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Development of Biosensors for Small Environmental Target Molecules Using Ce-Selex

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ABSTRACT

Microcystin is a naturally occurring liver cyanotoxin that can be found in water bodies around the world. Cyanobacterial blooms occur worldwide and can potentially contaminate municipal drinking water supplies with various toxins. These events are hazardous, naturally occurring processes that can be worsened by anthropogenic activities. The past 50 years have shown notable changes in global climate, likely due to environmentally detrimental human activities. Our environmental impacts have increased the frequency and severity of these events in the past decade. Developments in water quality control and maintenance have greatly reduced exposure to contaminated water, but current methods to detect microcystin are expensive and take several hours to complete. Aptamers are single-stranded DNA sequences with a high affinity to bind to a specific target. A simple to use, aptamer-based method could reduce water quality testing time and cost and allow more frequent testing by quality technicians and consumers alike. Selection of aptamers can be done using an in vitro selection process, Systematic Evolution of Ligand Exchange, combined with capillary electrophoresis.

INTRODUCTION

Humans live on continents, which are geologically temporary dry patches in a world of water. Our continents have always been impermanent when contrasted with the seemingly ever-abundant presence of water. Water and life on earth are linked as cycles that drive each other, and the vitality of every culture and civilization pivots on the development and maintenance of

methods for collecting and utilizing water. Human populations increase as technology develops, but our water needs have become even more crucial to our survival. Through innovation we have been able to utilize water from the earth's vast oceans, small streams, and the water trapped in deep underground aquifers.

Math Matters

Life on Earth should be untroubled in the pursuit of water, considering the abundance of this resource. However, a mere 3% of the earth's water is freshwater. Most of the freshwater is locked up in ice and in deep aquifers, leaving less than 1% of the freshwater in easily accessible surface waterways. Oceans cover 70% of our planet and contain 97% of the earth's water, but not a single drop is fit for our immediate consumption. Less than 0.1% of the water on earth is potable surface water; yet this is what keeps over 7 billion of us alive.

Water Contamination

Municipal water contamination

Municipal water treatment has significantly improved our ability to operate as a functioning and healthy civilization. Water treatment typically involves filtering out organic matter, sanitizing common bacterial contamination, and regulating the concentration of ions present. Public water sanitization allows us to trust that we will not be harmed while using municipal water and, in the United States and other developed countries, we can trust public sanitization the majority of the time. Regardless of Americans being avid consumers of water, utilizing 80–100 gallons a day (USGS, 2016), not including the water needed for food production, very little can be actively done to regulate and ensure the safety of the water we consume. Boiling water is not a common practice for Americans, unless bacterial contamination has been detected and reported to the community. Personal carbon filtration is more common among higher socioeconomic status consumers, where municipal water sites are better regulated than in lower socioeconomic communities (Koskei et al., 2013). Home

water testing kits can be costly, inaccessible, and have significant room for user error. The truth is that communities with fewer resources to test their water for safe consumption are more prone to experience water contamination (Yang et al., 2013; United States EPA, 2015). There is a need to give people the power to test their water in a simple and affordable way.

Small molecules as environmental contaminants

An environmental contaminant is a potentially harmful substance in the soil, air, or water. These substances can be produced anthropogenically or naturally and introduced deliberately or accidentally into the environment. Environmental toxins have the potential to cause cancer, disrupt endocrine functions, or generally harm human biological functions at various levels of severity. Environmental toxins can be relatively small molecules such as volatile organic compounds, pharmaceuticals, mycotoxins, and cyanotoxins. The vast range of environmental toxins reflects the importance of sustaining a healthy local and global ecology. Not only are monitoring and regulating toxins important, but having the ability to perform these tasks with speed, efficiency, and accuracy is crucial.

Cyanobacterial Blooms

Cyanobacterial blooms have received an increasingly intense review in the past few decades. Research regarding cyanobacterial blooms focuses on a variety of factors about the cause and effect of these events. In August 2014, a cyanobacterial bloom in Lake Erie contaminated the drinking water for the city of Toledo with various toxins, including microcystin. Half a million people were unable to use the municipal water supply for two days. Such events place humans and wildlife that inhabit affected areas at risk. Developments in water quality testing and treatment have greatly reduced the risk of exposure to contaminated water, but current methods to detect toxins such as microcystin can be time-consuming, limiting the speed with which a contamination event can be addressed (Ward et al., 1997). Tests for specific toxins usually require technicians to travel to the site, take samples, and then travel back to the laboratory to conduct sample analyses.

Microcystin

Microcystin is a liver toxin produced primarily by two genera of cyanobacteria, Microcystis and Planktothrix, and can be found throughout the world. The World Health Organization guideline value for microcystin is 1 μg/L, with a tolerable daily intake of 0.04 μg/L (United States EPA, 2015). If this liver toxin is present in greater concentrations, significant health risks could follow. Microcystis and Planktothrix blooms do not always produce toxins; however, the frequency of toxic events has increased in recent years. Additionally, one of the primary toxins involved in the 2014 water contamination event in Lake Erie were microcystins, as seen in **Figure 1**.

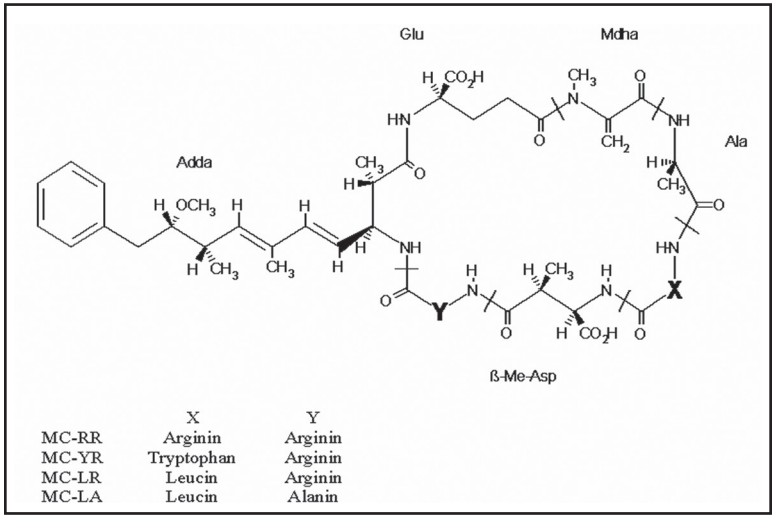

Figure 1. The general structure of a microcystin molecule with most common variations depicted, sites X and Y, are marked to represent the location of amino acid variations.

The general structure of microcystins remains the same with variations in amino acid composition at positions X and Y. I will be discussing microcystin-Leucine-Arginine (microcystin-LR), shown in **Figure 2**. This is the most common and one of the more detrimental of the many different permutations of microcystins. Microcystin-LR has been posed as the surrogate of detecting any microcystin, of microcystin producing cyanobacterial-bloom events (United States EPA, 2015).

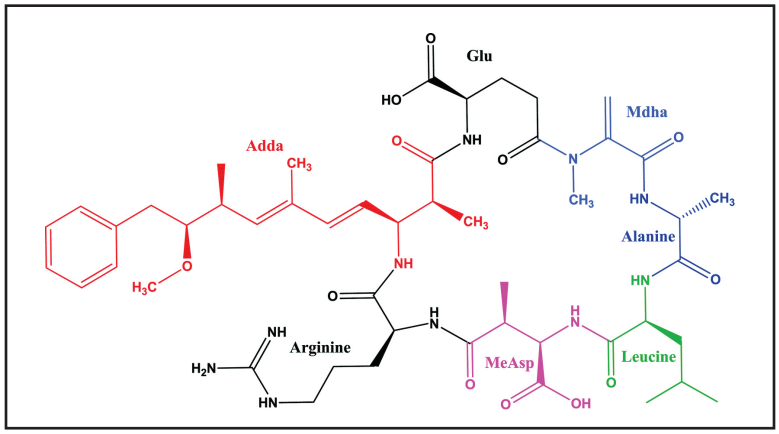

Figure 2. Structure of microcystin-LR with each component highlighted.

Persistent molecules, microcystins are structurally stable at the high temperatures and typical ranges of pH in natural surface waters. These factors make them particularly difficult to remove from contaminated waters (United States EPA, 2015). Water contaminated with microcystin cannot be used to drink, cook, or bathe in. Microcystins are not removable by boiling water. Municipal water plants can utilize carbon filtration to remove microcystin from water. However, due to the nature of cyanobacterial blooms, filtration becomes more difficult when the water quality is low during large events. This is because the activated carbon filter becomes plugged with everything else in the water, which leaves consumers powerless in their ability to take action to prevent the consumption of microcystin-contaminated water.

It is important to reiterate that not all Microcystis and Planktothrix blooms are toxic, but current data has suggested that the frequency of toxic blooms has been on the rise (Hudnell, 2008). This change can be due to a variety of reasons, but one of the main causes to this phenomenon is anthropogenic impact. Global climate change leading to an increase in precipitation and fertilizer runoff foster an environment for algal blooms to occur. Additionally, older lakes can be more prone to severe events due to eutrophication, which is the natural aging process for fresh surface waters. Cultural eutrophication—human impact causing

eutrophication—increases the speed of the aging process. While certain human impacts are likely the cause of this phenomenon, exact sources cannot always be identified.

The most common methods for testing the presence of microcystin in a sample are biological assays and instrumental techniques. The enzyme-linked immunosorbent assay (ELISA) is a biological assay that utilizes an antibody selected for an antigen present on microcystin (Carmichael and An, 1999). An instrumental technique commonly used to detect microcystin is HPLC, high-performance liquid chromatography, which separates molecules by polarity (El Semary, 2010). Eluted components in HPLC can also be analyzed by their UV-Vis spectrum for characteristic peaks. This method can take hours to days to complete, as it requires samples collected at the site to be transported back to the laboratory before analysis.

Biosensors

Biosensors are a class of analytical devices made of biological materials that have the ability to signal the presence of a chemical, or to change of composition of a solution. This is often due to some sort of binding activity that allows for detection. The most common materials used for the sensitive biological component are tissues, microorganisms, enzymes, antibodies, and nucleic acid. Biosensors have current applications in glucose monitoring, ozone detection, industrial food processes, etc. Biosensors are desirable compared to instrumentation due to their ease of use, speed, and low cost; additionally, methods that utilize biosensors are often portable, which can greatly increase the efficiency of the analysis.

ELISA.

Enzyme-linked immunosorbent assay (ELISA) is a popular analytical technique in wet biochemistry laboratories. It is commonly used to detect and quantify a variety of biologically relevant molecules such as hormones, proteins, peptides, and antibodies. There are several ELISA methods, as depicted in **Figure 3.**, though the basic principles still apply across all techniques. Each method is plate-based, meaning that the biosensor component is attached to a solid surface. The target is detected by antibody

recognition. Coloring agents turn these recognition events into a visible color, which can be used to determine the concentration of the target in the sample. While ELISA is a common and effective method for detecting targets, utilizing the technique can be time consuming and not as cost effective.

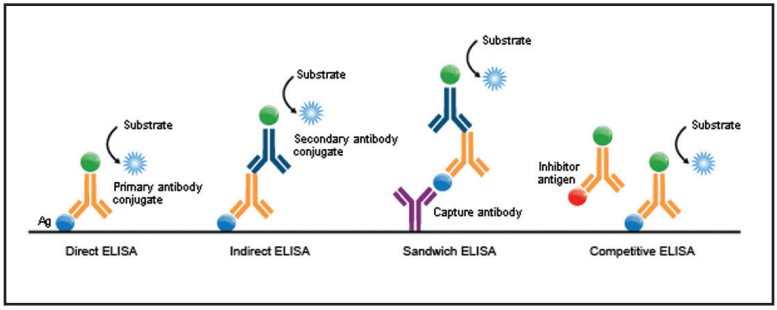

Figure 3. Various ELISA techniques.

Aptamers.

A class of biosensors made of a single-stranded oligonucleotide, much like antibodies, aptamers bind to their targets with high affinity. They have the ability to fold about a target molecule via hydrogen bonds, which allows them to be used to detect target molecules in complex mixtures (Upadhyay et al., 2013; Walter and Engelke, 2002).

Though antibody science is a well-known and highly developed field, aptamers have the potential to be more reliable than antibodies, due to their pH and temperature stability. Additionally, once an aptamer sequence is known, it can be easily synthesized in the lab and therefore does not suffer from batch-to-batch variability, which may occur with antibodies that require a host animal to facilitate formation.

Previous research has shown that aptamers have been successful in binding to targets of varying sizes, from large proteins to smaller antibiotics (He et al., 2012; Fredriksson et al. 2002). Our goal is to improve the method for selecting aptamers that have a high affinity and selectivity for small target compounds, specifically microcystin. This research hopes to employ a combination of techniques that will enable us to develop an aptamer for a small molecule, such as microcystin.

EXPERIMENT

CE-SELEX

SELEX

Systematic Evolution of Ligands by EXponential Enrichment (SELEX) is an *in vitro* process for aptamer selection. The SELEX process thins out a large library of random DNA sequences until only the most desirable sequences remain, as seen in **Figure 4**. This is accomplished by taking a very large library of random sequence DNA and incubating it with a target molecule. DNA that binds to the target can then be partitioned off from the fragments that did not bind. The bound sequences are then subjected to the polymerase chain reaction (PCR) in order to greatly increase the number of copies of the favorable DNA sequences for use in the next round of selection. This process continues until the aptamer with the highest affinity and selectivity for the target is found.

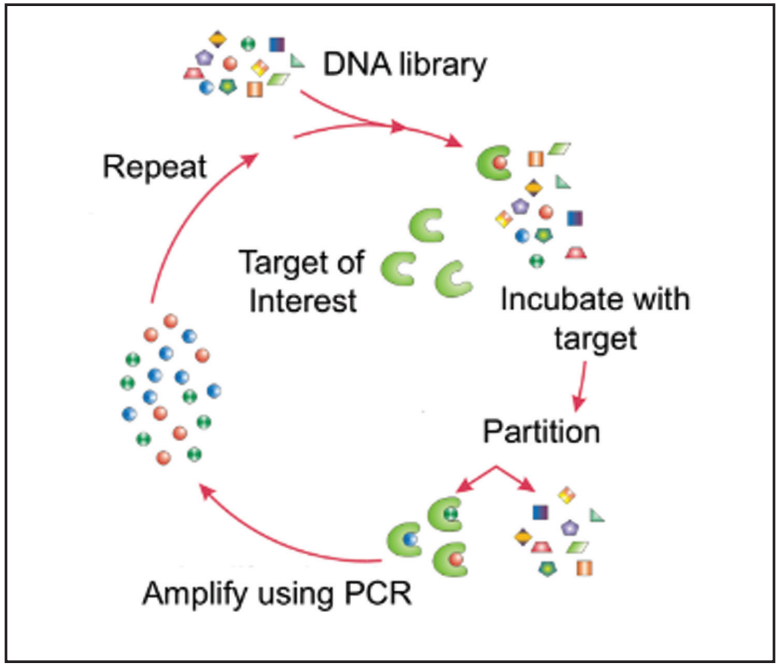

Figure 4. SELEX process of selecting desirable bound sequences from unbound sequences from a library.

Capillary electrophoresis

For the partitioning step of this process, we will be utilizing capillary electrophoresis (CE). When DNA binds to the target, both the size and charge is different, which allows partitioning between the bound and unbound sequences to occur. CE is a highly efficient separation method that works by separating the molecules based on their size-to-charge ratio. When DNA binds to a large target, such as a protein, the size-to-charge ratio of the DNA-protein complex is much different than that of the DNA alone, which allows partitioning between the bound and unbound sequences to occur.

Microcystin Modification for Selection

Cysteamine as a link

Microcystin-LR contains many shielded functional groups that make reacting the compound with other molecules difficult. Cysteamine can be used as a biologically compatible spacer between microcystin and another molecule. This reaction was carried out with a 2000–4000 molar excess cysteamine in carbonate buffer at a pH of ~9.6. The reaction was mixed at 30°C for 30 min. to an hour (Feng et al., 2012). The thiol group on cysteamine reacted with the alkene as depicted in **Figure 5**.

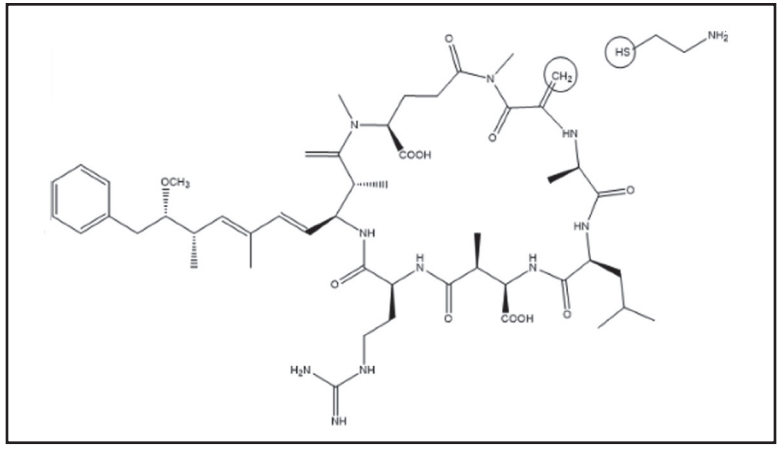

Figure 5. Microcystin and cysteamine with reactive functional groups circled.

Upon completion of the reaction, the reaction solution undergoes extraction to separate the product from excess cysteamine. To detect the presence of unreacted cysteamine, gold nanoparticles were used during column rinsing step, as gold and thiol groups have an extraordinarily strong interaction. Gold nanoparticles are a deep red color in solution, and when in the presence of the thiol groups on the unreacted cysteamine, the solution immediately turns dark blue. Excess cysteamine was considered completely removed when no additional blue color was observed. The extraction solution, 100% acetic acid and 0.1% TFA, was evaporated off with rotovap, taking care to place potassium carbonate in a bumper bulb to neutralize the TFA vapor. Cysteamine attached to the microcystin can facilitate a reaction with a larger molecule, effectively increasing the size-to-charge ratio of the target molecule. This increase in electrophoretic mobility allows for a larger difference in migration time between the DNA bound to the target and the DNA that did not bind.

Synthesis of Concepts

CE-SELEX has only been very successful when the target is a fairly large molecule, such as a protein. The target used in this research, microcystin toxin, is much smaller. CE-SELEX is difficult to achieve with small particles because DNA is a very large compound and the molecular targets in question are extremely small in comparison. This makes it very difficult to use CE to separate the bound DNA from the unbounded DNA, since the DNA-target complex is not much different in size and charge compared to the unbound DNA. To solve this problem, and make separation using CE possible, we can modify the electrophoretic mobility of the microcystin by conjugating the small molecule to a larger molecule. This effectively makes the target larger, and thus the DNA that binds to the target-complex will be more easily partitioned from the DNA that does not bind (White et al., 2010). The larger molecule facilitates an increase in size and charge to allow for partitioning from the DNA that does not bind to the target.

Once the microcystin has been successfully conjugated to the larger molecules, we can use it for CE-SELEX. This is done by taking random sequence (RS-60) DNA and incubating it with the microcystin conjugate. This solution is injected into a CE system. Running a buffer through the capillary, to allow the sample to run from the injection side to the collection side, carries out the collection. Both the DNA library and the targets are fluorescently tagged, and since they fluoresce at different wavelengths, separation can be monitored visually by shining lasers of different wavelengths at the capillary; when the targets and DNA pass by the laser and fluoresce, a detector measures the relative intensity and plots it against migration time. **Figure 6.** depicts how we are able to discern when to start and stop collection; because the DNA-target has a more positive charge than the unbound DNA, it will move faster through the capillary. This will be recognized by being the first peak with a significant amount of abundance.

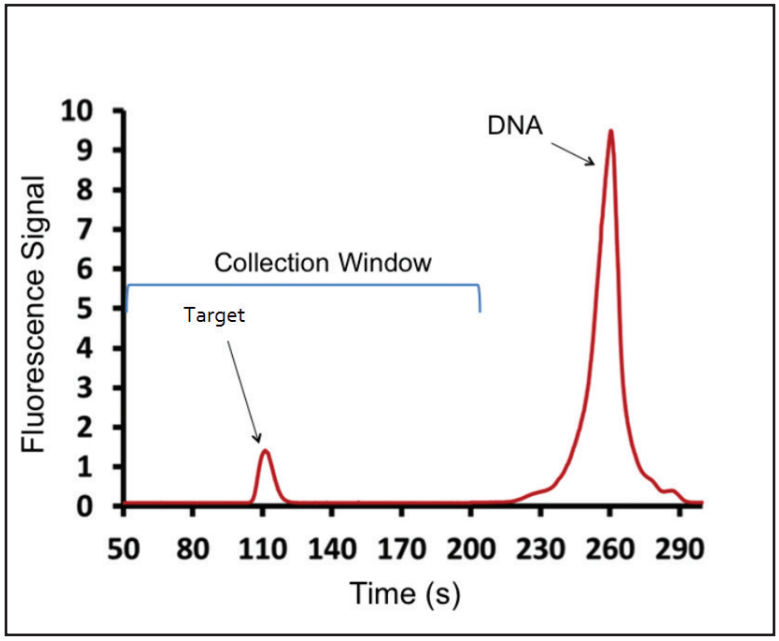

Figure 6. Electropherogram representing the difference in migration time between the target with bound DNA and unbound DNA. The collection window cuts off unbound DNA to ensure that only bound sequences are being selected.

When the complex has migrated all the way through the capillary, collection stops and the RS-60 DNA that did not bind is washed out of the capillary. This unbound DNA shows another much larger peak at longer migration times. The bound sequences are then put through PCR to increase their abundances. This results in a new, enriched library that will be put through another round of SELEX. After a few rounds of SELEX, the remaining DNA should be the most strongly binding sequences. Negative selection is run against the conjugate molecule to which the target was attached in order to ensure the aptamers remaining have a high affinity for the target, rather than the conjugate molecule.

CONCLUSION

SELEX is a well-established method that has mainly been used for large targets, such as proteins. Because the goal is to select for very small targets using a target-conjugate, we are approaching the problem in a novel way. This research will help present more efficient methods for selecting aptamers for small targets and is the first step in the development of aptamer-based biosensors for water quality testing. Not only can this research be applied to environmental targets, but it may also be applied to other areas of research, including physiology and medicine. There are many harmful environmental molecules; if this method is successful, there could be an aptamer for each one.

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