Evolving methods for rational de novo design of functional RNA molecules

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Abstract

Artificial RNA molecules with novel functionality have many applications in synthetic biology, pharmacy and white biotechnology. The de novo design of such devices using computational methods and prediction tools is a resource-efficient alternative to experimental screening and selection pipelines. In this review, we describe methods common to many such computational approaches, thoroughly dissect these methods and highlight open questions for the individual steps. Initially, it is essential to investigate the biological target system, the regulatory mechanism that will be exploited, as well as the desired components in order to define design objectives. Subsequent computational design is needed to combine the selected components and to obtain novel functionality. This process can usually be split into constrained sequence sampling, the formulation of an optimization problem and an in silico analysis to narrow down the number of candidates with respect to secondary goals. Finally, experimental analysis is important to check whether the defined design objectives are indeed met in the target environment and detailed characterization experiments should be performed to improve the mechanistic models and detect missing design requirements.

Keywords: RNA design, rational de novo design, synthetic biology, artificial RNA devices, mechanistic models, sequence sampling, experimental validation, RNA design tools

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RiboNucleic Acid (RNA) sequences are the perfect building blocks to reprogram cellular behavior as they are known to regulate gene expression at almost every step and in all domains of life. Features like environment-sensing abilities, enzymatic reactivities and a cost effective in vitro or in vivo synthesis make RNA a Swiss army knife in synthetic biology and its related fields such as (white) biotechnology and personalized medicine. Example applications include self-assembling RNA scaffolds that were designed to form lattices and tubular structures mimicking cytoskeletal proteins [1, 2] and RNA scaffolds that increase metabolic production by co-localizing related enzymes [3, 4]. Artificial RNA molecules can also be made to be highly stable by incorporating modified nucleic acid analogs, which circumvent rapid degradation by RNase enzymes.

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Early clinical trails utilizing such molecules as drugs show that RNA-based therapeutics might be an alternative route to cure so far untreatable (genetic) diseases [6]. However, these medical applications are still in their infancy and challenges, such as intracellular delivery across membranes to target specific tissues, need to be tackled before RNA-based personalized medicine can become a standard approach, as recently reviewed by Lieberman [6]. Naturally occurring RNA regulators and the regulatory mechanisms they employ — for instance miRNAs, riboswitches, trans-activating RNAs and ribozymes — often serve as templates for designing artificial counterparts or to fill specific gaps in the available repertoire of RNA devices. The diverse set of designed functional mechanisms was extensively reviewed [7, 8, 9, 10, 11, 12, 13] and thus is not discussed here. Advances in experimental technologies, such as high-throughput techniques to investigate functional variants or determine RNA structure, and in computational biology make RNA design a growing and fast developing research field.

As summarized by various reviews [14, 15, 16], artificial RNA molecules can be generated by several experimental strategies, such as selection and screening approaches, and by computational rational design. In this contribution we will focus on how rational de novo design of single- and multi-stable RNA sequences was accomplished in the literature. Hence, computational methods and experimental strategies to investigate the designs including their essential interplay are summarized, but we generally disregard the actual mechanisms of the novel RNAs in this review.

Figure 1: Overview of a general de novo design process. The initial design model development comprises a detailed investigation of the biological testing system, how to interface with the target environment, compile properties and parameters of RNA components to be utilized and drafting the functional mechanism of the overall design. Computational design is necessary to connect components while maintaining their functionality and gain the novel desired functionality. Software tools require design constraints and design goals, which are formulated from the model, as input. An iterative optimization loop of sequence sampling, objective evaluation and decision-making is performed. A subsequent filtering and in silico analysis step helps to include secondary design goals, properties which could not be taken care of in the optimization procedure. Functional testing and detailed experimental analysis in the lab are intended to identify working devices and to deliver valuable details and measured parameters to improve the design model.

We previously argued [17] that attempts to rationally de novo design RNA molecules frequently follow a common construction that we view as a pipeline, see Figure 1. Approaches to design RNAs consistently include initial analysis of the biological target system and the desired components followed by the application of one or more computational methods to derive candidate RNA sequences. Many design approaches also include further in silico analysis to reduce the list of candidates that must be tested and characterized in the laboratory. Thus, we found many similarities within the various design studies, for example, which algorithms and computational methods are used as
well as the experimental setup applied for validation and analysis. We therefore decided to review the three pipeline steps individually in the subsequent sections, describe how they were accomplished and highlight state of the art methods and novel ideas.

1. Characterization of the utilized biological system and components

Any rational de novo approach needs a priori knowledge about the relevant biological systems and components in order to reliably make decisions that finally lead to functional designs. Missing knowledge about the biological system and components, or premature design decisions will cause an extended experimental testing phase or might even make the project unfeasible. Unfortunately, this phase of information gathering is rarely described in detail. We therefore devote this section to summarizing what kind of information needs to be collected for a successful RNA design project.

The essential first step of designing functional RNA molecules is a detailed characterization of the in vitro or in vivo system in which the RNA must function. Typically, the novel RNA molecule is designed to interact with its environment in a specific manner. However, it might also influence — and be influenced by — other factors. Thus, it is not only important to examine the mechanism and properties of the desired interaction between the RNA device and its environment, but even more crucial to consider unwanted secondary interactions. Examples therefore might be off-target binding of the artificially designed RNA, interactions with proteins or other molecules, or degradation by RNases. If known, such disruptive factors can be taken into account at the various design steps. To decrease such influences during testing and debugging experiments, in vitro approaches such as the PURE cell-free system [18] and microfluidics devices [19, 20] can be used to approximate in vivo conditions while utilizing a well-defined reaction environment.

Moreover, researchers face many design decisions, from whether to use specific reporter genes or vector systems, to the selection of auxotrophic markers or resistant genes, and all these decisions impact the cell, the constructed plasmid and the functionality of the novel RNA device. For example, many strains exist for common model species, such as Escherichia coli (E. coli) strains like TOP10, DH5α or MG1655. They exhibit distinct features which affect the applicability of vectors or detection methods. For instance, TOP10 cells — in contrast to MG1655 or DH5α — combine the inability to metabolize arabinose with the deletion of the lacZ gene. Thus, TOP10 cells allow for systems that use arabinose-inducible araBAD-promoters and β-galactosidase as a reporter system. Therefore, it is important to carefully consider such issues about the biological testing system before any computation, as these early decisions affect subsequent steps in the project. For example, the sequence context of the vector or the reporter gene sequence must be taken into account, either directly — as input for design programs — or indirectly — as secondary design goals. The latter could be seen as good-to-have goals, where possible design candidates are sorted and filtered by their predicted RNA-RNA interaction with the context, in this example. Moreover, early decisions such as bacterial strains constrain what experimental analysis and characterization is possible, as explained in more detail in section 3.

Another example of a parameter which needs to be included as a design goal for the in silico computations are RNA concentrations. The relative amount of RNAs present in the cell is critical for proper functionality. This issue is especially crucial when an artificially introduced RNA must interact with other molecules, e.g., a riboswitch that is induced by a small RNA. RNA concentrations can be specified by using strong or weak promoters, whose strength is determined by their affinity for a specific σ-factor or RNA polymerase. Additionally, gene expression levels are also affected by the copy number of the chosen vector, which is regulated by the origin of replication (e.g., high
Figure 2: **A)** Artificial RNA devices with novel functionality are usually constructed by combining various components, e.g., an aptamer, a Ribosome Binding Site (RBS) and a reporter gene. **B)** The various properties of these components, and thus the functionality, need to be provided to computational models. Directly measured properties of a single sequence (top) — e.g., the binding affinity of the aptamer — fulfills this task, however with no design flexibility for that component. A catalogue of various sequences increases the flexibility (middle), while a dedicated model — constructed or trained by a big dataset — allows for maximum flexibility, as the properties can be predicted for any arbitrary sequence.

copy ORI pMB1 vs. low copy ORI p15A). When multiple plasmids are used, their origins of replication need to be compatible, as cloning procedures, e.g., for special resistance mechanisms, otherwise become difficult.

Additionally, during the design phase, information needs to be collected about biological components which will be part of the novel RNA device such as promoter, aptamer, catalytic centers, Ribosome Binding Site (RBS) or Shine Dalgarno (SD) sequences. The iGEM Registry of Standard Biological Parts is a web resource which collects well-specified, standard, and interchangeable components that can be used to design and construct integrated biological systems. Detailed information on these components is crucial for computational design. Such properties include structural conformations, essential nucleotides and the reaction mechanism (e.g., activity levels, reaction rates, binding affinities, ligand chemistry or reaction intermediates). The sequence context of a component is also important, and must be included as component property. For example, a stretch of consecutive uracil nucleotides in front of a SD sequence can boost translation initiation [21], while stable structural elements can cause the opposite effect [22].

In addition to extracting this information from the literature, it is important to perform experiments to characterize individual components with respect to the selected target system, in order to verify their functionality in the novel environment. For example, an aptamer needs its ligand binding affinity measured and the binding structure confirmed in conditions similar to the target environment, Figure 2. These measurements deliver the valuable sequence to function relations, which are crucial parameters for computational models.

Many studies crafted RNA designs in the way, that fixed nucleotide sequences served as RNA components and were embedded within random linker regions to derive novel functions. For example, a fixed aptamer sequence with its known binding affinity [23, 24] or an RBS sequence and its translation efficiency [25] were used. Although this approach provides a perfect sequence to function mapping for the individual components, it leads to very restrained flexibility for the computational design algorithms and thus to RNA devices with presumably bad overall functionality. For more flexibility, components should be described with as few fixed nucleotide positions as possible.

\[^{1}\text{http://parts.igem.org/}\]
To increase the number of possible sequences for a component, while still employing a directly measured sequence to function relation, a catalogue of various sequences for the component and their corresponding function could be used. The in silico design algorithm could then select a component sequence which fits the overall RNA device best.

Ideally, the desired components are described by an individual mechanistic model that maps any nucleotide sequence to a functionality score and vice versa, given user-supplied environmental factors such as temperature, Figure 2B). These models could be derived through high-throughput experiments and statistical learning or through extensive experimental characterization. Such models have the big advantage, that we might be able to predict the function for any given sequence, even for those which were not directly measured. An impressive example of a successful design approach based on detailed mechanistic models is the riboswitch design by Espah-Borujeni et al. [26]. The underlying mechanistic model to estimate translation initiation efficiency has been developed over years and is reported in several successive publications [27, 28, 22, 29]. Based on this model they designed riboswitches and accurately predicted their overall functionality.

Yet, how much flexibility is indeed necessary to obtain good design results depends a lot on the complexity of the desired functional mechanism and on the degree of novelty. The latter ranges from small performance improvements of an existing device, where solutions are likely to be highly similar to a previously established device, to a device with novel functionality designed completely from scratch.

2. Computational design of RNA molecules

After determining all the details of the target system and the set of components involved, it is necessary to use computational methods. This design step connects the selected building blocks, and adds additional functionality, e.g., ensuring that the desired regulation will take place. All of this computational analysis must also take the target biological environment into account.

Many previous designs were assembled by combining fixed sequences for the desired components [30, 31, 32, 23, 24] and inserting variable regions or changing various nucleotides, to achieve the desired functionality. Especially the latter approach is prone to the generation of dysfunctional devices, as even single nucleotide mutations can greatly alter the structure [33] and thus disrupt the functionality of the components. Therefore, after these alterations are introduced, computational prediction is needed to ensure that components still work in the new environment and that they interact as specified by the design goals.

Alternatives to a computational design approach are in vitro and in vivo screening or selection pipelines [14], where specific component sequences are concatenated with additional variable or random regions, leading to a mixed pool of different candidates [34, 35, 36]. Experimental measurement of the overall functionality of the individual devices leads to the elucidation of well-performing candidates. However, these approaches do not scale well with the complexity of the design mechanisms. Early design decisions, such as where random regions are placed, which exact component sequences are chosen or where sequence variability is allowed, will inevitably limit the solution space. Thus, when bad initial decisions are made, the likelihood of finding optimal devices is limited or — for complex mechanisms — can be vanishingly small. Furthermore, these screening or selection pipelines only allow the measurement of the overall functionality coupled to a readout mechanism, and not the functionality of the individual components. If mechanistic insights are desired, additional experimental analysis as outlined...
In section 3 is needed. Moreover, in the absence of a high-throughput readout for the
desired functionality, a screening or selection requires extensive laboratory effort.

In contrast, computational algorithms are capable of testing thousands to billions of
sequence combinations. Such algorithms can even use the predictive power of models to
evaluate the functionality of any arbitrary sequence, in order to find an optimal overall
solution in a huge solution space. The capabilities of computational approaches are, of
course, limited by the accuracy of the underlying models and parameters. For instance,
structure prediction is only as good as the underlying experimentally measured energy
parameters and the actual fold of a molecule might depend on environmental parame-
ters that are not included in the model. Thus, although computational approaches are
powerful tools to estimate RNA device performance, the predictions certainly need to
be verified experimentally.

2.1. Design Goals

Rational design starts by using the previously collected set of requirements, parameters
and functional mechanisms to define design objectives which can be understood by
design software and prediction algorithms. Many design programs focus on secondary
structures as their main input, as an RNA’s secondary structure is closely related to its
function. This simplification facilitated the development of many design programs, as
it allowed complicated conditions to be broken into two main design goals, called posi-
tive design and negative design. Positive design means that the target structure must
be thermodynamically stable, while negative design ensures that contrary structures
are less stable and thus less probable in the ensemble of structures. A sequence
which folds into the desired target structure is found, if both conditions are fulfilled.

Early design programs, such as RNAinverse [37], incorporate secondary structure
or features based on the secondary structure as the major design goal [38]. Designing
functional RNAs is therefore often reduced to solving the so-called inverse folding prob-
lem, which is the problem of finding a sequence that adopts a given secondary structure
as its most stable, i.e., minimum free energy (MFE), state. Many programs addressed
this by searching for sequences which minimize the distance between the target and the
actual MFE structure [37, 39, 40, 41] or the distance to the desired shape [42]. Zadeh
et al. [43] called this general approach MFE defect optimization and emphasized its
limitation: the probability of the target structure, which is proportional to its Boltz-
mann factor [44], can still be small, due to alternative structures with similar stability
in the ensemble of structural conformations. To circumvent this effect, the probabil-
ity of the target structure in the ensemble can be directly computed and maximized
[37, 45]. Unfortunately, optimizing for this so-called probability defect aims to remove
any structure in the ensemble that is not the exact specified target structure. Thus,
even very similar — and thus probably desired neighboring structures — are removed.
In contrast, the ensemble defect, which was implemented as quality measurement in
NUPACK:Design [46], ensures that structures similar to the target are favored, while
distant or contrary structures are prohibited. Although the latter is a perfect mea-
sure to derive robust single-stable RNA molecules, specifying additional goals might
be helpful. For example mutational robustness [42] aims to retain the desired target
conformation and thus the functionality despite sequence mutations. Such design goals
are perfectly suited to built static RNA molecules like transfer RNAs (tRNAs) which
should reliably fold into a single conformation.

Moreover, many applications require that the designed RNAs are able to change
their conformation upon an external signal. Wolfe et al. [47] introduced a reaction
pathway approach, where the ensemble defect is minimized for multiple RNAs in a
reaction well [48]. The addition of trigger RNA molecules induces structural changes
leading to a multi-state system by multiple RNA strands. Designing a single RNA
molecule which adopts multiple structural conformations is also possible, however it
requires more complicated objective terms as the ratio and the conversion between the
states has to be specified. A bi-state objective was introduced by Flamm et al. [49]
in the switch.pl program and extended to multi-state by Höner zu Siederdissen et al.
[50] and Hammer et al. [51]. It maximizes the probabilities of the target structures in
the ensemble while keeping the free energies of the target states similar. Structural
change upon an external trigger such as temperature change or the addition of ligands
can be achieved by including the external factor in the various terms of one target
state [49, 52]. Flamm et al. [49] proposed objectives that also design the conformation
landscape by defining energy barriers between states or the energy difference of target
states.

Recent studies refrain from solving the inverse folding problem as the only design
objective and thus relying solely on the close structure-to-function relationship of RNA.
They rather specify the characteristics of their desired mechanistic model directly by in-
cluding sequence-to-function measurements, crucial properties of the used components
and the desired functional mechanism [53, 54, 55, 56, 57]. These models might still
include structural features required at specific states, but should encompass much more
than structure prediction at the thermodynamic equilibrium. Pyle and Schlick [58] as
well as Carlson and Lucks [59] suggest that the process of co-transcriptional folding
and other kinetically driven events such as temperature change or ligand interaction
should be incorporated directly into the design process. Also 3D structural motifs or
3D structure prediction could be beneficial due to the closer function relationship. A
kinetic model for co-transcriptional folding and RNA-ligand interaction was recently
included in the ViennaRNA program barriers [60, 61] and could be used to predict
this important design goal. However, a more detailed model usually comes with higher
computational costs and kinetic simulations for thousands of candidate sequences are
rarely feasible as a design objective. Therefore, computationally fast thermodynamic
features dominate current design objectives and more detailed analyses are usually
postponed to a in silico analysis and verification step.

In summary, when formulating design objectives two conditions are crucial. The
main goals should explicitly ensure that the functionality of the individual components
is not disrupted when combined into a novel RNA device and the overall intended
functionality should be specified as directly as possible.

2.2. Generate a sequence towards design objectives

To obtain a sequence which fulfills the desired goals, various techniques were ap-
plied, including stochastic optimization approaches, constraint programming [62, 63] or
lately also machine learning [64, 65]. Some of them were recently reviewed by Churkin
et al. [38]. Basically, all approaches have in common that they require a method
for sequence generation. As the solution space for RNA sequences is exponentially
big, constraints or weights are applied to exclude solutions that do not have essential
properties. Subsequently, the quality of the obtained sequence candidates is evaluated
according to previously specified objectives and a strategy to decide whether to keep
or discard a solution is defined. This strategy might be an advanced optimization al-
gorithm and/or simple rules on how to sort and filter candidate sequences with respect
to certain threshold boundaries.

2.2.1. Generating sequences with given constraints and features

Unconstrained sequence sampling, where one out of four nucleotides is chosen per
position, leads to a search space which is exponentially growing with the sequence
length ($4^n$, were $n$ is the sequence length). Thus, sequence constraints are usually introduced to exclude parts of the sequence space. These can be hard constraints such as specific nucleotide patterns at fixed positions, e.g., for binding motifs and transcription start sites, as well as soft constraints specifying nucleotide compositions like GC-content or a coding sequence for specific amino acids. Sampling towards a probabilistic sequence model could be also applied, e.g., to obtain variable binding motifs. Negative constraints which aim to avoid sub-sequences in the complete design are also desirable, e.g., to avoid specific restriction cut sites.

Furthermore, introducing structural constraints is highly beneficial as they tend to decrease the search space enormously [50, Fig. 2]. For example, ribozymes, terminators or aptamers need to adopt a specific secondary structure to be functional. Thus, nucleotides in such a region must be able to form the correct base-pairing pattern. However, due the complexity of the underlying problem, we need to distinguish between the design of single-stable and bi- or multi-stable RNA molecules. Satisfying a single secondary structure constraint is easy as pairs of nucleotides which can form base-pairs can be simply picked at the corresponding positions. In contrast, respecting multiple structure constraints, maybe even in combination with certain sequence constraints is computationally complex [49, 66]. It is worth noting that crossing base-pairs such as in pseudo-knots can be easily handled as a structural constraint [67, 51].

The first program that could uniformly sample sequences able to adopt two structural conformations was switch.pl [49]. Thus, the corresponding contribution introduced many definitions and problem statements which are still used. Successive programs — multiSrch [68], Frnakenstein [69] and MODENA 2.0 [67] — then implemented decision tree based enumeration methods which enabled to include more than two structure constraints. However, it was shown that such methods introduce undesirable sequences biases [51]. Therefore, Höner zu Siederdissen et al. [50] introduced a graph-coloring counting algorithm — implemented in RNAblueprint [51] — which allowed to uniformly draw sequences with respect to multiple structural constraints. Recently, it has been proven that this problem is #P-hard [66].

As a compatible sequence can, but does not necessarily fold into the given target structure(s), it is beneficial to include RNA energy parameters in the sampling procedure and thereby obtain sequences that are more likely form the desired base-pairs in their structural ensemble [39, 40, 70, 66]. Respecting positive design directly in the sampling procedure was first introduced by Andronescu et al. [39] and implemented in RNA-SSD. The authors enhanced the RNAinverse algorithm by sampling the initial seed sequence concerning a probabilistic model to favor low energy target structures. Subsequently, Busch and Backofen [40] developed a dynamic programming approach for the seed generation in INFO-RNA to find the sequence that adopts the target structure with lowest energy possible. The IncaRNAtion approach [70] extended this to achieve Boltzmann sampling, where a partition function over all sequences given the target structure is calculated. This allowed global weighted sampling where sequences with a stable target structure are more likely to be drawn from the solution space than others and thus a bias toward the desired solutions is gained. By introducing a weighting term to adjust the nucleotide content, they got rid of previously observed GC-biases [70]. However, all these approaches were only capable of generating single-stable molecules. Only recently, RNAredprint [66] introduced a sampling algorithm which basically combines and extends the methods of IncaRNAtion and RNAblueprint [51]. Additionally, RNAredprint allows to include many of the previously mentioned constraints in an computationally efficient way and thus makes sequence sampling more powerful.

A different approach to gain similar functionality was introduced with RNAiFold [62] and its multi-state design successor [63]. The used constraint programming frame-
work allows to define the desired constraints in a convenient way in terms of programming. These include structural constraints as well as sequence soft-constraints such as compatibility to amino acid sequences. They can be specified either exactly or based on BLOSUM62 similarity\(^2\). However, the generally applicable constraint programming framework has the disadvantage of having limited run-time performance as the constraint dependencies are getting more complex. Nevertheless, RNAifold allows to either enumerate all sequences of the solution space, or with the extension of the Large Neighborhood Search (LNS) to explore unreasonable big solution spaces partially. RNAredprint as well as RNAifold are able to count the number of possible solutions and thus report if no sequence exists for the chosen inputs.

2.2.2. Optimization approach for finding desired solutions

By constraining and weighting the sequences during sampling, design goals like the presence of specific sequence patterns, structure compatibility or even positive design are achievable. However, more complex goals cannot be directly accomplished yet. A prominent example is negative design, which aims to get rid of competing states in the ensemble of structures to achieve high occupancy of the target state(s). Thus, RNA design is often described as a combinatorial optimization problem, which can be solved by iteratively tackling the forward problem, i.e., RNA structure prediction. Such approaches can be generally dissected into a sequence generation method (subsubsection 2.2.1), an objective function (subsection 2.1) to evaluate the generated sequences and an optimization strategy which decides whether to keep or reject a generated solution.

The first design programs mainly used the adaptive walk optimization, where a new sequence variant is only accepted if it has a better objective score than the currently best candidate [37, 49]. Zadeh et al. [46] included a rejection list of unfavorable mutations in NUPACK:Design to save computation time. INFO-RNA and RNA-SSD also accept worse scoring sequences with a fixed probability to be able to escape local minima [39, 40]. Simulated annealing, where this acceptance probability decreases during the optimization run, was implemented in RNAexinv, ARDesigner and RiboMaker [71, 53, 42].

Kleinkauf et al. [72] implemented antRNA, a nature inspired ant colony optimization algorithm. Here, ants traverse a decision tree which lists all allowed nucleotides for each position of the RNA sequence and leave a pheromone trail depending on the quality of the overall solution. Successive ants are then weighting their decisions by evaluating the pheromone amount along the trail. Other nature inspired methods follow principles of evolution theory. Here, a population of sequences is evolved by applying mutation and recombination to generate new offsprings, which are subsequently evaluated and selected by their overall fitness. Frnakenstein [69] implements such an optimization approach and in MODENA a multi-objective genetic algorithm based on these principals is applied [45, 67]. Only recently, Rubio-Largo et al. [73] also use evolutionary computation and a multi-objective strategy to optimize for standard design goals. Ramlan and Zauner [68] even developed multiple versions of their design tool with different optimization techniques, including a sorting-bins multi-objective optimization multiSrch and a non-deterministic stochastic variant StochSrchMulti.

Although various optimization strategies were applied for the RNA design problem, there exists to our knowledge no study that compares the efficiency or applicability of the different methods in equivalent context. The latter would require to use the same sampling method, objective function and inputs and only vary the applied optimization
algorithm to observe the differences in terms of run-time and quality. Furthermore, only little is known about the characteristics of the solution landscapes and it is hard to define a proper neighborhood relationship that connects RNA sequences with similar properties. Thus, it is not obvious how to traverse the solution space efficiently. Hammer et al. [51] tested the performance of optimization runs using different neighborhood relations and step sizes. Interestingly, a random mixture of small and large step-sizes performed best on a selected set of design instances. We know that properties of individual sequences are closely connected to the secondary structure and that the relationship between sequence and structure is quite complex. Thus, it might be possible to enhance optimization methods by pursuing the studies on sequence structure maps, neutral networks and shape space covering [33, 74, 75, 76] with respect to RNA design criteria.

2.2.3. Filtering and in silico analysis

Ideally, any design goal can be achieved by solving an optimization problem, which means that all generated candidates would perfectly fulfill the given requirements. However, as this is not the case, a subsequent in silico analysis step is often needed for various reasons. For example, some studies did not use an optimization approach at all and instead enumerated all their candidates. Thus, they rely on such an analysis step to select solutions by ranking and filtering with respect to certain criteria and threshold boundaries [23, 24]. Also, for Garcia-Martin et al. [62] an exhaustive quality determination is essential after the candidate enumeration step of the applied constraint programming approach.

A big advantage of this step is the possibility to sort and filter the candidates with respect to secondary design goals, which could not be included in the main optimization approach. These goals might be computationally too demanding to be iteratively calculated for each candidate sequence during the optimization. Examples are genome wide off-target searches for designed trans-RNAs or exhaustive kinetic folding experiments [77, 57]. By ranking the solutions accordingly it is possible to emphasize parts of the design objectives and choose candidates which fulfill various goals best, an approach which is similar to solve a posteriori multi-objective problem [78]. There, many Pareto optimal solutions are generated and thus the user can choose a posteriori which goal is important and heavily weighted. A careful specification of design goals and constraints is often missing and thus an enormous list of computationally generated candidates has been produced. A ranking and filtering is then the only option to reduce the amount of candidates to a number that can be handled in the laboratories.

Many of the mentioned tools deliver a complete package to convert the desired features of the novel RNA molecule into resulting sequences by doing many complex tasks on the way. In this section we dissected these programs, collected and classified the underlying methods, and highlighted the algorithmic advances recently made. As design problems are usually very diverse, delivering a complete software package with specific design goals, sampling methods and optimization strategies is not very useful for biologically relevant applications. Thus, we think that computational tools for rational de novo design only succeed if they are built as software components which can be flexibly combined to solve a specific design task [51, 57]. It should at least be possible to adapt the objectives, constraints and other prerequisites of the tools to real world scenarios in order to serve biologically meaningful applications.
3. Experimental analysis of the generated candidates

The main goal of the previous computational design was to connect the characterized components and add novel functionality with respect to the biological target environment. With functional testing assays we can investigate whether the candidates exhibit proper overall performance. However, the causes for frequent discrepancies between computational predictions and the biological testing results are manifold. Important influences of the target environment could have been missed in the model, utilized building blocks might have been disrupted due to the new context, or the model failed to describe the new mechanism. Detailed biological and biochemical characterization of the novel RNA device is required to be able to distinguish these aspects and to react by fine tuning parameters of the computational design or by identifying and including missing design objectives.

3.1. Functional testing

The experimental strategy for the initial functional evaluation strongly depends on the basic regulatory mechanism of the device to be tested. In general, RNA-based regulators either adopt alternative structures in order to present or mask certain regulatory elements (riboswitches) or to fold into an active or inactive ribozyme-based endonuclease (aptazymes) [79, 12]. A further possibility is to use trans-acting RNAs that specifically bind to a target transcript and thereby regulate expression. In the following, we briefly summarize experimental approaches for the functional testing of such RNA devices.

Testing at the protein level. Synthetic devices — riboswitches, aptazymes, trans-acting RNAs and others — are usually designed to regulate expression of a certain target gene. Hence, their overall functionality can be tested using a standard reporter like GFP, LacZ, auxotrophic or antibiotic resistance markers. While these genes are easy to handle and give reliable read-outs to measure the response ratio of the system, they have certain disadvantages to be considered. Analysis of green fluorescent protein (GFP) expression or other fluorescent proteins is rapid and straightforward, as it can be monitored without disrupting the cells and even allows for qualitative and quantitative fluorescence-activated cell sorting (FACS). A LacZ analysis is more labor-intensive, as suitable cell extracts have to be prepared. If the corresponding enzyme, β-galactosidase, is expressed it catalyzes substrate cleavage which results in an intense blue product that is easy to identify. The complex analytic method and considerable fluctuations of the β-galactosidase enzymatic activity makes GFP — despite a longer protein folding time — more suitable for functional testing. However, both reporter are perfectly suited for quantification [80, 81]. Auxotrophic and resistance markers can be tested in replica-plating, allowing for rapid selection of positive/functional constructs but a quantitative analysis of these markers is difficult. A very different read-out system was used by the Gallivan lab, where functional riboswitch constructs regulate the expression of CheZ, a protein involved in chemotaxis. As a result, active constructs could be identified by the migration of the bacterial host cells towards the source of the ligand molecule [82].

Testing at the RNA level. In most approaches, riboregulators are designed to control either transcription or translation of a certain target RNA. Yet, it is possible that the RNA construct follows a different regulatory principle than expected [83]. Accordingly, it is important to identify whether such devices act as intended. To identify regulation at the RNA level, several approaches are possible. One of the most obvious strategies is Northern blot, where specific oligonucleotide or antisense RNA probes identify the
expressed RNA. Depending on the experimental setup, this analysis provides further important information like expression level and — for instance in the case of aptazyme-mediated cleavage — size and abundance of individual RNA fragments. Similarly, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) can be used to investigate RNA expression and its turnover. A less labor-intensive approach is the usage of light-up RNA aptamer sequences as reporter. Here, Systematic Evolution of Ligands by EXponential enrichment (SELEX)-derived aptamers like Spinach, Broccoli or Mango can be used that mimic the fluorophore of GFP [84]. A disadvantage of light-up RNA aptamers is their rather low sensitivity. To overcome this problem, serial arrangements of multiple aptamer copies are used [85, 86]. Furthermore, the presence of high backgrounds that are not observed in vitro, necessitate proper control experiments and complicate the general in vivo applicability [87]. As an alternative, the Jäschke lab developed turn-on aptamers that either bind a fluorophore (like sulforhodamine B) or a quencher molecule conjugated to a fluorophore. If these ligands interact with the aptamer, the fluorescence signal strongly increases and can be measured without disrupting the host cell [88, 89]. However, the general applicability as a read-out system for synthetic RNA regulators still needs to be shown.

**Upscaling of functional testing.** The described read-out systems for functional RNA-based regulators are rather limited in terms of the number of samples to be tested. Computational design approaches can produce hundreds of equally good candidate sequences and valuable information about their prediction performance can only be gained if many of them are evaluated experimentally. Hence, it is desirable to test not only a few candidates but to scale up functional testing to several hundred or more. FACS is an ideal strategy to do so [35, 90]. However, the applicability of this approach is currently limited to fluorescence read-out on the protein level, because usage of the available light-up RNA aptamers suffers from the low sensitivity, as described above. If microfluidics systems such as the one applied to optimize the Spinach aptamer [20] are applicable as in vitro proxy needs to be tested.

### 3.2. Characterization of individual constructs

Independent of the outcome of the functional testing, it is important to understand and verify the mechanistic basis of the regulation in order to improve the computational design. Thus, it is mandatory to confirm and quantify the accomplishment of the initially specified design goals individually, which includes to assess the functionality of any utilized building block in the new context, to verify the mechanism of newly added functionality and to check for missing indispensable design goals. Especially the latter is a frequent cause of dysfunctional designs as there are currently many uncertainties in the predictions, which include inaccurate or missing model parameters as well as unconsidered influences of the target environment. Frequently missing aspects are degradation, RNases, RNA binding proteins and chaperons, off-target interactions, tertiary interactions and kinetic or co-transcriptional effects. In the following, we give a short — and definitely incomplete — overview about currently used investigations.

**Structure analysis of RNA devices.** In the case of riboswitches or aptazymes, the regulatory principle is based on the (predicted) structures and their ligand-dependent rearrangement. To investigate whether the intended structures are indeed dominating the structural ensemble in the target environment, several approaches are feasible. The most straightforward — but restricted to in vitro investigation — is in-line probing. End-labeled in vitro transcripts are incubated in a buffered solution over a long period of time, allowing that unpaired nucleotides in single-stranded regions adopt a conformation where the 2’OH group is in line with the phosphorus atom and the adjacent oxygen...
of the neighboring phosphodiester bond. This in-line configuration allows for a nucleophilic attack of the 2’hydroxyl, resulting in a specific cleavage of the single-stranded site [91]. Based on the resulting band pattern in polyacrylamide gel electrophoresis, the structural organization of such transcripts can be identified. Similarly, lead-induced cleavage can be used to generate band patterns indicative of single-stranded elements, where the hydrated Pb\(^{2+}\) acts as a Brønsted base and abstracts the proton from the 2’OH in the ribose, rendering it highly nucleophilic. An alternative is SHAPE, where single-stranded nucleotides are modified by chemical treatment (DMS and NMIA as examples) and detected by reverse transcription (RT) stops at these positions [92, 93].

While in-line probing can be used only in vitro, lead probing was successfully applied in vivo for individual transcripts, where the fragile positions were identified based on primer extensions in reverse transcription. Recently, also structure probing methods for high-throughput in vivo analyses were established. Methods like SHAPE-Seq, DMS-Seq, PARS, FragSeq, MOHCA [94, 95, 96, 97, 98] combine the sensitivity of single stranded RNA for cleavage or modification with massive parallel sequence analysis. While such approaches are more suited for whole structurome investigations, they can in principle also be used to determine the structural composition of individual RNA constructs.

An unavoidable fact for a switching RNA molecule is the side by side existence of multiple structures in the ensemble, which inevitably results in overlapping signals when analyzed structurally. Yet, based on SHAPE mapping data, a first approach to identify the individual conformations was recently developed [99], indicating that it might be possible in the near future to investigate RNA devices with multiple structures at equilibrium.

**Characterization of ligand interaction and recognition.** In the case of aptamer-dependent constructs, it is valuable to identify the actual affinity and interaction with its trigger molecule in the actual target environment. This includes experiments to verify the presence of the ligand in the actual reaction compartment, e.g., the bacterial cell in in vivo studies. Here, mostly in vitro investigations are performed, but in vivo approaches might also be applicable. Again, a rather easy approach is in-line probing in the absence and presence of the ligand [91]. Originally developed to determine the structure of the ligand-bound RNA and its affinity in term of K\(_d\), this method can be equally used for riboswitches [100]. Here, also lead probing as well as in vitro SHAPE should be applicable. Other approaches like surface plasmon resonance spectroscopy (SPR), electrophoretic mobility shift assay (EMSA) or microscale thermophoresis (MST) might also be usable. However, if the interacting ligand is a rather small molecule (as in most cases), the impact on the mobility of the transcript might be too small to be reliably detected.

**Determine RNA stability and degradation.** RNA stability and degradation are important factors when it comes to real-world applications of artificial RNA devices. As they are often ignored in current design models, detailed information about the stability and the degradation over time is essential in order to detect undesired effects. Previously described RNA quantification experiments can also be applied here, such as lifetime experiments with northern blot or qRT-PCR. These methods are commonly used to investigate and measure the RNA decay.

**Characterization of the transcript ends.** Previously mentioned experiments did not cover to verify that the complete RNA molecule, from its intended start nucleotide to the end, is present and appropriate. However, the actually occurring 5’- and 3’-ends of the designed RNA are of specific interest as they give information about, for example,
the transcription start site, termination site or cleavage positions in an aptazyme. As transcription start sites can differ from promoter to promoter, it is important to identify the exact sequence composition, since even a single unexpected nucleotide can lead to misfoldings and loss/reduction of function. The method of choice is 5'- (or 3') rapid amplification of cDNA-ends (RACE), where in the basic version of the protocol oligonucleotide adapters are ligated to the 5'- (or 3') end of the transcript followed by cDNA synthesis. The resulting product is PCR-amplified by the usage of a target RNA-specific primer. The adapter-ligation site (corresponding to the RNA end) is then identified by sequencing. Depending on the actual problem, many different RACE versions are available for RNA end investigation in prokaryotes as well as eukaryotes [101, 102, 103].

**Analysis of kinetic effects.** The actual functionality of multi-state RNA devices often depend on precisely timed kinetic effects, which are typically difficult to comprise in folding predictions. For example, an intrinsic terminator has to be present at the exact right time during the transcription process in order to stop the RNA polymerase efficiently. A fortiori, experimental examination of intermediate structural conformations during transcription is necessary to understand the function of many RNA devices, especially of riboswitches. Although Watters et al. [104] managed to obtain valuable structural folding information of the crcB fluoride riboswitch during transcription by utilizing co-transcriptional SHAPE-seq and Helmling et al. [105] successfully applied NMR spectroscopy to investigate co-transcriptional intermediate structures of the I-A type 2'dG-sensing riboswitch from Mesoplasma florum, such experiments are tedious and labor intensive and thus of limited use for design applications. Moreover, for a proper verification of the ligand-binding model not only the binding affinity, but also the speed of the reaction — the binding rate — is a crucial and game-changing factor. Therefore, Schaffer et al. [106] applied methods like in-line probing and SPR to investigate the kinetics and thermodynamics of the ligand to AdoCblRiboswitch interaction. Alternative fluorescent ligands with similar binding behavior and fluorescent nucleobases can be also used to obtain association rates by stopped-flow fluorescence [107, 108]. Nonetheless, for the purpose of a appropriate cost-benefit ratio, many of these characterization experiments are often spared. If so, at least functional testing assays with smartly designed control constructs to derive valuable characteristic details of the constructed RNA molecules should be performed. For example, proper controls for a transcriptional riboswitch could be a construct which comprises only the terminator or only the aptamer and the RBS with the otherwise exact same sequence as the designed construct. Also well-considered time series of standard RNA or protein read-out experiments can deliver important details about intermediates and kinetic effects. Furthermore, if it is not possible to derive worthwhile information from the characterization experiments, randomization and a subsequent screening or selection can be used to optimize the construct. However, it is important to investigate the optimized constructs and gain valuable knowledge about their (improved) functionality in order to enhance the design model for future applications.

**4. Conclusion**

Rational de novo design always requires some initial knowledge about the functional mechanism, the characteristics of components and the target environment. For a riboswitch regulating at the transcriptional level, for example, the most obvious
needed information is termination efficiency and mechanics of RNA structural refolding. However, other information is also important, e.g., ribosomal binding efficiency, which determines translation initiation for reporter gene expression. The combined information then makes up the functional model, which is part of the overall design goal.

Previously, this information was determined only for specific sequences, which limited the ability to optimize the overall computational design. The usage of a catalog of sequences for the individual building blocks will improve this situation to some extent. Currently, data-driven models are developed to provide enough detail and variability for this purpose. However, at the moment such models are rare and rational designs are often built with little knowledge about the biological environment and the functional mechanism. To compensate these uncertainties lots of subsequent laboratory work is required, such as functional testing and characterization experiments to verify the functionality of the individual components in the new context. Due to recent advances, well-designed high-throughput selection and screening pipelines are able to measure function of many varying RNA sequences, thus producing the amount of standardized data necessary to built detailed and extensive models [59, 22, 109, 110, 111].

Furthermore, we realized that rational de novo design was sometimes performed without much support from in silico calculations. We speculated that the reason might be a lack of trust in the available RNA design tools due to the limitations of computational prediction. Examples are the inability to consider pseudoknots, the exclusion of non-Watson-Crick pairings, the often neglected influence of ions in the predictions and models that do not accurately reflect ligand-RNA interactions. Furthermore, predictions might miss the structural influences of proteins and organic molecules in the cell and cannot explain possible structural differences between in vitro and in vivo experiments.

This mistrust is increased by the frequent lack of experimental verification or real-world synthetic biology applications. Those are often missing as experiments are time-consuming and expensive. Instead, the superiority of novel algorithms is often concluded with extensive benchmarks towards some seemingly arbitrary goals with no obvious biological applicability. We understand that benchmarking requires standardized inputs in form of an extensive test set and a precise definition of a perfect solution. These prerequisites are hard to be fulfilled by laboratory experiments. Nevertheless, in our opinion, the best benchmark of an RNA design tool is to show its ability to reliably produce functional RNA devices in real-world settings. NUPACK:Design, for instance, has been used to implement so-called “toehold” switches [25], small transcription-activating RNAs [112], small conditional RNAs to regulate RNA interference [113] and to control the formation of complex nanostructures [114]. The RNAiFold approach has been adapted and applied to design hammerhead ribozymes [115], regulate internal ribosome entry site (IRES) activity [116] and to realize temperature-sensitive IRES elements [117]. Thermoswitches have been designed utilizing switch.pl [118]. In most cases, the developers of the software are among the authors of all these application studies. This indicates that expert knowledge about the in silico design approach is beneficial for a successful in vivo implementation.

Of course, it is possible to completely skip computational design and instead utilize high-throughput screening or selection approaches to obtain complete RNA devices with the advantage that no model is required [83, 36, 119]. In such studies, the measurement directly indicates the functionality of the individual devices from the variable pool. However, the results of these studies do not generalize to other design problems. By contrast, if appropriate data can be collected in experimental design projects, this data could provide a basis for a computational model that might accelerate future design projects. In a successful example of this idea, Townshend et al. [120] use a
workflow of FACS and next-generation sequencing to achieve this [121].

On the experimental side, new approaches are needed to be able to measure and verify important parameters of \textit{in silico} models in an exact and high-throughput manner. This would allow the design and validation to form a stronger feedback loop in the design process, because directly measured data will lead to improved models.

Our vision is that it is possible to quickly and consistently design RNAs with the desired functionality for their use as artificial logic in gene-regulation, as scaffolding devices or as many other therapeutic applications. A fundamental stumbling block is the relative lack of effective collaboration between computational and experimental scientists. We believe that the inadequate level of communication between these fields is limiting the ability to develop quantitative mechanistic models. Moreover, individual pipeline steps are developed independently of one another, and thus fail to function optimally together. Finally, much research is ineffective in promoting progress, because it pursues arbitrary goals that are not biologically relevant.

To address this problem, there is a need to focus on the practical applicability of research, including novel algorithms, wet lab techniques and design projects. There is a need for computational tools that are useful for their actual purpose, experiments that measure properties needed for \textit{in silico} models and valuable data generated when performing screening and selection experiments.

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Abbreviations

- \texttt{tRNA} transfer RNA
- \texttt{RNA} RiboNucleic Acid
- \texttt{SELEX} Systematic Evolution of Ligands by EXponential enrichment
- \texttt{MFE} minimum free energy
- \texttt{RBS} Ribosome Binding Site
- \texttt{SD} Shine Dalgarno
- \texttt{GFP} green fluorescent protein
- \texttt{FACS} fluorescence-activated cell sorting
- \texttt{RACE} rapid amplification of cDNA-ends
- \texttt{qRT-PCR} quantitative reverse transcription-polymerase chain reaction
- \texttt{E. coli} \textit{Escherichia coli}
- \texttt{RT} reverse transcription
- \texttt{SPR} surface plasmon resonance spectroscopy
- \texttt{EMSA} electrophoretic mobility shift assay
- \texttt{MST} microscale thermophoresis
- \texttt{IRES} internal ribosome entry site
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