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Rapid generation of a nanocrystal-labeled peptide library for specific identification of the bacterium Clostrium Botulinum

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FY04 LDRD Final Report

Rapid Generation Of A Nanocrystal-Labeled Peptide Library For Specific Identification Of The Bacterium *Clostrium Botulinum*

LDRD Project Tracking Code: 04-FS-015
Jeffrey B.-H. Tok, Principal Investigator

Abstract

Several peptide libraries containing up to 2 million unique peptide ligands have been synthesized. The peptides are attached onto a 80 micron resin and the length of these peptide ligands ranges from 5 to 9 amino acid residues. Using a novel calorimetric assay, the libraries were screened for binding to the ganglioside-binding domain of *Clostridium Tetanus* Toxin, a structural similar analog of the *Clostridium Botulinum* toxin. Several binding peptide sequences were identified, in which the detailed binding kinetics are currently underway using the Surface Plasmon Resonance (SPR) technique.

Introduction / Background

Botulism is a potentially lethal disease caused by one of the seven homologous neurotoxic proteins usually produced by the bacterium *Clostridium botulinum*.[1,2] This neuromuscular disorder occurs through an exquisite series of molecular events, ultimately ending with the arrest of acetylcholine release and hence, flaccid paralysis. In light of the recent threats of bioterrorism, it is of significant interest and urgency to develop an ultra-sensitive method to rapidly detect the bacterium *Clostridium botulinum*.[3,4] Herein, a rapid *in vivo* biosensing technology is proposed which involves identifying peptide sequences that will recognize "protein signatures" unique to bacterium *Clostridium botulinum*.[5] The peptide sequences that are optimized through repeated rounds of screening a randomized peptide libraries attached to nanocrystals, will both penetrate

(i.e.. cellular uptake) and exhibit specific binding to protein fragments unique to the bacterium *Clostridium botulinum*. Application of metals and semiconductor nanocrystals had been highly focused upon lately due to their diverse yet unique magnetic, optical and electronic properties.[6] In particular, fluorescent quantum dots (QDs) had afforded numerous exciting diagnostic developments. QDs are monodisperse inorganic nanocrystalline particles made from semiconducting material and are typically in the size of 2 to 10 nm. As fluorescent probes, QDs have several advantages over conventional organic dyes: their emission spectra are narrow and symmetrical on the basis of their size and material composition, and their exhibit photostability.[7,8] In addition, they display broad absorption spectra, which make it possible to excite many QDs to different colors with a single excitation light source. This is certainly an advantage when studying multiple biological targets simultaneously in the bacterial cell.[9] The photostability of QDs also allow real-time monitoring or tracking of intracellular processes *in vivo* over extended periods. Therefore, we are interested in synthesizing libraries of randomized peptide sequences attached to QDs to identify optimized sequence that will function as (i) efficient nanoparticle-delivery agents for improved penetration of our target bacterial cells, and (ii) bind specifically to the target bacterium *Clostridium botulinum*.

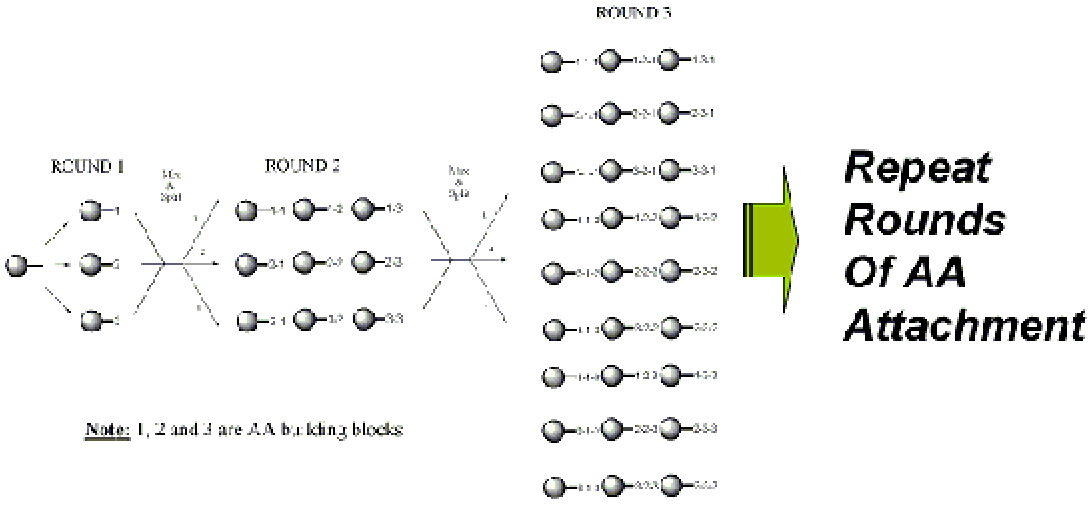
Research Activities / Research Outcome

1. Peptide library synthesis.

We have recently completed the synthesis of numerous peptide libraries using the "one-bead one- compound" approach (**Fig. 1**),[11-14] in which each library consists of approximately 2.5 million unique peptide sequences immobilized on Tentagel ® resins. We had synthesized numerous libraries of peptide sequences with different lengths, namely 5-mer to 9-mer. As an example, the diversity for the 7-mer and 9-mer libraries are 7^{19} and 9^{19} respectively (all twenty natural amino acids are employed in the library synthesis process, with the exception of cysteine).

Each of the libraries was subsequently interrogated with the binding domain of *Clostridium tetanus*, commonly known as the "fragment-C" due to its proximity to the carboxyl end of the neurotoxin protein. Applying the calorimetric assay approach (**Fig. 2**), which involves coupling the biotinylated protein target with streptavidin-linked Alkaline Phosphatase (AP), unique peptide ligands have been isolated based on a simple color change on the surface of the beads in the presence of the substrate BCIP. The darkest-stained beads were then isolated and sequenced through a peptide sequencer. We are currently re-synthesizing three of the peptide sequences for *in vitro* binding studies with a fluorescently-labeled *C. tetanus* "fragment-C" protein target. A similar approach will be utilized to approach the binding domain of BoNT/A.

Fig. 1. A schematic of "mix and pool" synthesis. As shown is a tri-peptide library in which 27 unique peptide ligands can easily be synthesized after 3 rounds with only 3 building blocks.



2. Fluorescent-based assay for screening peptide libraries.

Modified from a previously reported assay approach by Schreiber and coworkers, we had also successfully employed fluorescent microscopy-base assay to enable more efficient screening of our large collection of peptide-

coupled library resin beads. The fluorescently-labeled protein target allows us to identify binding peptidic ligands more efficiently through eliminating of the enzymatic labeling steps. Thus positive hits can easily be isolated through identifying the fluorescent beads as the binding of the fluorescently-labeled protein target confers fluorescent characteristics to the Tentagel ® resin beads.

3. Attachment of peptides onto Quantum Dots.

As mentioned, we had successfully completed the rapid parallel synthesis of the peptide library via standard Fmoc-chemistry on Tentagel ® resin. The positively identified peptides are currently being resynthesized in a larger amount (~3 mg), in which the crude peptides obtained were subsequently purified via semi-preparative Reversed-Phase HPLC. We are currently performing the following described experiments: The C-terminus of the peptides will be terminated with amide residues, while the N-terminus will be capped with a cysteine amino acid residue. The thiol (-SH) side chain of the N-terminal cysteine residue will allow efficient coupling to the functionalized QDs. As for the functionalization process for the QDs, attachment of the peptide library onto QDs requires that the surface of the nanoparticle be first functionalized with biocompatible dextran. The first step involve crosslinking the dextran-coated QDs with epichlorohydrin and reacting with ammonia to enable the primary amine groups.[10] The second step will be to activate the amino group by reacting it with N-succinimidyl 3-(2 pyridyldithio)propionate such that the various peptide sequences could be easily attached onto the QDs via disulfide bond formation (**Fig. 3**). The optimized peptide sequences will then be attached onto gold chips and be read with a portable Surface Plasmon Resonance (SPR) equipment to efficiently analyze and rapidly identify the presence of our target pathogen.[17-19]

Fig. 2. A schematic of calorimetric assay approach.

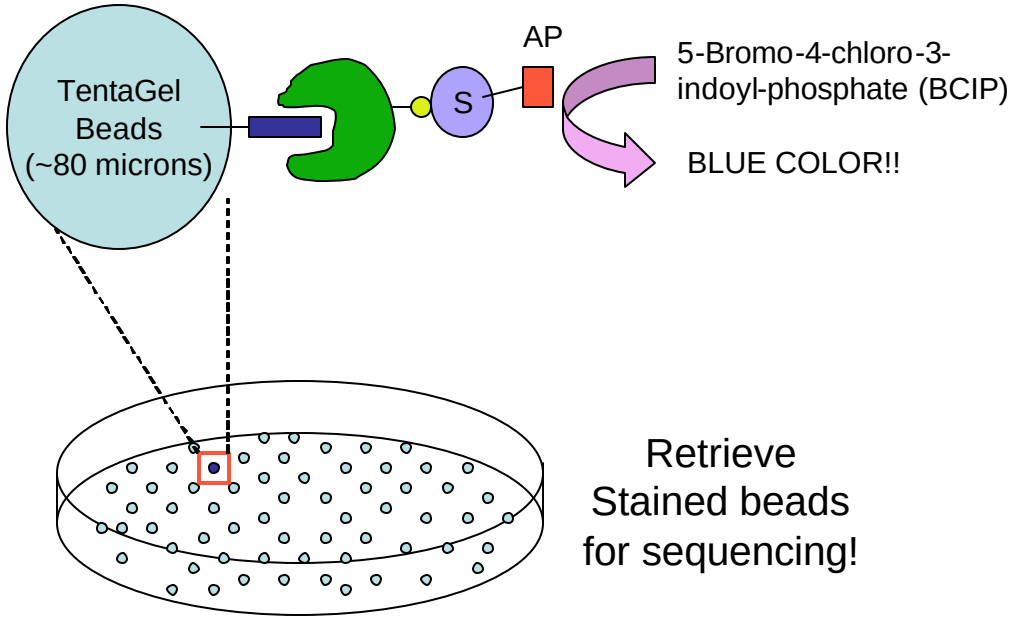
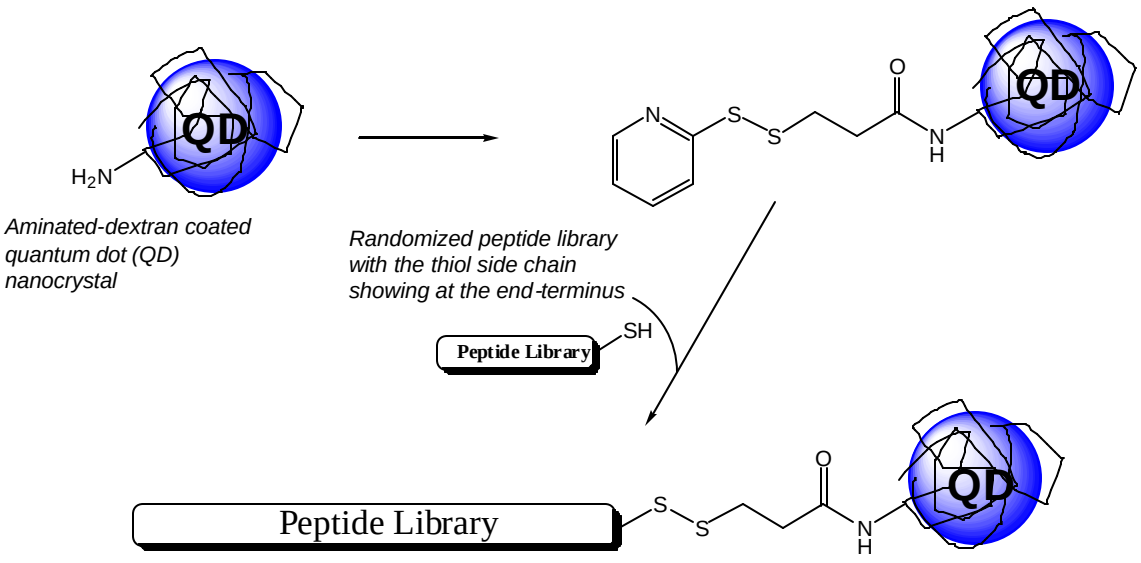


Fig. 3. Stepwise illustration in the parallel synthesis of surface-modified Quantum Dot (QD). The parent aminated-dextran coated QD nanocrystals was first activated with SPDP, and then reacted with thiol side chain of cysteine



Exit Plan

The above research effort has served to provide preliminary results which enabled the PI to recently submit an external grant:
PI: Tok. J. B.-H. "*Detection and Identification of Clostridum Botulinum Neurotoxin through Nanocrystals-Conjugated Peptide Aptamers*" NIH/NIAID, RCE for Biodefense and Emerging Infectious Diaseases. Proposed duration: 04/2005 to 03/2007. Total Cost: $500,000.

Summary

An extremely versatile and useful approach to synthesize peptide libraries has been demonstrated. The libraries have been successfully utilized to identify novel affinity binders against the ganglioside-binding protein domain of the bacterium *Clostridium tetanus*. Calorimetric assays to enable rapid screening of the peptide library have also been demonstrated and have subsequently afforded "lead" sequences to enable a second generation of peptide ligands. The incorporation of the peptide library with nanocrystals is currently underway to afford a real-time screening tool that will provide real-time temporal and sized resolution for direct observation of modes of action of the dynamic of peptide affinity ligand binding in individual pathogen cells. Such a tool will aid to discover when and where the inhibitory mechanism occur in the living pathogen cells, thus permitting one to better evaluate and design higher affinity and specificity binding ligands.

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References

(1) Josko, D. *Clin Lab Sci* **2004**, *17*, 30-34.

(2) Davis, L. E. *Curr Treat Options Neurol* **2003**, *5*, 23-31.

(3) Firmani, M. A.; Broussard, L. A. *Expert Rev Mol Diagn* **2003**, *3*, 605-616.

(4) Jain, K. K. *Expert Rev Mol Diagn* **2003**, *3*, 153-161.

(5) Simpson, L. L. *Annu Rev Pharmacol Toxicol* **2004**, *44*, 167-193.

(6) Josephson, L.; Kircher, M. F.; Mahmood, U.; Tang, Y.; Weissleder, R. *Bioconjug Chem* **2002**, *13*, 554-560.

(7) Nisman, R.; Dellaire, G.; Ren, Y.; Li, R.; Bazett-Jones, D. P. *J Histochem Cytochem* **2004**, *52*, 13-18.

(8) Olivos, H. J.; Bachhawat-Sikder, K.; Kodadek, T. *Chembiochem* **2003**, *4*, 1242-1245.

(9) Watson, A.; Wu, X.; Bruchez, M. *Biotechniques* **2003**, *34*, 296-300, 302-293.

(10) Schellenberger, E. A.; Reynolds, F.; Weissleder, R.; Josephson, L. *Chembiochem* **2004**, *5*, 275-279.

(11) Liu, R.; Marik, J.; Lam, K. S. *Methods Enzymol* **2003**, *369*, 271-287.

(12) Lam, K. S.; Liu, R.; Miyamoto, S.; Lehman, A. L.; Tuscano, J. M. *Acc Chem Res* **2003**, *36*, 370-377.

(13) Song, A.; Zhang, J.; Lebrilla, C. B.; Lam, K. S. *J Am Chem Soc* **2003**, *125*, 6180-6188.

(14) Lam, K. S.; Lehman, A. L.; Song, A.; Doan, N.; Enstrom, A. M.; Maxwell, J.; Liu, R. *Methods Enzymol* **2003**, *369*, 298-322.

(15) Daugherty, P. S.; Olsen, M. J.; Iverson, B. L.; Georgiou, G. *Protein Eng* **1999**, *12*, 613-621.

(16) Daugherty, P. S.; Iverson, B. L.; Georgiou, G. *J Immunol Methods* **2000**, *243*, 211-227.

(17) Wegner, G. J.; Lee, H. J.; Marriott, G.; Corn, R. M. *Anal Chem* **2003**, *75*, 4740-4746.

(18) Kobori, A.; Horie, S.; Suda, H.; Saito, I.; Nakatani, K. *J Am Chem Soc* **2004**, *126*, 557-562.

(19) Bae, Y. M.; Oh, B. K.; Lee, W.; Lee, W. H.; Choi, J. W. *Anal Chem* **2004**, *76*, 1799-1803.