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Patrick Daugherty

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University of California - Santa Barbara
Office of Research
3227 Cheadle Hall
Santa Barbara, CA 93106 -2050

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Report Title

Rapid Molecular Fingerprinting of Pathogens

ABSTRACT

The first aim of this project was to identify a set of ligands that bind specifically to each member of a panel of related alpha viruses, namely, equine encephalitis viruses (EEV). The second aim was to develop a bacterial cell-based array platform that can be used to detect viruses present in serum or other complex mixtures. During the course of this project we generated a set of peptides that bind to Venezuelan or Western EEV (VEEV, WEEV), and investigated a microfluidic array method to pattern these affinity reagent displaying cells into arrays, allowing their potential application for detection assays.

List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

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NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PHDs

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Title: Rapid Molecular Finger Printing of Pathogens
Award: W911NF-05-1-0083
Primary Investigator: Prof. Patrick S. Daugherty, Department of Chemical Engineering, Institute for Collaborative Biotechnologies, UC-Santa Barbara

Overview

The first aim of this project was to identify a set of ligands that bind specifically to each member of a panel of related alpha viruses, namely, equine encephalitis viruses (EEV). The second aim was to develop a bacterial cell-based array platform that can be used to detect viruses present in serum or other complex mixtures. During the course of this project we generated a set of peptides that bind to Venezuelan or Western EEV (VEEV, WEEV), and developed a microfluidic array method to pattern these affinity reagent displaying cells into arrays, allowing their potential application for detection assays.

Selection of Peptide Ligands for Equine Encephalitis Viruses (Trial 1)
We began by selecting peptide ligands for VEEV from a large bacterial display peptide library of the form $XXCX_7CXX$, where X is any of the standard amino acids. For these studies, our Army collaborators Randy Schoepp and Kara Schmid at USAMRIID provided unpurified virus samples of WEEV and VEEV. Our objective was to label each virus containing supernatent sample a different color, and then perform FACS screening. We used magnetic and fluorescence activated cell sorting to isolate a group of peptides that clearly exhibited binding using flow cytometry with whole cells.

VEEV binders from Trial 1

JR-267	ECCVYYYKWFCFS
JR-270	WSCMGYYWWTCVE
JR-269	WVCSYIYCYWCKA
JR-268	WRCNWWMCVWCDG
JR-271	WHCNMWACIWCEP

These ligands have been found to bind VEE, and to a lesser extent, WEE. However, the labeling of the entire cell lysate supernatant mixtures with biotin, complicated the selection of highly specific reagents. Thus, our collaborators invested significant effort to prepare purified samples of the entire panel, which were used for subsequent screening efforts below.

Selection of Peptide Ligands for Equine Encephalitis Viruses (Trial 2)

Our revised protocol was to screen a peptide library of 10^{10} members using magnetic selection (MACS) with biotinylated virus samples and streptavidin-coated beads. This

resulting cell population was then amplified by growth and induced to produce the cell surface peptides. Semi-purified and biotinylated virus samples of a known concentration were added to the cells and labeled with streptavidin-phycoerithryn (SA-PE) for scanning and sorted using FACS. These sorted populations were grown and the process was repeated until individual clones with effective binding to the virus were enriched.

Detailed Results

Stocks of two different viruses were provided including: 250uL of VEE TC83 at a concentration of $4x10^{11}$ pfu/mL, and 250uL of VEE1C at a concentration of $1x10^{10}$ pfu/mL. Samples were biotinylated, yielding concentrations of ranging from 5 to $7x10^{10}$ pfu/mL for VEE TC83 and 4.5 to $5.5x10^{9}$ pfu/mL for VEE 1C. Initially used the spin columns included in the kit to remove the access biotin, and found that the viruses got caught in the resin of the column. We performed a Western blot to compare the spin column and dialysis samples and found that the dialyzed sample had about two-fold more virus than the first sample.

We attempted screening experiments multiple times with varying ratios of virions to cells. The best results used a virus to cell ratio of 1:2 during the initial library screen with MACS. We did multiple rounds of sorting (labeled F1, F2 and F3 for FACS sorted population 1, etc) with a FACSAria, with increasing enrichment in the gated populations each round. The final sorted population was plated and 19 colonies were chosen to be sequenced. Of the 19 clones, eight sequences were unique, shown below.

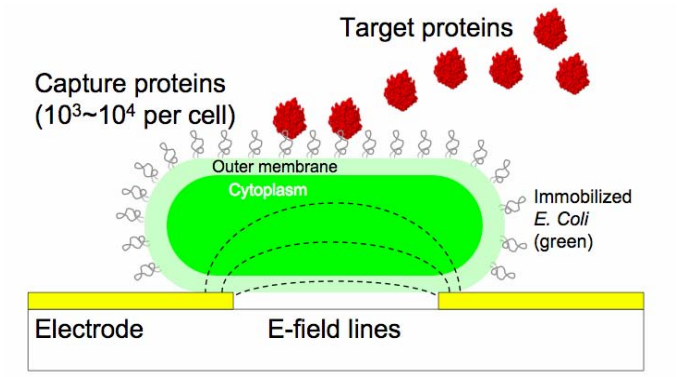
```
   VEEV-1 (1) --SLWYRPWCWLGYTEY----
   VEEV-2 (1) ---WWYGSMYQWWLEPEK---
   VEEV-3 (1) ---WWMGAWMDAEAYWT----
   VEEV-4 (1) ----WYGWWDTRVWWSGWD--
   VEEV-9 (1) --NWWAWAWMVEPQWET----
  VEEV-10 (1) --NYWGNWNLGGYMMVDREVQ
  VEEV-12 (1) WQGLWYYEWLDAGRE------
  VEEV-17 (1) --NWWGWMCLGGPQREK----
Consensus (1)    WWY   WL A
```

From the eight unique sequences, clones 1, 2, 3, and 5 were selected for further characterization for binding, and of these clones, 1 and 2 appeared to have the strongest virus binding strength at a cell to virus ratio of 1:20. Addition of human serum protein (5%) partially interfered with binding, though clone 1 retained it's ability to bind under these conditions.

In an attempt to alleviate viral aggregation, resulting from SA-biotin interactions, we directly labeled the remaining virus sample with the green fluroescent probe Alexa 488. However, Alexa labeling resulted in insufficient fluorescent signals for library screening.

Aim 2. Development of a cell-based array methodology for pathogen fingerprinting
During the course of this project we demonstrated, for the first time, a cell-based protein detection using genetically engineered *Escherichia coli* as probe cells that are spatially coordinated and immobilized to a glass/PDMS microsystem using dielectrophoresis (DEP). As a proof of principle, we achieved direct fluorescence detection of target molecules of streptavidin-phycoerythrin (SAPE) bound to the capture cells. This principle could be extended to enable virus detection in closed-system microfluidic devices.

E. coli expressing green fluorescent protein (GFP) in the cytoplasm and a streptavidin (SA)-binding peptide sequence (RLEICQNVCYYLGTL) on their outer membrane, were immobilized with DEP onto an array of interdigitated electrodes in a microfluidic device. The resulting cellular array elements exhibited fluorescence at 514nm and expressed ~10^3-10^4 capture proteins (per cell), which have a high-affinity for streptavidin (K_D ~ 10 nM). The device was fabricated on a glass substrate using PDMS4 with independent inlets for the probe cells, target proteins and wash buffer (Fig. 1). Efficient immobilization of the probe cells in a diluted PBS buffer (conductivity=0.1 S/m) was achieved using an AC signal of 3.3 Vpp at 5 MHz, and the probe cells uniformly covered the electrode within 10 minutes (Fig. 2). Non-specific binding was not observed in the upper electrode sets that were not activated.



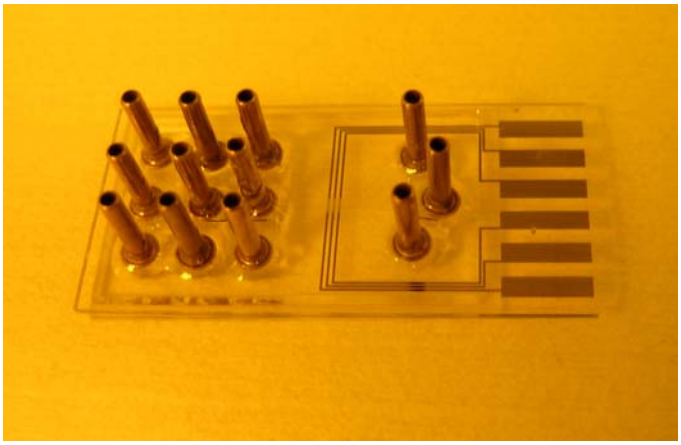


Fig. 1: (Top) Genetically engineered E. coli express ~10^4 capture ligands on their outer membranes. When coupled with DEP immobilization, these cells function as protein-sensing probes. (Bottom) A 3x3 protein-detecting microfluidic system with electrical and fluidic outlets.

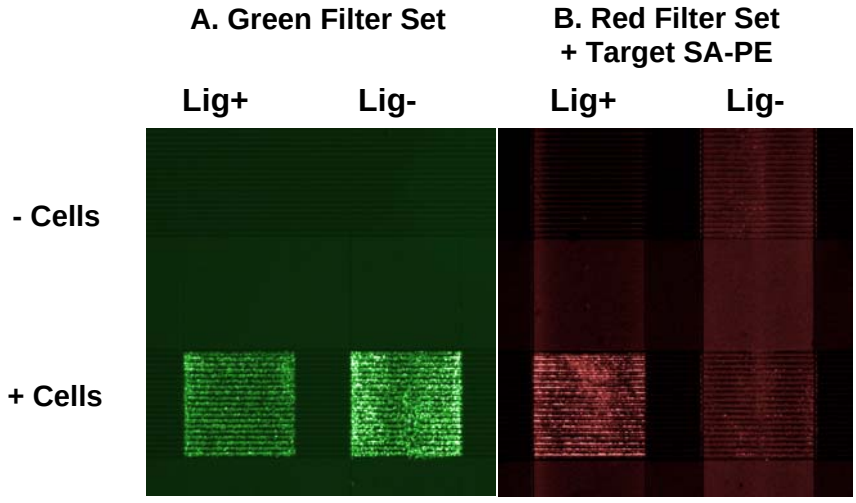


Fig. 2. (A) Fluorescence image of 2x2 electrode array in the presence of absence of cells that do or do not express the target binding ligand (Lig). GFP intensity is proportional to the cell density and thus capture ligand density. (B) After 10-minute incubation and 10-minute wash in microfluidic channel, the fluorescently tagged target protein SAPE (red) is detected only in the bottom-left electrode where SA-binding ligand displaying cells are immobilized.

The long term goals of this project are to apply the bacterial display selection approach to purified virus samples, to identify ligands that are maximally specific with each subtype. This set of ligands could be used to create a larger cell patterned array, which could in turn be 'trained' to recognize each virus species. As result of this work, one manuscript describing the cell based array platform has been submitted to Journal of Proteome Research.

Conclusions

The results presented here demonstrate the feasibility of identify peptides that bind to viral proteins that may have potential utility in diagnostic assays. However, unexpected difficulties encountered during the course of this project include establishing appropriate biotinylation conditions and the availability of sufficient quantities of purified virus for library screening. Additionally, the level of peptide display on the cell surface was found to be lower than desired for optimal library screening. This problem was corrected by evolution of an optimized bacterial display scaffold that enhances the efficiency of display of most peptides.