼5000, Supporting Information Figure 2) peptides on the array are positively charged under the assay conditions, yet only 54 peptides could be unambiguously identified as binders. Figure 3 shows that aside from charge another prominent feature of the selected sequences is the high frequency of aromatic hydrophobic Phe, and particularly, Trp amino acids. The role of hydrophobic interactions in carbohydrate recognition by aromatic amino acids is well documented. Such interaction occurs by stacking hydrophobic face of sugars against the flat hydrophobic plane of Trp or Phe through the C−H$\cdots\pi$ type interaction.[18] Many lectins (agglutinins), sugar binding proteins of nonimmune origin, often rely on these hydrophobic aromatic amino acids to achieve selectivity via a combination of hydrogen bonding and hydrophobic interactions.[19] Peptides selected by biocombinatorial strategies, for example, by phage display, to bind sugar epitopes have also been shown to have high prevalence of hydrophobic aromatic amino acids and bend-forming Pro in their sequences.[20,21] Although the link between binding targets and antimicrobial activity is not yet firmly established, most natural AmPs share traits similar to the glycan binding peptides selected by other means, namely positive charge and high content of hydrophobic residues.[22] This is not surprising because regardless of their mechanism of action, all AmPs must first interact with the bacterial cytoplasmic membrane mainly composed of carbohydrates.

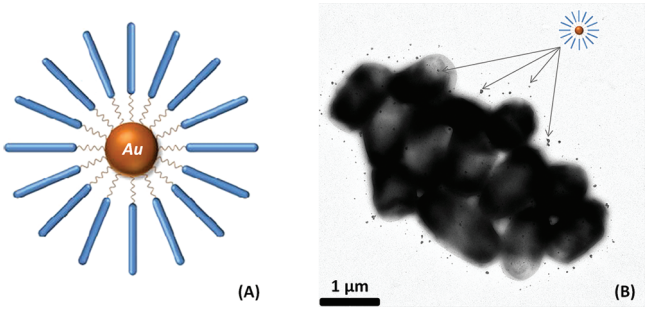

Figure 4. (A) Multivalent presentations of peptide QF8 (blue bars) on gold nanoparticle (QF8-AuNP) scaffold. (B) QF8-AuNP added to *E. coli* O111:B4 results in nearly instant agglutination of the bacterial cells. TEM image of the granular bacterial precipitate shows QF8-AuNPs (arrows) adhering to the outer LPS layer protruding from the bacterial membrane surface.

To test if the selected peptides are binding specifically to the outer saccharidic region of LPS and not through electrostatic attraction alone, we assembled two multivalent constructs with similar surface charge by using 20 nm gold nanoparticles as a scaffold. Since the dense layer of LPS on the surface of Gram-negative bacteria offers a multiplicity of binding sites, we posited that multivalent ligands would have a potential to engage multiple sites at the bacterial surface thus leading to bacterial aggregation similar to the action of natural lectins.

Our lead peptide QF8 (HRKPKFRHHHFKWKHWKGSC, Seq. 33, Supporting Information Table 1), which appeared roughly in the middle of the 54-peptide list of at least 2-fold inhibition by LPS and that was shown previously by us to bind glycan component of LPS,[11] was resynthesized and purified to >95% by HPLC. Using this peptide, multivalent construct QF8-AuNP that presents the peptide in a multimeric spherical fashion at the surface of nanosized gold particles (Figure 4A) was synthesized by self-assembly as described in Supporting Information. Cysteamine modified gold nanoparticles (Cys-AuNP) that had similar charge to QF8-AuNPs (ζ_{av} = +32 mV vs +36 mV, respectively) were similarly synthesized to test if the positive charge was responsible for binding.

Rapid agglutination of *E. coli* was observed in the presence of QF8-AuNPs, which occurred instantly and was visually manifested by a coarse granular bacterial clumping. The transmission electron micrograph (TEM) of the isolated and extensively washed bacterial precipitate showed bacterial cells cocooned by the nanoparticles evenly distributed throughout the outer LPS layer (Figure 4B). Addition of the same concentration of Cys-AuNPs (estimated by absorbance at 550 nm) to the same number of bacterial cells did not induce any agglutination. Instead it resulted in a red plasmon resonance shift of the gold nanoparticles, which indicated that the nanoparticles assembled at the surface of bacteria but did not agglutinate them. A similar effect was reported for gold nanoparticles assembling at the surface of a virus.[23] To test if hydrophobic component played a role in QF8-AuNP induced agglutination, the agglutination reaction was also performed in the presence of 0.25% Tween-20, a nonionic detergent known to disrupt hydrophobic interactions. The agglutination was significantly reduced by the detergent, suggesting that hydrophobic interactions also play important role in QF8-AuNP induced agglutination. This experiment demonstrate that the QF8 peptide is binding the LPS at the surface of the cells and cross-links them similar to the action of multimeric lectins and some glycan binding peptides.[24]

To summarize, in this Technology Note, we introduced a new general high-throughput screening platform to select peptide sequences that directly bind to live fluorescently labeled bacteria. We then demonstrated that the bacteria-peptide interaction can be efficiently disrupted in a competition assay by an excess of species-specific LPS. The LPS-binding peptide sequences selected by this method coincided with the sequences previously identified through direct binding of fluorescently labeled LPS and were compositionally similar to the naturally occurring antimicrobial peptides. This study not only directly supports our previous findings, but also extends it to the discovery of other cell surface binding ligands in a comprehensive fashion. As a proof-of-principle, we further demonstrated that multivalent conjugates of such peptides result in lectin-like agglutinating activity unlike similarly charged conjugates composed of lysine-like residues. Although this study focused on inhibition assays using free LPS, inhibition using intact unlabeled cells or other bacterial surface components is possible and is subject of our future reports. Areas of potential use of this technology are broad and include antimicrobial surface coatings, endotoxin removal, magnetic decontamination devices, biosensors, and infection-specific imaging.

■ ASSOCIATED CONTENT

Supporting Information. Detailed experimental procedures including slide preparation, screening assays, data analysis, and a list of peptides with physical properties. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Postal address: GlycoDots LLC, 5042 Wilshire Blvd 14030, Los Angeles, CA 90036, USA. Telephone: +1-(619)718-1347. E-mail: sergei.svarovsky@gmail.com.

Present Addresses

†GlycoDots LLC, 5042 Wilshire Blvd 14030, Los Angeles, CA 90036.

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