GENERATION OF A RECOMBINANT ANTIBODY LIBRARY FOR IMPLEMENTATION IN A QCM-BASED BIOSENSOR

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ABSTRACT

The potential threat of biological weapons necessitates the development of devices that can readily be employed by first responders and provide rapid detection of biological agents in the field. Our research focuses on the construction and implementation of a recombinant antibody-based biosensor that utilizes the quartz crystal microbalance (QCM) platform for the detection of bacterial targets. We have generated a single-chain Fv antibody (scFv) library from which bacteria-specific antibodies can be isolated. Methods have been developed to improve the affinity of the selected scFvs for the specified bacterial targets and to incorporate them into the QCM biosensor.

INTRODUCTION

A portable detector has been constructed and successfully used with a full-length monoclonal antibody to detect bacterial targets in a mixed solution. Target recognition is accomplished by antigen-antibody interaction, which is detected by an apparatus based upon a quartz crystal microbalance (QCM). QCM is a piezoelectric device capable of measuring miniscule changes in mass with an A-T cut quartz crystal coated with gold electrodes that permit attachment of protein through various chemistries. Changes in the oscillation frequency of the quartz crystal are indicative of changes in mass on the gold surface. These changes are interpreted by the software of the biosensor to determine the presence or absence of the target pathogen (*1, 2*). The QCM-based biosensor was designed to be used with minimal training by first responders. The small size of the individual units ensures that sample analysis can be performed on-site, alleviating the need for transport to laboratory facilities for prolonged analysis by ELISA and/or PCR.

To optimize the identification and production of target-specific antibodies, a recombinant scFv antibody library was used to select scFvs with high binding affinities for the target bacteria (*3-5*). Since scFvs are constructed from only the antigen-recognition domains of an antibody molecule, they can be produced in bacterial cultures allowing for a low-cost production method. The scFv gene can also be manipulated to improve affinity for the target bacteria by mutagenesis. These techniques permit the rapid isolation of scFv for specified targets.

EXPERIMENTAL

We have isolated target-specific scFvs from recombinant scFv libraries that were generated from both immunized and naïve rabbits. The immunized rabbit was inoculated with formalin-killed *Bacillus thuringiensis* to aid in the isolation of scFvs specific for this and other bacteria with similar surface characteristics. The naïve library contains greater scFv diversity with the ability to recognize a wider range of antigens.

The standard scFv selection protocol involves immobilizing target bacteria to a polystyrene microtiter plate and repeated wash steps to remove non-specific phage. To improve isolation of

monospecific scFv, a negative selection technique that uses non-target bacteria during the selection process is employed to remove phage that recognize surface epitopes common between bacterial species.

Target-specific scFv are improved through an affinity maturation process. ScFv are subjected to iterative rounds of random mutagenesis, which creates a pool of scFv genes with varying numbers of nucleotide substitutions (*6, 7*). This new scFv library is then used in the scFv selection process under increasingly stringent conditions. Clones isolated after a number of selection rounds are examined for improvements in binding characteristics and solubility compared to the initial scFv. Solubility of the scFv is assessed via immunoblot detection of scFv protein in the culture supernatant and the periplasmic space. Periplasmic proteins are isolated using an osmotic shock protocol (*8*) of the cell pellet following overnight induction of protein expression. Soluble scFv protein is blotted onto 0.45 um pore size nitrocellulose with the aid of a mild vacuum. ScFv is detected using a horseradish peroxidase (HRP) – conjugated anti-HA antibody through the generation of a chemiluminescent product.

Following scFv expression and purification, the scFvs are attached to the gold electrode of a quartz crystal via an amide bond formation. The gold is first treated with 11-mercapto-undecanoic acid solution to provide a terminal acid group on the surface for conjugation. The amide bond is formed between a free amine group of the scFv and the acid group on the alkane chain through an EDC/NHS reaction (*9*). For experimental purposes, the oscillation frequency of the quartz crystal is measured in real-time to determine binding of the target bacteria to the scFv. The actual biosensor, however, is designed to measure only the initial and final changes in frequency to determine the presence of the target bacteria and simplify field use of the device.

RESULTS

Recombinant antibodies specific for certain target bacteria have been isolated from both the immunized and naïve scFv libraries. Standard selection of scFv against the initial *Bacillus* target yielded scFv that also recognize non-target bacteria. Monospecific scFv were isolated by introducing negative selection steps, in which a non-target bacterium (*Escherichia coli*) was included in the later rounds of biopanning. This technique produced several scFvs that recognize only the target bacterium and display minimal interaction with non-target bacteria (**Figure 1**). By decreasing the concentration of the target bacteria during the selection process, we obtained an scFv for *Salmonella enterica* from the immunized scFv library. The naïve library is of greater diversity, thereby simplifying the isolation of scFvs specific for a variety of bacterial targets with the standard selection process. ScFvs from the naïve library will likely demonstrate a lower binding affinity and require affinity maturation through mutagenesis.

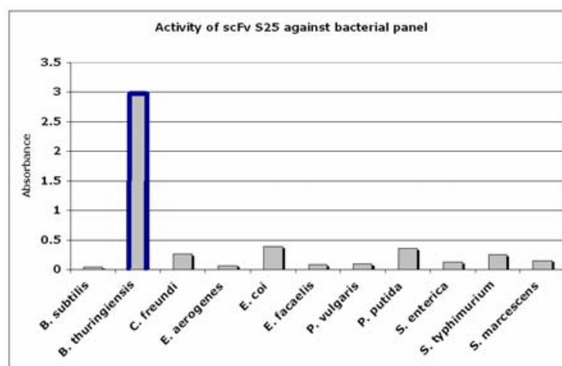

Figure 1 – Specificity of scFv S25
Binding specificity examined against a panel of bacteria via a whole-cell ELISA assay. The scFv is detected using a secondary antibody with a horseradish peroxidase conjugate (HRP) specific for a C-terminal HA-epitope tag. A colorimetric assay using a tetramethylbenzidine (TMB) substrate and absorbance measurement at 495 nm is used to quantitate bound scFv.

Mutagenesis of target-specific scFvs was conducted to generate random nucleotide substitutions in the scFv to alter the affinity and solubility of the antibody. Following mutagenesis, rounds of selection were conducted under increasingly stringent conditions to isolate scFvs for the target bacteria. Solubility was examined using a dot blot to detect scFv protein in the culture supernatant and the periplasmic space (**Figure 2**). Clones that demonstrated a significant quantity of protein in these fractions are being sequenced and tested for changes in specificity and affinity to the target bacterium via ELISA.

Recombinant antibodies have been immobilized on the QCM surface and used to detect *B. thuringiensis* in solution. A 50 Hz shift was measured within ten minutes after initial injection of the bacterial solution (**Figure 3**). The change in oscillation frequency was measured after washing of the surface with a buffer solution to verify that the change was not the result of transient surface interactions. The biosensor software measures the difference in the initial oscillation frequency and the final frequency following the buffer wash step. The frequency shift obtained for the scFv experiment was five times higher than the change required to generate a positive signal for detection of target bacteria. The same surface was also tested against non-target bacteria, which failed to produce a positive signal. Additionally, a bacteria/milk suspension was used to determine if a heterogeneous mixture would interfere with detection of the bacterial target. The frequency shift obtained was comparable to that obtained with neat bacterial suspension, producing a definitive positive signal.

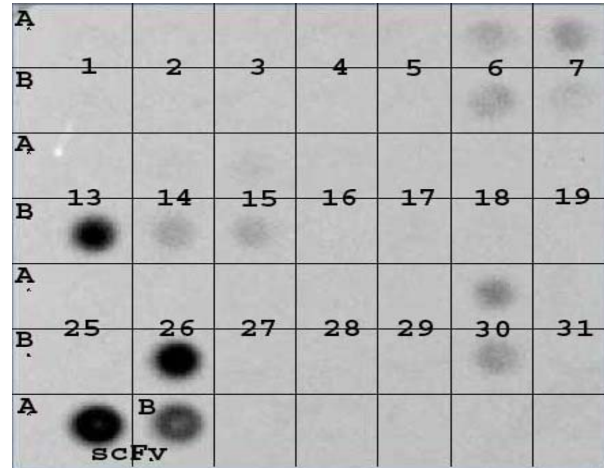

Figure 2- Dot blot solubility assay
200 ul of culture media (A) and the periplasmic fraction (B) from individual mutagenized clones are bound to nitrocellulose via vacuum. ScFvs are then visualized using an HRP-conjugated anti-HA antibody by chemiluminescence. Clones 13 and 30 contained considerable scFv in the periplasmic space. The original scFv is located in the lower left corner as a comparison

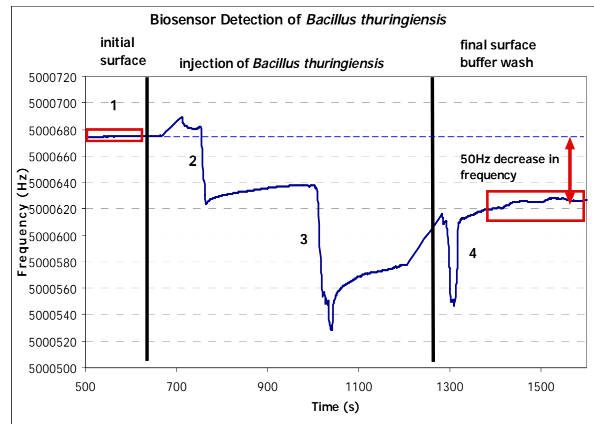

Figure 3 – Biosensor detection of target bacteria
Bacillus thuringiensis was suspended in a phosphate buffer and injected over the surface at a constant flow rate of 50 ul/min. Initial and final measurements (boxed) are compared for a positive signal by the detector. A 50Hz shift was recorded, 5x the value required for a positive signal.

(1) Initial oscillation frequency compared to final measurement for a positive or negative signal
(2) Frequency drop associated with the viscosity change from buffer to bacterial suspension
(3) Bacterial interaction with the surface
(4) Drop in frequency probably due to viscosity change

CONCLUSIONS

A system of generating antigen-specific antibodies for use in a field-based biosensor has been developed with the aid of a recombinant scFv library. The recombinant antibody system

alleviates the time-consuming process of isolation and production of full-length antibodies from mammalian cell cultures and provides methods for improving antibody affinity through simple gene mutagenesis. This system ensures that, as threats emerge, the biosensor can be configured to detect new pathogens by rapidly selecting and improving target-specific scFv

The QCM platform allows for the rapid on-site detection of pathogens without the need for transport to a distant facility for time consuming analysis. Current trials with the biosensor device suggest that a sample can be analyzed in as little as fifteen minutes compared to the hours required for conventional ELISA and PCR experiments. Our biosensor unit is designed to require only a minimal amount of user training. The prototype model requires only a series of user-prompted injections and quickly returns a positive or negative verdict, thereby making it ideally suited for use by first responders in the field.

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