# Multivalent Circular Aptamers: Versatile Nanostructures for Biomedical Applications

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## ABSTRACT

Nucleic acid aptamers are moving towards genuine competitiveness in therapeutic, diagnostic and biomaterials applications. We have introduced a major new aptamer class - multivalent circular aptamers or 'captamers' - that combines aptameric recognition with multitasking nucleic acid functions through the use of modular engineering principles derived from DNA nanotechnology. DNA captamers have been demonstrated in 2-, 3- and 4-headed versions, focussing upon the pleiotropic activity that can be obtained by combining different functions together. Several multitasking applications are described here. Development of captamer therapeutic candidates is moving towards a practical implementation of autonomous biomolecular computing that offers the potential to create a powerful class of smart nucleic acid drugs.

***Keywords***: nanobiotechnology, bioelectronics, biomolecular computing, rolling circle amplification.

## 1 BACK TO THE FUTURE

Fifty years after its discovery, the double helix continues to inspire fundamental science and new technologies. The field of structural DNA nanotechnology, which began with the engineering of basic three-way junctions [1], is producing an increasingly impressive range of sophisticated nanostructures and prototypical nanomachines. Over a similar time period, the compelling appeal of specific control over gene expression via 'anticode' technologies has generated cyclic waves of excitement with the successive development of antisense [2], nucleozyme and now RNAi technologies. Meanwhile, technological use of evolutionary principles has produced 'aptamers' [3-5] - folded nucleic acids capable of specific three-dimensional molecular recognition.

Seeking to unite these approaches - three technologies *must* be better than one - we have taken the simple but effective approach of using basic DNA nanotechnology principles to combine aptamer and other nucleic acid motifs together in novel and biomedically useful ways.

## 2 CAPTAMERS

Multivalent circular aptamers or 'captamers' are constructed by connecting two, three, four or more folded aptamer domains together with engineered nucleic acid duplex and junction structures [6]. This simple piece of engineering produces nucleic acids with a set of extremely useful properties that we have been slowly elaborating over recent years [7-13]. Figure 1 shows some schematics of basic multivalent aptamers.

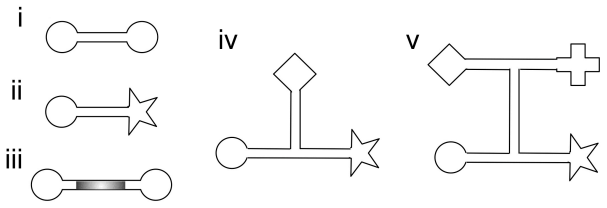


Figure 1: Representative captamers. Aptameric heads are depicted as different shapes. (i) Homodimer, (ii) Heterodimer, (iii) Homodimer with shaded internal transcription factor binding site, (iv) Trimer based upon a 3WJ scaffold, (v) Tetramer based upon two 3WJ scaffolds.

### 2.1 Stability

The relative instability of nucleic acids is a significant issue for technological applications in biology. Instability comes in two forms: (1) conformational heterogeneity due to kinetic and/or thermodynamic trapping of misfolded aptamer structures, and (2) degradation by ubiquitous nucleases, a particular problem for RNAs. The entropic properties of circular nucleic acids give them greater thermal stability as measured by melting temperature, which typically improves by 10-30 °C upon circularization. It also happens that the majority of physiological nuclease activity in mammals is of the 3'-exonuclease variety, so that removing the 3'-end by circularization produces a

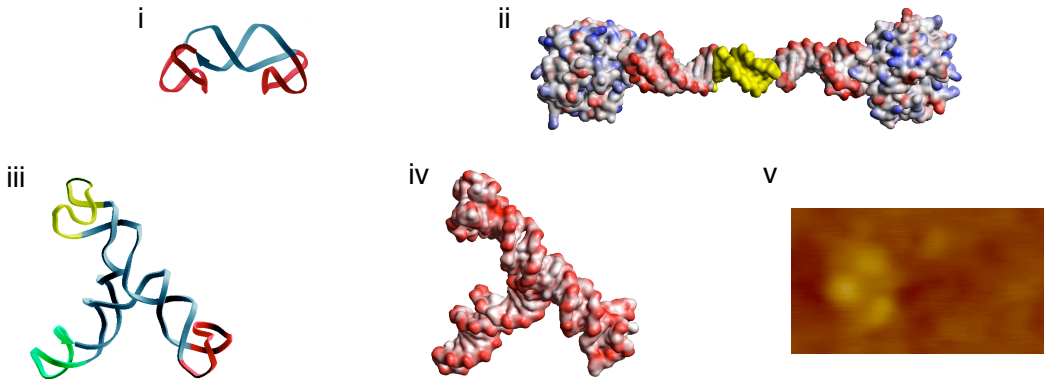


Figure 2: Three-dimensional captamer models. (i) cTT-2 dimer, (ii) cTT-6 dimer with internal CRE site (yellow) and two aptamer-bound thrombin molecules, (iii) cTUA-1 trimer, (iv) Space-filling model of cTUA-1, (v) AFM image of cTUA-1 on mica.

major improvement in nucleolytic stability. We have shown for example a 30-fold improvement in DNA aptamer half-life in human blood products [6].

## 2.2 Bioprocessing

An early captamer design goal was to allow for production by enzymatic methods instead of chemical approaches. In principle, provision of a suitable template to a sequential bioreactor consisting of (1) polymerase, (2) ligase and (3) exonuclease stages could achieve this goal at any necessary scale. Large-scale enzyme bioreactor production is not economically feasible at the moment, so chemistry and physical separation can be employed as for other aptamers, but these methods should become available in time.

## 2.3 Pleiotropy

Perhaps the most important property of captamers is their ability to multitask. This was initially conceived as the simultaneous action of multiple aptamer functions, such as might be required to inhibit two key enzymes involved in a disease process, but has evolved into a potentially powerful new framework for the development of smart nucleic acid drugs (see Section 3).

The first captamers combined two identical aptamer domains into a divalent dumbbell-shaped construct (Figure 2i) that displayed greater inhibitory potency towards thrombin than twice the amount of the monomeric GS-522/ARC183 aptamer under development by Archemix Inc. [6]. Engineering of selected three-way junction (3WJ) structures generated antibody-like aptamers with three alternative binding specificities or two binding sites and an effector/immobilization site that can carry a unique reporter group (Figure 2iii,iv) such as biotin, a fluorophore or other moiety.

The first demonstration of nucleic acid drug pleiotropy came with the introduction of a transcription factor decoy sequence into the central duplex of a dimeric aptamer (Figure 2ii). Such constructs can then modulate gene expression while simultaneously targetting other molecules.

For example, an E2F decoy sequence of the kind under development by Corgentech Inc. and two G-quartets like those of AGRO100 from Aptamera/Antisoma can be combined to create a synergistic more-selective antiproliferative. Since almost all nucleic acid drug formats - aptamer, transcription factor decoy, antisense, nucleozyme, immunomer and RNAi - are compatible with the captamer topology, a powerful set of complementary activities can be engineered as required.

## 2.4 Nanostructures

Because the intrinsic stability of captamers makes them attractive as Lego-like building blocks, we have begun to explore their self-assembly behaviour on surfaces (Figure 2v). Adopting tectoRNA-like principles may produce more robust nucleic acid assemblies than it has been possible to achieve to date.

## 2.5 Biosensing and Bioelectronics

The antibody-like behaviour of some captamers allows them to be used in straightforward sensing applications. Colorimetric and fluorescent sandwich assays with the expected performance characteristics have been shown.

We have recently demonstrated a sensitive and specific isothermal ‘proximity extension’ protein quantitation assay that exploits captamer circularity. In this assay dual simultaneous binding of a captamer and a 3’-tailed aptamer to a molecular target enables signal generation by polymerase-mediated rolling circle amplification (RCA). The proximity extension assay is some three orders of magnitude more sensitive than an equivalent aptamer sandwich assay and moves towards overcoming the inherent tendency of polyanionic aptamers to produce some

non-specific background signals in complex polycation-bearing mixtures. This avoids the works-in-buffer-fails-in-blood syndrome displayed by many novel assays.

Separately, we have been exploring the closer connection between electronics and molecular biology through the enzymatic introduction of electroactive groups into DNA and RNA [14,15]. The proximity extension assay has been implemented in an electrochemical format and other captamer-based bioelectronic applications are underway. The structural integrity of captamers makes them particularly useful in providing a solid structural framework for mounting electron carriers.

## 2.6 Biomaterials

Biomaterials applications of aptamers are oddly underdeveloped, especially when it is straightforward to create new surfaces with unique properties. We have for example produced a captamer called cTSA-2 that contains aptameric heads against thrombin and L-selectin plus an amino-labelled loop. This captamer can be immobilized via the amine loop to simultaneously perform anticoagulation and reversible leukocyte capture.

# 3 A BIOMOLECULAR COMPUTING PARADIGM FOR MODULAR NUCLEIC ACID THERAPEUTICS

The most technically advanced potential application of captamers lies in a computing-based model of nucleic acid drug development. Benensen and co-workers have recently described an elegant but biologically impractical approach to the control of gene expression through autonomous biomolecular computing mediated by multiple nucleic acid hybridizations [16]. In principle, multivalent captamers may provide a way to attempt a more feasible implementation of this concept.

The essential problem for any intracellular nucleic acid therapeutic is to first be delivered to the correct cells of the hundreds of cell types in the body, to localize to the correct cellular compartment such as the nucleus, cytosol or mitochondria, and then to interact with and either inhibit or activate its desired molecular target, which is typically one or more protein, RNA or DNA regions. In a biomolecular computing paradigm, these requirements can be naively (for emphasis, *naively*) described by binary IF-THEN-ELSE logic.

Captamers most suitable for this implementation are best viewed as an internal payload such as a transcription factor decoy for DNA or an RNAi sequence for RNA that is carried by aptameric delivery motifs. So-called 'escort' aptamers [17] have previously been proposed to transport agents specifically to cells. Payload-bearing captamers potentially capable of computing therapeutic requirements can be generated by the combination of selection experiments and rational nucleic acid engineering that is characteristic of these molecules. To take an example, for delivery of a single RNAi moiety targeted against a protein required to maintain proliferation of prostate tumor cells it would first be necessary to isolate RNA aptamer heads capable of directing selective uptake by the relevant prostate cell type, or preferably prostate tumor cells regardless of their bodily location to allow for targeting metastases. In principle this can be achieved by an aptamer domain that binds to an internalizing receptor and/or receptor cargo that is unique, specific or selective for the prostate cells. The aptamer must then escape the cell's vesicular trafficking system by piggybacking a ride to the nucleus, perhaps on the same or a different protein. This task is very difficult to achieve by rational means, but is potentially susceptible to cellular or *in vivo* selection of an appropriately constituted aptamer library. A great strength of aptamer selections is that they can be conducted in both positive and negative modes - selection for one binding property but not another, so that relatively greater uptake by the correct cell type is in principle selectable. We have developed circular DNA and RNA aptamer selection methods for particular application to the key technical challenge of selective uptake, and this work is underway.

Upon arrival in the nucleus, the RNA aptamer of our example should then enter the miRNA pathway, which commences with specific cleavage by the Drosha RNASE3L RNase and results in cytoplasmic expression of RNAi activity against a targetted mRNA, say of the MBD2 DNA demethylase. Ensuring correct cleavage of the given siRNA moiety from the aptamer construct is a relatively straightforward rational nucleic acid engineering problem that we are separately addressing. It may even be possible to require the presence or absence of a particular nuclear factor such as MINA before cleavage will proceed at an appreciable rate.

Taken together then, the "algorithm" for this captamer activity might then be expressed as:

IF (uptake by prostate cells)
  AND (NOT other cell types)
  AND (construct localizes to nucleus)
  AND (tumour protein MINA present)
THEN (initiate RNAi pathway for MBD2 mRNA)
ELSE (allow aptamer degradation)

This formulation ignores a range of biological complexities such as the inevitable partitioning resulting from mass action behaviour in complex environments, but does at least offer a clear statement of design goals for particular captamer constructs. Early attempts to implement this paradigm will no doubt fall far short of the magic bullet, but the general approach is well worth pursuing in as many aptamer and other formats as possible. The extremely poor potency of a recently heralded therapeutic siRNA demonstration in rats [18] highlights the

importance of this problem, making the prospect of universally constructed smart nucleic acid therapeutics too great to ignore.

## 4 PROSPECTS AND PERILS

Although obtaining uptake-specific aptamers is technically straightforward, different aptamer library formulations are likely to yield different results. The pioneering cellular uptake aptamer selections of Carson and co-workers [19] have provided an important view of the essential issues. It will be necessary for a number of groups adopting a variety of approaches to properly explore the potential of this phenomenon.

An additional lurking hazard for the development of natural captamer therapeutics lies in the innate immune system, where the TLR3/7/8/9 receptors mediate a cellular inflammatory response against certain extrinsic nucleic acids while PKR, OAS, ADAR and probably other proteins respond to nucleic acids in the cytosol or nucleus. A direct solution to this problem is to use modified nucleic acids to evade the receptors, but for captamers this would greatly complicate RNAi-based strategies like the one outlined above. It is quite likely that targeted, highly specific captamers could slip under the radar of the innate immune system at low concentrations, although this remains to be seen. Alternatively, we could make a virtue of necessity by targeting viruses and other diseases where an interferon response is naturally mounted. Encouragingly, DNA microarrays indicate that the first simple DNA captamers are mildly anti-inflammatory. Until we have a better understanding of innate immune 'epitopes', a case-by-case approach will be needed. Manifesting the therapeutic potential of these molecules will be an agreeable challenge for the next several years.

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