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Project Title: "New Strategies for Designing Bioadsorbants for Metal Removal: Selection of Antibodies and Peptides with High Specificity for Heavy Metals and Their Cell Surface Expression"

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Research Objectives

The broad, long term objective of the research plan is to develop exquisitely selective polypeptide metal chelators for the remediation of aqueous systems. A variety of polypeptide chelators will be developed and optimized ranging from antibodies to small peptides. Then, through unique molecular engineering approaches developed in our laboratories, the polypeptide chelators will be anchored directly on the surface of the cells that produce them. Thus, instead of using isolated biomolecules we will employ inexpensive genetically engineered whole cell adsorbents. Following a simple, easily scalable treatment, the engineered cells can be used to manufacture an inexpensive, particulate adsorbent for metal removal.

Research Progress and Implications

We have currently finished the final year of a three year program, including a six-month no cost extension. Work in the past eighteen months has been focused on enhancing the binding activity of the Ru(bpy)₃ antibodies in terms of both affinity and specificity. We were hampered in our efforts by the spectroscopic limitations of our Becton-Dickson FACSort FACS machine, in particular the inability to look at the

Ru(bpy)₃ emission directly. We had hoped we could use a workaround involving a different fluorophore attached to the Ru(bpy)₃ moiety, but after exhaustive attempts, this strategy proved non-viable. We have just purchased a new FACS machine that will be able to look at the Ru(bpy)₃ emission directly, and we will soon initiate a new round of studies.

- 1) We have isolated genes for the V regions of the heavy and light chains for the Ru(bpy)₃ specific monoclonal AC1106 (Shreder, K.S. Hariman, A. and Iverson, B.L., J. Am. Chem. Soc. 1996, 118, 3192-3201). This antibody binds Ru(bpy)₃ derivatives with high affinity, and will serve as our generic metal-complex binding pocket.
- 2) A -((gly)₃ser)₄- linker was added between the heavy and light chain to complete the construction on a so-called single chain Fv (scFv). The scFv is the smallest functional unit of an antibody that retains full binding activity.
- 3) The scFv has been cloned into a pET25b(+) vector for soluble expression.
- 4) We have synthesized various metal complexes to be used for a thorough binding analysis and optimization studies of the scFv.
- 5) We have prepared milligram quantities of the AC1106 scFv and characterized the binding properties using a fluorescence enhancement assay. We have measured binding constants for the scFv binding to various Ru(bpy)₃ derivatives.
- 6) We have cloned the scFv into our bacterial surface expression system in preparation for antibody engineering efforts. By using iterative rounds of randomization and selection via fluorescence activated cell sorting (FACS) the affinities for different metal ions will be evolved.
- 7) We have produced a sophisticated computer model of the scFv binding site to act as a guide for antibody engineering studies.
- 8) We have isolated Cu(II) and Ni(II) specific peptides using phage display technology. The isolated sequences contain histidine in the context of usually hydrophobic residues. These isolated sequences have improved physical properties compared to a simple polyhistidine peptide sequence.
- 9) We have initiated pilot studies to optimize the capture of metal complexes using the E. coli surface expressed scFv. The scFv reagent is prepared in "ready to use" form by simply growing a culture of cells. We are currently carrying out a systematic series of studies to find the best format for isolating metal complexes in a highly selective and efficient manner. This has proven successful on the small scale using a fluorescence binding assay, and we will be learning how to 'scale up' in subsequent investigations.
- 10) In advances related to the metal-binding work, we have been able to optimize further the efficiency of our bacterial surface expression/FACS selection system for isolating

interesting new antibodies. Using the optimized protocols on an scFv that binds the heart glycoside digoxin, we were able to produce an improved scFv with subnanomolar affinity after a single round of selection ("Antibody Affinity Maturation Using Bacterial Surface Display," Daugherty, P., Chen, G., Olsen, M., Iverson, B.L. and Georgiou, G. *Protein Eng.*, 1998, 11, 825-832.)! In addition, we have been able to combine multiple mutations isolated in different library screening studies. This new "supermutant" has an extremely high affinity, as the combined mutations conferred an additive increase in binding. These powerful new procedures will be employed in the upcoming metal-complex binding antibody evolution studies.

11) In another advance related to the metal-binding work, we have recently completed the saturation mutagenesis studies of an entire antibody binding pocket ("In vitro Scanning Saturation Mutagenesis of all the Specificity Determining Residues in an Antibody Binding Site," Chen, G., Dubrawsky, I., Mendez, P., Georgiou, G. and Iverson, B. L. *Protein Eng.* (1999) 12, 349-356). In other words, all of the amino acid residues that contact the bound antigen were changed one at a time to every other possible amino acid. Each mutant, 190 in all, was analyzed quantitatively for binding activity. The extremely high throughput of mutants required by this study was accomplished through optimized techniques we have developed for the in vitro production and analysis of antibody mutants (Burks, E.A., Chen, G., Georgiou, G. and Iverson, B.L. *Proc. Nat. Acad. Sci., USA*, 1997, 94, 412-416). Analysis of the data produced the first comprehensive model of the functional roles played by each amino acid in the binding pocket. This model will be used to steer future antibody engineering efforts.

Recent Activities

12) In the past year, we have been working to modify and optimize the antibody's binding affinity towards other metal complexes. To accomplish this task we used our bacterial surface display/FACS technology to screen and initially isolate antibodies with improved affinities from libraries produced by error-prone PCR of the starting wild-type anti-Ru(bpy)₃ antibody. However, we were not able to obtain an improved affinity mutant by this method. The low affinity of the initial starting antibody meant that we had to use high antigen labeling concentrations. In addition, the Ru(bpy)₃ reagent we were using had a relatively high level of cellular background labeling. As a result, we were not able to distinguish positive library members from negative ones because of reduced signal-to-noise.

13) To circumvent this problem, two approaches were examined. First, we cloned additional scFvs from the original AC1106-expressing hybridoma because that parental antibody has ~100-fold greater affinity for Ru(dmbpy)₃²⁺ than the scFv we initially cloned. A decrease in affinity on going from an IgG to a scFv is not unprecedented, however we decided to clone additional scFvs because, in the process of cloning from the hybridoma, mutations can arise from PCR errors. We isolated 6 additional scFv clones that had an ELISA signal against Ru(bpy)₃-BSA. Two of these were qualitatively better than the original scFv and were sequenced. These two clones differed from the original scFv by a few point mutations. Importantly, two of the amino acid differences occur in CDR3 of the heavy chain. Secondly, we were able to obtain collaboratively a scFv

isolated from naïve library that has a K_d of 29 nM. These additional Ru(bpy)₃-binding scFVs were cloned into our surface-display vector, and examined by FACS. As before, no usable FACS signal could be obtained with these new constructs.

14) One of the constraints of the Becton Dickinson FACSort that we use is that the only excitation wavelength available is 488 nm. Ru(bpy)₃ complexes are optimally excited at 450 nm. We now have a Cytomation MoFlo FACS instrument that will permit us to examine our better scFv clones at an optimal excitation wavelength. Unfortunately, despite valiant attempts using the MoFlo, we were unable to obtain usable FACS signals for any of the metal-specific antibodies displayed on bacterial surfaces. DRAT!

15) By continually trying to enhance expression of the scFv antibodies such as AC1106 we have been able to optimize our expression system. A non-trivial feat that will pay large dividends when we develop our affinity enhanced antibodies and prepare the cells to be used as immunoadsorbents. "Development of an Optimized Expression System for the Screening of Antibody Libraries Displayed on the E. coli Surface," Daugherty, P., Olsen, M., Iverson, B.L. and Georgiou, G., *Protein Eng.* (1999) 12, 613-621.

16) In work that is related to the antibody enhancement work, we have been able to use the same system to evolve an enzyme. In this case, a protease called OmpT was expressed on the E. coli surface, then selected by FACS to prefer an entirely new substrate that is not accepted by the original enzyme. In the process, we carried out what we believe to be the largest enzyme library screening ever based on the quantitative analysis of catalytic turnover from each library member. The lessons we have been learning through the Ru(bpy)₃ project were absolutely instrumental in our success here. Although an active protease with an entirely unprecedented cleavage specificity will not address the issue of metal decontamination, it will allow us to address many other issues of national need. For the first time we will have the ability to tailor enzyme substrate specificity in a powerful way! This work was recently published ("Function-based isolation of novel enzymes from a large library", Olsen, M.J., Stephens, D., Griffiths, D., Daugherty, P., Georgiou, G. and Iverson, B. *Nature Biotechnology*, (2000) 18, 1071-1074. This project has blossomed into an entire new focus of the group, and forms the basis for an NIH grant being submitted.

17) In the last six months of the project, we started the first comprehensive study to ascertain the optimum mutagenesis rate for enhancing antibody affinity through directed evolution. Our difficulty analyzing the metal binding antibody systems made it clear that we needed to learn much more about the directed evolution process. We discovered that in the rush to carry out "marquis experiments," the entire directed evolution field had not carried out careful, quantitative analyses that would serve as the foundation for optimizing protein activity. Our initial experiments yielded the surprising result that very high mutagenesis rates produce the best mutants, and many more active mutants than expected. This result has generated a great deal of interest in the directed evolution community, and will serve as the basis for a new thrust in our research. An NIH grant is being submitted on this topic. We are currently preparing a substantial manuscript that will describe these results in detail.

Conclusions

The bottom line is that we have proven the concept of inexpensive surface supported bioadsorbents in the form of metal complex specific antibodies (Accomplishments 1-9) and that these can be used to concentrate metal complexes. We have been challenged, however, by a recalcitrant system in which we unexpectedly lost antibody affinity when preparing the surface expressed constructs. This situation requires us to improve the properties of the antibody using our FACS-based antibody enhancement technology. Unfortunately, we are currently unable to get the FACS signals necessary to enhance the activity of the scFv antibodies and thereby optimize the metal binding system. A major problem is that the fluorescent probe we use for the FACS turned out to have an unacceptably high level of background binding to the cells, thereby reducing the signal-to-noise. Our new FACS machine will be assembled and running by the end of the month, and this will open up new opportunities to get the antibody enhancement studies back on track. We will now have the freedom to use entirely different FACS probes, such that the background binding problem can be eliminated.

The good news is that this project has taught us a great deal about our FACS system in that this has been by far our greatest challenge to date. Thus, in solving these problems we will have enhanced our protocols and antibody optimization systems to unprecedented levels as described in accomplishments 10, 11 and 15. In the mean time, we have been able to use our experiences to generate a very exciting new enzyme enhancement system that will form the basis for a major new scientific thrust within the group. Importantly, the ability to alter enzyme substrate specificity will have numerous applications in the bioremediation field, thus extending the theme of the original DOE grant.

Despite our enthusiastic efforts and failed attempts to produce bioadsorbents of practical value, the current project will be remembered as an important milestone in our laboratory. The inability of our system to produce better metal-specific antibodies uncovered some key issues that we had not focused on before, and encouraged us to pursue some entirely new directions with the technology. The results of these efforts will serve as the basis for our research program throughout the next decade. Thus, I consider this DOE funding to be the most important I have received to date, setting us upon a new and fertile path of discovery in the protein engineering field.

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