December 2, 2010 Linyi Gao

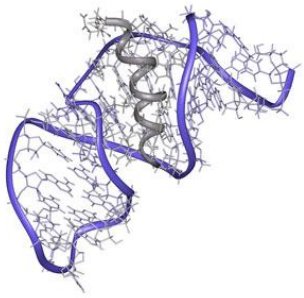
Narrative Slide Annotations

Drug Discovery with SELEX: Systematic Evolution of Ligands by Exponential Enrichment

Introduction

For an oligonucleotide of length *n*, the number of possible sequences is 4^n, which exceeds one trillion for $n = 20$.

Nucleotides (*n*)	Number of possible sequences (4^n)
5	1,024
10	1,048,576
15	1,073,741,824
20	**1,099,511,627,776**
25	1,125,899,906,842,624
40	1,208,925,819,614,629,174,706,176



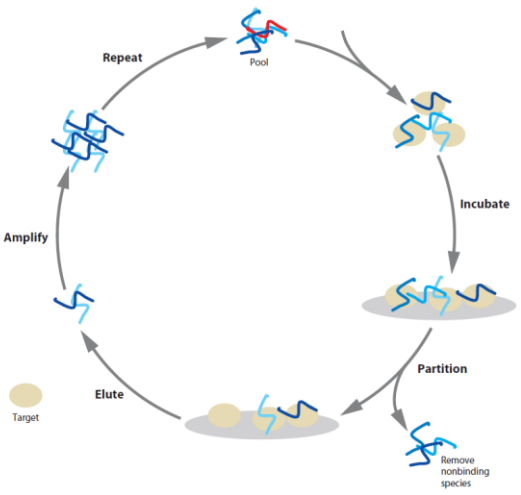
Most of these sequence possibilities probably never occur in nature and are never observed until synthesized chemically. Each sequence has a characteristic three-dimensional structure. Is it possible that a small number of them display very special properties?

There is reason to believe that a rare few among this multitude of possibilities may have special qualities. In cells, RNA composes tRNAs, siRNAs, ribosomes, and ribozymes, which have specific affinities for target substrates and perform specific actions. Could there not, by chance, be a few among the trillions that also have specific affinities for particular target molecules?

Among the many possible sequences, researchers have discovered that a select few have three-dimensional structures that allow them to bind very tightly and specifically to target proteins. These oligonucleotides are called aptamers. Each target protein likely has a small set of DNA or RNA aptamers that bind to it. Aptamers naturally lend themselves as drug candidates (Barbas 2009; NG 2006)—if an aptamer can be found that disrupts a protein interaction involved in a disease, it may be possible to administer that aptamer to the disease site to treat or cure the disease. Thus, it is of therapeutic interest to identify aptamers specific for disease-related proteins.

However, it is generally not possible to deduce, simply from analyzing the structure of the target protein, what the sequence of one of its aptamers will be. Moreover, aptamers are rare, occurring at a frequency of only a few in 10^{13} to 10^{15}. Identifying a desired aptamer seems like finding a needle in a large haystack. How can we find and make one? This is the objective of SELEX.

The key is to simultaneously screen most of the possible sequences for binding activity by mixing a random pool (library) of a large number of RNA sequences with the target protein in the same container all at the same time. Most RNAs will not bind the protein, but a small number will. The unbound RNAs can then be washed way, keeping only the desired aptamers. This is analogous to sifting sand in one's hands. Even though the sand is composed of many particles of silica, dust, rocks, shells, and other material, only the larger particles will get stuck between the fingers as the rest of the sand passes through. These larger particles are the aptamers. Although the precise composition of the sand is not known, the aptamers are readily isolated. Moreover, sifting all the sand ensures that no aptamer will be overlooked.



The canonical SELEX cycle (Bouchard et al. 2010) proceeds through 8-20 cycles of selection, isolation, and amplification. The cycle begins with a large random oligonucleotide library of 10^{13} to 10^{15} RNAs that consist of fixed sequences at their 5' and 3' ends and a variable sequence of 30-40 nucleotides in the middle (Bouchard 2010). The function of the fixed sequences is to allow PCR amplification of all the different reverse-transcribed DNA molecules simultaneously with the same primers. These RNAs are incubated together *in vitro* with the

purified protein target. Most of the RNAs will not bind to the protein, and these are washed away in the subsequent step. A small number of RNAs exhibit some affinity for the protein. These are eluted and isolated, reverse transcribed into DNA, PCR amplified, and transcribed back into RNA. This new RNA pool has now been enriched in the RNAs that bind the protein. In particular, the number of distinct sequences in the enriched RNA pool has decreased, but the quantity of each has increased. Using this new RNA pool, the SELEX cycle is repeated, and so on for 8-20 times. Each time the stringency of protein binding is increased so that fewer and fewer RNAs are collected, but the ones that are collected have the greatest binding affinity. By the end of 8-20 cycles, a small number of high-affinity aptamers are isolated. At this point, the aptamers can be sequenced, characterized, and further chemically modified.

SELEX: A Remarkable Feat

SELEX is a remarkable process. Such a massive screen requires that a large random pool that contains all the aptamers must made in a short time, stored in manageable space, cost a reasonable amount, and the vast majority of them discarded without extravagant waste. For ordinary objects, a collection of a few hundred trillion would be unimaginably large and difficult to make and manage. Yet for oligonucleotide aptamers, these four challenges can be overcome. Rapid synthesis (e.g. 1000 nucleotides/sec/DNA polymerase) can be achieved with DNA and RNA polymerase; the building blocks (A, T, C, G, and U) are readily obtained and abundant; and each aptamer is only a few nanometers in size, facilitating storage and disposal. SELEX takes advantage of these natural properties to enable massive screening. These advantages are summarized below:

Issue	Macroscopic (e.g. Legos)	Biochemical
Time	Slow, time-consuming to build	Fast—we can take advantage of the most efficient builders (RNA pol., DNA pol.)
Money &	$$$ required, wasteful	Free! And abundant (A, T, C, G)

Resources		
Space	Need lots for storage	Small—trillions in a test tube
	→Not possible	→SELEX

A Promising Method for Drug Discovery

SELEX is a very general method for drug discovery. In theory, any disease which relies on the action of a disease-specific signaling protein can be slowed down or stopped by administering the appropriate aptamer that binds tightly and specifically to that protein. This includes cancers, toxins, viruses, and pathogenic microorganisms (Khati 2010). Thus, in theory, SELEX could identify treatments for all these diseases. Moreover, SELEX is theoretically ideal for drug discovery because:

(1) It does not require knowledge of the sequences or structures of the components of either the random library or any of the enriched RNA pools. Sequence determination and structure characterization is performed only at the end of SELEX on the small number of aptamers that have been isolated.

(2) It requires no knowledge of the mechanism of aptamer binding and no prediction of aptamer sequence to narrow down the screen pool.

(3) The random library can be made arbitrarily large and inclusive of all sequence possibilities. Though more complex synthetic targets may be inaccessible in some assays, all oligonucleotide sequences are accessible through SELEX.

(4) It does not require extensive knowledge of the disease mechanism. For ordinary SELEX, only a target protein must be identified and isolated. For cell-SELEX (Pu et al. 2010), one does not even need to know which membrane proteins are different between disease cells and healthy cells—cell-SELEX can in fact be used to identify both the disease-specific protein markers (Ulrich 2009) and the appropriate aptamer simultaneously.

This ensures that, in theory, the search for aptamers is exhaustive—that is, our ability to find desired aptamers is limited only by their existence, not by our having sufficient knowledge to predict their binding or adequate resources to identify them.

Can SELEX be applied to molecules other than DNA and RNA?

The ability to extending a SELEX-like process of selection-isolation-amplification to molecules other than oligonucleotides depends on at least four criteria:

(1) We must be able to quickly and efficiently synthesize the large random library. For oligonucleotides, this is accomplished by a synthesizer; for small molecules, for instance, combinatorial chemistry provides a route.

(2) The large random library must be able to be assayed in the same container (test tube, filter, etc.) such that it does not damage itself through chemical reactions.

(3) Newly formed complexes between the target protein and a component of the random library must remain stable (i.e. not be interfered with by other components of the library) until isolation.

(4) After isolating the bound aptamers, we must be able to quickly and cleanly amplify them without any knowledge of their identity.

The fourth criterion perhaps provides the most difficult barrier toward extending SELEX to other classes of molecules. For DNA and RNA, we have remarkable tools for amplification—DNA polymerase, RNA polymerase, and reverse transcriptase. For molecules other than oligonucleotides, however, amplification is very difficult. For example, although SELEX is compatible with polypeptides through the first three criteria, it is probably not possible to amplify a polypeptide pool of unknown composition because there is no "reverse translase."

SELEX Aptamers as Drugs

SELEX aptamers are promising drug candidates. They are generally low in toxicity and non-immunogenic, making chronic systemic administration possible. However, several key problems of aptamers must be addressed (Bouchard 2010):

(1) Unmodified aptamers of pure DNA or RNA are metabolically unstable, undergoing degradation by nucleases before the aptamer can find and bind to the target.

(2) Non-protein-bound oligonucleotides may be rapidly eliminated through the kidneys and urine.

(3) Aptamers are rapidly distributed from the plasma and interstitial fluid into the tissues. This is undesirable because the action site of the aptamers is generally in the plasma and interstitial fluid compartments.

These concerns can be addressed by introducing chemical modifications to the aptamers, either after they have been identified or during the amplification process (Bouchard 2010). For example, a high molecular weight polyethylene glycol (PEG) moiety is often attached to the 3' or 5' –OH group of an aptamer after SELEX, improving its retention in the plasma. Also, the 2' –OH group of each nucleotide of RNA (or 2' –H of DNA) can be replaced with an $–OCH_3$ or –F to slow down nuclease degradation. This has recently been accomplished with a modified RNA polymerase, which can add 2' $–OCH_3$ nucleotides to the RNA chain (Bouchard 2010). Other site-specific modifications are also possible and may be the objective of next-generation aptamers.

Since SELEX is a relatively new method (first described in 1990), aptamer drugs have not yet become commonplace in the market. As of 2010, there is only one marketed aptamer drug, pegptanib (marked name Macugen®), which became FDA approved in December 2004 after ten years preclinical development (Ng 2006). Macugen® is an RNA aptamer that treats age-related macular

degeneration (AMD) that binding to vascular endothelial growth factor (VEGF)-165, which is a regulator of excessive blood vessel growth. Six other aptamer drugs are currently in clinical trials, including two others for macular degeneration, two for coronary artery bypass, one for thrombotic microangiopathy, and one for acute myeologenous leukemia (Bouchard 2010).

Overall, SELEX aptamers are a promising class of drugs whose potential has yet to be fully realized. The aptamer selection process uses a fruitful idea that takes advantage of several biochemical properties and tools. Through SELEX is a rich idea that can theoretically produce highly specific drugs for a wide of range of diseases, in practice many challenges must be overcome before these drugs can be introduced as viable therapies. In the future, overcoming these challenges and gaining more clinical familiarity with aptamer drugs should lead to the growth of aptamers as a rich source of disease treatments.

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Photo Credits

Front slide aptamer: http://aptamer.icmb.utexas.edu/images/aptamer-rre_rev.jpg

SELEX Cycle: Bouchard PR, Hutabarat RM, Thompson KM. Discovery and development of therapeutic aptamers. *Annu Rev Pharmacol Toxicol.* 2010; **50**:237–257.

Sand sifting through hand: http://www.istockphoto.com/file_thumbview_approve/9050527/2/istockphoto_9050527-sifting-sand-through-hands.jpg

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