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REVIEW

Riboswitches and synthetic aptamers: a head-to-head comparison

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ABSTRACT

Nucleic acid aptamers are unique molecular structures used for binding to diverse targets. There is a major challenge in adapting *in vitro*-selected RNA aptamers for building *in vivo* RNA devices that control cell function. In contrast, their natural nucleic acid counterparts, riboswitches, were deliberately evolved for efficient gene regulation and cellular programming. Encoded within cells, riboswitches exploit a natural aptamer module to bind to an intracellular small molecule target enabling regulation of fundamental metabolic pathways. Here, we review several key features of natural riboswitches that may account for their function in the cellular environment. We compared these features to those of *in vitro* selected RNA aptamers that bind to small molecule targets. Our analysis revealed that the aptamer structure and magnesium-dependence might be the largest contributors to failed synthetic RNA devices. Thus, we make several suggestions for forthcoming aptamer selections, which may improve the success of synthetic RNA design, implementation into cells, and ultimately expand their applications.

KEYWORDS: Aptamer, riboswitch, secondary structure, in vitro selection, small molecule, metabolite

INTRODUCTION

There is no shortage of novel applications for aptamers (Pfeiffer and Mayer, 2016; Ruscito and DeRosa, 2016; Etzel and Mörl, 2017). In the field of synthetic biology, RNA aptamers are frequently used to engineer RNA devices or biological circuits to probe and program cells (Findeiß et al, 2017). Such RNA-based control strategies offer several advantages including predictable tuning of gene regulation and biological responses (Chappell et al, 2015). As a result, RNA device applications are diverse, ranging from *in vivo* molecular-tracking to cellular therapy (McKeague et al, 2016).

Despite advances in RNA synthetic biology, and hundreds of examples of the application of RNA-based control, there is a major challenge in adapting *in vitro*-selected RNA aptamers for building new *in vivo* RNA devices (McKeague et al, 2016). For example, the theophylline aptamer overwhelmingly remains the biosensing component used for *in vivo* engineered RNA devices (Chang et al, 2014). We still do not understand why porting synthetic aptamers into cells (in particular eukaryotic cells) often fails and it is unclear which RNA features are critical for *in vivo* function (Findeiß et al, 2017). Furthermore, there are limited approaches to address this *in vitro* to *in vivo* challenge (Berens et al, 2015). Researchers have proposed that the aptamer selection environment is critical for successful implementation of synthetic aptamers inside cells (Carothers et al, 2010; McKeague et al, 2015). A second hypothesis is that *in vitro* selected aptamers lack the structural complexity needed for ligand binding and recognition (Ruff et al, 2010; Luo et al, 2017). Finally, it is suggested that many synthetic aptamers are folded into a pre-formed binding pocket and do not undergo the conformational change needed to act as a switch (Borujeni et al, 2016).

Riboswitches, on the other hand, have been explicitly evolved for gene regulation. Encoded within the genome of cells, riboswitches exploit a natural aptamer that binds

to an intracellular small molecule, enabling regulation of fundamental metabolic pathways in several organisms (Li et al, 2016). We sought to identify riboswitch features that permit their robust activity in the cell. In this review, we compare natural aptamers (riboswitches) to the current repertoire of synthetic aptamers. We first briefly summarize the current process of selecting novel aptamers *de novo* and riboswitch function. We next highlight some of the exciting applications and current challenges of synthetic RNA control devices. We then compare the target types, binding affinity, binding conditions, and structural features of synthetic aptamers and riboswitches. From the findings in our comparison, we conclude by providing several recommendations to improve the usage of synthetic aptamers to RNA-based *in vivo* regulation.

SELECTING SYNTHETIC APTAMERS

Aptamers are single-stranded oligonucleotides that display high affinity to their targets (Gold, 2015). Synthetic aptamers are isolated *in vitro* using the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (Kuwahara and Obika, 2013; Groher and Suess, 2015; Yang et al, 2016). The process involves iterative rounds of binding, partitioning, and amplification. To generate RNA aptamers, the additional step of transcribing the dsDNA template *in vitro* is required (Dolgosheina et al, 2014). For small molecules, the target must be immobilized onto a solid-phase support to facilitate partitioning (McKeague and DeRosa, 2012). In the past decade, there have been many modifications to the small molecule SELEX process to develop aptamers more rapidly, efficiently, or to enhance desired properties (Ruscito and DeRosa, 2016).

RIBOSWITCHES: NATURAL APTAMERS FOR GENE EXPRESSION CONTROL

Riboswitches are considered the natural analogues of synthetic aptamers because they form selective binding pockets for their target metabolites (Breaker, 2009; Nguyen et al, 2016). Riboswitches are commonly found in the untranslated regions (UTR) of mRNAs and modulate transcription or translation in a cis-fashion. Riboswitches are composed of two domains: the aptamer (for binding to a target), and an expression platform which interfaces with the transcriptional or translational machinery. Structural changes to the aptamer pocket induced by the binding of the target metabolite are communicated directly to the expression platform (see Figure 1) (Trausch and Batey, 2014). Then, through a variety of mechanisms, including transcription, translation and RNA processing (*e.g.,* splicing), the production of a protein is dynamically regulated. As a result, target metabolites within a cell can quantitatively regulate gene expression in cells (Breaker, 2009).

Since their discovery in 2002, scientists have identified more than 40 classes of riboswitches (McCown et al, 2017). Riboswitches exist throughout all kingdoms of life and have been identified in many genomes, including fungi, archea and plants; but most commonly in bacteria (Barrick and Breaker, 2007). In some cases, there are often thousands of representatives for some of these classes (McCown et al, 2017). Riboswitches, like synthetic aptamers, display high affinity and selectivity for their targets. As an example, the guanine riboswitches discriminate between adenine and guanine by a factor of 10,000 (Gilbert et al, 2009). As another example, the lysine riboswitches can differentiate between ornithine and lysine. The discrimination level between these molecules is 5,000 fold even though they differ by a single methylene group (Garst et al, 2011).

OPPORTUNITIES AND CHALLENGES USING SYNTHETIC APTAMERS FOR GENE CONTROL

Motivated by the diverse functions of natural RNA, researchers have created synthetic, RNA-based genetic control of cells and organisms (Isaacs et al, 2006). There are many successful applications of these RNA-based devices. Synthetic riboswitches have been employed to dynamically track metabolites in live cells and whole animals (Alsaafin and McKeague, 2017). Metabolic engineering has also seen the successful implementation of RNA-based technology. In particular, enzymes and microbial strains have been optimized for the fermentative production of drugs and biofuels using ribozyme-based switches (Wang et al, 2016). Finally, there has been a significant effort in exploiting RNA devices for mammalian gene expression control. In particular, trans-gene expression control of viral replication factors, serving as safety switches has been useful in viral gene therapy approaches. Additionally, cis-gene expression

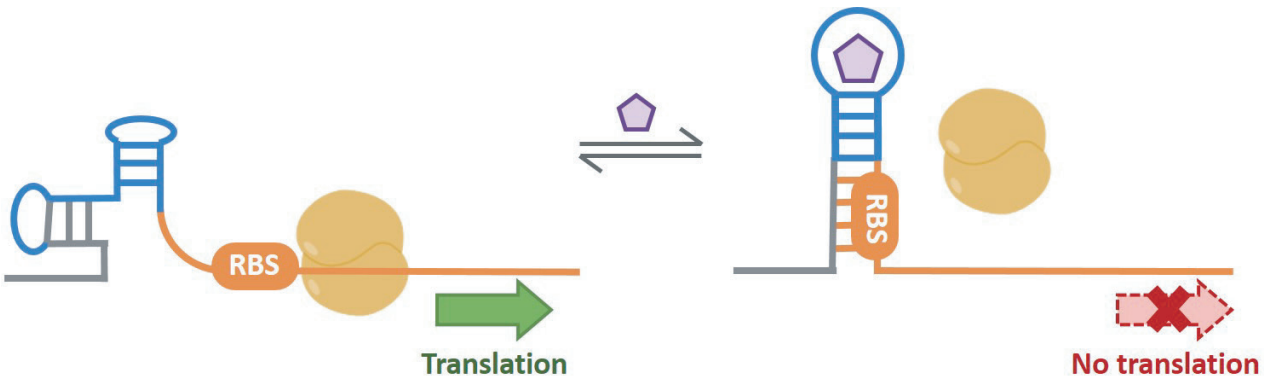

Figure 1. Riboswitches can regulate gene expression through a variety of mechanisms. As one example, the target ligand (purple pentagon) binds to the aptamer domain (blue). Binding results in a structural rearrangement that is communicated to the expression platform (orange). In this case, ribosome binding to the ribosome binding site (RBS) is prevented and therefore translation of the gene cannot be initiated.

control using ribozyme-based switches and synthetic riboswitches has been implemented for proliferation, apoptosis, and cell cycle control in mammalian and human cells (Auslander and Fussenegger, 2017).

There are several challenges to address before the full potential of RNA-based genetic devices can be realized. It is proposed that the biggest bottleneck is the general lack in available RNA aptamers that bind to small molecules (McKeague et al, 2016). Indeed, the same few aptamers (theophylline, tetracycline, and neomycin) have been employed in the past ten years (Berens et al, 2015). However, there are over 60 characterized RNA aptamers that have been selected to bind to small molecules (McKeague and DeRosa, 2012; Ruscito and DeRosa, 2016). Therefore, it is not so much the lack of available parts, but it is likely that these parts often fail when implemented *in vivo*, particularly within eukaryotic systems.

There are several possible reasons why RNA aptamers fail when transferred into the cellular environment. First, during cellular RNA synthesis, co-transcriptional folding may have an impact on the functional structure (Draper D, 2004; Meyer and Miklos, 2004; Lai et al, 2013). There has been significant progress aimed at addressing this challenge (Thimmaiah et al, 2015). Second, the epigenetic status and nucleic-acid protein interactions may play a role in functionality: to-date this has been difficult to study and test *in vitro*. Furthermore, the magnesium concentration may be a critical parameter or it is possible that aptamers lack the necessary structural complexity for function within the complex cellular environment.

It is also proposed that many *in vitro* aptamers are folded into a pre-formed binding pocket and do not undergo the necessary conformational change for switching activity (Borujeni et al, 2016). "Structure-Switching" *in vitro* selection methods address this problem by allowing for the isolation of aptamers that experience a conformational change upon binding (Nutiu and Li, 2005). Unfortunately, these methods have not been tested for the development of new RNA devices. Combined with the shortage of experimental data describing the dynamic structures of the 60 small molecule aptamers, it is difficult to assess the importance of the target-induced conformational change. Also important to note, alteration of higher-ordered structural interactions as a mechanism for achieving allostery likely does not require structure-switching properties of the aptamer (Townshend et al, 2015); regardless, we have experienced similar challenges using *in vitro* selected aptamers with this platform. Therefore, in this review, we focus on the evaluation of the potential impact of aptamer binding requirements and predicted secondary structure on the resulting *in vivo* RNA function.

COMPARISON OF RIBOSWITCHES AND APTAMERS

Synthetic aptamer examples

A few key synthetic aptamers are typically used in RNA device design and application (namely theophylline, tetracycline, and neomycin, Table 1). Dye-binding aptamers, such as the Spinach family, and Mango have also been successfully implemented several times in RNA device platforms. The sample set of "successful" aptamers, including a few more recently described examples (folinic acid, 5-hydrotryptophan), is very diverse. For example, the target type is highly varied: ranging from antibiotics to fluorescent dyes with molecular weights as low as 180gm/mol and as high as 615gm/mol. While on average the affinity of these aptamers is considered "high", the reported K_d value range varies between micromolar and nanomolar. Finally, the predicted structure varies in both stability and complexity (for details see section on "Structure and stability"). Given that no obvious trends emerged, we sought to expand our exploration of properties to all synthetic aptamers and key natural riboswitches.

To generate a list of synthetic aptamers binding to small molecules, we searched PubMed using the keywords "aptamer" and "SELEX". To generate a list of riboswitches, we searched PubMed using the keyword "riboswitch". For the sake of the comparison, we used the aptamer or riboswitch candidate/data with the highest affinity (lower K_d value). We compared our list to those previously described (Ruscito and DeRosa, 2016).

Target molecule

It is well known that synthetic aptamers can bind to a diverse range of targets. While the majority of aptamer targets are proteins, they can also bind to cells, peptides and viruses. Due to technical challenges, it is not surprising that fewer aptamers exist to small molecules (McKeague and DeRosa, 2012). Here, we first review the landscape of different small molecules that bind to synthetic aptamers and riboswitches (see Table S1). Interestingly, nucleosides and other nucleic acid components make up a large portion of targets for both synthetic aptamer targets and riboswitch targets. Furthermore, aptamers and riboswitches share several other classes of small molecules including cofactors, amino acids, and ions.

There are several important differences between targets that bind to synthetic aptamers compared to those that have been naturally-evolved as riboswitches. First, the targets that bind to aptamers are much more diverse. For example, there are a number of synthetic drugs and dyes that bind to aptamers. It is not surprising that organisms have not evolved riboswitches against synthetic targets such as dyes and small molecule drugs. In contrast, aptamers are frequently used in biomedical research (Ruscito and DeRosa, 2016).

On the other hand, cofactors make up the bulk composition of riboswitch targets. Cofactors are small molecules that enhance the catalytic activity of enzymatic reactions. They are responsible for energy production, redox reactions, and prevent cell damage (Cochrane et al, 2008). Given their importance for cell metabolism and health, it is not surprising that many riboswitches bind to cofactors. Cofactor-responsive riboswitches are capable of regulating the genes responsible for their synthesis or their import. Indeed, naturally occurring riboswitches respond to several of the most ubiquitous coenzymes found in proteins including adenosyl cobalymin (AdoCbl), flavin mononucleotide (FMN), thiamine pyrophosphate (TPP), and S-adenosyl methionine (SAM) (Cochrane et al, 2008; Edwards et al, 2010; McCown et al,2014).

Table 1. Examples of synthetic aptamers that have been successful implemented into RNA devices *in vivo.*

Aptamer	Junction # predicted	Predicted MFE	Aptamer Affinity	Reference
Theophylline	2	–9.1	100nM	Jenison et al, 1994
Tetracycline	3	–14.3	1μM	Berens et al, 2001
Neomycin	1	–10.8	8nM	Weigand et al, 2008
Spinach	1	–13.6	464nM	Paige et al, 2011
Mango	0	–0.7	3.2nM	Dolgosheina et al, 2014
Folinic acid	2	–11.6	16nM	McKeague et al, 2015
5-hydroxytryptophan	2	–12.9	3.9μM	Porter et al, 2017

It is possible that cofactor targets are well-suited for binding RNA inside a cell and result in better gene control. Cofactors typically have larger molecular weights (300 to 1000gm/mol) as compared to other small molecules. For example, amino acids are typically only 100gm/mol; nucleobases and nucleosides are between 100 and 300gm/mol; and metals/ions are well under 100gm/mol. This approximately order-of-magnitude difference in molecular weight could potentially result in an improvement in binding affinity or specificity – and account for the high function of riboswitches.

Binding affinity

While synthetic aptamers are known for high affinity, small molecule synthetic aptamers are notoriously challenging to select (Groher and Suess, 2016). In many cases, small molecule synthetic aptamers bind in the micromolar range (McKeague and DeRosa, 2012). One potential reason for the low activity of synthetic aptamers *in vivo* could be related to this low affinity. Therefore, we next compared the affinity of riboswitches and synthetic aptamers.

We first compared the average binding affinity of all aptamer-target pairs versus all riboswitch-target pairs. We included the best dissociation constants for each target. One important limitation is that different researchers and different affinity determination techniques yield different results (even for the same aptamer or riboswitch) (McKeague et al, 2015). Future binding affinity analysis of aptamers using the same platform may be useful to provide a true "head-to-head" comparison. Nevertheless, the comparison indicated that the average binding of synthetic aptamers is 150 ± 580μM, and for riboswitches is 26 ± 57μM. Therefore, we can conclude that riboswitches display approximately ten-fold (one order of magnitude) higher binding affinity than synthetic aptamers. This lower affinity of synthetic aptamers might partially explain why they fail when implemented for *in vivo* genetic control.

Given the broad distribution of K_d values, we also compared the median value for aptamers and riboswitches. The median value for synthetic aptamers is 2μM, and the median value for riboswitch targets is 580nM. Furthermore, when all currently-reported dissociation constants for riboswitches and synthetic aptamers were included, the range of measured affinities is broader. In fact, many riboswitches bind to their targets with very high K_d values. For example, the *glms* riboswitches in *B. anthracis*, the glutamine riboswitch in *S. elongatus* and the fluoride riboswitch in *T. petrophila* have binding affinities of 200μM, 150μM and 134μM, respectively.

We next performed a head-to-head comparison of the binding affinity of synthetic aptamers and riboswitches that bind to the same or similar targets (Table 2). In most cases, the K_d values are drastically different for aptamers and riboswitches. Several riboswitches display significantly higher affinity than the corresponding synthetic aptamer. As an example, the riboswitch for guanine has an affinity more than two orders of magnitude higher ($K_d = 0.0047\mu M$) than the synthetic aptamer ($K_d = 1.3\mu M$). This result is not surprising given that the majority of synthetic aptamers are selected using targets that are immobilized to a solid-support. As a result, less functional groups are available for binding to the aptamer. This is in contrast to natural riboswitches that are evolved with molecules in solution and exposed; potentially allowing additional chemical interactions with the nucleic acid.

Surprisingly, half of the targets considered in Table 2 display better binding affinity with their synthetic aptamers compared to the natural riboswitch. As one example, the direct comparison of cyanocobalamin indicates that the synthetic aptamer binds approximately three-fold higher than the riboswitch. Even more impressive is the aptamer for folinic acid that binds to its target almost three orders of magnitude better ($K_d = 0.0192\mu M$) than the natural THF riboswitch ($K_d = 18\mu M$) that binds to its target THF that differs by a single formyl group.

The differing results may be explained by the fact that riboswitches have evolved affinities that that meet the intracellular concentration of their targets, where in some cases these targets are at high concentrations. Furthermore, different binding affinities are required depending on the riboswitch mechanism. For example, transcriptional riboswitches require aptamers with very low K_d values since there is a limited time frame to interact with the target before transcription proceeds. Taken together, the results suggest that high affinity may be beneficial for achieving efficient *in vivo* genetic control; however, function is not solely dependent on the high affinity.

Binding conditions (magnesium)

It is commonly accepted that the binding conditions are critical for maintaining high aptamer affinity (Chang et al, 2014; Ruscito and DeRosa, 2016). For example, aptamer affinity is dependent on pH, buffer composition, and metal cation concentration (Geng et al, 2013). For this reason, selection experiments should be performed in the same conditions in which the aptamer will be applied. Unfortunately, most SELEX experiments reported-to-date use divalent cations,

Table 2. Binding affinity of synthetic aptamers and riboswitches against similar targets.

Aptamer target	Riboswitch target, species	Aptamers K_d, μM (reference)	Riboswitches K_d, μM (reference)
Adenine	Adenine, *B. subtilis*	10 (Meli et al, 2002)	0.354 (Lemay et al, 2006)
Guanine	Guanine, *B. subtilis*	1.3 (Kiga et al, 1998)	0.0047 (Mulhbacher and Lafontaine, 2007)
Cyanocobalamin	Cyanocobalamin, *E. coli*	0.088 (Lorsch and Szostak, 1994)	0.3 (Nahvi et al, 2004)
Flavin mononucleotide	Flavin mononucleotide, *E. coli*	1 (Burgstaller and Famulok, 1994)	3.6 (Pedrolli et al, 2015)
Folinic acid	Tetrahydrofolate, *S. mutans*	0.0192 (McKeague et al, 2015)	18 (Trausch and Batey, 2014)
S-adeosyl homocysteine	S-adenosyl homocysteine, *R. solanacearum*	0.2 (Gebhardt et al, 2000)	0.032 (Edwards et al, 2010)
Citrulline	Glutamine, *S. elongatus.*	62–68 (Mannironi et al, 2000)	150 (Ames and Breaker, 2011)
Valine	Glycine, *V. cholerae*	2900 (Majerfeld and Yarus, 1994)	3.5 (Huang et al, 2010)

typically magnesium (Mg^{2+}), at concentrations ranging from 5mM to 20mM. However, inside a cell, the Mg^{2+} concentrations are on the order of 0.1–1mM (Kennedy et al, 2014). Magnesium is important because the negative charge of RNA prevents folding into compact structures. Thus, positive ions promote folding by reducing the repulsion between phosphates. However, Mg^{2+} strongly stabilizes the tertiary structures of RNA even in the presence of an excess of monovalent ions (Draper, 2004). Therefore, it is possible that many of the synthetic aptamers do not function in the physiologically low Mg^{2+} environment due to improper folding of the functional structure needed for binding to the target. As an example, the high affinity theophylline aptamer requires >1mM of Mg^{2+}; at lower Mg^{2+}, the K_d is in the micromolar to millimolar range (Zimmermann et al, 2000). Moreover, other studies have shown that there is a strong correlation between the Mg^{+2} concentration and the stability of tRNA tertiary structure (Misra and Draper, 1999).

Therefore, we were interested if Mg^{2+} concentration played an important role in the function of synthetic aptamers. Here we first compared the average Mg^{2+} concentrations in which synthetic aptamers and natural riboswitches were selected or characterized. Interestingly, the average Mg^{2+} concentration for riboswitches is 17 ± 8mM, while the average for aptamers is 6 ± 5mM. These results potentially indicate that there is no significant difference in the Mg^{2+} concentration for the characterization of synthetic aptamer activity (or affinity) of the aptamers and riboswitches.

We next reviewed whether Mg^{2+} concentration played a role in the affinity of the synthetic aptamers and riboswitches. While affinity does not necessarily imply function inside cells, it is one indicator of aptamer function; thus, we used it as a metric for our comparison. In 2015, McKeague et al. examined the relationship between the concentration of cations used in a selection experiment and the resulting dissociation constants of the selected synthetic aptamers. The results indicated a general correlation suggesting that higher affinity aptamers were obtained with lower metal cation concentration. Here, we specifically focused on Mg^{2+}, and compared the affinity of small molecule interactions with synthetic aptamers and riboswitches. In Figure 2, the affinity vs the Mg^{2+} concentration is shown for both aptamers and riboswitches.

Figure 2 displays the mean of the K_d values compared to the concentration of Mg^{2+} used in their characterization. An initial comparison revealed there is a significant difference between the K_d values of at least two different Mg^{2+} concentrations in the aptamer comparison (Kruskal–Wallis: $p<0.05$); however, there is no significant difference (Kruskal–Wallis: $p=0.9093$) when comparing the Mg^{2+} and affinity of riboswitches.

In general, the Mg^{2+} concentration used to characterize riboswitches are relatively high. For example, the FMN riboswitch is characterized at 40mM Mg^{2+} and many riboswitches are characterized at Mg^{2+} concentrations in the range of 10 and 20mM. However, Chang et al (2014) reported the K_d values of several riboswitches using an SPR platform at 5mM Mg^{2+}. At these lower Mg^{2+} concentrations, most of the riboswitches maintained high affinity. For example, the cyclic-di-GMP class I and the TPP (*thiM*) riboswitches bound in the picomolar range (Smith et al, 2009; Kulshina et al, 2017). This supports our results in Figure 2 that riboswitch affinity is probably not dependent on Mg^{2+}. This may be explained because riboswitches were evolved within the cell, where folding is typically assisted by RNA binding proteins to compensate for charge repulsion.

In contrast to the riboswitch results, the Mg^{2+} concentration may have an impact on the affinity of aptamer binding. We performed a post hoc analysis using Dunnett's test to compare the mean rank of the K_d values for each Mg^{2+} concentration used with synthetic aptamers. In this case, the K_d values for aptamers characterized at low Mg^{2+} concentrations (less than 2mM) displayed significantly better affinity (median = 300nM; $p<0.05$) than aptamers that were selected and characterized at Mg^{2+} concentrations greater than 5mM (median = 10μM). These results support the finding by Carothers and colleagues that indicate that tighter binding aptamers show less dependence on magnesium than weaker-binding aptamers (Carothers et al, 2010).

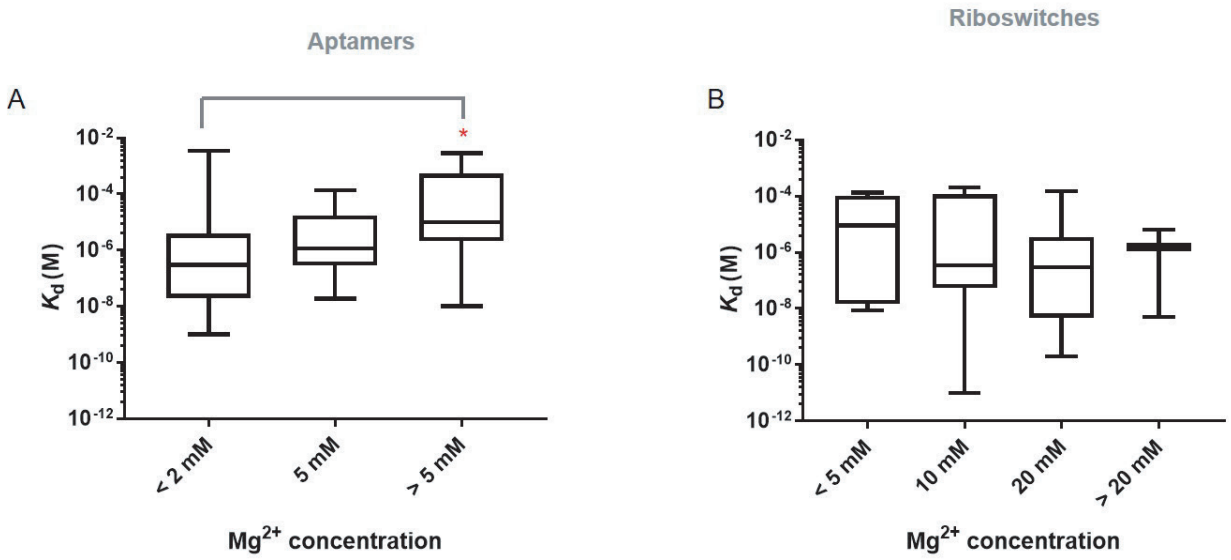

Figure 2. Box plot indicating the relationship between the binding affinity (Kd) and Mg2+ concentration for (A) synthetic aptamers and (B) riboswitches binding to small molecules. p=0.03 (*).

McKeague et al (2015) demonstrated that the affinity of high affinity aptamers that were selected at high concentrations (5mM) of Mg^{2+} is drastically reduced upon characterization at lower Mg^{2+} conditions. Given that the majority of aptamers continue to be selected at high Mg^{2+} concentrations, it is likely that their high affinity is reduced once inside the low Mg^{2+} cellular concentration. Therefore, we propose that the *in vitro* Mg^{2+} concentration should be lowered in future selections (McKeague and DeRosa, 2014).

Structure and stability

It is known that most synthetic aptamers tend to fold into simple structures such as hairpins – also known as "1-way junction" structures (Luo et al, 2010). In fact, less than 1% of synthetic aptamers are predicted to fold into complex structures such as 4-way and 5-way junctions. The fact that selection favors the simplest solution continues to be an ongoing challenge in the field (Joyce, 2004; Ellington et al, 2009). In contrast, riboswitches are known to form 3-way junction structures or higher. Therefore, we next examined the structural complexity of small molecule aptamers compared to riboswitches. To achieve this, sequences were analyzed using the RNAstructure package (Mathews et al, 2004). The number of junctions was determined using the *CountJunctions* algorithm as previously described (Luo et al, 2010). Figure 3 shows the distribution of junction structures.

The analysis indicates that riboswitches form more complex structures compared to aptamers. Almost 44% of riboswitches are predicted to form 3-way or 4-way junction structure (32% 4WJ; 12% 3WJ), whereas less than 20% of aptamers fold into these complex structures (only 5% are 4WJ and 14% are 3WJ). We also used the QuadBase2 (Dhapola and Chowdhury, 2019) and QGRS Mapper (Kikin et al, 2004) but found no trend in the prediction of G-quadruplexes (see Figure 3). Therefore, there is no clear benefit for the formation of RNA G4s from our data set, however, it is possible that more complex structures are better-suited for *in vivo* function. One caveat is that riboswitches (mean length = 97 ± 50nts) are often longer than synthetic aptamers (mean length = 67 ± 34nts) which may partially explain the higher propensity of complex structures in the riboswitch group (Luo et al, 2010).

It has been previously suggested that RNA structural complexity may afford the functional RNA molecule with higher affinity (Luo et al, 2010). Therefore, we investigated whether structural complexity correlated to the binding affinity of aptamers and riboswitches. Specifically, we compared the mean K_d values of different structural groups. The only significant difference was found when comparing synthetic aptamers that are predicted to fold into 1-way junction structures (mean K_d = 2.9μM) compared to all other more complex aptamers, including 2-way, 3-way, and 4-way junction structures (mean K_d = 0.11μM) (Figure 4: Mann-Whitney test, p=0.0045). While this comparison does not necessarily address the challenge of applying synthetic aptamers *in vivo*, it confirms previous suggestions that selecting for more structurally-complex sequences may be a useful strategy for ensuring high affinity of the aptamer-target complex (Carothers et al, 2004). In line with this, recent work has shown that using natural RNA scaffolds with rich structural complexity resulted in successful selection of new synthetic aptamers (Porter et al, 2017).

While our analysis suggests that structural complexity may only have a small impact on binding affinity, the results confirm that natural riboswitches typically exhibit higher structural complexity compared to *in vitro* selected aptamers. Therefore, structural complexity may play other roles in function, such as achieving highly selective binding (Garst et al, 2011). In many cases, aptamers selected *in vitro* have not been tested for their specificity against *in vivo* metabolites. Indeed, several metabolites are present at drastically high concentrations inside the cell including for example, amino acids, nucleotides, central carbon intermediates, and redox cofactors (Bennett BD, 2009). While riboswitches have been evolved to selectively bind to their targets within

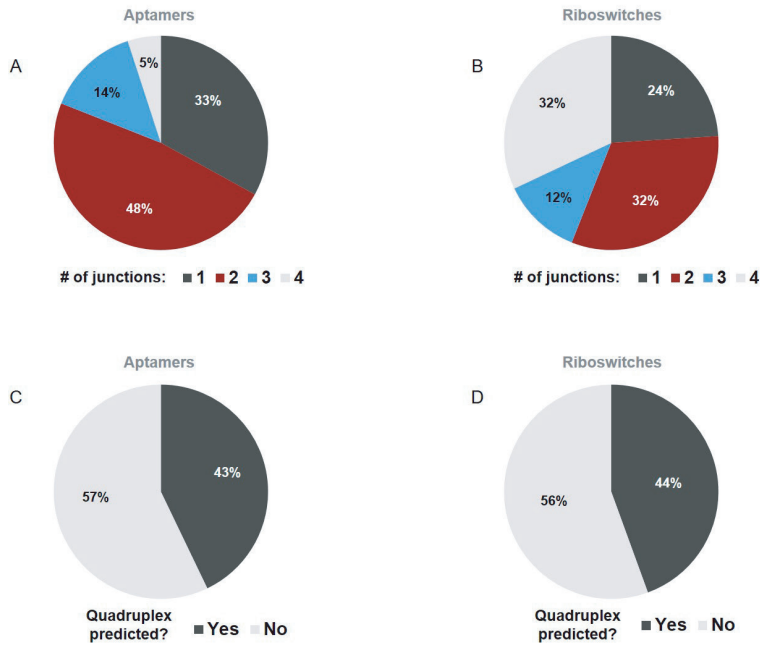

Figure 3. Comparison of the predicted structural distribution of synthetic aptamers and riboswitches. Distribution of predicted structures according to "number of junctions" for (A) aptamer sequences and (B) riboswitches predicted using RNAstructure and the "CountJunctions" Algorithm. Distribution of predicted structures that contain at least one quadruplex for (C) aptamers and (D) riboswitches according to the QuadBase2 software.

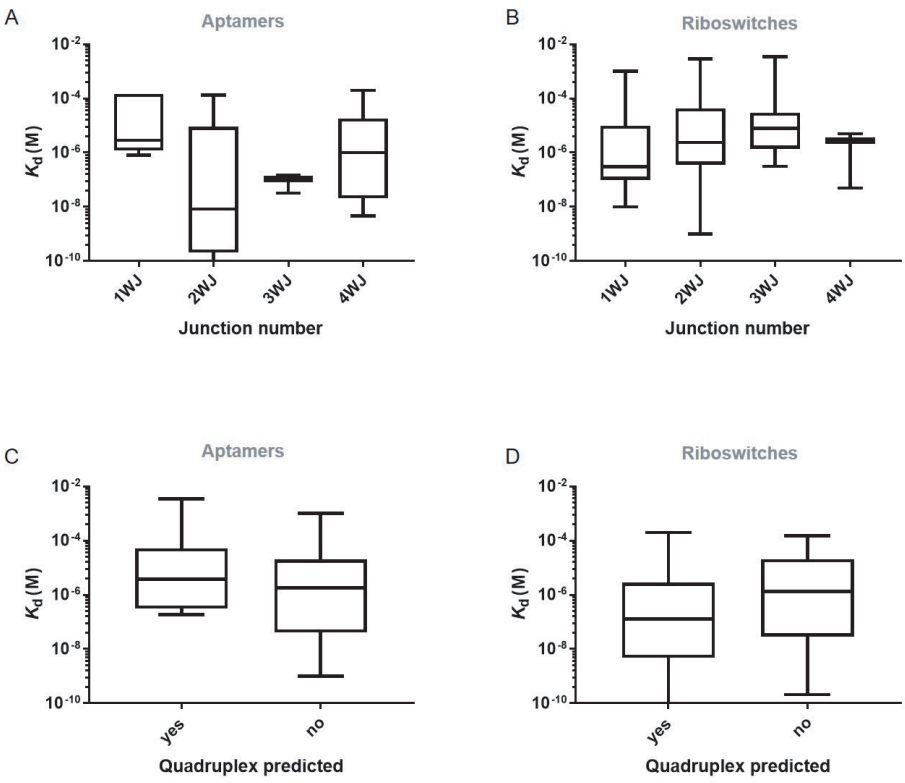

Figure 4. Average binding affinity according to the complexity of the predicted structure reported as either the total number of junctions (1 WJ = a one junction structure) for all (A) aptamers and (B) riboswitches; or based on whether a G-quadruplex structure was predicted for all (C) aptamers and (D) riboswitches.

this complex metabolic environment, it is possible that synthetic aptamers non-specifically interact with many metabolites, resulting in high basal activity and off-target effects; this specificity challenge is elegantly described by Gold et al (2012). Before implementing aptamers into cellular applications, we suggest that the selectivity is more thoroughly tested. An even better solution would be to perform *in vitro* selection experiments within a more complex metabolite environment, ensuring that the most "specific" synthetic aptamers are selected.

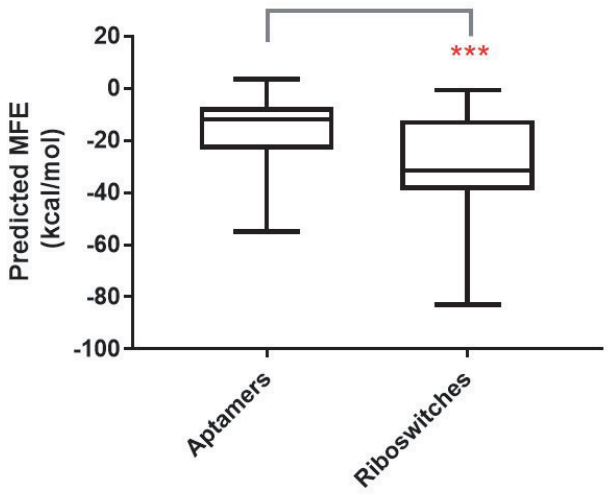

Figure 5. The mean stability (MFE) for synthetic aptamers and riboswitches binding to small molecules, predicted in RNAstructure. p = 0.0002 (***).

We next compared the "stability" of the predicted structures. To do this, we computed the lowest minimum free energy (MFE) using the RNAstructure package. On average, the predicted aptamer structures are less stable (higher MFE): we calculated a mean MFE of -12kcal/mol compared to the more stably-predicted riboswitch secondary structures (mean MFE = -31.4kcal/mol) (Figure 5: Mann-Whitney test, p=0.0002). The relatively low stability of aptamers may result from the selection experiment itself. First, aptamer libraries are shorter in length and second, putative aptamer sequences must be accessible to reverse transcriptase at moderate temperatures. Thus, the selection likely favors structures with only modest stability. Regardless of why this result occurs, the difference in stability may indicate that more stable structures are better suited for RNA device activity. For example, perhaps a higher stability helps prevent the formation of alternative folded structures within the larger context of the mRNA sequence. Inside the cell, where the aptamer conformation is communicated to the expression platform, it may be critical that there are limited alternative structures available to allow proper binding of the target and activity withing the switch platform. One complication is that many aptamers (both natural and synthetic) adopt a less stable structure (than the predicted MFE) (Domin et al, 2017). Further work is required to understand this relationship and how to import synthetic aptamer designs *in vivo*. In particular, more examples of experimentally-derived structure of aptamers, including additional examples of crystal structures, would be valuable.

CONCLUSIONS

Here we reviewed the ongoing challenge of developing new RNA-based cellular devices. To better understand why *in vitro* selected aptamers often fail when implemented into the cell, we compared features of naturally-evolved riboswitches and *in vitro* selected small molecule aptamers for the first time. Our study reveals several important differences between riboswitches and synthetic aptamers that researchers should take into consideration prior to the design of new synthetic RNA devices. First, we were surprised to uncover that the binding affinity of riboswitches was not necessarily a critical parameter for ensuring their function *in vivo*. Of course, higher binding affinity may be a useful feature, but it is insufficient to fully account for the differences observed in the high functioning of riboswitches compared to aptamers. Our review also highlighted that aptamer affinity is dependent on the magnesium concentration. This hypothesis must be more rigorously tested, but we nonetheless suggest that new aptamer selections performed at magnesium concentrations that are relevant *in vivo*. We also propose that the structure (either the complexity or the stability) of the folded RNA aptamer or RNA riboswitch plays an important role in function. The structure is likely important for i) maintaining specific binding, ii) ensuring robust genetic control, and iii) facile design of new switch candidates. As such, new aptamer selections may benefit from biased libraries or libraries that were computationally-designed to yield more highly-structured putative aptamers; with particular emphasis on using natural RNA scaffolds. Finally, to ensure the aptamer binding results in a significant structure rearrangement, we suggest employing a "structure-switching" based selection.

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COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

1WJ: 1-way junction
AdoCbl: Adenosylcobalamin
cyclic-di-GMP: Cyclic diguanylate
FMN: Flavin mononucleotide
G4: G-quadruplex
glmS: Glucosamine-6-phosphate
K_d: Dissociation constant
Mg^{2+}: Magnesium cation
MFE: Minimum free energy
PCR: Polymerase chain reaction
RBS: Ribosome binding site
SAM: S-Adenosyl methionine
SELEX: Systematic evolution of ligands by exponential enrichment
THF: Tetrahydrofolic acid
TPP: Thiamine pyrophosphate

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