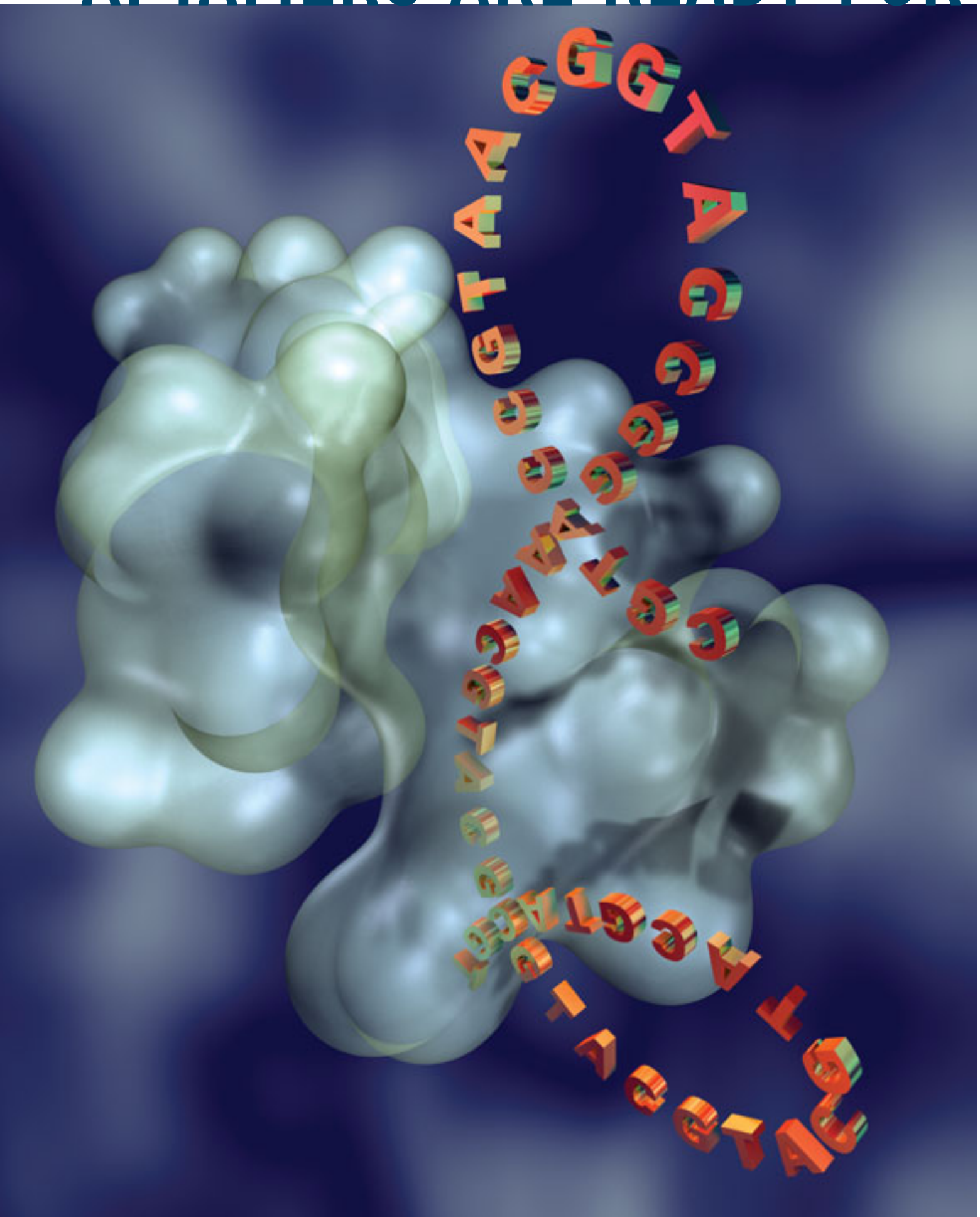
APTAMERS ARE READY FOR



THE SPOTLIGHT

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Aptamers are set for center stage. First discovered in 1990 (*1*, *2*), these 3-D structures of DNA or RNA are capable of binding to a variety of targets (*3*). Because of their binding abilities, aptamers are starting to appear in a series of applications—biosensors, imaging probes, MALDI targets, and drugs, to name a few. "I think [aptamers] were viewed as novelty molecules for a long time, and now they're beginning to show their stuff," says Andrew Ellington at the University of Texas at Austin. But aptamers do face some hurdles. To prove themselves worthy over the next few years, aptamers must be easier to produce, become more commercialized, and dislodge antibodies from their exalted position as ubiquitous affinity probes.

Although discovered 15 years ago, aptamers are now starting to show their versatility in roles as diverse as biosensors and drugs.

Antibodies vs aptamers

Aptamers inevitably are compared with, and compete against, antibodies because both are involved in specific recognition of molecules. Antibodies got a head start in the 1950s and are now deeply entrenched in labs. Getting scientists outside the aptamer community to switch from their tried-and-true antibodies to aptamers will take quite a bit of coaxing. Robert Kennedy at the University of Michigan, Ann Arbor, says people will probably only work with aptamers "if there are things that an aptamer can do that you would be hard-pressed to do with an antibody."

Fans of aptamers argue that there are a host of reasons for using aptamers over antibodies. For one, antibodies must be produced biologically, which involves messy cell cultures and lab animals. Jack Szostak at Harvard Medical School says, "Aptamers are small nucleic acids that can be selected under very general conditions and produced by chemical synthesis, without the constraints imposed by having to be selected or produced in a living organism."

Systematic evolution of ligands by exponential enrichment (SELEX) is the original method for producing aptamers in vitro. It consists of exponentially selecting and amplifying aptamers from a combinatorial library of 10^{15}–10^{18} oligonucleotide molecules. Once the sequence of a particular DNA aptamer is known, it is easy and inexpensive to create

more of the aptamer in a DNA synthesizer. (RNA aptamers are generally harder and more expensive to generate.)

Because aptamers are chemically synthesized, additional chemistries can be tacked on, usually without a loss in function, giving aptamers a leg up over antibodies. Chemical groups can be attached to the ends of aptamers to increase their life spans in the bloodstream, target them to particular locations, or help immobilize them to a surface. And immobilizing aptamers is a lot easier than immobilizing antibodies, because there aren't problems with potential loss of function.

In addition, Ciara O'Sullivan at the Universitat Rovira i Virgili (Spain) points out that aptamers can recover from exposure to undesirable conditions. She says that the in vitro selection process for aptamers can be carried out under conditions akin to those used in the assay for which the aptamer is being developed. This means the aptamer will maintain its structure and function in the final assay and not fall apart, a problem that haunts finicky antibodies. Aptamers can also be used for the development of sandwich assays for small molecules or modified into a molecular beacon format (a DNA strand with a fluorescent tag and a quencher) for reagentless assays. Antibodies cannot meet these challenges.

Weihong Tan at the University of Florida thinks the molecular weight of aptamers, which is low compared with that of antibodies, is helpful in cell studies. "When the molecular weight is less, it [helps] with tissue penetration and shorter residence time in blood," he says. And Linda McGown at the Rensselaer Polytechnic Institute says that aptamers "have a smaller footprint when you attach them to a surface [and] get a higher binding density, which in some cases may be important."

When it comes to debating the use of aptamers over antibodies for diagnostic applications, Larry Gold of SomaLogic says if he thought ELISAs could be easily scaled up for large diagnostic tests, he wouldn't have worked with aptamers. But because antibody-based sandwich assays present inherent limits to scalability, Gold says it's worthwhile to pursue aptamer-based arrays for diagnostic applications.

One criticism of aptamers is that they are nucleic acids and therefore are highly susceptible to degradation by nucleases. Researchers have solved this problem by using modified bases, such as 2′-O-methyl and 2′-fluoro derivatives, to make the resulting aptamer backbones resistant to degradation. Other drawbacks of aptamers include the lack of functionality within the basic building blocks. Proteins have a repertoire of 20 amino acids; aptamers can only use C, A, G, and T (or U in the case of RNA aptamers).

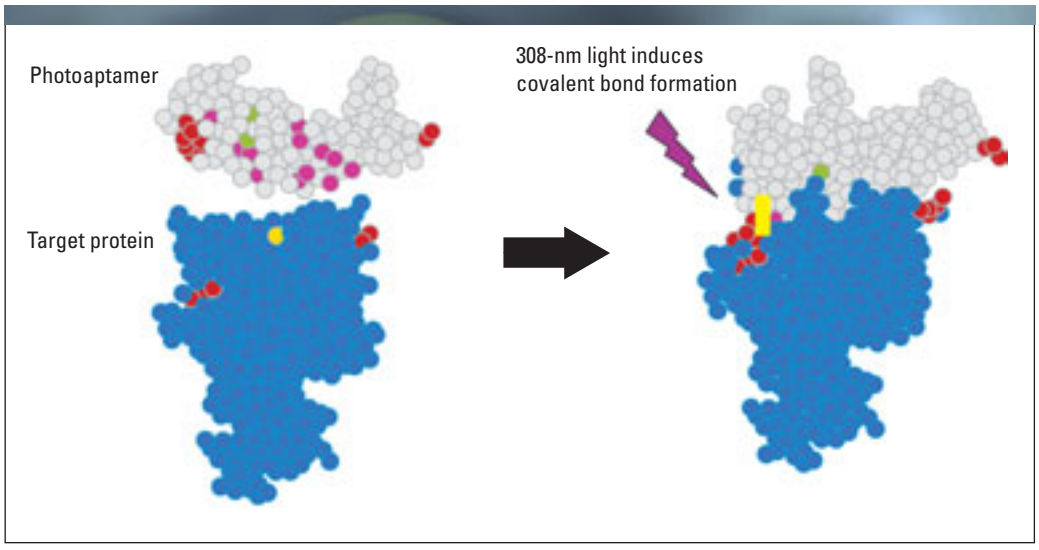
Experts are hoping that the landscape for aptamers will change as companies, such as AptaRes and NascaCell, spring up to produce customized molecules against targets provided by customers. The hope is that as the capabilities of aptamers gain more widespread recognition, the demand for them will drive the market and lower the cost of finding aptamers for particular targets.

Putting aptamers to work

When it comes to applications, the experts have a plethora of ideas. SomaLogic is developing aptamer proteomic chips as diagnostic tools that screen for biomarkers in serum. Ellington's group has demonstrated that aptamers can be immobilized on beads, introduced onto a sensor array, and used for the detection and quantitation of proteins (*4*). McGown and colleagues have used a thrombin DNA aptamer as a MALDI capture matrix to selectively capture thrombin for TOFMS (*5*). And Kennedy's group showed that DNA aptamers immobilized on chromatographic supports can selectively bind and separate adenosine monophosphate (AMP), cyclic-AMP, adenosine diphosphate, adenosine triphosphate, NAD^+, and adenosine from mixtures as complex as tissue extracts (*6*).

Tan and Yingfu Li at McMaster University are both using aptamers as signaling probes in their labs. Tan has modified molecular beacons to form aptamers. "An aptamer can recognize a protein, but a molecular beacon aptamer can recognize a protein [and] at the same time give you a signal," explains Tan. Although fluorescent tags are more common, Tan says his lab is also developing radioactive tags for its molecular beacon aptamers.

Li and his colleagues use a different mechanism for signaling. When not bound to its target, the aptamer forms a duplex structure with a complementary oligonucleotide that is modified with a quencher. Thus, in the duplex form, the aptamer's fluorophore is quenched. When the protein target appears, the aptamer releases the complementary oligonucleotide, takes on



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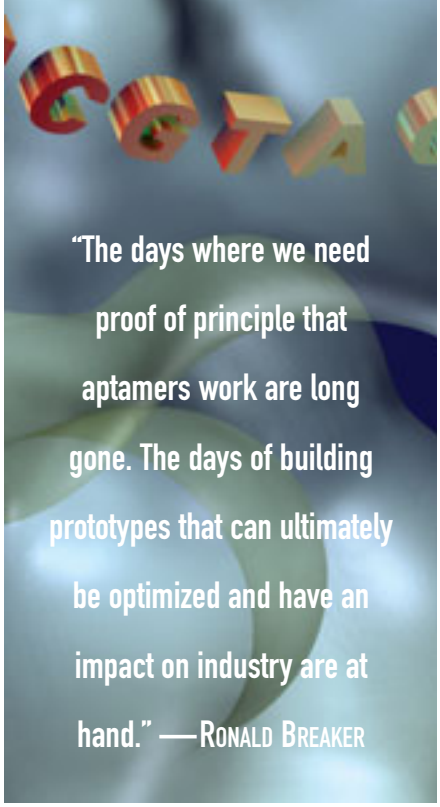
During photoSELEX, the bromodeoxyuracil in the photoaptamer cross-links to an electron-rich amino acid in a target protein in the presence of UV light.



its 3-D structure, and preferentially binds the target. The fluorophore on the aptamer then generates a signal to show binding.

Perhaps the biggest buzz of excitement over aptamers is in the therapeutics arena. Eyetech, in collaboration with Pfizer, is developing Macugen, an aptamer-based drug for the treatment of age-related macular degeneration (AMD). The aptamer binds and inhibits a particular isoform of vascular epidermal growth factor, a protein known to play a critical role in AMD. Upon approval from the U.S. Food and Drug Administration, Macugen will be the first aptamer to be commercialized for therapeutic use.

Bruce Sullenger and colleagues at Duke University Medical Center, the University of Michigan Medical Center, and Regado Biosciences have reported the use of aptamers as anticoagulants in animal models (*7*). Their anticoagulant aptamer's function can be reversed by the addition of an RNA antidote that disrupts the aptamer structure. This drug–antidote pair could give clinicians control over the aptamer's function once it has been administered to a patient.

Sullenger states that aptamers have to make their special mark as drugs. "You don't want [aptamers] to be 'me-too' drugs. You want to take advantage of their properties that make them unique. One thing that is unique about aptamers is that it's easy to rationally design antidotes to them. That's not true with antibodies and small molecules."

The development of aptamer-based drugs is extremely beneficial for the field. Sergey Krylov at York University (Canada) says, "It shows potential investors that there is a drug [like Macugen] based on aptamers. The big money stayed away from [aptamers] because there was no demonstration of something practical." Besides Eyetech and Regado Biosciences, other companies, such as Archemix, are also pushing for aptamer therapeutics.

Nature does it better

In the early days of aptamers, researchers were hounded at conferences by the following question: If aptamers are so wonderful and have so many advantages over proteins, why doesn't nature use them?

Ronald Breaker and his colleagues at Yale University answered the question with an elegant demonstration of naturally occurring aptamers (*8*). Riboswitches consist of two domains, an aptamer domain and a functional domain that controls gene expression. When a ligand binds to the aptamer, the aptamer undergoes a conformational change that results in either increased or decreased gene expression. Breaker's lab even found an example in which two aptamers worked back to back in a cooperative fashion in a riboswitch. Both aptamers bound glycine, and the binding of glycine at one aptamer improved the binding affinity for the amino acid at the second aptamer (*9*). Breaker says, "It's like hemoglobin, but only made of RNA."

The discovery of these in vivo aptamers presents some new applications. Breaker says that aptamers can now be considered as in vivo drug targets; derivatives of target molecules could be designed to trick riboswitches into artificially turning gene expression off or on.

The demonstration of in vivo aptamers also has implications for how in vitro aptamers can be created. Ellington says, "I think we've settled for simple [in vitro] structures for too long. Now we're probably going to go about trying to make ever more complex structures, just as nature has in the past."

But there might be some challenges in trying to increase the length of the in vitro aptamers to catch up with the longer lengths of in vivo ones. Michael Bowser at the University of Minnesota points out that limits exist to the lengths of oligonucleotides made by DNA synthesizers. Beyond 100–120 bases, the purity of the synthesis declines, the yield drops off significantly, and the synthesis becomes more expensive.

Tinkering with SELEX

Some researchers argue that the process of in vitro production of aptamers has been the bottleneck in the field and has prevented widespread use of aptamers. A push is on now to streamline SELEX to make it simpler and quicker. In its traditional form, the process takes 15–18 rounds of selection and amplification to identify an aptamer. Each round takes 2–3 days to complete. Further, the first few rounds are conducted blindly because the number of aptamer molecules is so low that they cannot be detected.

"It's sociologically distressing to watch students and postdocs do these kinds of procedures, because they have to be nursed along!" says Gerald Joyce at the Scripps Research Institute. "[Aptamers] are very special, rare molecules in a population. They are literally one in a billion. It takes a while to harvest molecules like that."

Conventionally, SELEX is carried out using either a filter assay or affinity chromatography. For the filter assay, the oligonucleotide library and target are mixed together and run through a filter. The sequences that bind to the target can't pass through the filter, and the ones that don't bind do pass through.

For the affinity chromatography approach, the target is attached to an affinity column and the oligonucleotide library is passed over the column. Those sequences that can bind to the target are retained on the column, while the remaining oligonucleotides pass out of the column.

The Ellington lab has pushed to automate the SELEX process. They have combined liquid handling systems and other accessories with a PCR machine so that a human being becomes unnecessary for most of the process. With the automated system, the usual 3-day period to manually do a single round of selection is replaced by 6 rounds of selection in 22 h. The system is even ca-

pable of simultaneously selecting aptamers to eight different targets in a given run.

Some labs, like those of Krylov and Bowser, are now using CE to reduce the number of selection cycles. Bowser and colleagues' technique, called CE-SELEX, involves incubating a limited amount of protein target with the oligonucleotide library. The mobility of free nucleic acids in CE is significantly different from that of those bound to protein. The method uses this difference in mobility to separate aptamers from non-aptamers, and the selection for an aptamer takes as few as two rounds. Bowser states the advantages of CE-SELEX: "The selection takes place free in solution . . . and the separation power of CE is much higher. The first advantage limits nonspecific interactions. The second reduces the number of rounds of selection necessary."

Krylov and colleagues have developed what they call a Swiss Army knife approach to selecting aptamers. Krylov says their method can select aptamers in a single cycle. The investigators use CE to do three things: partition aptamer–protein complexes from unbound oligonucleotides, measure equilibrium binding constants of the aptamers to their targets, and apply the kinetic profile to select for those aptamers within a predefined range of binding parameters.

Even within the conventional SELEX method, researchers are approaching aptamer production with some planned design to only pick those aptamers with highly defined characteristics. Gold and colleagues at SomaLogic use a designed selection process called PhotoSELEX. The investigators introduce bromodeoxyuracil into the otherwise normal oligonucleotides of a library. They then proceed with the conventional selection cycle, but with the additional requirement that the bromodeoxyuracil in the photoaptamer is within an angstrom or two of an electron-rich amino acid in the target protein.

To select for the photoaptamer–target pairs that meet the requirement, UV light is briefly shone on the mixture. The photoaptamer–target pairs that have the bromodeoxyuracil adjacent to a tyrosine, or a similarly electron-rich amino acid, at the time the UV light is turned on become covalently cross-linked to each other. Gold says that imposing the photocross-linking requirement increases specificity by ~1000-fold. When used in single-photoaptamer assays (which avoid the limitations of antibody-based sandwich assays), stringent washes remove excess or nonspecific proteins once the photoaptamer is covalently bound to its target protein, a critical advantage over sandwich assays.

Positive and negative selections also help researchers sculpt aptamers and make the molecules highly specific. Tan's group is selecting a panel of aptamers only toward tumor cells. An oligonucleotide library is incubated with a tumor cell; those oligonucleotides bound to the tumor cells are eluted and amplified by PCR, and the amplified pieces are then incubated with a normal cell. Those pieces that bind both to the tumor cell and to the normal cell are discarded. Hence, only the oligonucleotide pieces that bind to the tumor cells are chosen for further rounds of selection and amplification. Tan says the use of cell-based aptamers has the potential to generate a series of molecular probes for tumor diagnosis and, eventually, therapy. O'Sullivan says her group is involved in a project that takes a similar approach to produce aptamers that discriminate between maternal and fetal cells in blood. The hope is to use those aptamers for the isolation of fetal cells in maternal blood as part of a noninvasive prenatal diagnostic test that will some day make amniocentesis unnecessary.

What's next?

O'Sullivan cautions that the cart must not be placed before the horse. A systematic analysis of aptamer structures and behaviors is needed to understand whether any overarching rules govern aptamer function in assays, as is the case with antibodies. "We're trying to see if there are any universal rules," O'Sullivan says. "Is every aptamer completely different? No one has systematically looked at what parameters are important for optimal aptamer performance in assays and detection systems."

McGown points out that only a limited number of aptamers are currently available, making it difficult to press ahead with more general applications. She says, "I think one of the obstacles to further developments has been the lack of aptamers to broad ranges of targets. There have been a number of aptamers generated primarily to targets that are more therapeutic rather than of general analytical interest."

The feeling is that more investment is needed to help the community grow so that more aptamers to various targets can be produced. Greater investment will encourage more researchers to develop applications that take advantage of the versatility of aptamers. "The biologists have demonstrated that aptamers can do a lot of interesting things. Now it's up to people who are more focused on technology development to get involved," says Breaker. "The days where we need proof of principle that aptamers work are long gone. The days of building prototypes that can ultimately be optimized and have an impact on industry are at hand."

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