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A TWO-STEP PHAGE DISPLAY PANNING METHOD FOR SELECTING PEPTIDES TO DETECT HMGB1 AND ITS POTENTIAL FOR DEVELOPING PEPTIDE-BASED BIOSENSORS

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DECLARATION OF INTEREST

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ABBREVIATIONS

µl	Microliter
°C	Degrees Celsius
1°	Primary
2°	Secondary
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
HMGB1	High Mobility Group Box 1
HRP	Horseradish peroxidase
IPTG	Isopropyl β-d-1-thiogalactopyranoside
M	Molar
mM	Millimolar
NNK	Nucleic acid code for two of any base pair (NN) and one guanine or thymine (K)
OD_{450}	Optical density for light with wavelength 450 nanometers
OD_{600}	Optical density for light with wavelength 600 nanometers
PBS	Phosphate buffered saline
PFU	Plaque-forming units
pH	Negative of the base 10 logarithm of the activity of the hydrogen (H) ion
RCF	Relative centrifugal field
RPM	Rotations per minute
S+	With affinity for saliva

S-	With no affinity for saliva
S+H+	With affinity for both saliva and HMGB1
S+H-	With affinity for saliva and not for HMGB1
S-H+	Without affinity for saliva and with affinity for HMGB1
S-H-	Without affinity for saliva or HMGB1
SAW	Surface acoustic wave
TBST	Tris-buffered saline with polysorbate 20 (Tween)
X-gal	5-bromo-4-chloro-3indoyl-β-D-galactopyranoside

EXECUTIVE SUMMARY

Background: Expeditionary and deployed personnel are exposed to the widest variety of biological, chemical, environmental, and biomedical threats. Biosensors, devices that combine a biological element with a technological sensor platform to detect an analyte, have great promise and are urgently needed to detect and identify threats in real-time. Unfortunately, biosensors are hampered by a historical reliance upon animal-generated antibodies as their biological recognition elements. Antibody production and characterization are slow, difficult, and sequential processes that rely upon inducing an immune response in a laboratory animal.

Objective: We characterized an alternative method for producing biological recognition elements: phage display. Phage display leverages small viruses (bacteriophages) which display short, random peptides. We intended to develop a phage panning protocol that excludes phages with peptides that bind a background solution and collects only those which bind a desired analyte.

Methods: Phage panning was conducted by a two-step method where phages which bound to the background solution (human saliva) were excluded before the remaining phage pool was panned again to collect only those which bound the analyte (recombinant human high mobility group box 1 protein). Phages collected by this scheme were then amplified, tested for affinity to the analyte, and sequenced to determine the identity of their displayed peptide.

Results: The phage panning scheme collects phages that do not bind the background solution, but do have a high affinity for the selected analyte. These phages bound the analyte with even greater affinity when presented with the analyte in the background solution. The peptides displayed on these phages are all unique and have no conserved motifs, secondary structures, or hydrophobic regions.

Conclusions: This two-step panning scheme can be tuned to include almost any analyte or background solution. It produces many unique peptides with desirable characteristics for use as biological detection elements in biosensors. The entire pipeline can be completed in approximately ten weeks and the peptides it yields have many advantages as recognition elements in biosensors when compared to more traditional, antibody-based elements.

INTRODUCTION

Deployed or expeditionary forces are necessarily at risk, but some risks are easier to identify than others. In situations where military personnel may contact natural or weaponized bacterial pathogens, lethal viruses, or toxic and debilitating chemicals, there is an urgent need to detect and identify these hazards immediately. This is especially true with viral and bacterial pathogens (Centers for Disease Control and Prevention, 2018), that are invisible to the naked eye and can cause harm with only a small number of particles (Lonsdale, Taba et al. 2013). Biosensors, devices that use a biological element (*e.g.* cells, enzymes, antibodies, peptides, or DNA aptamers) to detect the presence of an analyte, are a common tool for identifying these types of hazards (Walper, Lasarte Aragones et al. 2018).

Biosensors combine a peptide, antibody, enzyme, etc. biorecognition element with a sensing platform that can detect and quantify the biorecognition element's signal. An ideal biosensor is one that is: (1) label-free, meaning that it recognizes the analyte directly and without some added fluorophore, dye, or other marker, (2) real-time, meaning that it measures the analyte and reports its findings immediately, (3) rugged or fieldable, (4) accurate and precise, and (5) simple to operate, read, and produces an actionable output (Drake and Levine 2005, Coimbatore, Presley et al. 2008, Shabani, Zourob et al. 2008, Ahmed, Rushworth et al. 2014).

Unfortunately, antibodies, the typical biorecognition element for many biosensors, have several problems that make these sensors less than ideal (Coimbatore, Presley et al. 2008, Tolba, Ahmed et al. 2012). First, antibodies are generated in animals, which means they will not be useful against any highly toxic or contagious analytes, since these may kill or harm the animal before the antibodies could be produced (Wesolowski, Alzogaray et al. 2009, Walper, Anderson et al. 2012, Rahim, Wang et al. 2019). Antibodies are often used with other labels, secondary antibodies, and after sample processing steps (Soelberg, Stevens et al. 2009, Billingsley, Riley et al. 2017, Selvam, Wangzhou et al. 2017), making them not label-free. Also, antibodies are relatively massive when compared to aptamers or peptides, which can lead to off-target binding (Mukundan, Kumar et al. 2012) and signal-to-noise problems in some detector types. Finally, antibodies often degrade and lose their affinity for the analyte in temperatures outside a narrow range (Walper, Lee et al. 2013, Raina, Sharma et al. 2015), so they may not be rugged enough for field use. Our approach to address the problems of antibodies as biorecognition elements

(Walper, Lasarte Aragones et al. 2018) is to use phage display-derived peptides (Goldman, Pazirandeh et al. 2000) to bind and recognize a specific hazard.

Phage display is the technique of inserting the sequence for a short protein, also called a peptide, into one of the genes coding for an exterior protein of a bacteriophage virus (Tikunova and Morozova 2009). These bacteriophages (phages) display the inserted peptide on their viral coat. A phage display library is created by inserting randomized peptides into a large collection of phages (Ledsgaard, Kilstrup et al. 2018). These libraries can then be screened, or "panned," against any desired analyte to isolate only phages with peptides that have affinity for the analyte selected (positive panning) (Fralick, Chadha-Mohanty et al. 2008). Panning can also be done to exclude any phages with peptides that have affinity for a sample (negative panning), for example whole blood (Arap, Kolonin et al. 2002) or saliva.

We developed a two-step approach for phage display panning. First, we panned the phage library against whole human saliva to exclude any phages with peptides that have affinity for saliva. Next, we panned the non-saliva binding fraction of the phage library against human High Mobility Group Box 1 (HMGB1), a potential biomarker for traumatic brain and cranial nerve injuries (VanPatten and Al-Abed 2017, Aucott, Lundberg et al. 2018, Paudel, Shaikh et al. 2018), and collected the fraction that bound HMGB1. This gave us a pool of phages with peptides that have affinity for HMGB1, but no affinity for human saliva to use as a proof-of-concept for our two-step panning approach. These phages bind the selected analyte (HMGB1) *in vitro* even in the presence of the background solution (saliva). We tested several phages and found many displayed increased affinity for their analyte when the background solution is present. These phages were all distinct from one another, suggesting our approach is a rapid and simple way to isolate large pools of phages with unique peptides that bind only the analyte selected and not a pre-screened background solution. This approach is much faster and less expensive than antibody production, and its peptide products are easier to modify than antibodies. It also has the benefit of not requiring an animal or successful immune response to generate large numbers of useful peptides for biorecognition elements in much-needed biosensors.

MATERIALS AND METHODS

Materials

Tubing. Microcap capillary micropipettes (32 mm, Drummond Scientific Company, Broomall, PA) and 0.8 mm bore × 1.6 mm wall, size #13 Marprene tubing (Watson-Marlow, Wilmington, MA) were used during phage panning.

Equipment. A Pumpdrive 5201 peristaltic pump (Heidolph, Schwabach, Germany) was used for phage panning. A 50TS microplate washer (BioTek, Winooski, VT) and a Synergy H1 hybrid plate reader (BioTek) were used during the enzyme-linked immunosorbent assays. Two centrifuges were used; a benchtop 5430R microcentrifuge (Eppendorf, Hamburg, Germany) for slower spins at 4,300 ×relative centrifugal field (RCF) and a Avanti JXN-30 high-speed centrifuge (Beckman Coulter, Brea, CA) for faster spins at 20,800 ×RCF.

Bacteria and phage propagation. A Ph.D.-12 commercial phage display library kit (New England BioLabs, Ipswich, MA) provided all phages and the *Escherichia coli* ER2738 K12 host bacterial strain. All bacterial cultures were grown in Luria-Bertani liquid medium (1% tryptone, 1% sodium chloride, 0.5% yeast extract by weight).

Procedures

Phage display panning – crosslinking panning target to microcapillary tube interior: Panning procedures were conducted as previously reported by Titus et al. (Titus, Kay et al. 2017) with some modifications. Acetone was wicked into a 10 µl microcapillary tube (32 mm Microcap capillary micropipette, Drummond Scientific Company, Broomall, PA) to clean it, then wicked away with a paper tissue. After the tube air dried for 30 minutes, freshly prepared 2% aminosilane in acetone was wicked into the tube and incubated at room temperature. After 30 seconds, the aminosilane solution was wicked out of the tube and the tube was allowed to air dry for at least 30 minutes. Next, freshly prepared 10 mM sulfo-LC-SPDP (sulfosuccinimidyl 6-(3'-(2-pyridyldithio)propionamido)hexanoate, ThermoFisher Scientific, Waltham, MA) in 1×phosphate buffered saline (PBS) + 10 mM ethylenediaminetetraacetic acid (EDTA) was wicked into the capillary tube and incubated at room temperature for one hour. The tube was rinsed twice by wicking with 1×PBS + 10 mM EDTA before the panning target solution, HMGB1 (recombinant human HMGB1 protein CF, R&D Systems, Minneapolis, MN) or whole human saliva (saliva, normal, pooled human donors, Lee Biosolutions, Maryland Heights, MO) diluted in PBS, was wicked into the tube and incubated overnight in a humidified container at 4

°C. The following day, the panning target solution was wicked out and the tube was again rinsed twice with 1×PBS + 10 mM EDTA before proceeding to phage library recirculation.

Phage display panning – phage library recirculation: One end of the microcapillary tube with the panning target crosslinked to its interior surface was mated with tubing (Marprene Tubing, 0.8 mm bore × 1.6 mm wall, size #13, Watson-Marlow, Wilmington, MA) and the entire assembly was rinsed with 250 µl 70% ethanol from one end by peristaltic pump (Pumpdrive 5201, Heidolph, Schwabach, Germany) at 20 rotations per minute (RPM). The assembly was air dried for 30 minutes at 5 RPM then rinsed with 1×PBS + 10 mM EDTA and filled with blocking buffer (SuperBlock, ThermoFisher Scientific, Waltham, MA). The blocking buffer was incubated for one hour at 4 °C then drained at 5 RPM, and 1.5 ml tris-buffered saline with 0.1% polysorbate 20 detergent (TBST) was washed through the tubing and microcapillary assembly at 5 RPM. The phage display library (Ph.D.-12 Phage Display Peptide Library, New England BioLabs, Ipswich, MA) was diluted 1:100 in TBST and approximately 250 µl was used to completely fill the assembly. The other end of the microcapillary tube was mated with the tubing and the phage library was recirculated at room temperature for one hour at 5 RPM.

After recirculation, the binding fraction (the portion with phages with high affinity for the crosslinked analyte) was adsorbed on the interior surface of the microcapillary tube while the non-binding fraction (the portion with phages with low or no affinity for the crosslinked analyte) remained in circulation. The non-binding fraction was collected, then the assembly was rinsed with 2.5 ml TBST. The binding fraction was collected by filling the assembly with 0.2 M glycine hydrogen chloride (pH 2.2) elution buffer and recirculating it at room temperature for ten minutes at 5 RPM. The binding fraction was collected in a clean tube and neutralized with 17 µl tris-buffered saline pH 9.2. This binding fraction was stored at 4 °C for up to two weeks or at -20 °C for longer if needed.

We used a two-step panning scheme (Figure 1). First, the phage library was recirculated through a microcapillary tube with a biological background solution (whole human saliva) crosslinked to its interior surface. After panning, two fractions were collected. The non-binding fraction contained phages with low or no affinity for the saliva, the biological background solution (S- phages). The binding fraction contained phages with high affinity for saliva, the biological background solution (S+ phages). Each fraction was panned again, through a

microcapillary tube with the desired analyte (HMGB1) crosslinked to its interior surface. This yielded four fractions: the S+ phages without affinity for the analyte (S+H-), the S+ phages with affinity for the analyte (S+H+), the S- phages without affinity for the analyte (S-H-), and the S- phages with affinity for the analyte (S-H+). The S+H+ fraction was retained for use as a positive control. The S-H- fraction was retained for use as a negative control. The S-H+ fraction was retained for future use as biorecognition elements for detecting the analyte (HMGB1) in a biological background solution (saliva). All three fractions were separated into individual phage clones, amplified, titered, and characterized.

Isolating individual phage clones: Non-binding and binding fractions from phage display panning contain many, many individual phages. To amplify and characterize an individual phage, it must first be isolated from the others. We used the double agar overlay technique to separate phages from each other and identify individual phage clones. First, an overnight culture of ER2738 K12 *E. coli* started the day prior was subcultured by adding fresh media in a 1:1 ratio and further incubating at 37 °C with shaking. After 30 minutes to one hour, the culture's OD_{600} was measured, the bacterial cells were spun down at 4,300 ×RCF for 5 to 10 minutes at room temperature and resuspended in sterile media to OD_{600} 0.4 to 0.6. The phage display fraction was serially diluted in sterile media from $10^{-1}\times$ to $10^{-12}\times$ its original concentration. Next, 3 µl of 5% isopropyl β-d-1-thiogalactopyranoside (IPTG) and 4% 5-bromo-4-chloro-3indoyl-β-D-galactopyranoside (X-gal) in dimethyl sulfoxide (DMSO) (ChromoMax IPTG/X-Gal solution, Fisher Scientific, Waltham, MA) and 250 µl resuspended bacterial culture were added to 100 µl of the diluted phage fraction. This entire mixture was then added to 3.5 ml of molten agar media in a pre-warmed glass tube, and the tube mixed by rolling vigorously between palms. The tube's mixed contents were then poured onto a pre-warmed solid agar media plate, which was swirled to distribute the molten agar uniformly. The plate was incubated in a biosafety cabinet with the lid ajar until the molten agar gelled, then the new overlay plate was incubated at 37 °C overnight. This process was repeated in duplicate or triplicate for each phage display fraction dilution. The following day, individual phage clones from the phage display fraction were visible as small clearings with blue-green halos or hazy blue-green circles (phage plaques) within the bacterial overgrowth. These plates were sealed and stored at 4 °C for up to two weeks.

Phage amplification: Once separated from other phages in the phage display fraction, unique phage clones on a double agar overlay plate can be picked and amplified. First, an overnight culture of ER2738 K12 *E. coli* started on the day prior was diluted to OD_{600} 0.09 and incubated at 37 °C with shaking. After 15 minutes incubating in a baffled flask with vented (or loose) cap, 100 ml of the liquid *E. coli* culture was inoculated with a single phage plaque picked from a double agar overlay plate with a sterile transfer pipette (if amplifying from an already amplified stock, cultures were inoculated at a multiplicity of infection < 1.0) and incubated at 37 °C with vigorous shaking. After 4.75 to 5 hours, the infected *E. coli* culture was aliquoted into 50 ml conical tubes with approximately 35 ml of infected culture per tube. These tubes were centrifuged at 4,800 ×RCF for 10 minutes at 4 °C to pellet bacterial cells. The supernatant was removed (the pellet was discarded) to another sterile 50 ml conical tube, one-sixth volume 20% polyethylene glycol in 2.5 M sodium chloride was added, and the tubes were incubated overnight at 4 °C. After at least 8 to 12 hours, the tubes were centrifuged again at 4,800 ×RCF for 55 minutes at 4 °C to pellet the phages. The pellets were retained (the supernatants were poured off and discarded) and 1 ml 1×PBS + 10 mM EDTA was added. This was incubated at least one hour at room temperature to soften the pellet. Vortexing periodically during and after incubation helped to resuspend the pellet. Repeated gentle pipetting also sped resuspension. Newly resuspended pellets were transferred to sterile microcentrifuge tubes and centrifuged at 20,800 ×RCF for 10 minutes at 4 °C. The supernatant was removed and discarded and the pellets were finally resuspended in 50 µl 1×PBS + 10 mM EDTA.

Determining amplified phage stock concentrations: Stocks of amplified phage clones must be titered to reveal their concentrations. The protocol for titering phage stocks was identical to the protocol for isolating individual phage clones described above, except more replicates were performed across a smaller range of stock concentrations. Since amplified phage stocks typically had between 10^8 and 10^{14} plaque-forming units (PFU)/ml, performing triple replicates of 10^{-7}, 10^{-8}, and 10^{-9} dilutions was usually sufficient. Counting the phage plaques on each plate and multiplying to correct the dilution factor revealed the concentration of the amplified stock per 100 µl. Further multiplying by 10 to correct the amount added revealed the number of PFU/ml.

Enzyme-linked immunosorbent assay and analysis: High titer phage stocks were characterized for affinity to the desired analyte by enzyme-linked immunosorbent assay (ELISA). First, 96-well plates were coated with the desired analyte. The desired analyte (recombinant human HMGB1) was diluted to 10 µg/ml in 0.1 M sodium bicarbonate (pH 8.6), and 100 µl was added to each well. Plates were then incubated overnight at 4 °C in a humidified air-tight container. The following day, the analyte solution was removed and each well rinsed six times with TBST before being incubated with 200 µl of blocking buffer per well at 4 °C for two hours. After incubating the blocking buffer, it was removed, each well was rinsed six times with TBST, and 100 µl of phage (in place of 1° antibody in a traditional indirect ELISA) diluted in PBS was added to each well. After incubating the phage dilution at room temperature for two hours (with agitation at ~240 RPM), the phage solution was removed, each well was rinsed six times with TBST, and 100 µl of horseradish peroxidase (HRP)-conjugated 2° antibody (Anti-M13 g8p Monoclonal Antibody (HRP), Antibody Design Laboratories, San Diego, CA) in PBS was added to each well. The secondary antibody was incubated at room temperature for one hour (with gentle agitation at ~240 RPM). Before developing the color reaction to indicate phage 1° binding to the target analyte, the 2° antibody was removed, each well was rinsed six times with TBST, and 100 µl of HRP substrate (*o*-phenylenediamine dihydrochloride, SIGMA*FAST* OPD tablet set, Sigma-Aldrich, St. Louis, MO) diluted 1:20 in TBST was added. The color reaction was incubated in the dark at 25 °C and each well's OD_{450} was measured every five minutes for 75 to 90 minutes.

OD_{450} values for each combination of phage concentration and analyte concentration were pooled and the means compared (plus or minus standard error of the mean) with other combinations and controls as appropriate. Each 96-well plate was set up such that every batch of experimental ELISA data was accompanied by a blocked negative control (blocked with blocking buffer but no 1° phage or 2° antibody) and unblocked negative control (never blocked with blocking buffer, incubated with 1° phage, or incubated with 2° antibody) to reveal baseline values for the color reaction. Greater OD_{450} indicated a relatively greater amount of 2° antibody bound to 1° phage, which we interpreted as higher affinity for the target analyte by that 1° phage's displayed peptide. Monitoring changes in OD_{450} over time, as a function of target analyte concentration, revealed which phages had relatively higher affinity for the target analyte or bound the analyte at relatively lower analyte concentrations.

Sanger sequencing and sequence analysis: Once a phage was found to have a relatively high affinity for the analyte or to bind the analyte at a relatively low analyte concentration, the phages were reserved for Sanger sequencing to reveal the identity of their displayed peptide (Figure 2). We used the -96 sequencing primer reverse strand (5'-CCC TCA TAG TTA GCG TAA CG-3' or 3'-GCA ATG CGA TTG ATA CTC CC-5') from the Ph.D.-12 phage display library kit. Since the sequencing primer was on the reverse strand, all results had to be converted to the reverse complement such that the first base read became the last base in the final sequence and the complementary base pair was used instead. This made reading and analyzing the sequencing results simpler. In the reverse complement of the sequencing results we looked for signature sequences directly upstream and downstream of the inserted phage display peptide. The upstream signature sequence was 5'-TCT CAC TCT-3' and the downstream signature sequence was 5'-GGT GGA GGT-3'. Finding both signature sequences in the reverse complement of the sequencing results revealed the 36 base pairs coding for the 12 amino acids in the inserted peptide (Figure 3). These 36 base pairs were parsed into 3 base pair codons and translated into amino acids (Wernersson 2006). By design of the phage display library, each codon should be made up of base pairs NNK (any base pair, any base pair, guanine or thymine).

Peptide analysis: Once peptide identities were extracted from Sanger sequencing data, they were compared using the PRALINE multiple sequence alignment tool (ibi.vu.nl/programs/pralinewww) (Heringa 1999, Heringa 2002, Pirovano, Feenstra et al. 2008). Peptide sequences were aligned to check for any conserved sequences or motifs, conserved patterns of similar amino acid type, conserved patterns of similar hydrophobicity, and conserved secondary structures.

RESULTS

Selection of high specific peptide probes: Our panning scheme (Figure 1) eliminated many phages without peptides that have affinity for the chosen analyte. After two successive rounds of negative panning, where phages that do not bind the substance immobilized inside the glass microcapillary tube were collected (*e.g.* S-H- phages), we collected on the order of 10^9 PFU/ml (data not shown). When a double panning strategy to collect phages that bound only one of the two substances immobilized inside the glass capillary tube were collected (*e.g.* S-H+ or S+H- phages), we collected on the order of 10^5 PFU/ml (data not shown). When the panning

strategy collected only phages that bound to both substances immobilized inside the glass capillary tubes (*e.g.* S+H+ phages), we collected on the order of 10^3 PFU/ml (data not shown).

Confirmation of probe specificity by ELISA: Both positive (selecting for phages with peptides that do bind a given substance) and negative (selecting for phages with peptides that do not bind a given substance) panning protocols succeeded. When phages with peptides that do not bind to either the biological background solution or the chosen analyte (S-H- phages) were tested by ELISA for the ability to bind the analyte, we found the mean OD_{450} values changed very little over time as the ELISA color reaction was developed (Figure 4A). Conversely, when phages with peptides that do not bind the biological background solution but do bind the chosen analyte (S-H+ phages) were tested by ELISA using the analyte as a target, we found that mean OD_{450} values rose markedly over time (Figure 4B). This effect seems to be dose-dependent with the amount of analyte used as the target, as 10 μg HMGB1/ml wells reached the highest OD_{450}, followed by 5 μg HMGB1/ml (Figure 4B). Mean OD_{450} values for 0.1 and 0.0 μg HMGB1/ml were approximately equivalent (Figure 4B).

Advantage of panning to eliminate phages with background affinity: The strategy (see Figure 1) of one negative then one positive round of phage panning yielded multiple phages with peptides that bind the chosen analyte even better when these phages, the target analyte, and the background solution were all incubated together during the phage-as-primary-antibody step of an ELISA (Figure 5). The increase in phage binding was less evident at higher concentrations of the target analyte but became clear when increasingly smaller amounts of the analyte incubated in a 10% dilution of the background solution were used as the ELISA target.

Unlike the S-H+ phages, which bind their analyte well even when incubated with the biological background solution (Figure 6A-B), phages with affinity for both the analyte and the background solution (S+H+ phages) do not bind the analyte well when it is incubated with the background solution (Figure 6C-D.

Analysis of amino acid sequences of selected probes: After collecting multiple phages with measured affinity for the chosen analyte even when incubated with a biological background solution, we used Sanger sequencing (see Figures 2-3) to reveal the identity of each displayed peptide. When the sequences for nine S-H+ phages were checked for conserved residues (Figure

7), residue types (Figure 8), hydrophobicity (Figure 9), or secondary structure (Figure 10), we did not detect any common patterns or conserved motifs.

DISCUSSION

The potential of phage display for peptide probe selection: The development of peptide probe-based biosensors is an emerging field (Kim, Cho et al. 2019, Wasilewski, Szulczyński et al. 2019). Phage display is an excellent and underutilized tool for screening probe peptides that can be used as biorecognition elements in biosensors. Phages with peptides that bind the analyte (Figure 4B), but not a biological background solution (Figure 6B), can be preferentially selected very rapidly. Though we report on only a small number of phages here, at the end of our panning process (Figure 1) we collected 50 µl of S-H+ phages at 10^5 PFU/ml concentration. This gives us a large pool to draw upon for further phage and peptide characterization should one or more of our (randomly) selected peptides fail as biorecognition elements. The ten S-H+ phages we characterized are all unique with no conserved motifs (Figure 7), residue types (Figure 8), hydrophobicity or hydrophilicity regions (Figure 9), or secondary structures (Figure 10). This gives us confidence that within the remaining pool of roughly 2500 S-H+ phages (25 µl of 10^5 PFU/ml S-H+ phages) there are a high number of other unique peptides with potential for use in biosensors.

The advantage of phage display: These large numbers of phages and their peptides, along with the speed of panning and amplification procedures, give phage display-derived peptides tremendous advantages over antibodies. Many phages (on the order of 10^9) are screened at once during one hour of phage display *in vitro* recirculation, making this technique highly parallel in contrast to the lengthy in series and *in vivo* techniques required to isolate a new monoclonal antibody (Wesolowski, Alzogaray et al. 2009, Walper, Anderson et al. 2012, Rahim, Wang et al. 2019).

The advantages of phage display over antibody selection: Phage display has other advantages over antibody production beyond those of speed and number. At 7-12 amino acids in length, phage display-derived peptides are much easier and less expensive to produce (Newton, Kelly et al. 2006). The peptide approach is also animal-independent and does not depend upon the immune response, making it possible to use phage display even against analytes which would otherwise be lethal or dangerous to a production animal (Leenaars and Hendriksen 2005, Rahim,

Wang et al. 2019). Because peptides are much shorter and smaller than antibodies, it is easier to optimize and test each amino acid on a peptide (Otvos and Wade 2014). Their short lengths also make them easier to produce synthetically en masse than antibodies (Wylie, Wylie et al. 2018), more thermostable (Raina, Sharma et al. 2015), and much easier to customize during production (Goldman, Pazirandeh et al. 2000). With only 12-20 amino acids, peptides have less bulk to be responsible for off-target binding and produce correspondingly less background (Tawil, Sacher et al. 2012). Finally, in sensing platforms that rely on an increase in mass to detect the analyte (*e.g.* surface acoustic wave (SAW)- or quartz crystal microbalance-based platforms), the small size of a peptide being used as biorecognition element means a much greater signal-to-noise ratio than if a small analyte was bound by a relatively massive antibody (Tawil, Sacher et al. 2012).

Combining phage display-derived peptide probes and SAW sensors: The ease of collection, characterization, and customization, and the advantage in signal-to-noise ratio suggests phage display-derived peptides are best suited for use in SAW-based biosensors. Since SAWs produce a signal based on a change in mass density on their piezoelectric surface (Tawil, Sacher et al. 2012), they are primed to exploit the greater signal-to-noise ratio inherent in a relatively small and stiff peptide (Mukundan, Kumar et al. 2012). Since peptides like the ones described here can be selected with low affinity to the background solution, they are ready for use in a continuously sampling, label-free sensor like a SAW (Mujahid and Dickert 2017). The real-time capabilities of a SAW sensor would be wasted on a biorecognition element that requires sample preparation or labeling steps. Instead, a biological fluid can flow over the SAW's sensitive surface, an analyte can bind to the phage display-derived peptide tethered on the surface, and the SAW will detect this binding event within microseconds. Such a sensor might even be implanted into the body (Murphy, Bahmanyar et al. 2013) or other sensitive location and interrogated wirelessly (Fachberger and Erlacher 2009, Shevchenko, Kukaev et al. 2018, Tao, Hasan et al. 2018).

MILITARY SIGNIFICANCE

Applying peptide probes to developing a biosensor for traumatic brain and cranial nerve injuries: Beyond serving as a proof-of-concept for our phage display pipeline, the peptides we characterized and identified also have functionality for detecting traumatic brain and cranial nerve injuries. U.S. military personnel suffered more than 383,000 traumatic brain injuries from

2000 to 2018 (Defense and Veterans Brain Injury Center 2020), and modern warfare is increasingly expeditionary (O'Brien 2020). Future naval conflict with near-peer adversaries will produce large numbers of blast casualties and the requirement for prolonged field care. Reverberating shockwaves in confined areas, such as shipboard compartments, light armored vehicles, and amphibious assault vehicles, will amplify a blast's effects (Leibovici, Gofrit et al. 1996). An increase in blast-related injuries will necessitate new ways for corpsmen to identify, diagnose, triage, and treat a corresponding increase in traumatic brain and cranial nerve injuries. Quantitative testing will eliminate the need for Sailors and Marines to identify and self-disclose their own symptoms, which they may loathe to do for fear of disrupting their own mission. In the near-term these peptides will enable rapid, objective, and quantitative testing for traumatic brain and cranial nerve injuries. In the long-term these peptides will help to address multiple Naval operational gaps including: (1) the lack of ability to use imaging or biomarkers in diagnosis, (2) the inability to objectively and definitively identify traumatic brain and cranial nerve injuries, (3) the lack of understanding of these injuries and the insufficient understanding of their short- and long-term effects, (4) the lack of screening criteria for milder injuries, and (5) the need for Commanders to have real-time insight into the mental health, physical health, and medical readiness of their Sailors and Marines.

While the SAW sensing platform we propose is a relatively mature technology (Coimbatore, Presley et al. 2008, Fachberger and Erlacher 2009, Rocha-Gaso, March-Iborra et al. 2009, Mukundan, Kumar et al. 2012, Mujahid and Dickert 2017, Chen, Wu et al. 2018, Tao, Hasan et al. 2018), it is hindered by a reliance on antibodies as primary detectors. Our approach, which leverages phage display-derived peptides, has many advantages over antibody-based methods (Goldman, Pazirandeh et al. 2000, Kehoe, Velappan et al. 2006, Tolba, Ahmed et al. 2012, Raina, Sharma et al. 2015). The entire pipeline from securing a sample of the desired analyte to peptide sequences ready-for-synthesis takes less than one month of laboratory time. In this case, our methods were developed and refined by one undergraduate student and one contract research scientist over the course of a Naval Research Enterprise Internship Program 10-week internship. Conversely, antibodies can take months to produce and isolate, much less characterize and produce in high quantities (Committee on Methods of Producing Monoclonal Antibodies 1999, Leenaars and Hendriksen 2005). The shorter timelines enabled by our phage display pipeline will accelerate sensor development to meet the needs for monitoring

environmental samples for contamination (Suk, Zmorzynska et al. 2011, Luka, Samiei et al. 2019), disease surveillance and diagnosis (Gallego, Sintchenko et al. 2009), for biomarker quantification (Sage, Besant et al. 2015), and biological threat warning systems (Coimbatore, Presley et al. 2008).

There are many threats U.S. military personnel must be prepared for, and the list is growing longer. Breaking the historical reliance on antibodies as biorecognition elements for sensors will let us produce tests and sensors much more rapidly and without production animals. We should eliminate the antibody bottleneck by incorporating phage display into the biosensor development pipeline. Phage display-derived peptides, especially when paired with SAW-based sensor platforms, are poised to replace traditional antibodies and should be embraced. These sensors with phage display-derived peptides will be useful in operational environments, for environmental monitoring, and for biomedical purposes in the military health system.

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FIGURES

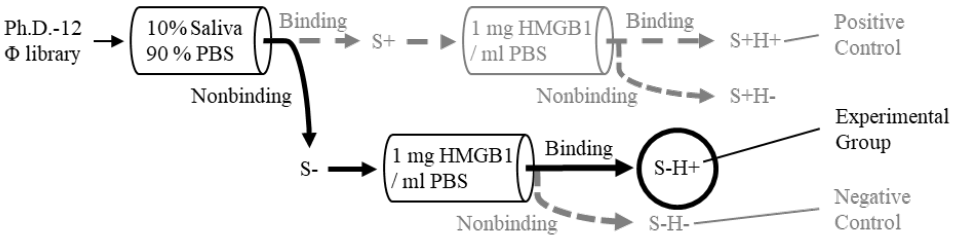

Figure 1. Example panning scheme for collecting phages that do not bind a biological sample matrix (saliva) but do bind an analyte (HMGB1). Panning the phage display library against the biological sample matrix (saliva) first and collecting the nonbinding phages eliminates any phages with strong affinity for the matrix solution. These phages are then panned against the desired analyte (HMGB1) and the phages that bind the analyte are collected. This process can also be adapted to collect phages for use as positive (binding both the matrix and the analyte) and negative (binding neither the matrix nor the analyte) controls.

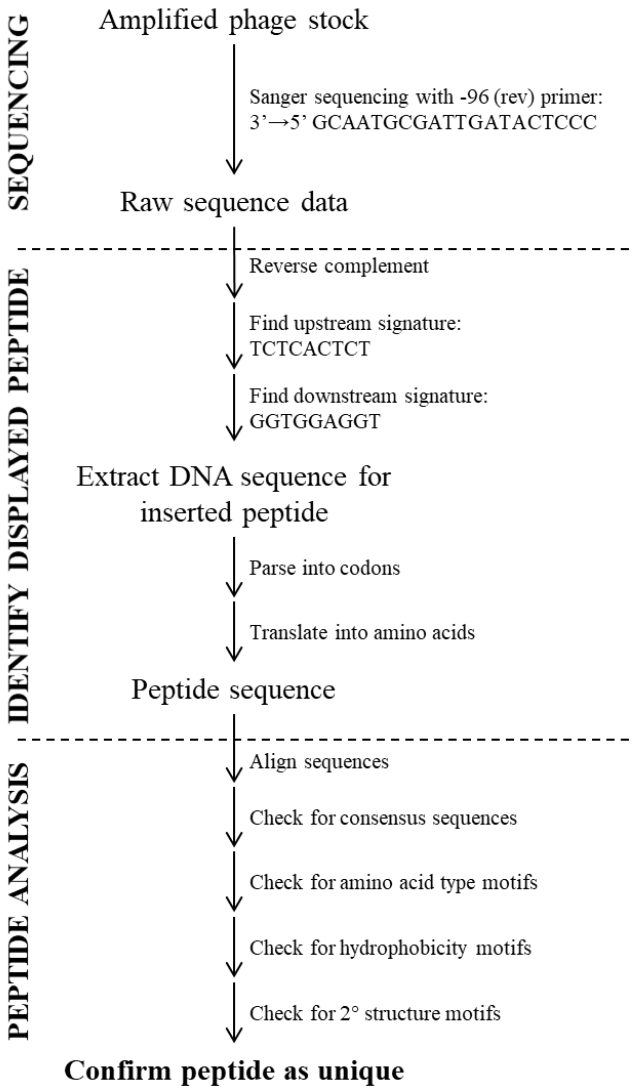

Figure 2. Pipeline from HMGB1-binding phage to unique peptide sequence. After phage affinity for HMGB1 is confirmed by ELISA, the inserted phage display peptide's identity is revealed by Sanger sequencing. Peptides are compared with one another to confirm each is unique.

Example (from S-H+9):

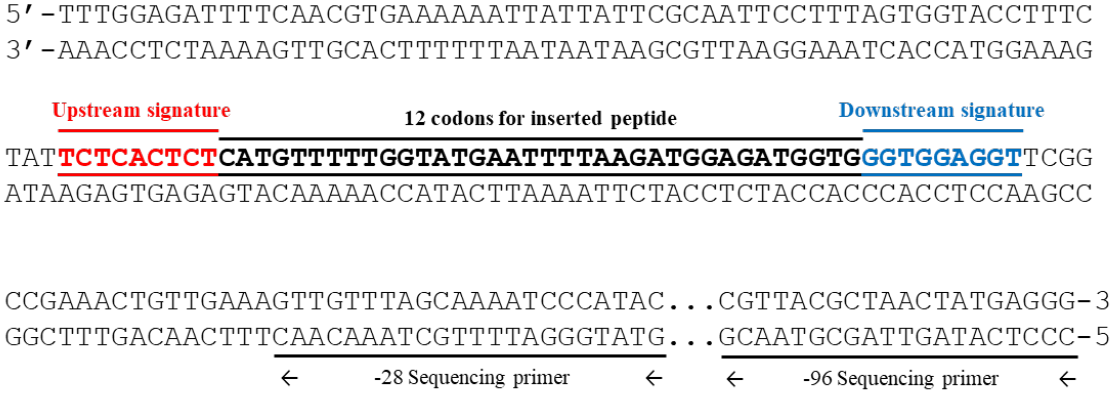

Figure 3. Example sequencing result using the -96 sequencing primer. After Sanger sequencing with the recommended -96 sequencing primer (3'-GCAATGCGATTGATACTCCC-5'), the 12 codons of the inserted phage display peptide are revealed by searching for the upstream (red) and downstream (blue) signature sequences which flank them. The 12 codons for the inserted peptide can also be found by identifying the -28 sequencing primer (3'-CAACAAATCGTTTTAGGGTATG-5') downstream and counting 28 base pairs upstream. In actual sequencing results, the -96 sequencing primer and many base pairs upstream of it will not be present, though the -28 sequencing primer may be and the downstream signature should be.

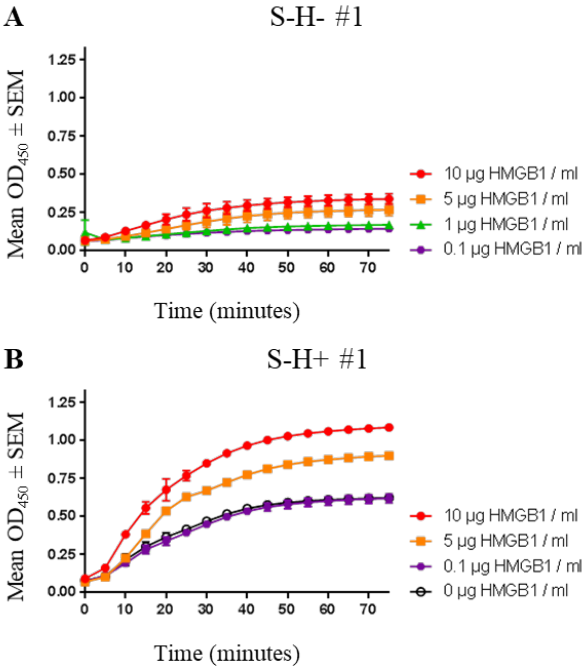

Figure 4. Panning separates phages by their affinity for the analyte (HMGB1). Phages displaying peptides with no (or low) affinity for a chosen analyte (HMGB1) are not retained inside the glass microcapillary tube during panning, and are easily separated from those which do bind the chosen analyte. A representative nonbinding phage (S-H- #1, shown in A) does not bind to HMGB1 with high affinity and produces low optical density (OD_{450}) during ELISA tests. A representative HMGB1-binding phage (S-H+ #1, shown in B) does bind to HMGB1 and produces a correspondingly higher OD_{450}.

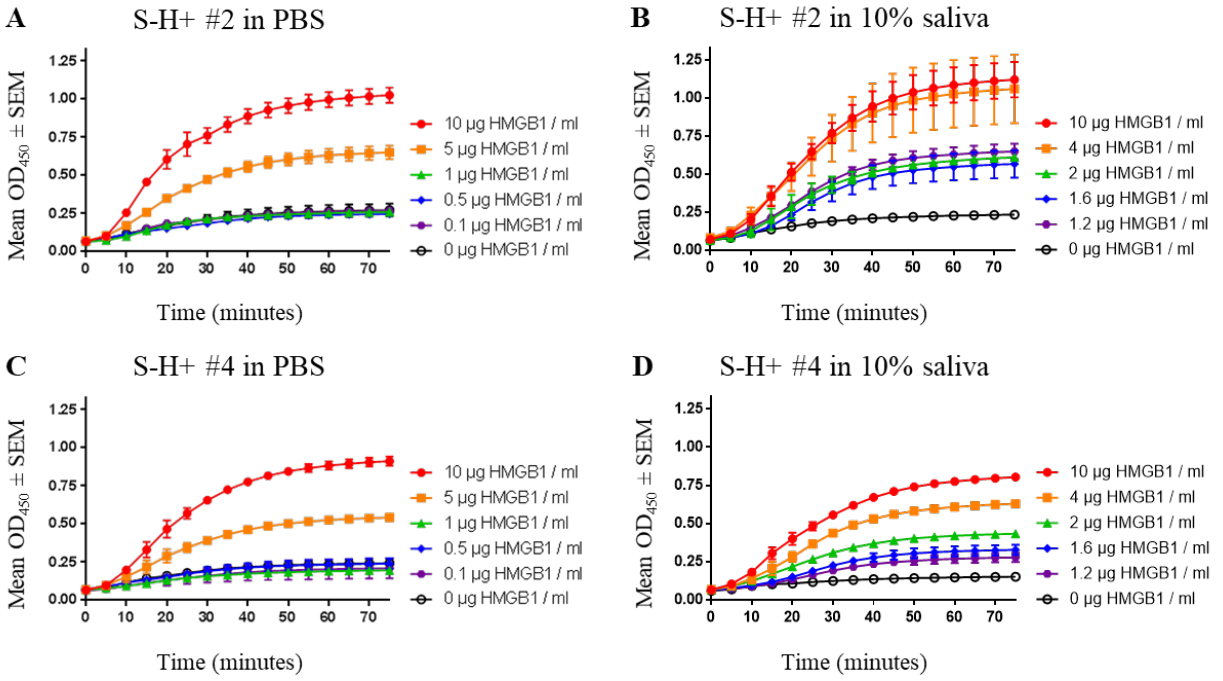

Figure 5. Phages bind their analyte (HMGB1) even in the presence of a biological fluid (saliva). Phages selected for their inability to bind a biological fluid (saliva) and ability to bind an analyte (HMGB1) bind the analyte even when incubated with the biological fluid. Two representative phages, S-H+ #2 (A-B) and S-H+ #4 (C-D) are shown. Both phages bind their analyte with greater affinity at lower analyte concentrations when incubated with the biological fluid.

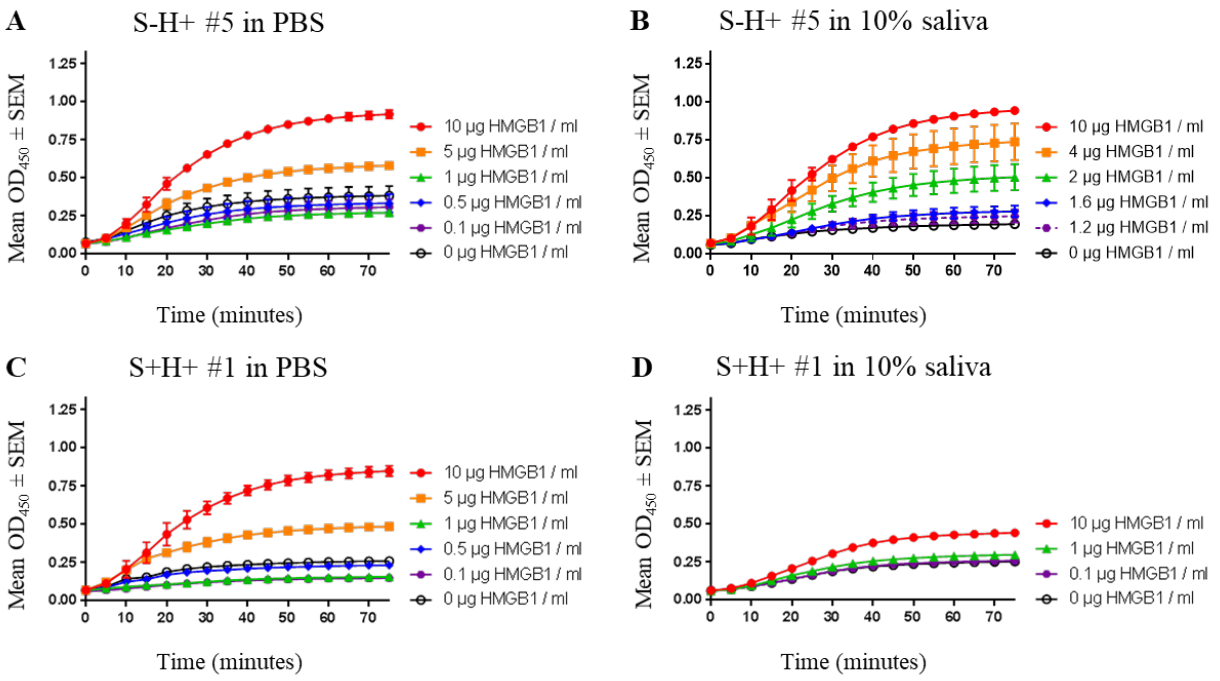

Figure 6. Negative panning to remove phages with affinity for the biological solution results in higher affinity for the analyte when incubated in the biological solution. The panning scheme eliminated phages with affinity for the biological solution (saliva) before collecting phages with affinity for the chosen analyte (HMGB1). These S-H+ phages (representative phage S-H+ #5 shown) bind the analyte well in buffer (A) and in buffer plus saliva (B). S+H+ phages with affinity for both the biological solution and the analyte bind the analyte well in buffer (C) but do not bind as well when incubated in buffer plus saliva (D).

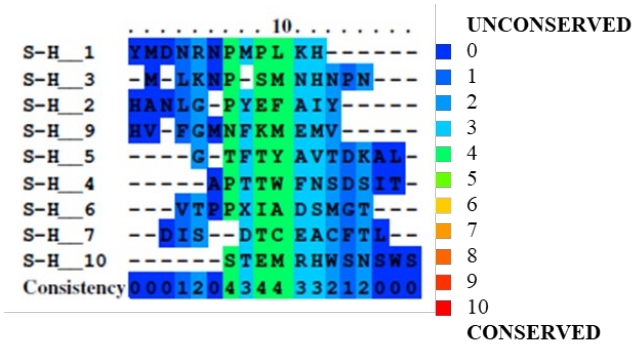

Figure 7. Alignment and residue conservation. S-H+ peptides aligned and marked for the conservation of each residue relative to the other residues at the same position in other peptides. Relatively conserved residues are marked with redder colors, while less conserved residues are marked with bluer colors.

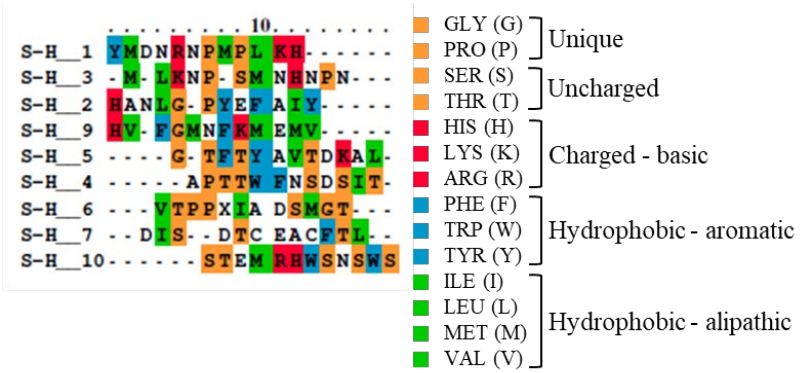

Figure 8. Alignment and residue type. S-H+ peptides aligned and marked for their residue type and frequency (ClustalX color scheme).

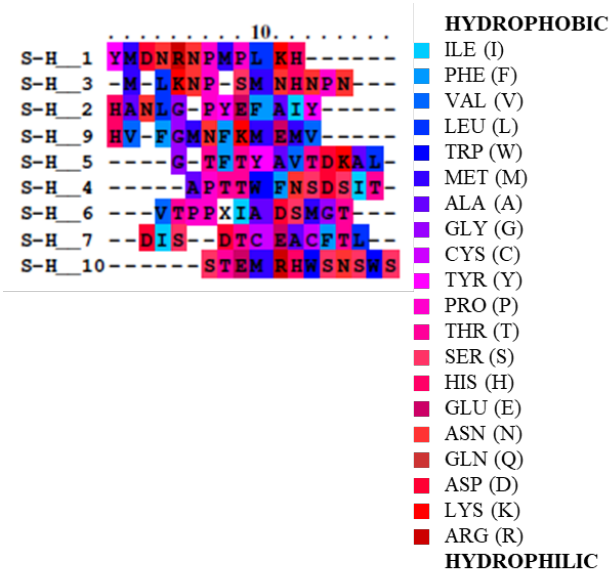

Figure 9. Alignment and residue hydrophobicity. S-H+ peptides aligned and marked for the hydrophobicity (bluer colors) or hydrophilicity (redder colors) of each residue.

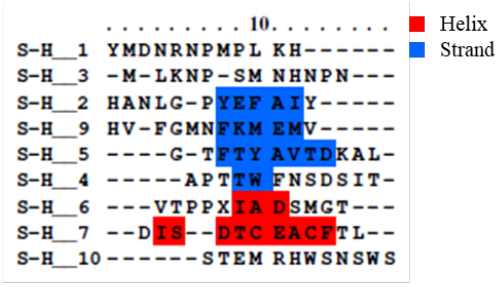

Figure 10. Alignment and secondary structure propensity. S-H+ peptides aligned and marked for their propensity to form helix-shaped (α-helices, red) or strand-shaped (ß-folds, blue) secondary structures.

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14. ABSTRACT

Expeditionary and deployed personnel are exposed to the widest variety of biological, chemical, environmental, and biomedical threats. Biosensors, devices that combine a biological element with a technological sensor platform to detect an analyte, have great promise and are urgently needed to detect and identify threats in real-time. Unfortunately, biosensors are hampered by a historical reliance upon animal-generated antibodies as their biological recognition elements. Antibody production and characterization are slow, difficult, and sequential processes that rely upon inducing an immune response in a laboratory animal. We characterized an alternative method for producing biological recognition elements: phage display. Phage display leverages small viruses (bacteriophages) which display short, random peptides. We intended to develop a phage panning protocol that excludes phages with peptides that bind a background solution and collects only those which bind a desired analyte. This two-step panning scheme can be tuned to include almost any analyte or background solution. It produces many unique peptides with desirable characteristics for use as biological detection elements in biosensors. The entire pipeline can be completed in approximately ten weeks and the peptides it yields have many advantages as recognition elements in biosensors when compared to more traditional, antibody-based elements.

15. SUBJECT TERMS
Biosensors, biorecognition, phage display

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