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Biodiscovery of Aluminum Binding Peptides

by Bryn L. Adams, Deborah A. Sarkes, Amethist S. Finch, Margaret M. Hurley, and Dimitra Stratis-Cullum

ARL-RP-0453 **August 2013**

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14. ABSTRACT

Cell surface peptide display systems are large and diverse libraries of peptides (7-15 amino acids) which are presented by a display scaffold hosted by a phage (virus), bacteria, or yeast cell. This allows the selfsustaining peptide libraries to be rapidly screened for high affinity binders to a given target of interest, and those binders quickly identified. Peptide display systems have traditionally been utilized in conjunction with organic-based targets, such as protein toxins or carbon nanotubes. However, this technology has been expanded for use with inorganic targets, such as metals, for biofabrication, hybrid material assembly and corrosion prevention. While most current peptide display systems employ viruses to host the display scaffold, we have recently shown that a bacterial host, *Escherichia coli*, displaying peptides in the ubiquitous, membrane protein scaffold eCPX can also provide specific peptide binders to an organic target. We have, for the first time, extended the use of this bacterial peptide display system for the biodiscovery of aluminum binding 15mer peptides. We will present the process of biopanning with macroscopic inorganic targets, binder enrichment, and binder isolation and discovery.

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peptide, synthetic reagent, affinity reagent, bacterial display, biosensing, biomineralization, solid

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ABSTRACT

Cell surface peptide display systems are large and diverse libraries of peptides (7-15 amino acids) which are presented by a display scaffold hosted by a phage (virus), bacteria, or yeast cell. This allows the self-sustaining peptide libraries to be rapidly screened for high affinity binders to a given target of interest, and those binders quickly identified. Peptide display systems have traditionally been utilized in conjunction with organic-based targets, such as protein toxins or carbon nanotubes. However, this technology has been expanded for use with inorganic targets, such as metals, for biofabrication, hybrid material assembly and corrosion prevention. While most current peptide display systems employ viruses to host the display scaffold, we have recently shown that a bacterial host, *Escherichia coli*, displaying peptides in the ubiquitous, membrane protein scaffold eCPX can also provide specific peptide binders to an organic target. We have, for the first time, extended the use of this bacterial peptide display system for the biodiscovery of aluminum binding 15mer peptides. We will present the process of biopanning with macroscopic inorganic targets, binder enrichment, and binder isolation and discovery.

Keywords: peptide, synthetic reagent, affinity reagent, bacterial display, biosensing, biomineralization, solid binding peptides, aluminum, biopanning,

1. INTRODUCTION

Biological molecules (e.g., polypeptides, DNA, etc.) exhibit a high level of selectivity, allowing for the controlled formation of complex composite structures. The construction of these hybrid organic/inorganic structures is directed by the inherent molecular recognition and self assembling nature of biological molecules. Advanced materials composed of biomolecule-coupled inorganics are of considerable interest in the development of nano- to macro-scale fabricated technologies, including biosensors. Of the biological molecules, peptides have the most potential for use because they act as molecular erector sets to control spatially oriented biomaterial synthesis. Peptides have been developed that bind to a variety of inorganic materials, including metals[1-6], oxides[7, 8], alloys[9], metal salts[10], and semiconductors[11-13], and have been incorporated into diverse applications, such as organic scaffolds that mediate surface mineralization[14-16] and material functionalization[12, 17]. They have also been used for immobilization in affinity chromatography[18], and metal sequestration[19-23]. Such peptides have been primarily derived through the biopanning of peptide display libraries. Biopanning is an affinity-based selection technique in which high affinity peptide binders are enriched from a peptide library consisting of several steps, including binding, washing, and enrichments. The exact steps vary, depending on the host organism, display scaffold, and target of interest.

Although most peptide libraries are phage (virus) -based, several *Escherichia coli* display libraries have been developed and used to produce inorganic metal binders[24-28]. However the most recent and promising *E. coli* display system (Figure 1), developed by Daugherty and colleagues in 2008[29] has never before been used to develop bulk metal binding peptides. This novel library has the greatest estimated diversity ($3x10^{10}$ discreet random peptides) to date and is comparable to diversity estimates for phage display libraries. The unconstrained 15mer peptide library is displayed on the cell surface in the engineered display scaffold protein, eCPX. This system has been utilized to develop affinity

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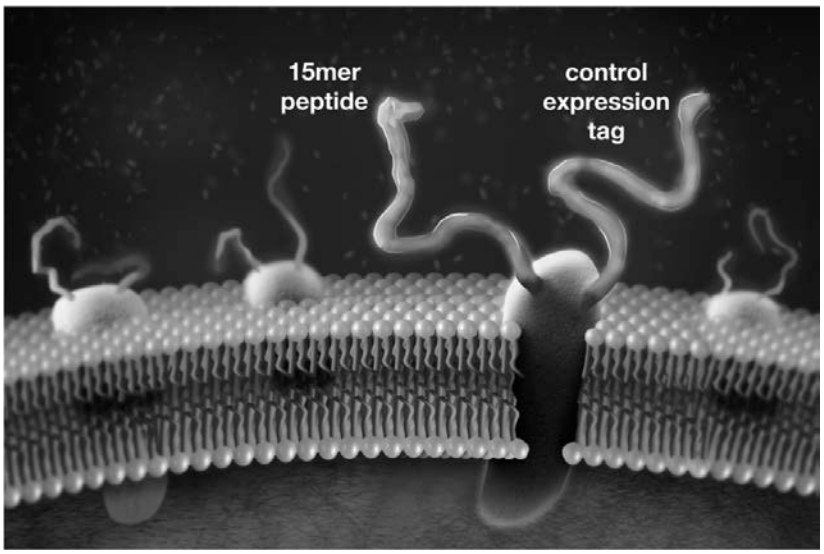

Figure 1. Schematic diagram of the eCPX peptide display system. The eCPX scaffold is anchored in the *E. coli* outer membrane and the 15mer peptide is located on the N-terminus. An expression control peptide, PX2, is located on the C-terminus.

binders for various protein targets[29-40], including some through semi-automated methods. To our knowledge, the eCPX peptide display system has never before been used in biopanning to develop affinity peptides for any bulk solid. This presents a unique challenge because the eCPX system biopanning methodologies were developed on the nanoscale against a protein target. Additionally, the protein target is often labeled or conjugated in order to facilitate sorting or quantify target binding. Since this technique cannot be performed with a bulk solid, these traditional biopanning methods cannot be employed at a macroscale level. It was therefore necessary to develop novel, alternative techniques and methodologies.

Bulk aluminum was used as a model target material to develop a bulk solid biopanning method for use with the eCPX display system. This methodology would be extensible to affinity peptide development for other bulk solids, including many nontraditional materials. Such peptides could be used in composite materials for varying applications, such as biosensors, biomaterials, engineered tissues, electro-optical magnetic and photonic devices.

2. MATERIALS AND METHODS

2.1 Bacterial strains and culture conditions.

E. coli MC1061 cells were used in all cloning and peptide display experiments, and are commercially available through ATCC (ATCC 53338). All cells were grown in LB Miller (LB) broth at 37°C with continuous shaking at 250 rpm. All cells containing a pBad33-derived plasmid were supplemented with 25µg/mL chloramphenicol (LB+Cm). Occasionally, cells were cultured on solid LB Miller agar plates supplemented with chloramphenicol and incubated in a stationary 37°C incubator.

2.2 Construction of negative control display scaffold and phage derived aluminum binding peptides A1-S1 and Al-S2

To create a control eCPX display system, the eCPX display scaffold gene sequence was custom synthesized in a pUC57 commercial vector (Genscript) with an omission of the 15mer peptide sequence. The gene and recipient vector, pBad33, were both digested with *SacI* and *SalI*, and ligated together using T4 ligase. The resulting plasmid, pB33-nl, was

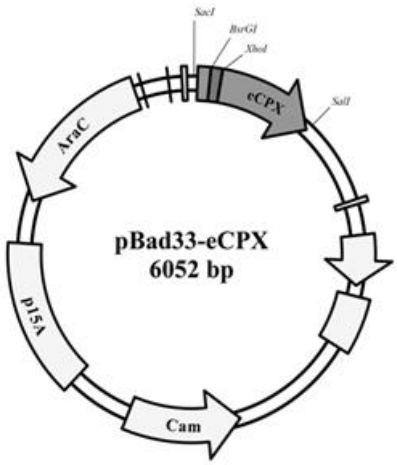

Figure 2. Plasmid map of the eCPX peptide display scaffold. This plasmid was engineered to have a plug-n-play configuration, allowing peptide sequences to be commercially synthesized and easily inserted into the scaffold using unique *BsrGI* and *XhoI* restriction enzyme sites. The gene encoding eCPX is indicated in light gray and the region encoding the peptide is indicated in dark gray.

transformed into chemically competent *E. coli* MC1061 cells. Plasmid pB33-nl was further modified to create a plug-n-play vector such that any peptide sequence could easily be cloned into the eCPX display scaffold. These modifications removed the *SfiI* restriction enzyme site downstream of the eCPX gene and replaced the *SfiI* restriction enzyme within the eCPX gene with *BsrGI*. This new plasmid is shown in Figure 2 and was named pB33-nl3. Additionally, it also served as a negative control because the encoded eCPX display scaffold was identical to all other eCPX display scaffolds in the library with the exception that it displayed no peptide. The full eCPX sequence was verified by DNA sequencing (Genewiz).

In order to utilize aluminum binding 12mer peptides previously developed from a phage library[9], peptides Al-S1 (VPSSGPQDTRTT) and Al-S2 (YSPDPRWSSRY) were synthesized (BioBasics) for cloning into pB33-nl3 using standard molecular biology methods. Primers used to amplify the peptide insert for cloning were as follows: Forward 5' TTCCGTAGCTTGTACATGTGGCCAG 3' and Reverse 5' CACCGCTGCCACCGCT 3'. The 83bp inserts were ligated into pBad33-nl3, which was digested with *BsrGI* and *XhoI*. The resulting plasmids, named pBad33-AB1 or pBad33-AB2, were then transformed into chemically competent MC1061 cells and insertion of the peptide sequences verified by DNA sequencing (Genewiz).

2.3 Flow cytometry analysis of plug-n-play peptide display system

An overnight cell culture was diluted 1:100 in 5 mL fresh LB+Cm and grown to an OD_{600} 0.50-0.55. At that time, the eCPX production was induced by the addition of 0.04% arabinose and continued growth for an additional 35-45 min. After induction, 5 µL cells were added to 25µL 250 nM YPet-Mona for 45 min. on ice. Cells were then pelleted and resuspended in 1 mL ice cold BD FACSFlow immediately prior to flow cytometric measurements, and proper eCPX expression was indicated by increased FITC signal.

2.4 Wash protocol optimization

The effectiveness of Tween20 and TritonX as components of the wash buffer was compared. An eCPX peptide display library was inoculated into 500 mL LB+Cm and grown to an OD_{600} =0.5. Peptide expression was induced with 0.04% arabinose for 1 hour. Eight 1 cm x 5 cm sterile bulk aluminum alloy strips were added to the induced culture and incubated for 1 hr at 4°C, with gentle rocking. The aluminum strips were removed, and briefly rinsed by gently dipping into 25 mL sterile PBS. The strips were then washed in 25 mL sterile PBS with either 1% TritonX-100 or 1% Tween20

for 1 min. at 100 rpm. The strips were then removed to 0.2% glucose supplemented media and incubated for 5-7 hours. At this time, the aluminum strips were removed and the OD_{600} measured.

The optimal concentration of Tween20 in the wash buffer was determined by cell growth measurements in various concentrations of Tween20. An overnight culture of MC1061 pB33-nl3 was diluted 1:100 into fresh LB supplemented with 0, 0.01, 0.05, 0.1, 0.5, 1, 1.5, or 2% sterile Tween20. The cultures were allowed to grow for 18 hours, at which time the OD_{600} was measured.

The effectiveness of a PBS with 1% Tween20 (PBST) wash buffer and PBS alone was compared to determine if Tween20 increased the stringency of non-specific peptide binder removal. An eCPX peptide display library was grown and induced as described in the paragraph above. After rinsing samples briefly in PBS, the aluminum samples were placed in either 25 mL PBS or PBST and washed 3 times, for 5 min each at 100 rpm. The samples were then removed to 0.2% glucose supplemented media and incubated for 5-7 hours. At this time, the aluminum strips were removed and the OD_{600} measured.

2.5 Development of aluminum binding peptides through biopanning with the eCPX peptide display

The eCPX bacterial display library was inoculated into 500 mL fresh media, then grown and induced as described in section 2.3. After induction, cells were chilled on ice for 15-30 min. Sterilized aluminum samples were added to the induced library and placed on a shaker at 4°C for 15 min. The aluminum samples were briefly rinsed in sterile PBS and transferred to PBST. The samples were washed for 5 min. and additional washes were added with each round of sorting to remove loosely bound cells. After washing, bound cells were recovered by removing the aluminum samples to 0.2% glucose supplemented media overnight. This overnight culture was then used in the following sorting rounds, for a total of 4 rounds. Ninety-six randomly selected colonies from round 4 were sequenced using the pBAD Forward universal primer (Genewiz) and the peptides were identified from the sequences using the InsertMultiSeek analysis tool (www.sequencetools.com). Peptide sequences containing stop codons or with frame-shift mutations were discarded as these display scaffolds would not be properly displayed on the cell surface.

2.6 Characterization of individual aluminum binding peptides

The 17 aluminum binding peptides were characterized by first comparing their growth rate. Overnight pure cultures of each isolate were diluted 1:100 in 5 mL fresh media and the OD_{600} was measured every 30 min. for three hours (the time required to reach OD_{600} =1.0). A growth curve was generated for each strain, and from this, the doubling time was calculated using an exponential growth nonlinear fit. The growth rates were compared using a one-way ANOVA to determine if the mean growth rate of any isolate was significantly different from the rest.

A previous study that developed aluminum binding peptides[9] identified the highest binding affinity peptides through a competition assay. Overnight cultures of the 17 isolates were grown and induced as described in section 2.4. After induction, the cultures were mixed in equal amounts and incubated with the aluminum samples for 15 min. at 37°C. The aluminum samples were briefly rinsed in sterile PBS and transferred to 25 mL PBST, where they were washed 3 times for 5 min at 150 rpm. The aluminum samples were removed to 6 mL 0.2% glucose supplemented media and incubated for 1 hour. Serial dilutions were made from the initial mixed isolate cultures and the binder recovery cultures, and duplicate 100 µL spread plates were prepared to obtain single colonies. Plates were incubated at 37°C for 24 hours, at which time 50 colonies were randomly selected from each plate and sequenced to identify the peptide. Bulk sequence analysis was performed on the sequences using the InsertMultiSeek analysis tool (www.sequencetools.com). The peptide composition from the initial culture was then compared to the final binder recovery culture.

The results were validated by determining the aluminum binding propensity of each isolate individually. An overnight culture was diluted 1:100 in 5 mL fresh media, grown and induced as described in section 2.4. After induction, the cells were chilled on ice for 15-30 min to stop cell growth and sterilized aluminum samples were added to the culture tubes. The samples were then returned to 37°C for an additional 15 min to allow cell binding to occur. The aluminum samples were briefly rinsed in sterile PBS, transferred to 30 mL PBST, and shaken at 150 rpm at room temperature for 30 min. The aluminum samples were removed to 6 mL 0.2% glucose supplemented media and incubated for 1 hour. The number

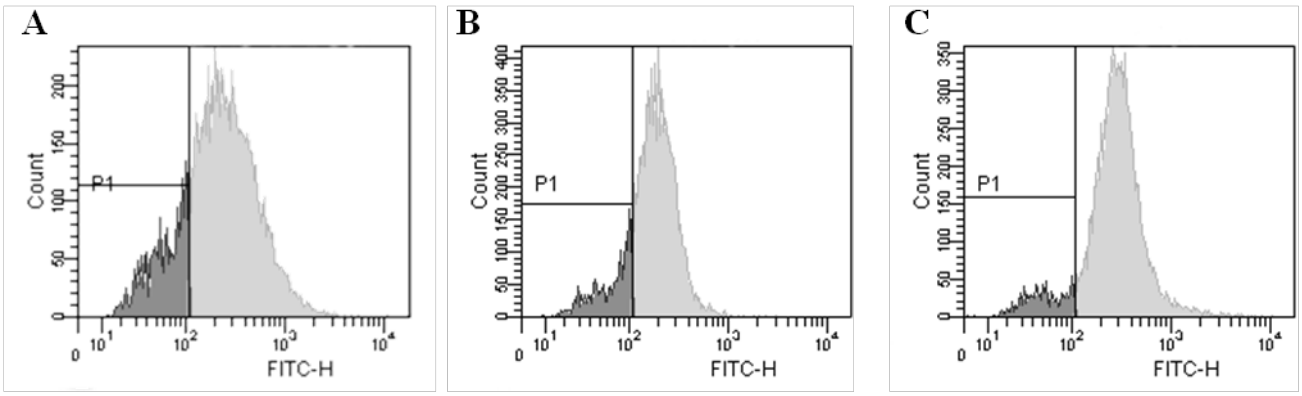

Figure 3. Flow cytometry analysis of various engineered eCPX display scaffolds including A) the plug-n-play scaffold, which also serves as a peptide negative control construct, and the phage-derived 12mer aluminum binding peptides, B) Al-S1 and C) Al-S2. In all panels, the scaffold expression cell population is indicated in light gray.

of cells/mL recovered from the aluminum was determined by serial dilutions on LB agar plates. Plates were incubated at 37°C for 24 hours before colonies were counted.

3. RESULTS AND DISCUSSION

We have previously demonstrated the use and effectiveness of the eCPX peptide display library in the development of affinity peptide binders for the Protective Antigen component of the *Bacillus anthracis* Anthrax toxin[35]. These studies employed a micromagnetic sorter (MMS), magnetic activated cell sorting (MACS), and fluorescence activated cell sorting (FACS), using a biotinylated protein target with 1µm magnetic beads or a secondary fluorescent label. These techniques, and the protocols that utilize them, were quite successful when the target was a protein or other nanoscale molecule. Unfortunately, similar success does not translate to macroscale targets, such as bulk metals, using the same techniques largely due to the increased scale of sorting and affinity characterization. As a result, there is a need for new techniques and protocols to facilitate biopanning of bulk materials, specifically with this display library.

3.1 Synthesis and evaluation of control eCPX scaffolds

In order to develop a bulk material biopanning protocol, several control constructs were required. A negative control eCPX display system was synthesized such that it was identical to the display scaffolds found in the library, except it lacked a 15mer peptide. Furthermore, the vector was also designed for easy plug-n-play of synthetic peptides, resulting in novel peptide expression on the eCPX scaffold. Such was the case for phage-derived 12mer aluminum binding peptides, Al-S1 and Al-S2. These peptides were custom synthesized and cloned into the negative control, pB33-nl3, and expressed on the *E. coli* cell surface. The expression level of the negative control scaffold and the two phage-derived aluminum binding peptides were verified using FACS. The results showed that alterations to the eCPX display scaffold N-terminus, which was necessary to facilitate the display of the negative control, Al-S1, and Al-S2 peptides, did not affect the expression or display of the scaffold itself (Figure 3). In addition to the 15mer peptide displayed on the N-terminus of the scaffold, there is an internal control peptide located on the C-terminus (Figure 1). This peptide, PX2, is specifically labeled by the fluorescent tag, YPet Mona. Analysis of this tag allows expression and display efficiency of the eCPX scaffold to be monitored, and indirectly measure the 15mer peptide display levels. Cells displaying the negative control eCPX scaffold bound 79% YPet Mona, and indicates that 79% of cells displayed the scaffold. Similarly, Al-S1 had 81% scaffold displayed and Al-S2 had 90% scaffold displayed. These values are consistent with maximum scaffold display routinely

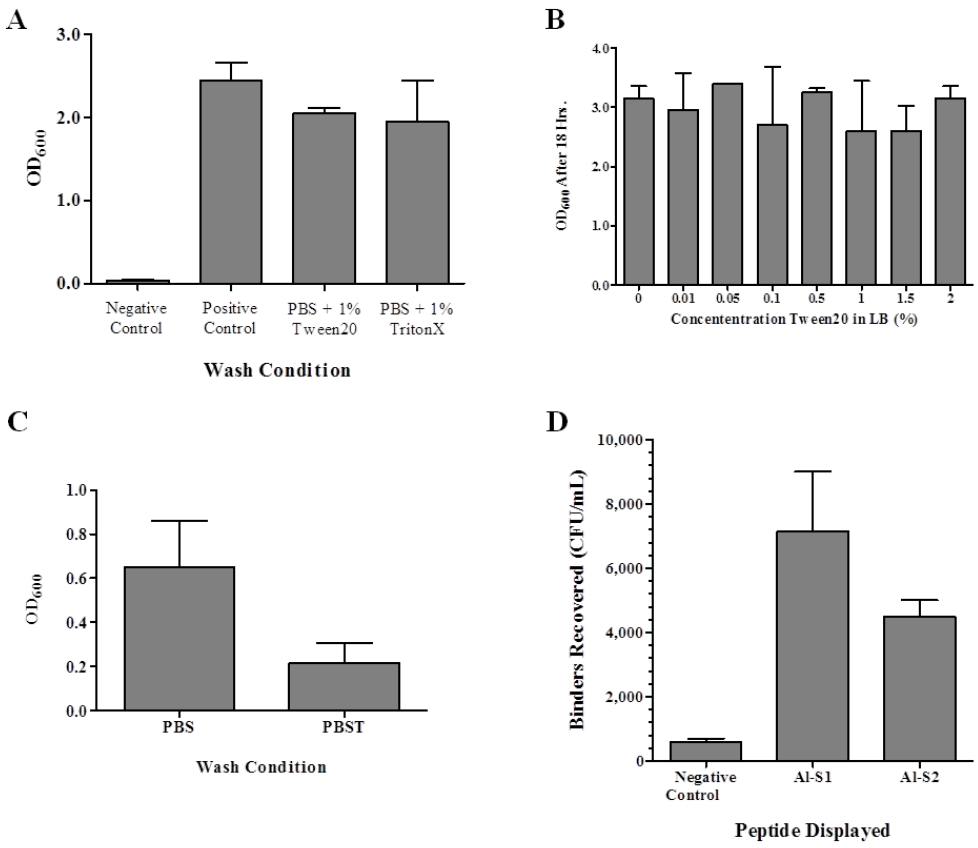

Figure 4. Optimization of sample washing conditions. A) Comparison of two common wash buffer detergents, Tween20 and TritonX. B) Evaluation of various Tween20 concentrations on cell growth. C) Comparison of wash buffer efficiency using PBS with 1% Tween20 (PBST) and PBS. D) Aluminum binding capability of the scaffold alone (negative control) and 12mer phage derived aluminum binding peptides displayed on the eCPX scaffold. Error bars represent the standard error of the mean (SEM) for two replicate samples.

seen in this laboratory. These results confirm the utility of the eCPX system for functional expression of foreign peptides.

3.2 Development of biopanning wash protocol

The negative control scaffold, Al-S1 and Al-S2, were used to develop a biopanning protocol utilizing a bulk metal as a target. We first began by developing the washing step, as this step is most critical for the removal of non-specific binders. Two non-ionic detergents commonly used at low concentrations in wash buffers for many laboratory assays, Tween20 and TritonX, were surveyed.

After washing the bulk aluminum samples in PBS solutions containing these detergents, Tween20 and TritonX reduced the numbers of cells binding by 16% and 20%, respectively, as compared to the control (no washing) (Figure 4A). Although there was no difference in non-specific cell removal between the two detergents, we decided to use Tween20 in future experiments because it is a milder detergent that generally does not affect protein activity. Also, Tween20 was a wash buffer component used during biopanning for aluminum peptide binders from a phage library[9]. Although Tween20

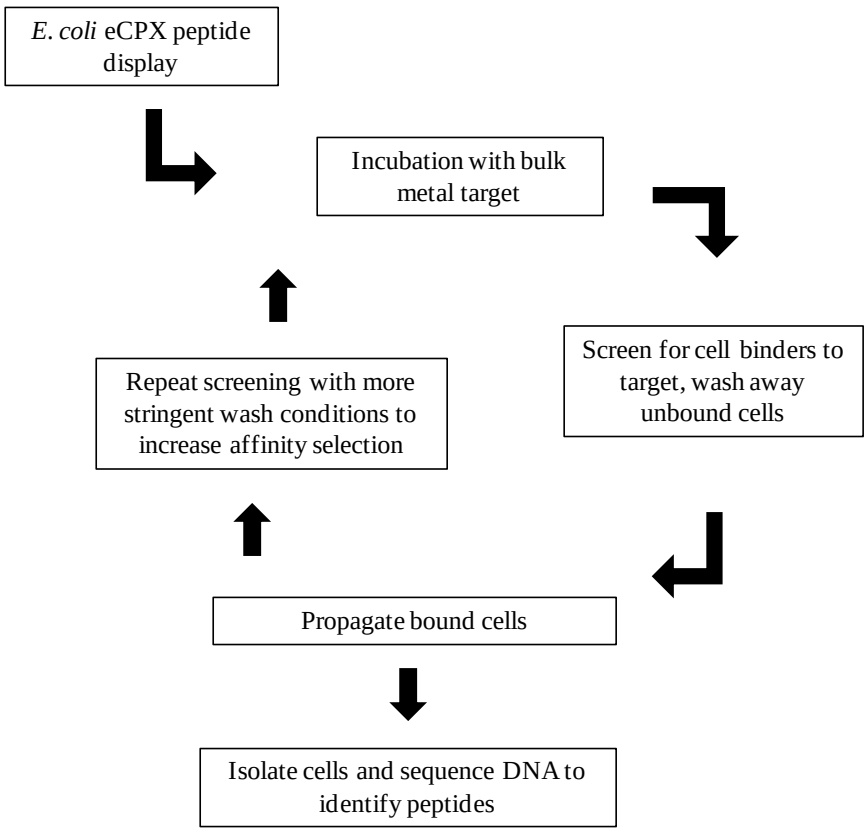

Figure 5. A schematic diagram of the process of biopanning with a bulk solid target. The library is incubated with the target material (in this case, a bulk aluminum alloy) and cells displaying the affinity peptides bind. Loosely bound or unbound cells are washed away and the bound cells are cultured to form a binder enriched population. The cycle is repeated 3-4 times, with increasing wash stringency. The best target binders compose the final population and the peptide sequences are identified through DNA sequencing of the eCPX gene.

is generally considered to be non-toxic to cells, it can cause mammalian cell lysis at higher concentrations. Therefore, *E. coli* cells were grown in LB supplemented with a range of Tween20 concentrations in order to determine if growth could be affected. We examined Tween20 concentrations between 0.01% and 2%, and found no effects on cell growth (Figure 4B). Cultures containing Tween20 were observed to grow to the same density as cultures without Tween20, indicating that the Tween20 concentrations tested did not negatively impact the growth. Based on these results, a 1% Tween20 wash buffer was used in subsequent washing protocols. Furthermore, the addition of Tween20 was found to significantly reduce non-specific cell attachment to the aluminum samples. Samples washed in PBS alone were found to have a 67% higher amount of cell attachment after washing than samples washed in PBS 1% Tween20 (PBST) (Figure 4C).

Aluminum binding in experiments, up to this point, had been assessed by measuring the cell density re-grown from the aluminum surface over 5-7 hours. As an alternative to this time consuming and less accurate method, a binder recovery assay (described in section 2.6) was developed. This assay allowed cells bound to the aluminum surface to be enumerated after 1 hr recovery incubation. Such a short growth period likely facilitated only 1-2 cell divisions, and thus cell counts were likely very true to the actual cell number on the aluminum surface after washing. Using this assay, the aluminum binding capability of Al-S1 and Al-S2 was compared to the negative control. After washing, there was a significant difference in the cell numbers recovered from aluminum samples incubated with the two phage-derived aluminum binders as compared to the negative control. Figure 4D shows that Al-S1 and Al-S2 had approximately 10-fold more cells recovered than the negative control. The low number of negative control binders recovered is also noteworthy because it demonstrates that binding was most likely facilitated by the displayed peptides, and neither general bacterial cell adhesion

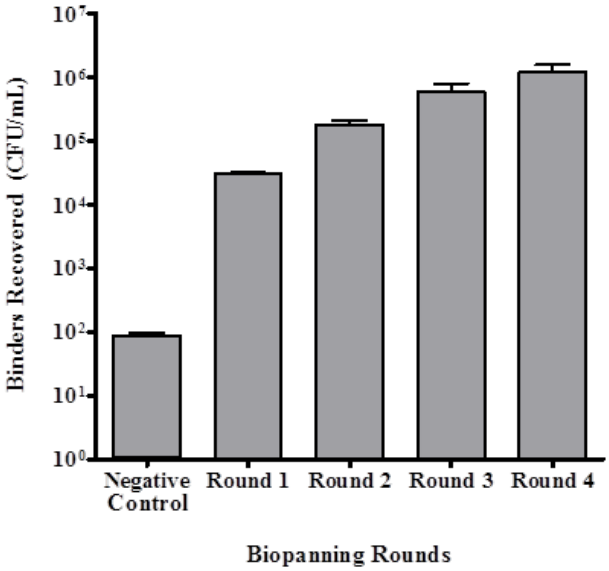

Figure 6. Results from aluminum affinity binder biopanning using the eCPX peptide display library. Enrichment of the cell population is shown with successive biopanning rounds. Error bars represent the SEM for two replicate samples.

elements nor the display scaffold itself had a significant contribution to aluminum surface binding. This bulk solid amenable assay confirmed that the insertion of synthetic aluminum binding peptides into the eCPX yielded a functionally displayed affinity peptide on the eCPX scaffold.

3.3 Biopanning the eCPX library for aluminum binding peptides

Based on the traditional biopanning protocols used previously with this eCPX library as well as protocols established by other investigators utilizing a bulk metal target, we developed experimental techniques specifically designed for bulk solid affinity peptide discovery with the eCPX display library. The process is detailed in section 2.5 and described schematically in Figure 5. We preformed four rounds of biopanning with the aluminum samples and measured the aluminum binding propensity after each round. There was a marked increase in the number of cells recovered from the aluminum surface as the wash conditions became more stringent and the high affinity binder population was enriched (Figure 6). There was a 40-fold increase in cells recovered from aluminum samples incubated with the fourth (and final) round, as compared the first, and substantial increases in recovered cells from each successive round. In order to identify peptides enriched through biopanning, DNA sequencing analysis was performed on colonies sampled from round 4. Seventeen unique sequences were found, with a peptide designated DBAD1 being identified 49 times, while the other 16 sequences were identified only once. In general, these sequences had a high frequency of hydroxyl or sulfoxyl containing residues, and are consistent with aluminum affinity peptides from a phage library[9].

3.4 Affinity characterization of aluminum binding peptides

In a previous report using a phage library, Zuo *et al.*[9] identified the strongest aluminum binding peptides through a competition assay. After a stringent washing regime, the peptides isolated from the aluminum samples most frequently were said to be the strongest binding sequences. In order to perform a similar competition assay using our 17 aluminum affinity peptides, we first had to ensure the growth rates of cells displaying these peptides were equal. Significant growth rate differences would skew the competition assay because the resulting predominate peptides would be due to rapidly growing cells rather than better binding. A comparison of the average doubling time for cells displaying each peptide showed essentially no differences and indicated that all isolates grow at the same rate (Figure 7A). The competition

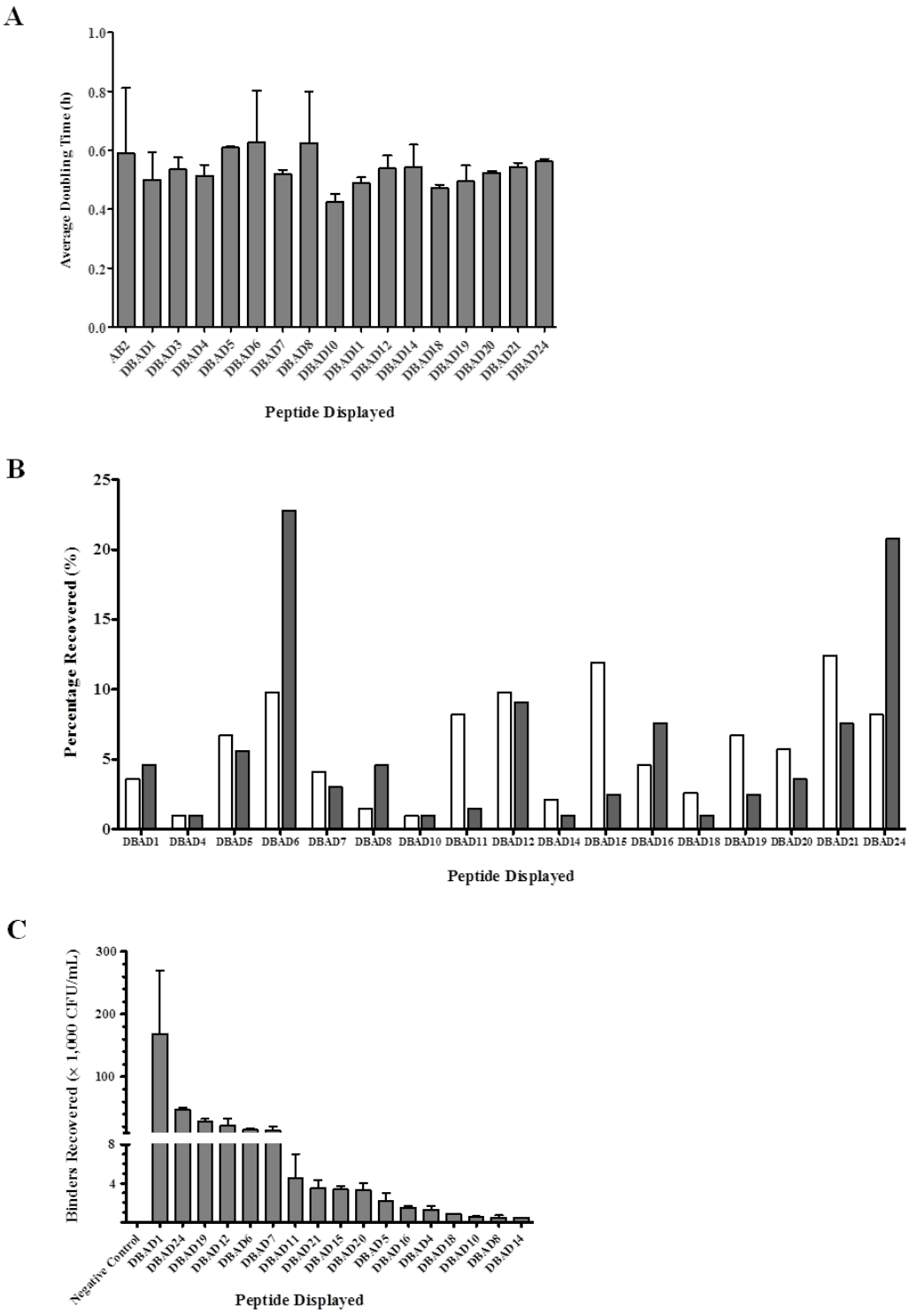

Figure 7. Characterization of novel aluminum binding peptides. A) Comparison of doubling time (growth rate) of cells expressing the aluminum binding peptides. B) Binding competition assay comparing the change in composition of each isolate in the population before (□) and after (■) aluminum binding. C) Comparison of aluminum binding by each of the 17 isolates. In all panels, error bars represent the SEM of two replicate samples.

assay showed that the dominate peptides present after aluminum binding and washing were peptides DBAD6 and DBAD24 (Figure 7B). These peptides occurred 153% and 132%, respectively, more in the aluminum bound population as compared to the starting population. No other peptides became markedly enriched in the recovery culture; in fact, most peptide occurrences remained unchanged or decreased. These results suggested that peptides DBAD6 and DBAD24 were the highest affinity binders. This was somewhat surprising since peptide DBAD1 was highly enriched in the round 4 sampling and suggests DBAD1 may have the higher aluminum binding affinity. For this reason, it was crucial to determine the aluminum binding affinity of each peptide isolate in pure culture. Figure 7C shows the number of binders recovered from bulk aluminum individually incubated with cells displaying each of the 17 peptides isolated. The peptide binding affinities varied widely, spanning a 2-log difference in values. Peptide DBAD1 clearly had the most cells recovered from the aluminum samples and was markedly higher than all other peptides. DBAD1 was 3.6-fold higher than the second most recovered binder, DBAD24, and 360-fold higher than the lowest, DBAD14. It is noteworthy that peptide DBAD24 was the second best binder when assayed individually and was found to be a high affinity binder using the competition assay. DBAD6, the other high affinity binder according the competition assay, was in the top five best aluminum binders when measured individually. Although the competition assay failed to identify the best binder (DBAD1), it did find two of the top 5 binders. These data suggest that a competition assay may not an optimal method for assessing the peptide binding affinity to the target. Also of interest was that the negative control had a 40-fold and 16,000-fold difference in cells recovered, as compared to DBAD14 and DBAD1, respectively. Taken together, these data further support our assertion that bulk aluminum binding is specifically facilitated by the displayed peptide.

4. CONCLUSION

In conclusion, we developed a method for biopanning the eCPX peptide display library for affinity binders to bulk solids, using aluminum as a model material. This was the first time such a target was used with this library, and as part of the biopanning methodology development, we also created a plug-n-play vector platform. We showed the utility of the platform by expressing functional phage-derived aluminum binding peptides on the eCPX scaffold. We successfully used our novel bulk material biopanning method to identify unique high affinity aluminum peptide binders. Although a competition assay, which was used in other metal binding peptide biodiscovery reports, failed to delineate the aluminum binding propensity of the peptides isolated here, individual characterization identified six high affinity peptide binder candidates. We are continuing to characterize these peptides and others from the literature in order to gain an understanding of the principles that govern peptide binding to metals and other bulk surfaces.

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6. REFERENCES

[1] Cetinel, S., *et al.*, "Peptides to bridge biological-platinum materials interface," Bioinspir. Biomim. Nanobiomater. 1(3), 143-153 (2012).

[2] Oren, E. E., *et al.*, "Metal recognition of septapeptides via polypod molecular architecture," Nano Lett. 5(3), 415-419 (2005).

[3] Khoo, X., *et al.*, "Directed assembly of PEGylated-peptide coatings for infection-resistant titanium metal," J. Am. Chem. Soc. 131(31), 10992-10997 (2009).
[4] Naik, R. R., *et al.*, "Biomimetic synthesis and patterning of silver nanoparticles," Nat. Mater. 1(3), 169-172 (2002).
[5] Van Dorst, B., *et al.*, "Phage display as a novel screening tool for primary toxicological targets," Environ. Toxicol. Chem. 29(2), 250-255 (2010).
[6] Huang, Y., *et al.*, "Programmable assembly of nanoarchitectures using genetically engineered viruses," Nano Lett. 5(7), 1429-1434 (2005).
[7] Chen, H., *et al.*, "QCM-D analysis of binding mechanism of phage particles displaying a constrained heptapeptide with specific affinity to SiO_2 and TiO_2," Anal. Chem. 78(14), 4872-4879 (2006).
[8] Rothenstein, D. C., *et al.*, "Isolation of ZnO-binding 12-mer peptides and determination of their binding epitopes by NMR spectroscopy," J. Am. Chem. Soc. 134(30), 12547-12556 (2012).
[9] Zuo, R., *et al.*, "Aluminum-and mild steel-binding peptides from phage display," Appl. Microbiol. Biotechnol. 68(4), 505-509 (2005).
[10] Lee, Y. J., *et al.*, "Fabricating genetically engineered high-power lithium-ion batteries using multiple virus genes," Science 324(5930), 1051-1055 (2009).
[11] Whaley, S. R., *et al.*, "Selection of peptides with semiconductor binding specificity for directed nanocrystal assembly," Nature 405(6787), 665-668 (2000).
[12] Estephan, E., *et al.*, "Sensing by means of nonlinear optics with functionalized GaAs/AlGaAs photonic crystals," Langmuir 26(12), 10373-10379 (2010).
[13] Peelle, B. R., *et al.*, "Design criteria for engineering inorganic material- specific peptides," Langmuir 21(15), 6929-6933 (2005).
[14] Chung, W.-J., *et al.*, "Evolutionary screening of collagen-like peptides that nucleate hydroxyapatite crystals," Langmuir 27(12), 7620-7628 (2011).
[15] Dickerson, M. B. A., *et al*., Peptide-induced room temperature formation of nanostructured TiO_2 and $BaTiO_3$ from aqueous solutions**,** at ACS National Meeting, Philadelphia, PA. (2008)
[16] Forbes, L. M., *et al.*, "Tunable size and shape control of platinum nanocrystals from a single peptide sequence," Chem. Mater. 22(24), 6524-6528 (2010).
[17] Sanghvi, A. B., *et al.*, "Biomaterials functionalization using a novel peptide that selectively binds to a conducting polymer," Nat. Mater. 4(6), 496-502 (2005).
[18] Beitle, R. R. and Ataai, M. M., "One-step purification of a model periplasmic protein from inclusion bodies by its fusion to an effective metal-binding peptide," Biotechnol. Progr. 9(1), 64-69 (1993).
[19] Sousa, C., *et al.*, "Metalloadsorption by *Escherichia coli* cells displaying yeast and mammalian metallothioneins anchored to the outer membrane protein LamB," J. Bacteriol. 180(9), 2280-2284 (1998).
[20] Pazirandeh, M., *et al.*, "Development of bacterium-based heavy metal biosorbents: enhanced uptake of cadmium and mercury by *Escherichia coli* expressing a metal binding motif," Appl. Environ. Microbiol. 64(10), 4068-4072 (1998).
[21] Bae, W., *et al.*, "Enhanced bioaccumulation of heavy metals by bacterial cells displaying synthetic phytochelatins," Biotechnol. Bioeng. 70(5), 518-524 (2000).
[22] Xu, Z. and Lee, S. Y., "Display of polyhistidine peptides on the *Escherichia coli* cell surface by using Outer Membrane Protein C as an anchoring motif," Appl. Environ. Microbiol. 65(11), 5142-5147 (1999).
[23] Samuelson, P., *et al.*, "*Staphylococcal* surface display of metal-binding polyhistidyl peptides," Appl. Environ. Microbiol. 66(3), 1243-1248 (2000).
[24] Brown, S., "Engineered iron oxide-adhesion mutants of the *Escherichia coli* phage lambda receptor," Proc. Natl. Acad. Sci. U. S. A. 89(18), 8651-8655 (1992).
[25] Kjærgaard, K., *et al.*, "Sequestration of zinc oxide by fimbrial designer chelators," Appl. Environ. Microbiol. 66(1), 10-14 (2000).
[26] Hnilova, M., *et al.*, "Peptide-directed co-assembly of nanoprobes on multimaterial patterned solid surfaces," Soft Matter 8(16), 4327-4334 (2012).
[27] Hall Sedlak, R., *et al.*, "Engineered Escherichia coli Silver-Binding Periplasmic Protein That Promotes Silver Tolerance," Appl. Environ. Microbiol. 78(7), 2289-2296 (2012).
[28] Thai, C. K., *et al.*, "Identification and characterization of Cu_2O-and ZnO-binding polypeptides by *Escherichia coli* cell surface display: toward an understanding of metal oxide binding," Biotechnol. Bioeng. 87(2), 129-137 (2004).

[29] Rice, J. J. and Daugherty, P. S., "Directed evolution of a biterminal bacterial display scaffold enhances the display of diverse peptides," Protein Eng. Des. Sel. 21(7), 435-442 (2008).
[30] Thomas, J. M. and Daugherty, P. S., "Proligands with protease-regulated binding activity identified from cell-displayed prodomain libraries," Protein Sci. 18(10), 2053-2059 (2009).
[31] Sun, J., *et al.*, "Reconstitution and engineering of apoptotic protein interactions on the bacterial cell surface," J. Mol. Biol. 394(2), 297-305 (2009).
[32] Zhang, S. and Link, A. J., "Bcl-2 family interactome analysis using bacterial surface display," Integr. Biol. 3(8), 823-831 (2011).
[33] Kenrick, S. A. and Daugherty, P. S., "Bacterial display enables efficient and quantitative peptide affinity maturation," Protein Eng. Des. Sel. 23(1), 9-17 (2010).
[34] Dane, K. Y., *et al.*, "Cell surface profiling with peptide libraries yields ligand arrays that classify breast tumor subtypes," Mol. Cancer Ther. 8(5), 1312-1318 (2009).
[35] Kogot, J. M., *et al.*, "Screening of peptide libraries against Protective Antigen of *Bacillus anthracis* in a disposable microfluidic cartridge," PLoS ONE 6(11), e26925 (2011).
[36] Stratis-Cullum, D., *et al.*, Rapid peptide reagent isolation in a disposable microfluidic cartridge, ARL-TR-5357 (2010).
[37] Stratis-Cullum, D., *et al.*, *Bacterial display peptides for use in biosensing applications*, in *On Biomimetics*, D.L. Pramatarova, Editor, InTech, Online. (2011)
[38] Stratis-Cullum, D. N., *et al.*, "Development of bacterial display peptides for use in biosensing applications," Proc. SPIE. 8358, Chemical, Biological, Radiological, Nuclear, and Explosives (CBRNE) 8358 (2012).
[39] Sooter, L. J., *et al.*, "Hand held biowarfare assays: rapid biowarfare detection using the combined attributes of microfluidic *in vitro* selections and immunochromatographic assays," ACS Symposium Series 101673-83 (2009).
[40] Pennington, J. M., *et al.*, "Isolation and characterization of anti-SEB peptides using magnetic sorting and bacterial peptide display library technology," Proc. SPIE 8358, Chemical, Biological, Radiological, Nuclear, and Explosives (CBRNE) Sensing XIII, 8358 83581Z (2012).

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