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Advances in Synthetic Peptides Reagent Discovery

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15. SUBJECT TERMS
peptide, synthetic reagent, affinity reagent, bacterial display, biosensing, hybrid materials, advanced libraries

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Advances in synthetic peptides reagent discovery

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ABSTRACT

Bacterial display technology offers a number of advantages over competing display technologies (e.g, phage) for the rapid discovery and development of peptides with interaction targeted to materials ranging from biological hazards through inorganic metals. We have previously shown that discovery of synthetic peptide reagents utilizing bacterial display technology is relatively simple and rapid to make laboratory automation possible. This included extensive study of the protective antigen system of Bacillus anthracis, including development of discovery, characterization, and computational biology capabilities for in-silico optimization. Although the benefits towards CBD goals are evident, the impact is far-reaching due to our ability to understand and harness peptide interactions that are ultimately extendable to the hybrid biomaterials of the future. In this paper, we describe advances in peptide discovery including, new target systems (e.g. non-biological materials), advanced library development and clone analysis including integrated reporting.

Keywords: peptide, synthetic reagent, affinity reagent, bacterial display, biosensing, hybrid materials, advanced libraries

1. INTRODUCTION

Persistent surveillance of a variety of threat agents at trace concentrations, whether chemical or biological in nature, in air, food or water supplies is of critical to ensure the highest degree of survivability across the spectrum of operations of our nation's soldiers. One of the key challenges in hazard detection is that threat agents do not exist in an isolated environment, but are contained within a complex background of interfering molecules, which impedes rapid and accurate sensing. In order to overcome these challenges, there is a need to develop bioreceptors (i.e. affinity reagents) with specific recognition for the target of interest, that are robust and reliable. Many sensor technologies utilize antibodies, which have high affinity and specificity to a given target, but are very costly, difficult to mass-produce and typically have limited stability. Additionally, the current rate of antibody production (weeks to months) does not meet the rate the DOD requires for reagent development against new and emerging threats. Alternatively, synthetic affinity reagents have been developed from various synthetic recognition element technologies (e.g. bacterial display and phage display) in a matter of days or weeks [1].

Synthetic recognition elements (SRE) include various types of biological molecules, such as nucleic acids, peptides, and proteins, and have been extensively reviewed[2, 3]. Our work has primarily focused on the development and use of peptide-based SRE. Like the traditional antibody-antigen interactions, peptide SRE or peptide affinity reagents, selectively bind to a target with high affinity via a mechanism similar to antibodies. However, unlike antibodies, these reagents can be developed very rapidly and offer a level of stability not possible with standard antibodies. Specific affinity peptides are developed by screening a library of peptides for binders to a target of interest and once identified, those peptides can be mass produced through standard synthetic techniques.

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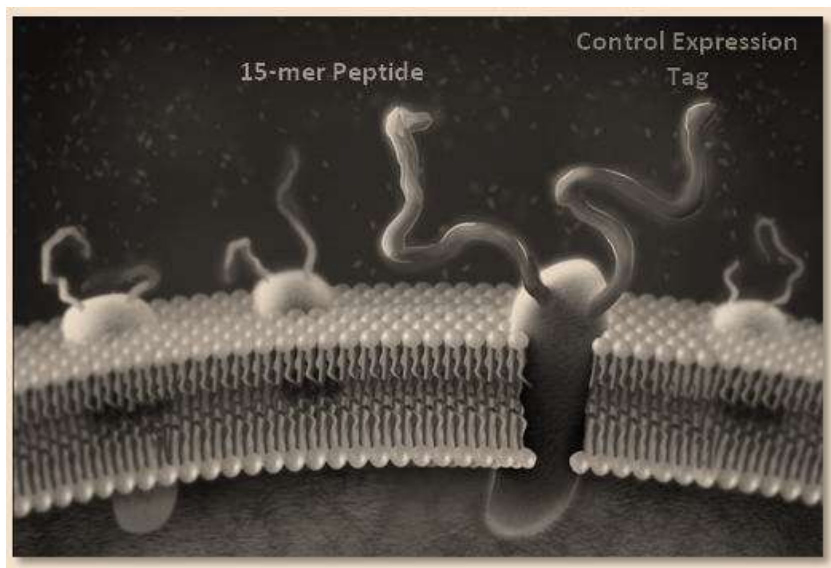

Figure 1. Conceptual diagram of an unconstrained bacterial display technology possessing an engineered, randomized 15-mer amino acid binding region and a simultaneous tag to monitor expression enabling quantitative affinity reagent screening.

Several types of cell surface display systems have been developed, including phage [4], bacteria [5-7], yeast [8, 9], insect cell/baculovirus [10], as well as mammalian cell culture [11]. Although each system has its unique advantages and disadvantages, they are all used in conjunction with biopanning to identify affinity peptides to a specific target. Biopanning is an affinity selection technique in which a library of cell surface displayed peptides is incubated with the target and binding sequences are screened and selected for further propagation. By re-screening this selected subpopulation of binders under more stringent conditions, higher affinity binders can be enriched. A final set of target binders can be identified through DNA sequencing, as the peptide sequence is DNA encoded [12]. Compared to the other display technologies, bacterial display, specifically *Escherichia coli*-based display systems, have an extremely fast replication rate and can easily be genetically modified for tailored biodiscovery applications. Thus, bacterial display is ideal for combating newly emerging threats that have no readily available or specific antibody bioreceptor.

E. coli has been engineered to develop affinity peptides by modifying natural cell components or systems. Brown [13] developed an *E. coli* concatamer library displayed on the external domain of a lambda phage receptor and the adherence appendage, fimbriae, was modified in another *E. coli* to form a display scaffold by inserting a peptide library into the structural component, FimH [14]. To date, the most widely used *E. coli* surface display system is FliTrx. This *E. coli* flagella-based display system has been used to develop a variety of peptide SRE's to diverse targets [7] [15-18]. Although these systems have been successfully utilized to produce affinity peptide reagents, they are often criticized for their lack of peptide diversity as compared to other display technologies (e.g. phage display). Recently, a novel, engineered *E. coli* peptide display library has been developed with a greater estimated diversity ($3x10^{10}$ discreet random peptides) [19, 20]. This library utilizes the engineered protein, eCPX, as a display scaffold. This is a circularly permuted form of the native *E. coli* outer membrane protein, OmpX. The eCPX protein is a membrane spanning beta barrel with a surface exposed C- and N-terminus. The C-terminus contains a P2X peptide, which binds the Mona-SH3 domain [21] and the N-terminus contains a flexible random 15 amino acid peptide library [19, 20] (Fig. 1). This system has been previously used to successfully screen for peptides SRE's to streptavidin [19], protease activated proligands [22], pro-apoptotic and anti-apoptotic peptides [23, 24], vascular endothelial growth factor (VEGF) [21], breast tumor type–specific peptide ligands [25], protective antigen (PA) protein of *Bacillus antracis* [1, 26-29], and staphylococcal enterotoxin B (SEB) [30].

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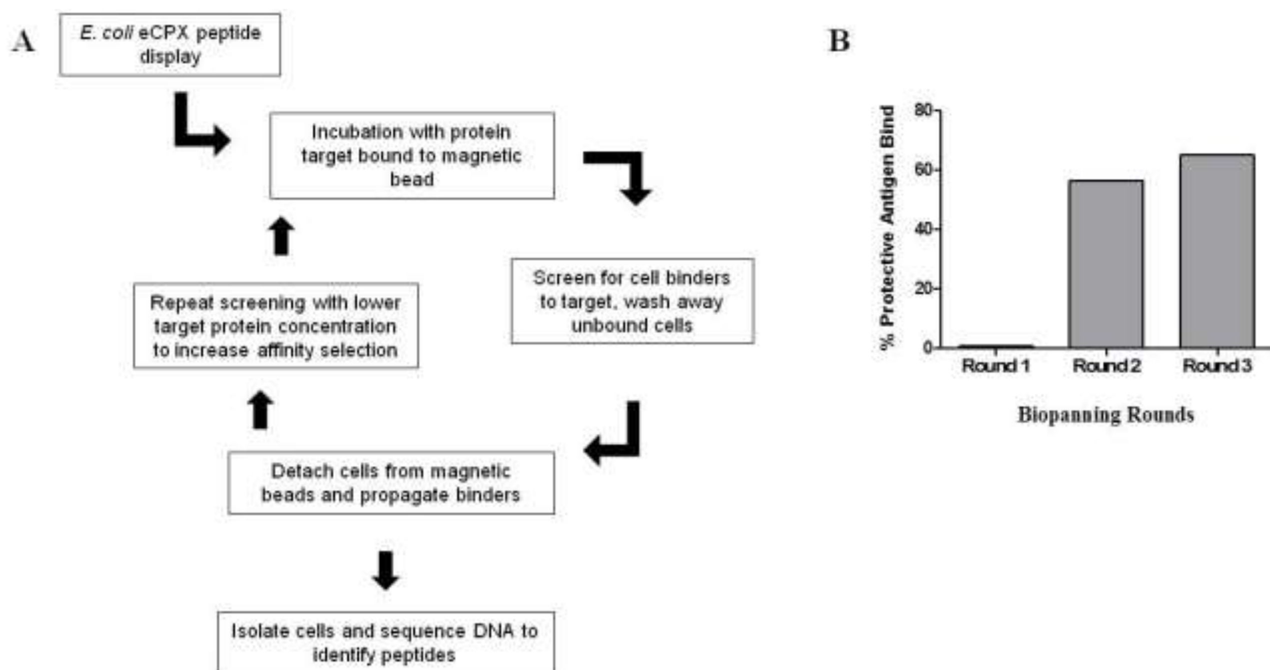

Figure 2. (A) Schematic diagram showing the steps involved in traditional biopanning of a display library including incubation with magnetic beads conjugated to the protein target, stringency wash steps to remove weakly bound library members, and enrichment of the bound population prior to identification of isolated colonies using DNA sequencing. (B) Shows a typical enrichment in the % of a library population that binds over the course of three rounds of biopanning.

2. ADVANCES IN BACTERIA SURFACE DISPLAY BIOPANNING

2.1 Traditional biopanning

Bacterial peptide display is most commonly used to isolate binders to proteins, and has been used for applications such as vaccine development [31], biocatalysts [32], and biosensors [33]. As such, many of the bacterial display libraries were develop and biopanning techniques optimized for use with a protein target. Although the exact steps may vary for each type of bacterial display system, the general biopanning process is very similar and is depicted in Fig. 2A. Biopanning consists of incubating the library with a protein of interest and selecting those cells that bound the target. Due to the small size and ease of "tagging" protein targets, a variety of commercial and custom technologies are available to aid in high throughput screening and selection of target binding cells. These steps are typically performed one of two ways: 1) the target protein is fluorescently labeled and fluorescence activated cell sorting (FACS) is used to extract those bound cells from the population or 2) the target protein is conjugated to magnetic beads, which retain the protein bound cells while non-binders are washed away. Both cell binder selection approaches have been shown successful in previous studies [20, 26]. A modified, self contained, automatic magnetic selection technique was develop and described by Kogot *et al.* [26]. A Micro-Magnetic Separation (MMS) platform was used to screen the eCPX library for peptide SRE's to the PA protein to improve the reproducibility, reduce the cost, reduce cross contamination, and minimize exposure to the target protein.

After selection of target binding cells, this target affinity subpopulation is then propagated to create a secondary cell population to further refine for higher target affinity binders. By repeating the screening and selection steps (rounds) utilizing sequentially lower concentrations of the target protein, the highest affinity peptides are selected. Typically, 3-5 rounds of biopanning are sufficient for the completion of affinity peptide biodiscovery. The affinity maturation of the enriched surface displayed peptide population can be seen by measuring the number of cells binding the protein target in each round. For example, in the development of PA peptide SRE's from the eCPX library, marked increases in the

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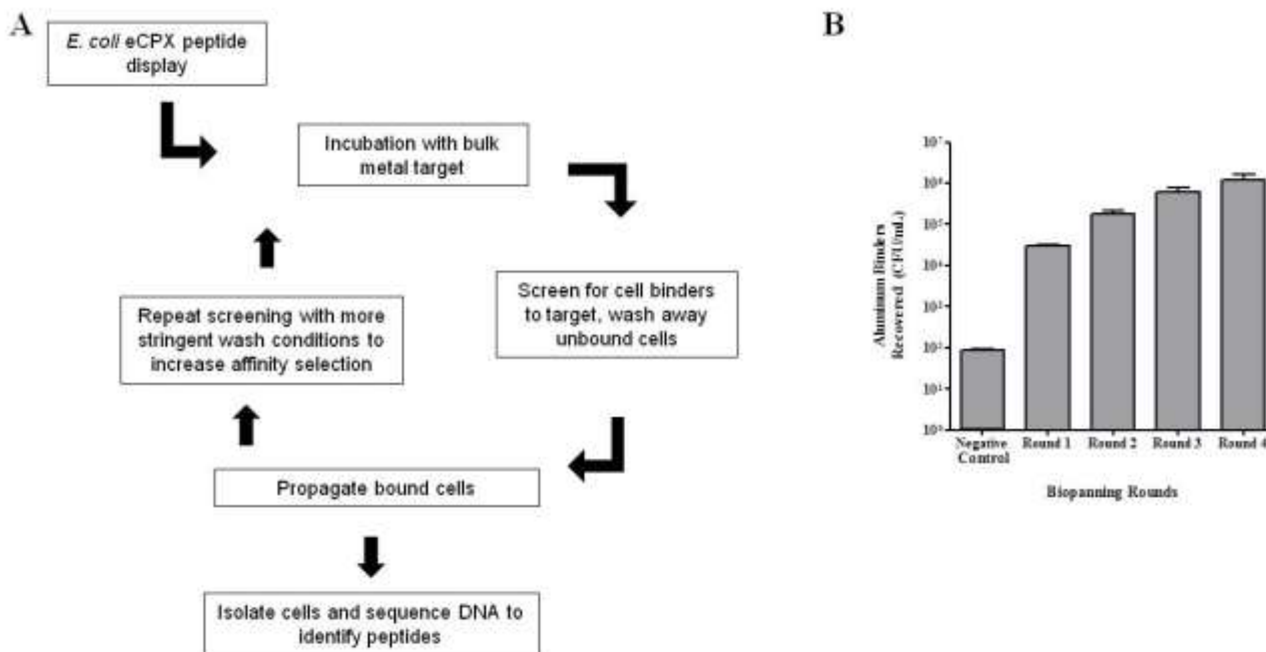

Figure 3. (A) Schematic diagram showing the steps involved in biopanning to bulk materials using a display library (B) Shows a typical enrichment in the % of a library population that binds over the course of four rounds of biopanning.

percentage of cells binding PA were observed through 3 rounds of biopanning (Fig. 2B). Such enrichment in target binders is typical of a successful biopanning technique. After the completion of biopanning, the final cell population is cultured on plates in order to produce individual colonies. The peptide display library is genetically encoded in the cells and the identity of the enriched affinity peptides is easily determined using standard DNA sequencing techniques.

2.2 Nontraditional biopanning

The use of a protein target to develop affinity peptides from bacterial libraries may yield peptides useful in a variety of applications, as noted in the previous section. However, there exists application for peptide development using a bulk, macroscale target. The incorporation of a bulk target material into the biopanning technique requires critical modifications to the process and is therefore referred to as nontraditional biopanning. Most often the bulk target utilized in nontraditional biopanning is a metal and the developed affinity peptides have far reaching applications in various fields of study. Interest in these types of peptides has persisted for some time and phage display has been used extensively to this end. Peptide SRE's have been developed with affinity to metals [34-39], oxides [40, 41], alloys [42], metal salts [43], and semiconductors [44-46] for use in the mineralization [47-49] and functionalization [45, 50] of surfaces. Some types of *E. coli* display systems have been used to develop metal compound binders. *E. coli* cell surface display has been utilized in conjunction a bulk metal target. Brown [13] developed an *E. coli* concatamer library displayed on the external domain of a lambda phage receptor and sorted for iron oxide binding.. The previously noted FimH library[14] was screened for zinc oxide binding and yielded several specific binding motifs. The *E. coli* flagella-based display system (FliTrx), developed by Lu *et al.* [7] has been used to sort for gold [15], silver [16], copper and zinc oxide [17] binders.

Nontraditional biopanning with a bulk metal target precludes the use of automated, high throughput technologies like FACS due to the large size of the target (typically on the order of cm). Therefore, alternative methods must be developed for the selection and screening of affinity binders. A general schematic for nontraditional biopanning is shown in Fig. 3A. In traditional biopanning, the bacterial display library is cleaned and concentrated through centrifugation and resuspension in a buffer before the protein target is added. Steps may also be taken at this time to block nonspecific interactions by the addition of bovine serum albumin (BSA). In nontraditional biopanning, the bulk target is simply added to the library in the growth medium. Issues with nonspecific binding are address in the washing steps and no

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blocking measures are taken during the incubation step. Also, incubation should be short (30 min or less) to promote specific and high affinity binding. Longer incubations may result in nonspecific attachment, such as early biofilm formation. Because target bound cells cannot be picked from the population, as is possible in traditional biopanning, the unbound cells must be efficiently removed and then the target bound cells isolated. With a bulk material, this is simply done by replenishing the media. Due to the importance of non-binder removal, the washing step is critical. A detergent, such as Tween20 or Triton X is often added to break non-specific attachments, and a vigorous agitation aids in the removal of loosely bound cells. By increasing the stringency of the wash conditions, the binder affinity can be enriched as the biopanning process progresses. As in traditional biopanning, the final steps consist of isolating individual colonies form the final biopanning round population and DNA sequencing analysis to identify the developed affinity peptides. Also similar to traditional biopanning, enrichment in cell binders to the target should be evident through the nontraditional biopanning process. We utilized the eCPX peptide display library to develop a nontraditional biopanning technique using a bulk aluminum alloy as a target material. Using a standard dilution plate count technique, we quantified the number of cells bound to the aluminum alloy surface after each round of biopanning. A clear trend in increasing cell attachment was observed, as shown in Fig. 3B. Although different from the traditional biopanning process utilizing nanoscale protein targets, successful affinity peptide development is possible using a nontraditional approach for bulk, macroscale targets, such as metal alloys.

3. ADVANCED BACTERIAL DISPLAY LIBRARIES

3.1 There is increasing interest in the development of affinity peptides f novel targets for a variety of applications in the research and development fields. The use of such targets will require biopanning approaches to be tailored to the specific target material and a cell surface display host capable of providing this level of flexibility will be necessary. *E. coli* is widely recognized for its relatively easy to manipulate genetic material that allows customized libraries to be generated and transformed into cells at very high efficiencies, both at a commercial scale and at the laboratory bench. Furthermore, the very rapid growth rate and simple culture requirement makes biodiscovery of novel peptides a relatively straightforward process that can be scaled-up for manufacturing. For these reasons, it is likely the use of *E. coli* as a cell surface display host and the new peptide libraries that utilize it will continue to expand, and it will play a role in the next generation of peptide reagent discovery. Survey of potential host *E. coli* strains

Table 1. Table of transformation efficiencies from three host strains of *E. coli.*

Strain	pB33-eCPX Transformation Efficiency (CFU/µg)
ER2738	4 x 10^8
MC1061	3 x 10^7
TG1	2 x 10^8

There are a number of commercially available *E. coli* host strains which have been used with phage display libraries, several of which can be purchased electrically competent, or ready to be transformed with a library. We obtained three of these electrically competent *E. coli* strains, ER2738, MC1061, and TG1 (Lucigen; Middlebrook, WI), and introduced the eCPX peptide display scaffold plasmid via electroporation according to manufacture instructions. After growing the transformed colonies on LB agar plates supplemented with 25µg/ mL chloramphenicol, the transformation efficiency for each strain was calculated using equation 1 and the results are displayed in Table 1.

$$Transformation\ efficiency\ (CFU/\mu g) = \frac{\#\ colonies\ on\ the\ plate}{ng\ DNA\ plated} \times 1000\ ng/\mu g \quad (1)$$

All three strains transformed with relatively good efficiency, 10^7-10^8 colony forming units (CFU)/ µg DNA, although the manufacture reported transformation efficiency of 4×10^9 CFU/µg with a pUC19 control plasmid was not obtained. The

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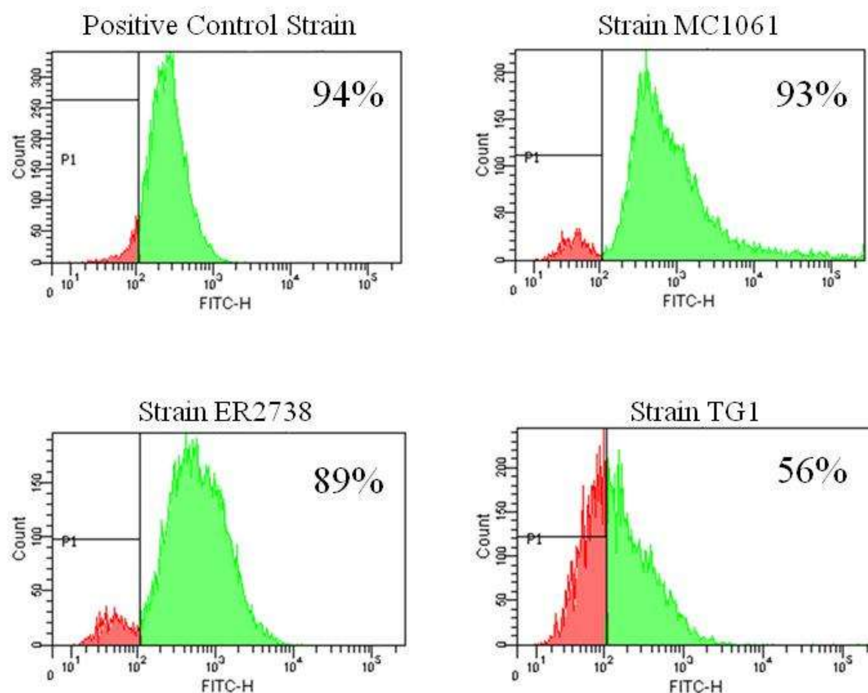

Figure 4. Evaluation of eCPX bacterial library expression monitored via the YPET-Mona expression tag and fluorescence activated cell sorting (FACS) analysis.

slightly lower efficiency is likely due to the size difference in the eCPX plasmid and the control pUC19 plasmid. Furthermore, the efficiency could likely be increased by optimizing the electroporation conditions specifically for the eCPX plasmid. The ability to effectively transform the host cell is critical for library construction because the ultimate library diversity can be negatively impacted by a poor transfer of the DNA library into the host cells, even if the DNA library is highly diverse.

3.2 Evaluation of eCPX display ability

Each of the three *E. coli* strains described in Section 4.1 has key genetic features that make it amenable to the surface display of engineered proteins. In order for a strain to be used as a host for an advanced eCPX peptide display library, it must have both high transformation efficiency and be capable of good eCPX display scaffold expression on its cell surface. In order to evaluate the expression and display level of eCPX in each strain, flow cytometry with 25µL 250 nM YPet-Mona was used as previously described[26]. The results showed that MC1061 had the highest eCPX surface display of all three strains and was almost the same as the positive control (Fig. 4). ER2738 also had good eCPX surface display, however TG1 demonstrated very poor display. Based on these findings, *E. coli* strain MC1061 was determined to be the best host for the advanced eCPX library.

3.3 Real time cell reporting in advanced peptide libraries

As the use of biopanning continues with new, bulk material targets it becomes increasingly important to visualize and measure cell binding in real time and to facilitate high throughput screening capabilities currently found with protein targets. Whereas the protein target is typically tagged in traditional biopanning regimes, it is not feasible to label a bulk target. However, it possible to label the cells themselves such that binding to the target material can be monitored in real time. Studies are currently underway to genetically engineer the eCPX peptide display system to produce a stable fluorescent reporter within and allow cells to be visualized by using current fluorescence spectrophotometry technologies. The commercially engineered red fluorescent protein, dsRed, is used in the current advanced library development, however another fluorescent protein can also be employed. The dsRed protein is very stable and is widely used in a variety of reporting-type applications and assays. The dsRed gene is inserted into the eCPX plasmid upstream

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of the eCPX-encoding gene, but under the same arabinose inducible promoter (Fig. 5A). The plasmid is then transformed into *E. coli* cells and co-expressed with the eCPX display scaffold. Cells which produce the dsRed protein are visible in the red region using confocal microscopy (Fig. 5B) and general UV light (Fig. 5C). Due to the high stability of dsRed, cells will remain fluorescent day after the protein is first produced and the fluorescence is not easily quenched. We believe that this is an ideal system for use in an advanced display library. Work is currently underway to produce a 15mer peptide library contain this dsRed reporter construct and examine its utility with nontraditional targets. This conceptualized real time cell reporter library would be incubated with a target of interest, like a bulk metal, unbound cells removed in a subsequent washing step, and the remaining bound cells visualized and/or their fluorescence intensity measured. The bound cells would then be propagated and additional biopanning rounds performed as described in section 3.2. The enrichment of target binding could be monitored in real time during the biopanning process and would be indicated by increasing fluorescence intensity. In addition to monitoring enrichment, the efficiency of the washing step could be monitored; a small change in fluorescence after washing would indicate an ineffective wash and

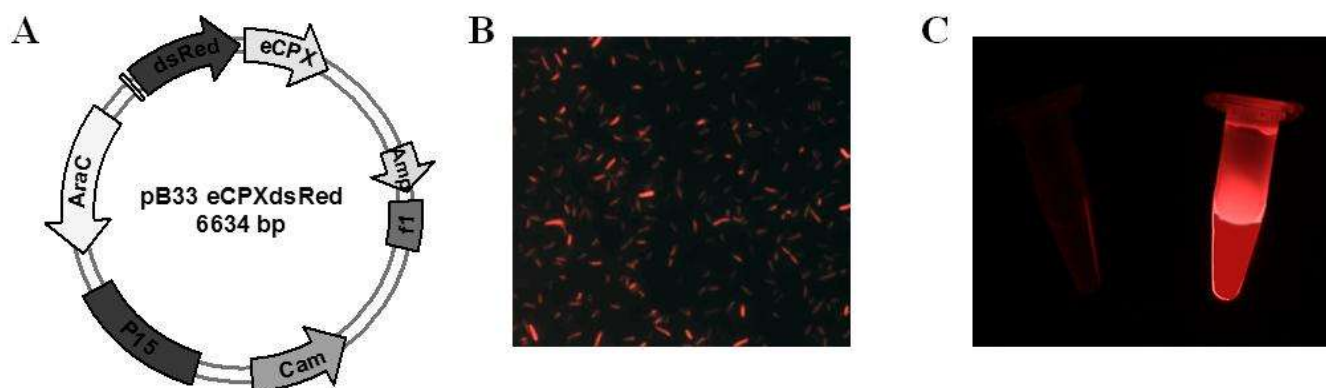

Figure 5. A) ds Red fluorescent protein gene insertion into the eCPX plasmid (B) epi-fluroescence microscopy image of integrated dsRed reporting with the e-CPX bacterial display library, and (C) UV illumination of two tubes of library stock, having no dsRed on the left, and integrated dsRed on the fluorescent image on the right acquired using a standard gel documentation system.

an excessively large change may indicated an overly aggressive wash. This can be useful when developing a nontraditional biopanning approach for a new target. Additionally, both epifluorescence and confocal microscopy (depending on the target material) can be used to directly visualize bound cells on the material. Unlike electron microscopy techniques, the cells remain viable for downstream steps using these techniques.

4. CONCLUSION

To conclude, we have previously shown the power of peptide discovery using bacterial display technology including rapid discovery with a low cost microfluidic sorting system. This work highlights our progress towards extending biopanning discovery of bacterial display libraries to peptide discovery for a wider range of material systems including bulk objects. Towards this end, advanced library development and clone analysis including integrated reporting will allow for simplified yet versatile discovery. The biopanning discovery for bulk materials using bacterial display offers a number of advantages with the most attractive being the rapid, simple discovery methodology empowered by the *E. coli* host. Also, the ability to directly propagate the portion of the library that is bound to the sample without any complicated desorption steps which are likely to lead to loss of the best binding population is a desirable feature.

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