Aptamer-Mediated Delivery and Cell-Targeting Aptamers: Room for Improvement

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Targeting cells with aptamers for the delivery of therapeutic cargoes, in particular oligonucleotides, represents one of the most exciting applications of the aptamer field. Perhaps nowhere has there been more excitement in the field than around the targeted delivery of siRNA or miRNA. However, when industry leaders in the field of siRNA delivery have tried to recapitulate aptamer-siRNA delivery results, they have failed. This problem stems from more than just the age-old problem of delivery to the cytoplasm, a challenge that has stymied the targeted delivery of therapeutic oligonucleotides since its inception. With aptamers, the problem is compounded further by the fact that many aptamers simply do not function as reported. This is distressing, as clearly, all published aptamers should be able to function as described. However, it is often challenging to recognize the details that might flag an unreliable aptamer from a viable one. As such, unreliable aptamers continue to be peer reviewed and published. We need to raise the bar and level of rigor in the field. Only then can we think about taking advantage of the unique attributes of these molecules and address the issues associated with their use as agents for targeted delivery.

Keywords: commentary, aptamer, cell surface, targeted delivery

A Brief Overview: Aptamer Potential and Setbacks

SINCE THEIR DISCOVERY [1,2], aptamers have held the promise of rapidly and easily generating synthetically tractable ligands for both diagnostic and therapeutic purposes. Sometimes, donning other names, including “nucleic acid ligands,” “oligobodies,” or “chemical antibodies,” for more than 25 years, these molecules have offered the promise of antibody-like function in a nonbiological, chemical package.

Over the last 10 years or so, the field of aptamers has grown from its initial focus targeting small molecules and soluble proteins, targets which gave rise to clinically relevant molecules such as the anti-vascular endothelial growth factor (VEGF) aptamer, pegaptanib (Macugen); the anti-platelet-derived growth factor (PDGF) aptamer, pegpleranib (Fovista); and the IXa aptamer, pegnivacogin (a component of Reg1), to include cell surface targets, an area in which our laboratory had been focused*.

As diagnostics targeting cell surface receptors, aptamers can serve as labeling agents to phenotype cells and tissues *ex vivo* or as capture agents to abet cell purifications in much the same capacity as antibody-based agents. As therapeutics

targeting cell surface receptors, an area that typically drums up significant excitement, aptamers can serve as direct inhibitors of receptor function (see e.g., Ref. [3]) or further engineered to activate function (see, e.g., Ref. [4]). The ability to engage cell surface targets has also led the field into the realm of targeted delivery of therapeutics, where the chemical nature of these molecules provides great advantages for developing drug conjugates, since conjugation of nucleic acids to small molecule drugs, proteins toxins, liposomes, or other nanoparticles is relatively straightforward.

Aptamers that bind cell surface receptors are readily endocytosed. As such, cargoes, such as membrane-permeable small-molecule drugs (for some examples see [5–8]), protein toxins which possess an innate ability to enter the cytoplasm [9], or nanoparticles such as liposomes that are capable of fusing with membranes and releasing at least a portion of their cargoes to the cytoplasm (for some examples see [10,11]) can all be delivered. However, as oligonucleotides themselves possess no innate mechanisms for endosomal escape, it is hard to understand how aptamers directly conjugated to therapeutic oligonucleotides, such as siRNA or miRNA, can deliver cargo to the cytoplasm. Indeed, when industry leaders in the field of siRNA delivery have tried to recapitulate aptamer-siRNA delivery results, they have failed and have now largely turned their backs on the technology.

These failures add insult to injury in a field where aptamers in general are being questioned and not just for delivery

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issues. There have been doubts raised as to whether aptamers can even be used as drugs at all. Indeed, despite the promise of being “antibody-like,” aptamers only have a single champion: the Food and Drug Administration (FDA) approved Macugen, which is essentially not used in the clinic anymore. Approved for clinical use in December of 2004 and specific for the VEGFA isoform, VEGF-165, this molecule, the first-in-class anti-VEGF therapy for the treatment for age-related macular degeneration (AMD), looked destined to be a blockbuster. However, shortly thereafter, the off-label use of the anti-VEGF cancer therapeutic, Avastin, an antibody that inhibits all isoforms of VEGFA, proved safe and effective [12]. Since then, Avastin, as well as the approval of other protein-based pan-VEGF inhibitors designed for ocular use (Lucentis and Eylea), which yield superior clinical results compared with Macugen, have relegated the aptamer drug to the sideline.

Two other high-profile aptamers have recently also failed to pass clinical trials, although it is important to note that the trials failed not because of the aptamers themselves. In the case of Fovista (anti-PDGF), the aptamer was tested in combination with the two most common anti-VEGF therapies for the treatment of AMD (Eylea and Lucentis) and showed no improvement over standard of care during the Phase III trial [13]. In the case of Reg1 (an antifactor IXa aptamer paired with its complementary reversal agent, reverseran) which specifically inhibits and subsequently restores blood clotting, the failure came as a result of complications due to polyethylene glycol (PEG) allergies [14], which led to one death during the Phase III trial [15]. This, coupled with the fact that no improvement over standard of care was observed at the time of the adverse event, led to premature termination of the trial.

Clinical failures, however, should not be considered unusual; many more drugs fail clinical trials than pass them. In fact, all of the aforementioned aptamers (pegaptanib, pegpleranib, and pegnivacogin) work exceptionally well; they bind tightly and specifically to their molecular targets and inhibit function. But, such results certainly contribute to the miasma that surrounds the field. Perhaps more disappointing, however, is the lack of aptamer-based drug candidates even advancing to the clinic (reviewed in Ref. [16]). With the tremendous success of antibodies and antibody-based drugs in the clinic, Big Pharma has their champion, and aptamer development has been largely relegated to academic laboratories. Additionally, even though aptamers that have advanced to clinical testing had their origins in academic laboratories (e.g., pegnivacogin), the fact that the onus will be on more poorly funded academics to move the field forward does not bode well. With the growing lack of faith in aptamers and with only one aptamer drug in the clinic, the utility and future of these molecules remains largely in question. The lack of reproducibility observed with aptamer-siRNA/miRNA delivery simply makes this all the more worse.

The Problem of Making Reliable Aptamers

On paper, the SELEX process appears simple. However, the practice of aptamer development is, in fact, significantly harder than it appears. Selections can be plagued by a variety of artifacts as well as other complications, which can confound the development process.

We have spent considerable time trying to validate cell surface targeting aptamer function of molecules developed both in our laboratory as well as others. This is not something we sought out to do, but something we wound up having to do to find molecules which would work reliably and reproducibly. Many of the molecules we pulled from the literature are reported to be capable of inhibiting tumor growth directly or delivering various therapeutic cargoes to cells, including siRNA or miRNA. However, many of these molecules simply failed to function in our hands in a straightforward assay format: target binding as assessed by flow cytometry (article in preparation). Under our assay conditions (see Validation assays with proper controls section), these molecules simply do not engage their reported molecular targets on the cell surface. Thus, it is difficult to understand how they could deliver appreciable amounts of a therapeutic cargo. It does not, however, rule out some of the phenotypic effects (e.g., cell growth, tumor inhibition, etc.) ascribed to these molecules, which could potentially be triggered by transient or low-affinity interactions.

This is not to say all aptamers failed in our assays. Several we have developed, tested, or have taken from the literature and used for further development performed well with cytometry. These include molecules that target the human transferrin receptor [10,17], the epidermal growth factor receptor [6], the prostate-specific membrane antigen [9], and others (see e.g., molecules described or tested in [5,18,19]).

We have presented these data in various forms over the years to very mixed responses. Some have told us we were not doing our experiments correctly, while others have told us that they observed the same results (or lack thereof). Others have given us a pat on the back and thanked us for doing and saying what others would not. Most notably from all this feedback, however, is that for almost every molecule we have tested that did not work, someone has told us that it did not work for them either. Of course, it is hard to prove a negative result unambiguously, so in general, we like to say that the function of such molecules are simply, “not robust,” and that better results could be expected if better molecules were used. However, the point is the same: if the molecules cannot or do not function reliably in other people’s hands or in assays, other than those in the original publication, this is a problem. Moreover, we are fooling ourselves if we think that these unreliable molecules could one day be a drug.

When aptamer selections work, they work relatively quickly. The bulk of the time is actually spent performing downstream characterizations and assessing function. Unfortunately, many researchers focus heavily on the function and the phenotypic effects of selected molecules, not on more basic characterizations, such as evaluating direct target engagement *in vitro* and *in vivo*. One perhaps somewhat cynical view is that one needs efficacy data to convince their peers that something is exciting enough to fund. The unfortunate net result is that experimental results observed or performed in one laboratory are often not robust. This is not to say, however, that the initial experiments were wrong, but simply that the observed phenotypes may have been the consequence of other factors of the study design that might have been more apparent if better validation had been performed up front. Of course, such validations come at a cost, one that academics often cannot afford. Such problems persist in all fields, but if the aptamer field wants to make meaningful contributions toward therapeutic development, this needs to change.

To date, the only cell surface-targeting aptamer to enter a clinical trial has been AS1411, an aptamer designed to bind to nucleolin, expressed aberrantly on the surface of cancer cells [20]. In cells grown in culture, this aptamer demonstrated an antiproliferative effect. *In vivo*, the molecule showed good safety in a Phase I clinical trial and a modest effect in a Phase II trial when used in combination with cytarabine in patients with AML [21]. However, clinical development has not continued. Interestingly, it has not been this molecule's antiproliferative properties, which typically garner the most interest in the field. Instead, the molecule has become commonplace as a reagent for cancer cell targeting, engaging cell surface nucleolin. This is despite the fact that while the aptamer may bind nucleolin, the presence of this protein on the cell surface is not necessary for cell uptake. As reported by the group that discovered the molecule, aptamer cell binding and uptake are nucleolin independent, whereas the antiproliferative effect of the molecule relies on nucleolin [22]. Yet, many in the field seem to overlook this fact, and articles continue to abound that use AS1411 to target nucleolin-positive cancer cells for the specific delivery of therapeutic cargoes.

In many respects, the field of cell surface targeting with aptamers has become a bit of the Wild West, with laboratories that develop these molecules using a variety of different approaches to assess the binding function of their selected molecules. There is little, if any, standardization. Because of this, there is no easy way to compare or validate results between different laboratories. Other laboratories often just take these initial experiments/publications as true when performing subsequent studies in their own systems without performing their own additional validations. Often, they omit critical controls and just cite that this was done in earlier work.

Some groups will take an aptamer selected to bind a mouse protein and use it to show phenotypic responses or delivery of cargoes to human cells without validating crossreactivity. Like antibodies, aptamers are not guaranteed to crossreact. From our group's work over the years, we know that aptamers can be highly specific, with molecules selected against a human protein sometimes even failing to crossreact with monkey homologs, which possess ~95% pairwise identity [17]. Of course, sometimes binding sites of cell surface receptors are well conserved among species even though they possess a low sequence identity. Indeed, there are reports of some aptamers crossreacting with proteins that contain less than 40% pairwise identity [23].

All these slights are likely not due to laziness or oversight, but may simply be an issue of resources. Many laboratories simply do not have the time and/or money to revalidate aptamers for their own purposes. However, if the field is to continue, a little more care, from the initial selectionologist to the end user, could make a big difference.

How to Restore Trust in Aptamers

So what can be done about this problem? How can we ensure that aptamer selections in the future and the aptamers developed will indeed function as we want them to?

There is not a single or simple answer to these questions, especially since each aptamer, each selection, and each molecular target is unique and has its own sets of issues to ad-

dress. As noted above, the issue of reproducibility plagues all fields, including antibodies [24,25]. However, there are some overarching points that researchers in the field need to mind if aptamers are to reach their full potential. The details of each point could make for whole chapters themselves. If researchers could heed some of the following abridged points, however, it would help to ensure the production of more reliable, robust molecules.

Validation assays with proper controls

The first and most important step in moving the field forward is to standardize the proper and thorough testing of the candidate aptamer with any and all appropriate controls. Some of this is starting to happen, but critical controls are still often overlooked.

Over the years, our laboratory has advised other laboratories on numerous selections, and many researchers have been surprised at the differences they see with a couple of added controls, a few more replicates, or even having a colleague repeat an assay. Choosing the right method of testing should help ensure that the selected aptamer will perform as necessary further downstream.

For soluble proteins, nitrocellulose filter binding with radiolabeled aptamers has been the mainstay for *in vitro* binding validations [26]. However, such assays typically require significant amounts of target protein (which can be prohibitive for cell surface proteins produced from mammalian cells) and may not correlate to actual cell surface-binding capabilities.

For aptamers that target cell surface proteins, the selected molecules need to be capable of engaging their targets on actual cells. This engagement is critical, essential, and needs to be confirmed. If an aptamer was selected using only the soluble form of a protein, there is no guarantee it will bind to the receptor on the surface of a cell, a reason my group began performing selections using recombinant proteins in combination with target cells (see e.g., Ref. [10,27]). Even for aptamers selected against proteins displayed on the cell surface (whether overexpressed or naturally), control experiments must be performed to demonstrate that the aptamer function is due to that interaction. This could be through negative or knockout cell lines and supported by *in vitro* binding experiments.

Over the years, different groups have made use of a variety of different assays to assess cell binding, including the use of polymerase chain reaction (PCR) to detect enriched libraries, specific clones on cells, or the use of radioactive or fluorescently labeled aptamers to assess uptake using scintillation counters or fluorescence plate readers. The use of PCR to monitor binding and uptake is an extremely sensitive approach, which provides a direct link with the selection process, which itself relies on PCR. As such, care needs to be taken in interpreting the results, as they are not an independent means of assessing aptamer function, but simply an extension of the selection process.

A similar case can be made for assays performed using radioactively or fluorescently labeled aptamers on cells. For all of these assay formats that are performed on "bulk" cells, a potentially significant problem with cell binding analysis is that there is no way to ensure that the observed signal is due to interaction with the entire cell population or a subset of cells within the population. Indeed, dead and dying cells have

previously been demonstrated to be an issue, often nonspecifically taking in aptamer and thwarting target-specific enrichment in cell-based selections [28].

In general, we believe microscopy is a very poor primary method of validating target engagement on the cell surface. Microscopy is best suited as a supporting method and makes for excellent follow-up experiments after the aptamer has been validated by other methods, especially when used to demonstrate localization or colocalization within a cell. Quantitation of the fluorescence signals and statistical methods are often not performed. For qualitative images, signals as low as twofold over background can appear highly significant. Additionally, only a very small number of cells are typically interrogated or reported, thus further compromising the potential for accurate statistical analysis. Add to this a missing critical control or inappropriate use of fixative (see Some factors to consider that could significantly affect assay results section), and one can easily misinterpret microscopy data.

From our own experience, we highly recommend the use of flow cytometry for testing and validating aptamer function on cells; our methods for doing this have previously been published [10,17,27]. Flow cytometry provides a means to detect fluorescence linearly over 6 logs and implicitly provides a means to compare and statistically analyze fluorescence signals easily. Unlike the bulk cell-based assays mentioned above, flow cytometry allows one to interrogate each cell in a population individually, thus seeing whether the observed staining is specific to all of the cells in a sample or to a subpopulation of cells. When combined with an appropriate live/dead stain, one can easily exclude dead or dying cells. Moreover, numerous well-validated, fluorescently labeled antibodies are available that can be used as controls not only to validate target expression on the target cells, but also to compare staining levels, which are important features. For example, if your target-specific aptamer only shows a 2- to 3-fold signal over background staining, but a target-specific antibody shows a signal ~ 2 logs over background, something is likely amiss (note: while aptamers typically only bear a single fluorophore, antibodies are typically labeled with between three and five fluorophores, so comparisons need to take this into account).

Typically, we perform flow cytometry experiments in two assay formats. In the beginning, we mimic standard assay conditions used for antibody-based flow cytometry, an immunophenotyping experiment, which typically make use of tissue culture grade phosphate-buffered saline (DPBS) or other isotonic phosphate-buffered saline solutions (see e.g., Ref. [29]). As a standard, we supplement these buffers with Mg^{2+} and/or Ca^{2+} at physiologically relevant concentrations (~ 1 mM). Additionally, because aptamers can potentially misfold at low temperatures, we perform our experiments at room temperature, not on ice. Finally, because of the potential issues mentioned below, we avoid fixing the cells; the assay is performed on live cells.

Alternately, we perform assays on live cells in cell culture media with serum. This latter format provides a means to look at the cells in a medium that is fully supportive of growth and provides a means to look at the ability of the target cells to more naturally bind their target and be endocytosed (provided this is consistent with the target biology or aptamer function). Both the bound and endocytosed fluorescent ligands can be detected by cytometry, although no distinction between what is outside the cell and inside the cell can be

made without additional controls and experimentation. Before addition of fluorescently labeled aptamer, the medium is supplemented with blocking agents (e.g., ssDNA). Following incubation (typically an hour to minimize nonspecific uptake; (see Some factors to consider that could significantly affect assay results section), the cells are lifted from the plate (if adherent), washed, and analyzed by flow cytometry.

Reproducibility

Thankfully, more and more groups are repeating assays and publishing auxiliary assay results in supplemental data sections. However, it is still important enough for a point to be mentioned here that all validations and assays should be repeated multiple times and, ideally, by different researchers.

In our group, one of the laboratory mantras was, “love everyone, trust no one,” and we made a point of testing and retesting any aptamer we were interested in using, including our own. Any time a condition was to be changed, we would retest the aptamer to see how its function might have changed. We were also fortunate enough to have collaborators with whom we could send sequences to for testing, a luxury not every group has.

In addition, we typically use a combination of cell profiling, siRNA knockdown, and engineered cell lines to further validate target binding and expression. We have found this approach very useful, especially when target cells proved finicky or particularly sticky. For example, in the case of the prostate-specific membrane antigen (PSMA), in addition to looking at aptamer binding to LnCAP cells, which naturally expresses this protein target, we have also looked at expression on 22Rv1 cells, another prostate line known to naturally express PSMA [9]. Furthermore, we engineered multiple cell lines to ectopically express PSMA: PC3-PSMA [9], HeLa-PSMA [19], and B16-PSMA (unpublished data). In all cases, the aptamer binding to cells was tested by flow cytometry using the reported, target-specific aptamer, and a target-specific antibody, which provide means to profile target binding. When target-specific aptamer staining goes up, the target-specific antibody staining should behave similarly. Finally, to further ensure target specificity, we made use of siRNA to knockdown the target of interest. Target knockdown was then monitored by flow cytometry (e.g., see Ref. [17,27])

Some factors to consider that could significantly affect assay results

On the road to standardization, there are many factors that should be addressed in assaying aptamer function. Even with our favorite assay format, flow cytometry, there are a number of concerns that researchers need to be aware of to perform successful assays.

First, in our experience, fixing cells either before or after staining with a cell-targeting aptamer can alter results. Fixing cells before staining can result in some cell permeabilization that can confound results. Additionally, as common fixatives, such as formaldehyde, chemically modify proteins, the process may fundamentally alter the nature of the aptamer target. Thus, as a rule, we typically perform analysis on live (often arrested) cells, although we and our collaborators have previously used fixatives on tissues [19] and cells [30] subsequent to aptamer staining with success. However, care needs to be exercised as prolonged incubations postfixing can lead to diffusion of the aptamer. Also, such experiments were

performed only after the aptamers were thoroughly validated using other approaches.

Second, incubation times need to be limited. Incubating labeled aptamers with live cells for extended periods of time can lead to significant levels of nonspecific uptake (article in preparation). For this reason, we typically limit staining to ~1 h. Longer incubations are permissible, but background nonspecific staining will increase; the use of an appropriate negative control sequence and/or blocking agents to confirm specific uptake become even more important.

Third is the need for control sequences. Regardless of the assay format chosen, it is important to include nonspecific controls in any experiment. A scrambled, defined sequence or random pool sequences need to be included to control for nonspecific cell binding, which can be significant even in the presence of blocking agents (see Some factors that could significantly affect assay results section). The best nonspecific control is a nonfunctional variant of the aptamer being tested or simply a defined sequence with a nucleotide composition not too different from the sequencing being tested that has been demonstrated to give minimal background in the given assay conditions. Control sequences, which use different sugar chemistry or that significantly alter the aptamer nucleotide composition, are ill advised (e.g., a common control employed for experiments conducted with AS1411, an aptamer composed entirely of dG and dT residues, was to convert all of the dG positions to dC), as they result in fundamental and significant changes to the molecules, which could lead to misleading results. The use of an appropriate nonspecific or nontargeting control sequence cannot be overstated. The inclusion of such controls is not only essential for validating *in vitro* function on proteins and cells, but also critical for validating *in vivo* function, where the presence of an oligonucleotide itself may have some impact on the biology or the model being tested.

Fourth, we highly recommend using blocking agents, such as ssDNA, and especially in experiments that involve cell surfaces. Aptamers are polyanions and can thus readily interact with targets nonspecifically through charge-charge interactions, especially at high concentrations (>1 μ M). We have found that some cell types are significantly more prone to nonspecific uptake than others (article in preparation). For example, in the absence of blocking agents, LnCAP cells will nonspecifically bind nucleic acids quite well, whereas their non-PSMA-expressing counterpart, PC3 cells, do not. In the presence of blocking agents, the nonspecific binding and uptake between the two cell types is significantly diminished. Thus, anyone testing a putative PSMA-binding aptamer without blocking agents could easily be led to believe that a higher staining in the LnCAP cells compared with PC3 cells is an actual signal. Similar problems likely exist for other targets.

In our experience, the addition of a nonspecific polyanionic competitor such as single-stranded DNA is often sufficient to block a significant fraction of the nonspecific interactions observed when aptamers are incubated with cells. Typically, we use this blocking agent at a concentration of 1 mg/mL, at which the ssDNA significantly inhibits nonspecific binding and uptake by cells, but has essentially no effect on the binding of aptamers that are specific for cell surface targets. We have also made use of tRNA. When compared with DNA, RNA-based blocking agents lose their potency over time more quickly due to more rapid serum degradation (unpublished data). Dextran sulfate, a blocking agent

used by SomaLogic and a reagent used to prevent aggregation in cell culture [31], would also likely be a good choice. However, care should be taken with any additive to ensure the conditions and concentrations used do not affect cell health.

Finally, a note on fluorescence labeling: a number of groups use random chemical alkylation of the nucleobases as means to label aptamers for experiments (e.g., Mirus Label IT), a technique commonly used to track siRNA. Aptamers are not siRNA; they rely on their bases for function and, more than likely, will suffer if chemically modified. Aptamers can easily be labeled site specifically during chemical synthesis. It is one of the great features of these molecules. After all, this is the strategy that will be used to append any diagnostic or therapeutic cargo to these molecules during future clinical development. Alternately, fluorescent tags can be easily appended to almost any molecule by hybridization to a 3' or 5' extension. This later approach sometimes raises concerns that the extension will interfere with function. However, as the extension ultimately takes part in a duplex, it is unlikely to affect aptamer folding. In our own group, we have made use of this approach not just for labeling individual aptamers following production by *in vitro* transcription, but for monitoring the selection itself. During the selection process, we block the 3' constant region of the library with the reverse primer, which places this region in the same structural context during the selection that it will have when used to carry a fluorescently tagged oligo or other cargo (see, e.g., Ref. [17] and [10]).

Conclusions

Despite any negativity that may be placed on them, aptamers are still remarkable molecules and still have that incredibly high potential for use both as a research or clinical reagent. However, published aptamers must function as promised and be robust enough to handle conditions in any given researcher's hand. To date, this has not been the case.

As selectionologists, we need to take a little more care in how we assess and use the aptamers that we make. The use of appropriate controls and standardizing the way aptamers are tested will help make it much easier for laboratories to check each other, to check any aptamer they wish to use, and to spot whether something is amiss. With just a little more diligence and collective effort, aptamers may soon regain their former promise. Then, with more reliable aptamers in hand, the aptamer community can finally move to overcome the big challenge for targeting agents: delivery of siRNA and other cargoes to the cytoplasm.

Author Disclosure Statement

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