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Morpholinos and PNAs compared

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Introduction

This chapter will compare and contrast the properties and applications of two leading antisense molecules, Peptide Nucleic Acids (PNAs)[1,2] and Morpholinos[3,4]. Where appropriate, I discuss the compelling advantages which these two advanced 'blocker' types provide relative to 'modifier' types of antisense molecules. Some of the properties which are compared include chemical synthesis strategies, chemical stabilities, backbone flexibilities, aqueous solubilities, target selection criteria, target binding affinities, and sequence specificities.

Since the mid-1980s, phosphorothioate-linked DNA oligos (S-DNAs) have dominated the antisense field. For many antisense applications, however, advanced non-ionic oligos provide a far better combination of properties, including stability in biological systems, high efficacy and specificity, lack of toxicity, and freedom from non-antisense effects. Prior to the discovery of PNAs by Nielsen *et al.*, I was the first person to conceptualize and to synthesize morpholinos, recognizing their advantages for antisense chemistry. Morpholinos and PNAs share a number of key properties, such as non-ionic backbones whose structures differ radically from that of nucleic acids, resistance to enzymatic degradation, and high (Morpholinos) or very high (PNAs) affinity for complementary RNA sequences. In the context of diagnostics, a particularly valuable property of both structural types is that they strongly pair to complementary genetic sequences under conditions which disrupt secondary structures of nucleic acids. Another particular advantage of morpholinos, and part of the original impetus to develop them, is the fact that they are relatively cheap to produce; the subunits of Morpholinos can be assembled into antisense oligos via simple and efficient coupling to the morpholine nitrogen, without the expensive catalysts and post-coupling oxidation steps required in the production of most nucleic acid analogs.

In spite of their many similarities, Morpholinos and PNAs also exhibit significant differences which translate to differing advantages in particular applications. Two key differences which bear on their preferred applications are: 1) PNAs have higher affinities for RNA than do Morpholinos, though both structural types form duplexes with RNA which are more stable than DNA/RNA duplexes, and much more stable than S-DNA/RNA duplexes; 2) Morpholinos are highly soluble in aqueous solutions, generally 5 to 30 mM for 25-mers, depending on sequence, whereas PNAs are typically several hundred-fold less soluble. As a consequence of these differing properties, it appears that PNAs are better suited for high-affinity applications such as targeting short sequences (e.g., the exposed segment of telomerase RNA) and for discriminating between single base differences, as in SNPs (single nucleotide polymorphisms). Conversely, Morpholinos excel in applications which require high aqueous solubility and exquisite discrimination between a targeted mRNA and tens of thousands of non-target mRNAs, such as *in vivo* applications with developing embryos and other complex systems.

Classification of antisense structural types

Sequence-specific nucleic acid-binding oligomers ('oligos') can be divided into two categories:

1) 'blocker' oligos which hydrogen bond to, but do not modify their complementary (targeted) genetic sequences;
2) 'modifier' oligos which H-bond to and then modify their targeted sequences, either directly by crosslinking or cleavage, or indirectly via RNase H-mediated degradation.

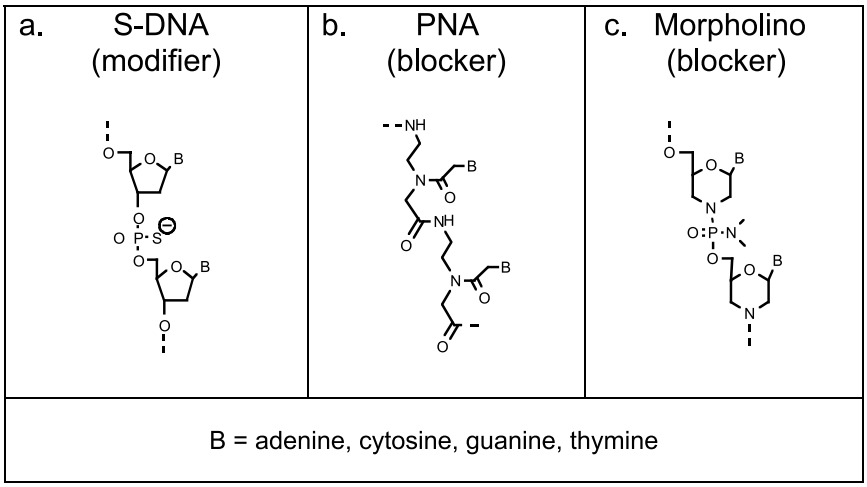

Figure 1. Representative oligo structural types.

Oligos in these two categories differ fundamentally in regard to which sequences they can target in an RNA transcript. Specifically, modifier oligos, exemplified by S-DNAs (Figure 1a), have the potential to target sites anywhere in an RNA transcript. In contrast, blocker oligos, exemplified by PNAs (Figure 1b) and Morpholinos (Figure 1c), are generally ineffective when targeted against intron sequences in a pre-mRNA, unless the target site is immediately adjacent to a splice site. Blocker oligos are also generally ineffective when targeted against amino acid-coding sequences more than about 40 bases 3′ to the AUG translational start site in an mRNA. This is probably because once a ribosome completes its assembly at the AUG translational start site, its ATP-dependent unwinding activity becomes capable of displacing nearly all blocker oligos from their targeted sites in the downstream amino acid-coding region.[1,4,6]

Figure 2a illustrates the limited regions of an RNA transcript which are potentially available for targeting by blocker oligos, currently estimated at about 5% of the total length of the average RNA transcript. Figure 2b illustrates the extensive region of an RNA transcript which is potentially available for targeting by modifier oligos of the types which effect cleavage or degradation of their targeted sequences, essentially 100% of RNA transcripts.

From a targeting perspective, at first glance the modifier oligos (e.g., S-DNAs) appear attractive because they offer the possibility of targeting sites throughout an RNA transcript (Figure 2b). However, in practice this apparent advantage is greatly lessened because selecting effective targets for modifier oligos is often highly unpredictable and requires much empirical experimentation,[7,8] presumably because most possible target sites are unavailable due to secondary structures in the RNA transcripts and/or due to other poorly understood factors.

In contrast to the case for modifier oligos, advanced types of blocker oligos (PNAs and Morpholinos) targeted against sequences within their targetable regions (Figure 2a) generally give predictable and effective results.[9] This is probably because the high RNA-binding affinities of advanced blocker types allow them to efficiently invade the extensive RNA secondary structures common to natural RNAs.[4]

Preparation of morpholinos and PNAs

Subunit synthesis

In the context of custom oligos for research applications, the costs of starting materials, ease and yield of key steps in subunit syntheses, and the costs of oligo assembly and processing are of lesser interest to both producers and users because labor expenses generally dominate the cost of custom oligos. However, for larger-scale applications, such as clinical diagnostics and therapeutics, these factors play a major role in the cost of the finished oligo. Because production costs will be an important factor in clinical applications, these costs will be discussed below.

PNA subunits are prepared from the standard purine (A and G) and pyrimidine (C and T) nucleobases. The key step in producing PNA subunits is selective alkylation on the nitrogen at the 9 position of the purines

and the 1 position of the pyrimidines, as illustrated in Figure 3a. Any lack of selectivity in this alkylation reaction will require careful purification of the desired product. In contrast to alkylation of the bases, adding the N-protected aminoethyl glycine backbone moiety and adding appropriate nucleobase protective groups should be relatively straightforward and should give good yields and purities.

Morpholino subunits are prepared from the natural rA, rC, and rG ribonucleosides. For the fourth subunit, we prefer to use synthetic rT instead of the natural rU because of the positive impact T bases have on RNA-binding affinities of the resulting oligos. The key steps in synthesis of Morpholino subunits are as follows: oxidative opening of the 5-membered ribose ring; closing the resulting dialdehyde on ammonia to give a 6-membered morpholine ring; and reductive removal of the original 2′ and 3′ hydroxyls, as illustrated in Figure 3b. These three steps are carried out sequentially in a single pot without intervening purifications. Once the ribose-to-morpholine conversion is accomplished, adding appropriate protective groups and adding the chlorophosphoroamidate moiety to the original 5′ oxygen are relatively straightforward and give good yields and purities. It is noteworthy that in the dry state the final protected/activated Morpholino subunits are stable for many months at –20°C.

Table 1 shows approximate relative costs of the key subunit starting materials for PNA and Morpholino subunits from representative chemical supply companies, and for comparison, subunits for DNA analogs such as S-DNAs.

Both PNAs and Morpholinos enjoy a significant advantage over DNA analogs in regard to cost of starting materials, although some of this cost advantage is lessened due to the costs and yield losses in converting the nucleobases and ribonucleosides to their respective PNA and Morpholino subunit structures.

Oligo assembly

In regard to assembling subunits into oligos, PNAs are typically assembled using a 3-reaction cycle: coupling, capping, and deprotection, as shown in Figure 4a. Including the intervening washes, the subunit addition cycle typically consists of 7 to 9 individual steps.[10,11,12]

For assembly of Morpholinos, we use a very simple 2-reaction subunit addition cycle analogous to that used for active-ester peptide synthesis. The two reactions are coupling and deprotection, as shown in Figure 4b. A capping step is not used because coupling and deprotection efficiencies are very high (estimated at about 99.7% for each reaction). Our newest subunit addition cycle for Morpholino oligos comprises these two reaction steps plus three wash steps, for a frugal 5-step assembly cycle carried out on a simple custom-made synthesizer. Through careful selection of solvent combinations, about 80% of the expensive solvent components can be recovered and, after distillation, reused. This recycling allows for cost saving on both the purchase of more solvents and disposal of used solvents. In principle, because of the good stability of the activated Morpholino subunits, much of the excess subunit used to drive the coupling to completion can also be recovered and reused. However, this is only practical in large-scale synthesis of an individual oligo. The recovery and reuse of both subunits and solvents has the potential to substantially reduce oligo costs in large-scale production of Morpholino therapeutics.

Properties of morpholinos and PNAs

Chemical stability

The PNA backbone is stable to strong bases (which would degrade RNA) and to strong acids (which would depurinate DNA). The only significant instability of a PNA chain is when it has a free aminoethyl N-terminus, which occurs briefly during each coupling cycle in oligo assembly[10] and would occur if one failed to cap the N-terminus on completion of the oligo. Because of favorable geometry, this aminoethyl moiety (pKa ∼10.5) can cause rearrangements and subunit deletions.[10] Capping or otherwise modifying the N-terminus after completion of oligo assembly effectively stabilizes the finished oligo.

A particular advantage arising from the exceptionally high chemical stability of the PNA backbone is that while the oligo is still on the synthesis resin, a series of amino acids can be added to the oligo to generate a peptide component suitable for enhancing delivery into cells, or suitable for diagnostic applications. The PNA component easily survives the rather harsh conditions required for removal of protective groups on the side chains of that added peptide.

The Morpholino backbone is also stable to strong bases, but in contrast to the acid stability of PNAs, the Morpholino backbone is cleaved by strong acids, such as trifluoroacetic acid. While the sensitivity of Morpholinos to strong acids does impose some limitations

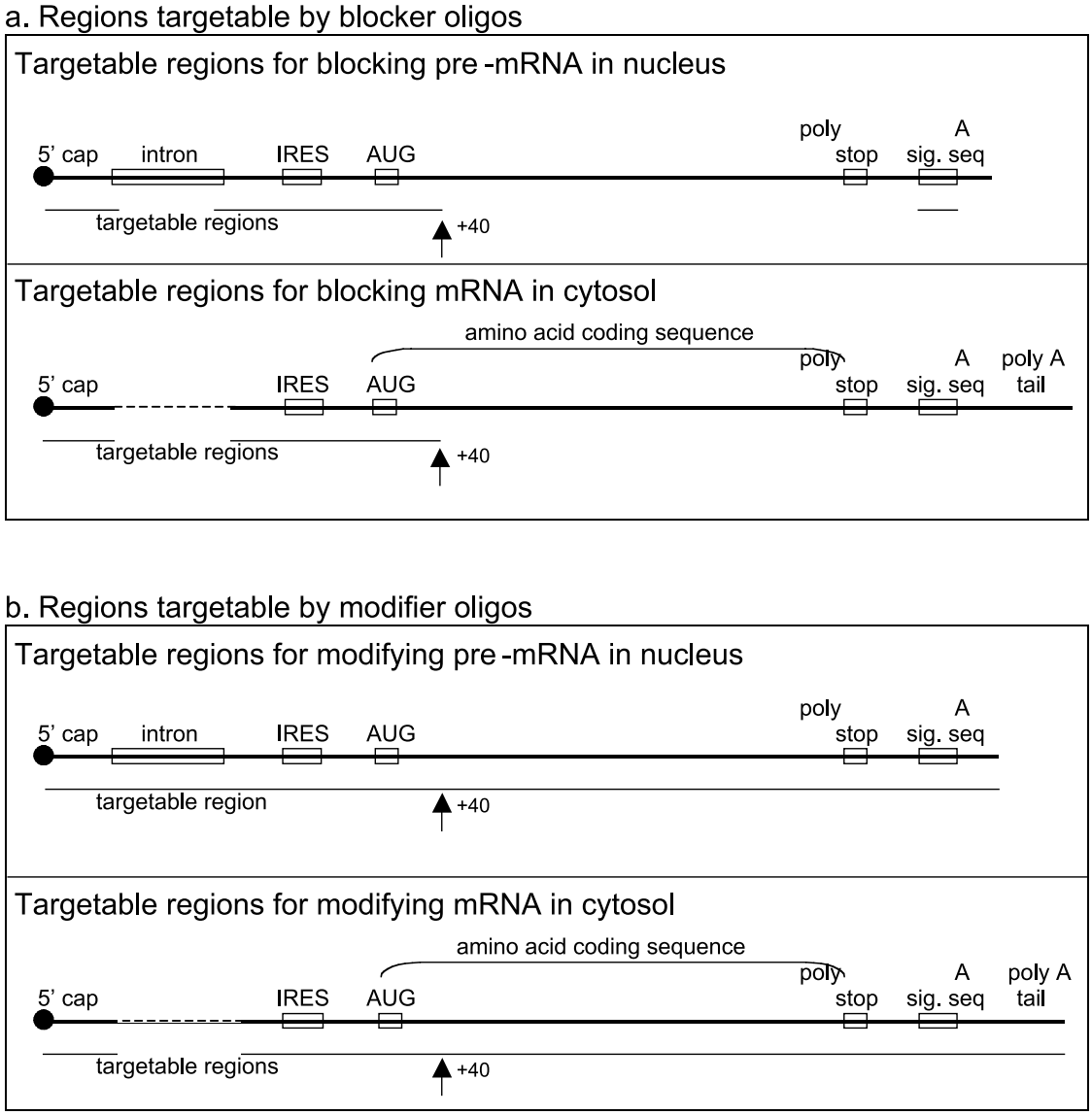

Figure 2. Targetable regions in RNA transcripts.

Table 1. Relative costs of starting materials

Oligo type	Starting material	Approximate relative cost per mole
PNA	Nucleobases	1
Morpholino	Ribonucleosides	2
DNA analogs	Deoxyribonucleosides	70

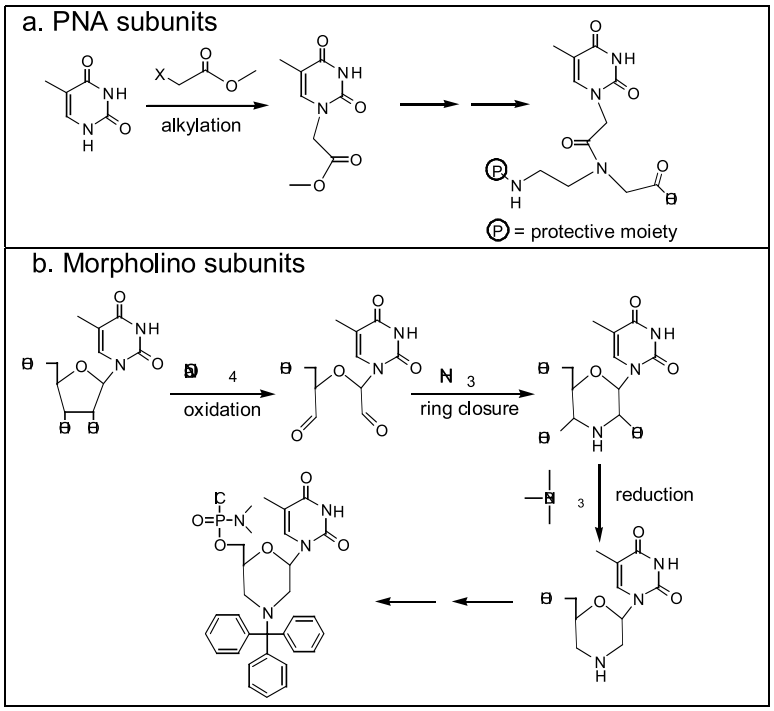

Figure 3. Key steps in subunit synthesis.

on possible chemical manipulations, that same sensitivity also affords at least one significant advantage – a fast and easy way to confirm sequence. In this method, a small portion of synthesis resin with completed oligo still attached is treated with neat TFA (40 minutes at room temperature) to generate on average of one cleavage per oligo chain. The resin is then washed free of TFA and 3′-terminal oligo fragments, and added to concentrated ammonium hydroxide to cleave the resin-bound 5′-terminal oligo fragments from the resin and to deprotect the nucleobases. A mass spectrum directly provides that oligo's sequence simply by tabulating the mass differentials between consecutive fragment peaks.

Biological stability

A major challenge in using antisense oligos has been retention of the integrity of the oligo for a sufficient length of time to achieve the desired experimental or therapeutic effect. For example, bare RNA and DNA oligos introduced into cells undergo substantial enzymatic degradation in a matter of minutes. Various modifications of DNA and RNA backbones (e.g., replacing a sp. pendant oxygen on the phosphorous with a sulfur, as in S-DNAs, or adding an alkyl group to the 2′ oxygen of RNA, as in 2′O-Methyl RNA) as well as modifying the termini of the oligos to block exonucleases[33] all serve to improve stabilities in biological systems. Nonetheless, for close analogs of DNA and RNA having anionic intersubunit linkages, enzymatic degradation generally remains a significant problem in longer-term experiments.

Unlike the biological instabilities of close analogs of RNA and DNA, the radical design departure from natural nucleic acid structure embodied in PNAs and Morpholinos, coupled with their non-ionic intersubunit linkages, renders them highly resistant to enzymatic degradation in blood and within cells.[14,15] This stability to enzymatic degradation provides a compelling advantage in applications requiring long-term activity in biological systems, such as in studies in embryos[16] and in therapeutics.

Backbone flexibility

A significant difference between PNAs and Morpholinos is that PNAs have significantly greater backbone flexibility. Specifically, as illustrated by the acyclic conformation on the left side of Figure 5a, PNAs have 7 bonds per subunit which can undergo relatively free rotations, though this may be transiently reduced to

5 freely rotatable bonds for some fraction of the subunits due to intramolecular H-bonding, as illustrated by the pseudo-ring conformation on the right side of Figure 5a. Molecular modeling suggests that the more flexible backbone of PNAs should favor applications such as triplex formation, including PNA/DNA/DNA and PNA/RNA/PNA triplexes. Indeed, PNAs are well known for excelling in such triplex applications.[17,18]

In contrast to the relatively flexible backbone of PNAs, Morpholinos have a more rigid backbone because they have only 4 bonds per subunit which can undergo relatively free rotations, as illustrated in Figure 5b.

We have found that a key requirement for achieving good antisense activity with high-affinity non-ionic oligos is that they must not contain undue self-pairing potential. Self-pairing is a particular problem for PNAs and to a lesser extent, Morpholinos, because in contrast to the case for oligos having ionic backbones, with non-ionic oligos there is no electrostatic repulsion between the backbones of paired segments to help counterbalance the Watson/Crick pairing. Consequently, when significant self-pairing potential is present in such an oligo, self-pairing is likely to dominate to the exclusion of the desired oligo/target pairing.

In the context of relative backbone rigidities, a likely consequence of the greater flexibility of the PNA backbone, compared to the more rigid Morpholino backbone, is that the PNA oligo can more readily adopt a conformation suitable for self-pairing. To put this in semi-quantitative terms, from our experience in targeting many antisense oligos, it appears that the more flexible PNAs can have no more than about 8 contiguous Watson/Crick H-bonds of self-pairing before they start to suffer a reduction in antisense efficacy. In contrast, the more rigid Morpholinos can have up to about 11 contiguous Watson/Crick H-bonds of self-pairing without suffering significant loss of antisense activity. It should be noted that these numbers of allowable H-bonds are estimates, but may vary up or down by about one H-bond for particular sequences. Table 2 illustrates representative sequences having the approximate maximum amounts of self-pairing which appear to still allow good antisense activity for each of the structural types. Note that an A/T or A/U pair contributes 2 Watson/Crick H-bonds, while a G/C pair contributes 3 Watson/Crick H-bonds.

This modest difference in allowable amount of self-pairing potential for the two structural types results in considerable latitude in selecting effective targets for Morpholino oligos[19] as compared to rather severe restrictions imposed in selecting effective targets for PNAs.[20] The greater latitude in picking Morpholino targets is particularly advantageous when one wishes to target a relatively long RNA sequence (20 to 30 bases) in order to maximize the chance of complementing a suitable single-stranded region of the RNA, which appears to be needed for efficient initiation of oligo/target pairing.

Table 2. Approximate maximum allowable amounts of self-pairing

PNA:	-GCA-	-ATTA-
	-CGT-	-TAAT-
Morpholino:	-GCAC-	-ATTAC-
	-CGTG-	-TAATG-

Aqueous solubility

Conventional wisdom has long held that oligos having non-ionic backbone structures invariably exhibit poor aqueous solubility. Indeed, until recently there was much support for this view, evidenced by the poor aqueous solubilities of the multiple non-ionic structural types shown in Figure 6. Solubilities of these structural types typically are limited to only about 10 to 100 μM, depending on length and sequence.[21,22,23,24,25]

While a variety of solubilizing moieties have been added to these oligos to improve their limited aqueous solubilities (e.g., terminal phosphate on Methylphosphonates, polyethylene glycol on DNA carbamates and Morpholino carbamates, one or more lysines on PNAs), nonetheless, the inherent low solubilities of the core oligos often leads to aggregation and precipitation in many biological applications.

Surprisingly, non-ionic Morpholino oligos with freely rotatable bonds in the intersubunit linkage have been found to have excellent water solubility. To illustrate the importance of freely rotatable bonds, at 37 °C carbamates are known to exhibit restricted rotation, while sulfamides and phosphoroamidates are known to exhibit relatively free rotation. In this context, we have found that a Morpholino oligo having the more rigid carbamate intersubunit linkages (Figure 7a) is several hundred fold less water soluble than a corresponding Morpholino oligo containing the more flexible sulfamide or phosphoroamidate intersubunit linkages (Figures 7b–c).

The likely reason for this great difference in water solubility between these Morpholino subtypes is that the restricted rotation of the carbamate linkage largely prevents stacking of the bases,[26] so that dissolution in an aqueous environment then requires an energetically unfavorable insertion of the hydrophobic faces of the unstacked bases into water. Conversely, molecular modeling suggests that the free rotations of the phosphorodiamidate and sulfamide linkages should allow excellent stacking of the bases, and this has been confirmed experimentally for the phosphorodiamidate linkage.[26] This base stacking translates into excellent water solubility, presumably because the stacking effectively hides the hydrophobic faces of the bases from the aqueous environment.

One practical consequence of the excellent aqueous solubility of phosphorodiamidate-linked Morpholinos (typically 5 to 30 milliMolar for 25-mers) is that a minimal volume of a highly concentrated oligo solution can be injected into quite small eggs or early-stage embryos (e.g., zebrafish), as is required for developmental studies. This high water solubility, combined with exquisite sequence specificity, negligible toxicity, lack of non-antisense effects, and stability in biological systems, have made Morpholino oligos the preferred tools for selective gene knockdown studies in developmental biology.[9,27]

Salt dependence of binding

An important consequence of an oligo having a non-ionic backbone, as is the case for PNAs and Morpholinos, is that their binding affinity for complementary genetic sequences is relatively insensitive to the ionic strength of the medium. Figure 8a compares T_m (melting temperature) values for 20-mer DNA/DNA and corresponding Morpholino/DNA duplexes as a function of salt concentration. Figure 8b compares T_m values for 20-mer DNA/RNA and corresponding Morpholino/RNA duplexes as a function of salt concentration.

PNA/DNA and PNA/RNA duplexes also exhibit similar independence between T_m and salt concentration.[1]

As will be described in the section below on 'applications', this independence between T_m and salt concentration provides a dramatic advantage over classical anionic DNA and RNA oligomers and polymers in probe diagnostic applications.

RNA-binding affinity

Blocker-type oligos must tightly bind to their targeted RNA sequences in order to prevent RNA processing (e.g., splicing), readout (translation), or other functions (e.g., extension of telomers) of their targeted RNA transcripts. Figure 9 shows thermal transitions of various oligo/RNA duplexes (20-mers) at physiological salt concentration.

For comparison, we have found that at physiological salt concentration, PNA/RNA duplexes typically have thermal transitions similar to that of 2′O-Methyl RNA/RNA duplexes.

From Figure 9, one can see that Morpholino/RNA duplexes are more stable than corresponding DNA/RNA duplexes, and much more stable than corresponding S-DNA/RNA duplexes. While not included in Figure 9, under the same conditions we have found that PNA/RNA duplexes typically have substantially greater stability (about 8° to 10°C for 20-mers) than the already high stability of Morpholino/RNA duplexes.

It has been postulated that it is their high affinity for RNA which allows Morpholinos, and probably PNAs as well, to efficiently invade even quite stable RNA secondary structures,[4] while the much lower affinity of S-DNAs (cf. Figure 9) necessitates an exhausting search for suitable RNA target sequences.[7,8] Such searches are probably necessarily extensive because in natural RNAs unstructured regions and regions having secondary structures of only minimal stability are relatively rare. Thus, the widely differing RNA-binding affinities between S-DNAs and the advanced non-ionic Morpholinos and PNAs may account for why Morpholinos, and probably PNAs, typically have an exceptionally high targeting success rate[9] (on the order of 75% to 85%), while S-DNAs generally have a much poorer targeting success rate[7,8] (on the order of 10%).

Minimum Inactivating Length (MIL)

A useful measure of an antisense structural type is its 'Minimum Inactivating Length' (MIL), which may be defined as the shortest length of oligo of a given structural type which achieves substantial inhibition of its targeted sequence at a concentration typically achievable within cells. It should be noted that the measured MIL value for a given structural type varies somewhat as a function of sequence, G+C content, and concentration of the oligos tested. Nonetheless, by testing a range of oligo lengths targeted against the same target

Table 3. MIL values for different structural types

Structural type	MIL value
S-DNA	8
PNA	10
Morpholino	15

region, one can obtain reasonable comparative MIL values for various structural types of interest.[4]

Figure 10 shows the results of experiments carried out to estimate MIL values of S-DNAs, PNAs, and Morpholinos in a cell-free translation system (with added RNase H to afford good S-DNA activities). In these activity-versus-length experiments a set of oligos of the three structural types ranging in length from 8 bases to 30 bases were targeted against a region of rabbit alpha-globin leader sequence (Figure 10a), and a second set were targeted against a region of the Hepatitis B virus (HBV) leader sequence (Figure 10b). These two sets of oligos at a concentration of 300nM were assessed in a cell-free translation system for their abilities to inhibit translation of a downstream luciferase-coding sequence. The experimental procedures, oligo sequences, and RNA targets used in this study are detailed elsewhere.[4,28]

Table 3 gives the approximate MIL values derived from this length-versus-activity study.

As will be discussed in the next section, these differing MIL values strongly influence the preferred applications of PNAs and Morpholinos, particularly in regard to applications in complex systems.

Specificity

The first strategy as regards specificity may be called 'short-is-good'. A widely accepted criteria for specificity of an antisense oligo is how well it can distinguish between its targeted sequence and a non-targeted sequence differing by only one base. In general, an oligo's ability to discriminate on the basis of a single base mismatch increases as its length decreases, reaching maximum discrimination at a length corresponding to, or just slightly greater than the MIL value for that structural type. Thus, a PNA of about 10 or 11 subunits in length would be expected to have maximum single-base mis-pairing discrimination for that structural type, while a Morpholino of about 15 or 16 subunits in length would be expected to exhibit maximal single-base mis-pairing discrimination for that structural type. Since a single base mis-pairing in a 10-mer PNA/RNA duplex (10% of bases mis-paired) has a substantially larger impact on duplex stability than a single base mis-pair in a 15-mer Morpholino/RNA duplex (7% of bases mis-paired), the higher-affinity PNAs provide a substantial advantage over lower-affinity Morpholinos for applications requiring single-base mis-match discrimination. Such applications include targeting single nucleotide polymorphisms (SNPs) and targeting point mutations.

Another strategy is that 'longer-is-better'. While the challenge of discriminating between a single base difference has received much attention in the antisense field, and is well met by short, high-affinity oligos such as PNAs, for antisense applications in complex systems the principal challenge is quite different. Such mainstream applications include determining the function of newly-sequenced genes; generating morphants[9,27] in embryos; validating targets in drug development programs; and developing therapeutics for viral diseases and cancers. The challenge in these complex systems is to strongly inhibit a targeted RNA (achieve high efficacy) without inadvertent inhibition of any other RNAs in the system (i.e., to achieve high specificity). Stated differently, what is needed in complex systems is an oligo which affords high efficacy, while also rigorously discriminating between its target RNA and thousands to tens of thousands of non-target RNAs.

To appreciate the challenge of obtaining both high efficacy and high specificity in a complex system such as a human, first consider the extreme case of a hypothetical ultra-high affinity 5-mer modifier-type oligo that has an MIL (Minimum Inactivating Length) of 5. Most RNAs in the pool of RNA transcripts will contain an average of about 4 copies of any given 5-mer sequence, based on an average RNA transcript length of about 4000 bases. Thus, if all sequences in the RNA transcripts were targetable, then one would expect this 5-mer oligo not only to inactivate its desired targeted RNA species, but also to inactivate nearly all other RNA species in the system.

For the case of a blocker-type oligo (such as a PNA or Morpholino), the specificity situation is not quite as bleak. This is because blocker-type oligos are generally only targetable against about 5% of the bases in a typical RNA transcript (cf. Figure 2a). As a consequence, an ultra-high affinity 5-mer blocker oligo is only expected to inadvertently inhibit about 20% of the non-targeted RNAs in the system – but this is still far

too little specificity for most applications in complex systems.

This brings us to what I believe are two crucial design requirements for an antisense oligo suitable for achieving both high efficacy and high specificity in a complex system. First, the oligo's MIL (Minimum Inactivating Length) value must be sufficiently large that the oligo has little chance of inadvertent inactivation of non-targeted species in the system's entire pool of RNA transcripts. Second, in order to achieve high efficacy the oligo's length should be appreciably longer than its MIL.

In regard to a lower limit for the MIL of oligos suitable for use in a complex *in vivo* system, for human use the current estimates are that the pool of RNA transcripts (before splicing) comprise about 30,000 species[29]. If pre-spliced RNA transcripts average on the order of about 4,000 bases in length, this gives approximately 120 million unique-sequence bases in the RNA pool, of which an estimated 5% (about 6 million bases) are targetable by advanced blocker type oligos (see Figure 2a). Table 4 below gives estimated numbers of RNA species in a human which would be inadvertently inhibited by oligos having the indicated MIL values and lengths corresponding to those values.

The values in Table 4 suggest that in order to have a reasonable chance of no inadvertent targets in a complex system comprising about 6 million bases of targetable unique-sequence RNA, an oligo should have an MIL value of about 12 or greater.

A 12-mer oligo with an MIL of 12 should have excellent specificity in a system as complex as a human; nonetheless, that 12-mer also would have only marginal efficacy. Since increasing binding affinity in order to increase efficacy would decrease the MIL below the value needed for high specificity in this complex system, the remaining option is to increase efficacy by increasing the oligo's length. As for the suitable length for achieving high efficacy, we generally find that Morpholino oligos 25 bases in length can provide high efficacies (90% to 100% target inhibition) at modest concentrations (100 nM to 1000 nM) in cell-free test systems where actual oligo concentration is known.[3,4,30,31] However, increasing the length of the oligo in order to increase its efficacy also leads to a modest increase in the number of potential inadvertent targets for that oligo. To illustrate, if the MIL for an oligo of a given structural type is about 15 (the case for Morpholinos) and the oligo length is 25, that oligo actually contains 11 different 15-mer sequences, each with its own potential for inadvertent inhibition of non-targeted sequences. Thus, when the greater length needed for high efficacy is factored in, estimated numbers of inadvertent targets in the human RNA pool as a function of MIL are calculated as:

Table 5. Estimated numbers of inadvertent targets in RNA pool of 6 million targetable bases

MIL value	Estimated number of inadvertent targets for 25-mer oligos
8	1650
9	390
10	92
11	21
12	5.7
13	1.5
14	0.4
15	0.1

$$\text{Inadvertent targets} =$$

$$(\text{pool complexity} / 4^{\text{MIL}})\,(\text{oligo length} - \text{MIL} + 1)$$

Using this equation, estimated numbers of inadvertent targets in the human RNA pool are tabulated for 25-mer oligos as a function of the oligos' MIL values.

The values in Table 5 suggest that to achieve high specificity in a human, a high-efficacy 25-mer should have an MIL of about 14 or greater. Thus, I predict that to achieve both high efficacy and high specificity in a human one should use an oligo structural type with an MIL value of about 14 or greater, and the oligo length should be on the order of 50% to 100% longer than the MIL value. Not unexpectedly, the lower-affinity Morpholinos (MIL ~15) fit these design criteria appreciably better than the higher-affinity PNAs (MIL about 10).

To test this theoretical prediction concerning how to achieve both high efficacy and high specificity in a complex system, we have carried out experiments to assess relative specificity of S-DNA, PNA, and Morpholino oligos in a test designed to emulate a high-complexity system.[4,28] In these experiments, two oligos of each structural type were used. One oligo of each type was perfectly complementary to its targeted mRNA (globin leader sequence) to provide a measure of total inhibition achieved by that structural type as a function of oligo concentration. The other oligo

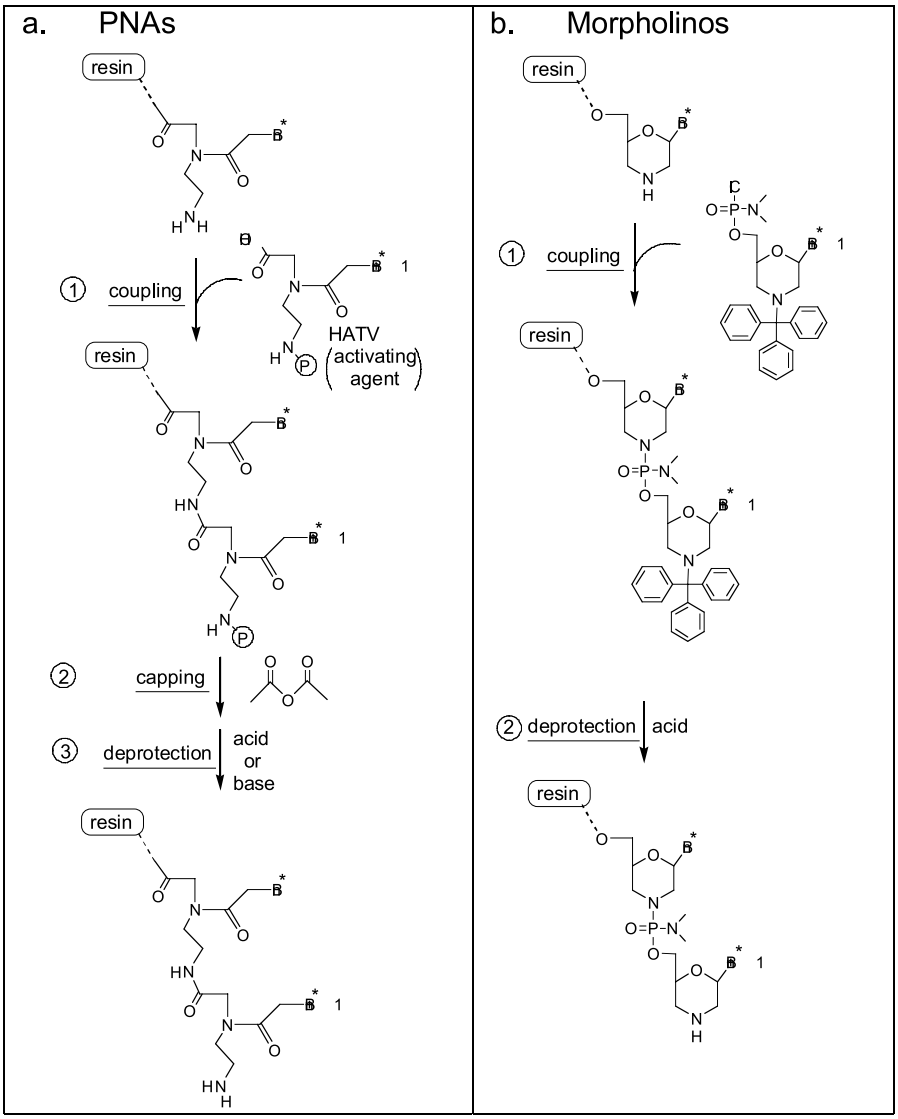

Figure 4. Representative oligo assembly cycles.

Table 4. Estimated numbers of inadvertent targets in RNA pool of 6 million targetable bases

MIL value (X)	Sequence permutations (4^X)	Estimated number of inadvertent targets (6,000,000 / 4^X)
8	65,500	92
9	262,000	23
10	1,050,000	6
11	4,190,000	1.4
12	16,800,000	0.4
13	67,100,000	0.09
14	268,000,000	0.02
15	1,070,000,000	0.006

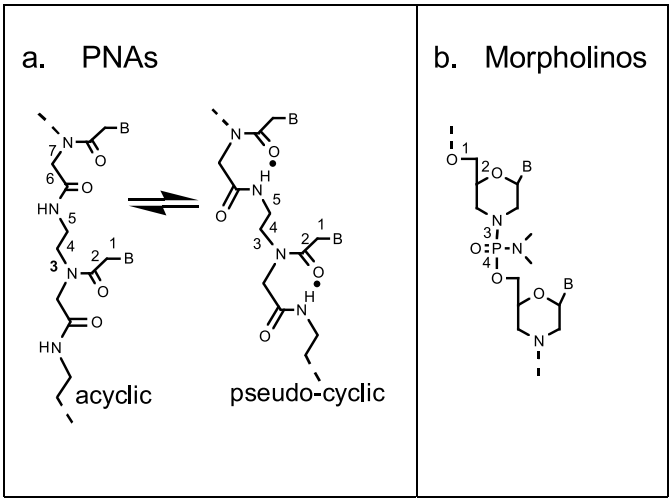

Figure 5. Backbone bonds with relatively free rotation.

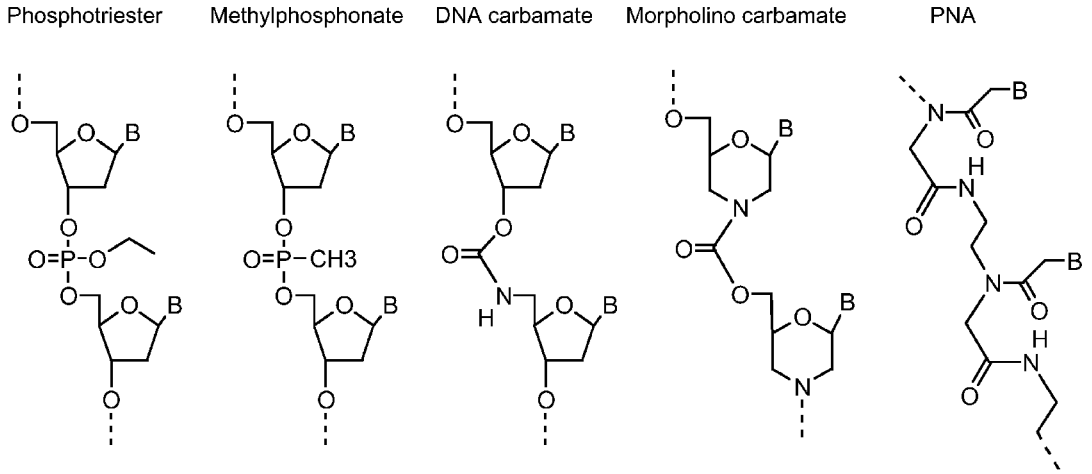

Figure 6. Non-ionic oligos with low aqueous solubilites.

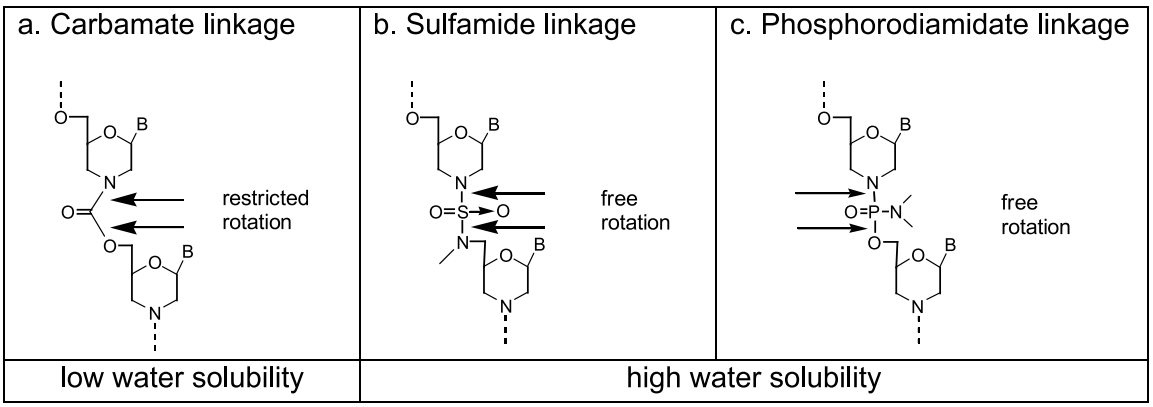

Figure 7. Morpholino oligos with varying aqueous solubilities.

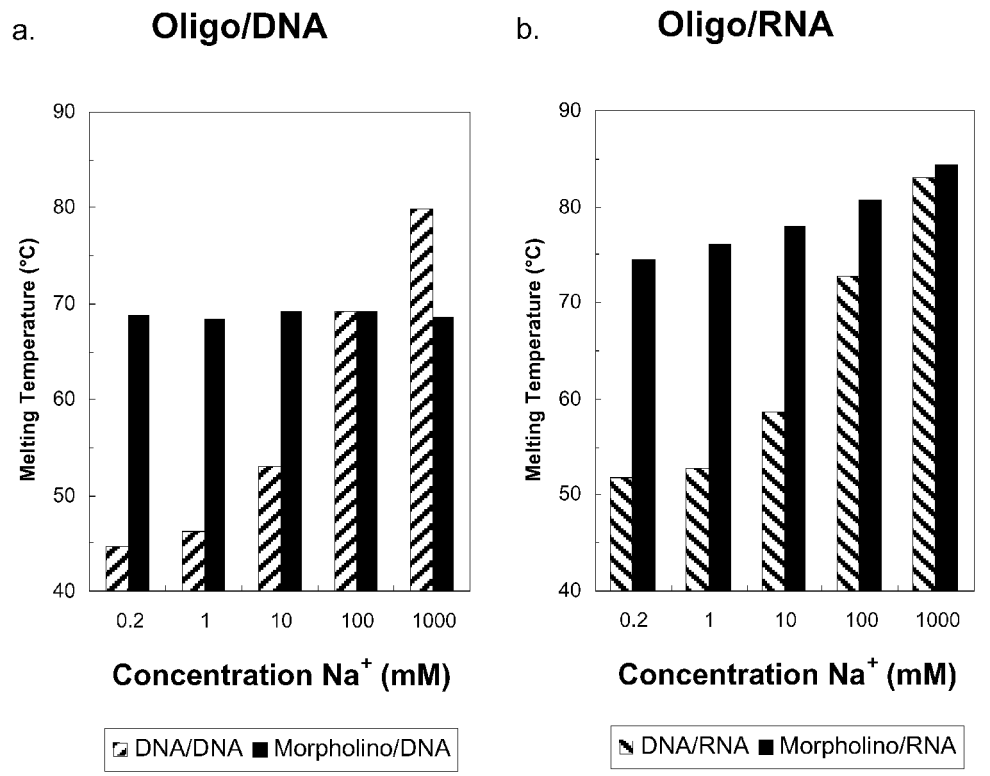

Figure 8. Salt dependence of Tm values for oligo/DNA and oligo/RNA duplexes.

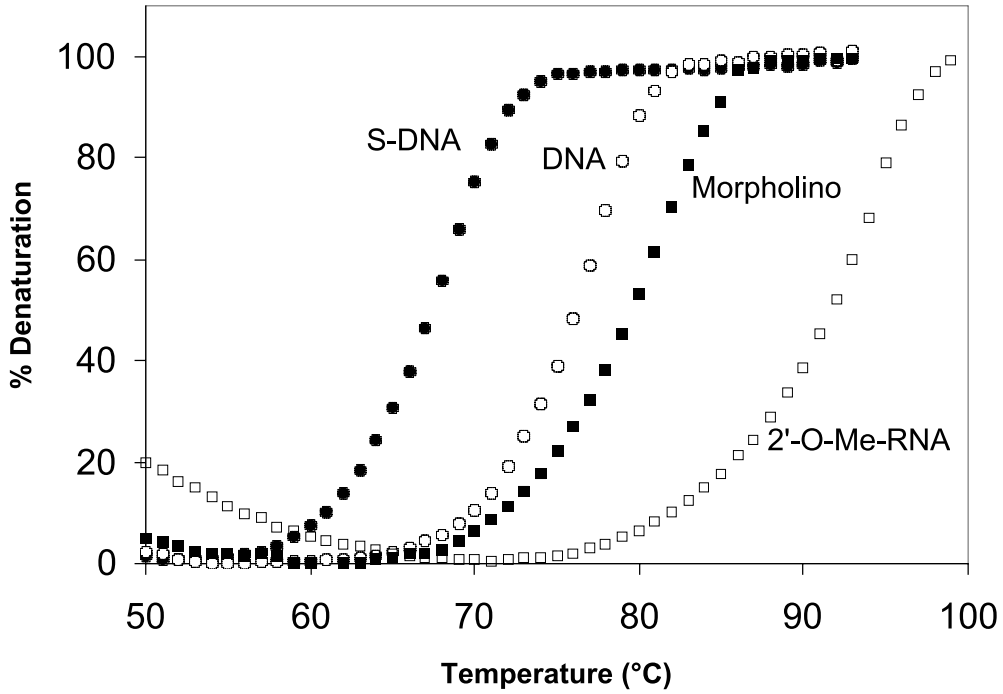

Figure 9. Thermal transitions of 20-mer oligo/RNA duplexes.

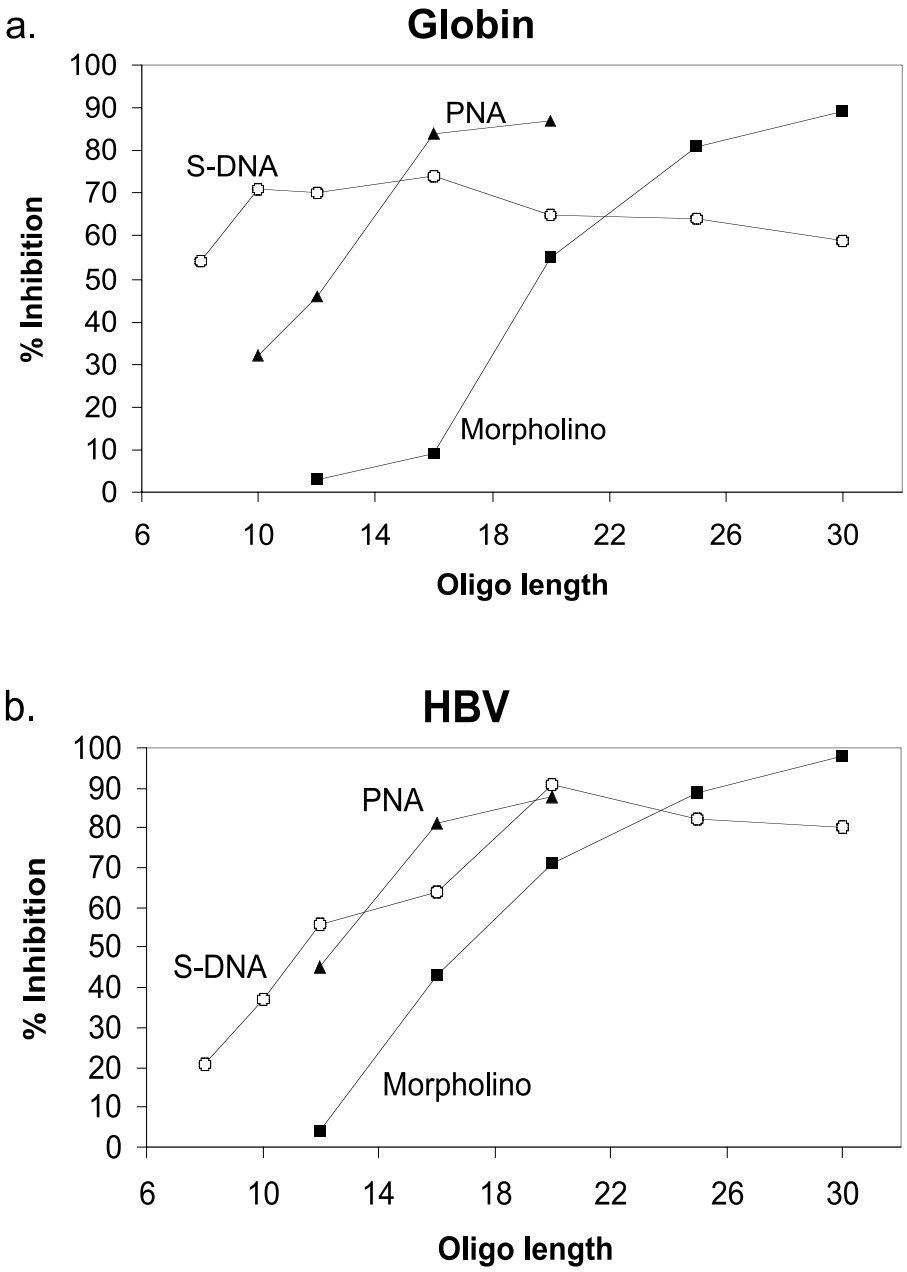

Figure 10. Antisense activity as a function of oligo length.

of that type incorporated mis-pairing to that same target sequence, with the longest run of perfect pairing comprising 10 contiguous base-pairs, to provide a reasonable emulation of the estimated level of sequence homology likely to be encountered in the RNA pool within a representative human cell. For the S-DNA and Morpholino structural types 25-mer oligos were used and the mis-paired oligos contained four mis-pairs to the target sequence. For the PNA structural type 20-mer oligos were used and the mis-paired oligo contained three mis-pairs.

Specific inhibition was calculated as the difference between the inhibition value for the perfectly-paired oligo and the inhibition value for the mis-paired oligo

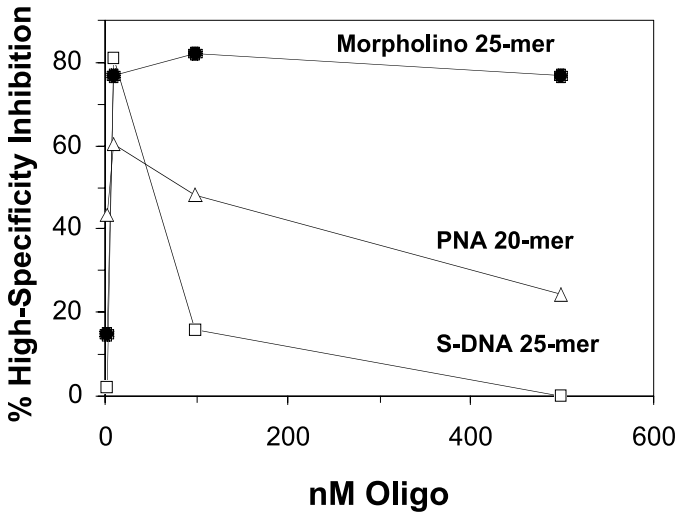

Figure 11. High-specificity component of inhibition by three structural types.

at each concentration. This provides a measure of the high-specificity inhibition achieved by that structural type as a function of concentration. Figure 11 shows a plot of this high-specificity component.

In accord with the length-versus-activity results in Figure 10 and the calculated data in Table 5, the experimental results in Figure 11 demonstrate that in this test emulating a high-complexity system, the high-MIL Morpholinos (MIL ∼15) indeed exhibit substantially better sequence specificity over a wider concentration range than the lower-MIL PNAs (MIL about 10), which in turn exhibit substantially better sequence specificity over a wider concentration range than S-DNAs (MIL ∼8). These results provide support for Morpholinos being a preferred structural type for applications in complex systems.

Delivery into cultured cells

Until the mid-1980s most antisense experiments were carried out in cell-free test systems where the focus was on assessing prospective structural types for directly inhibiting translation of their targeted mRNAs. By the late 1980s and early 1990s, however, the antisense field had evolved to a stage where experiments were being carried out with cultured cells, at which point serious problems were encountered. Studies by several groups elucidated one particular problem – in cultured cells, neither ionic nor non-ionic antisense oligos can diffuse across cell membranes at any reasonable rate.[32,33] Instead, much evidence suggests that antisense oligos enter cultured cells via endocytosis and subsequently most or all of the oligos are degraded, remain trapped in the endosome/lysosome compartment, or are exocytosed back to the extracellular medium.[34] Thus, under normal conditions antisense oligos fail to attain entry into the cytosol/nuclear compartment where their targeted sequences reside.

This delivery challenge has led to wide-ranging efforts to develop effective methods for delivering antisense oligos into the proper subcellular compartment of cultured cells. To a large extent these efforts have been successful, though toxicity from the delivery reagents remains a significant limitation for most of the delivery methods. Of particular note, in the past few years a wide range of components have been investigated for delivering PNAs into the cytosol/nuclear compartment of cells[35−42] and some of these may also hold promise for *in vivo* delivery.

In our own experience, delivery methods which work with Morpholinos generally also work well with PNAs. For example, scrape delivery is one method that is simple, effective and reliable for delivering both PNAs and Morpholinos into adherent cells.[43,44] It is also one of the few methods which work in the presence of high concentrations of serum.

A new and even more effective method suitable for delivering both Morpholino and PNA oligos entails complexing the non-ionic oligo with a partially complementary DNA oligo and then mixing this partial duplex with the weakly-basic polyamine, ethoxylated polyethyleneimine (EPEI), after which the composite complex is added to cells.[45] The EPEI, which is only partially ionized at pH 7, serves both to bind electrostatically to the negatively-charged DNA component

of the oligo/DNA duplex, and to bind electrostatically to negatively-charged cell surfaces, effecting rapid endocytosis of the oligo/DNA/EPEI complex. It is believed that when the pH drops within the endosome, the EPEI is further ionized to the point where its charge density is sufficient to permeabilize the endosomal membrane, allowing release of the oligo into the cytosol of the cell. This 'Special Delivery' method (see also: www.gene-tools.com) is effective with a broad range of cell types, is quite efficient with both adherent and non-adherent cell types, and is less damaging to cells compared to most other delivery methods. Regrettably, the method does not work well in the presence of high concentrations of serum.

Delivery in vivo?

While methods for delivering antisense oligos to the cytosol/nuclear compartment of cultured cells are now fairly well developed and reliable, most or all of those methods appear to be ineffective and/or too toxic for use *in vivo*. In light of these limitations in applying successful cultured-cell delivery methods *in vivo*, it came as a considerable surprise to many in the antisense field when reports began to circulate in the mid-1990s that by some as-yet-undefined mechanism, antisense oligos are able to gain entry into the cytosol/nuclear compartment of cells *in vivo*,[46,47] particularly in the liver and kidney. Subsequent to these reports, there have been additional reports implying successful *in vivo* delivery. In this context, it is widely touted that one S-DNA oligo (Vitravene) has been approved by the Food and Drug Administration of the USA for *in vivo* therapeutic application in humans,[48] and this is commonly construed to be definitive proof that antisense oligos (or at least S-DNAs) readily gain entry into the cytosol/nuclear compartment of cells *in vivo*. However, this 'proof' of effective cytosol/nuclear delivery *in vivo* is less impressive than it may appear, particularly in light of evidence that this particular S-DNA (ISIS 2922) does not function by an antisense mechanism within cells, but instead probably functions largely or solely in the extracellular milieu as an immune stimulatory agent due to CpG-containing sequences at each end of the oligo.[49]

Further evidence for effective cytosol/nuclear delivery *in vivo* has come from reports from AVI BioPharma. This company developed a Morpholino oligo (Resten NG) which was reported to be effective in inhibiting restenosis following balloon angioplasty.[50,51] However, it should be appreciated that in this particular application, connections between cells in the artery wall are seriously perturbed during the course of the angioplasty procedure, and such perturbations have been reported to permeabilize plasma membranes of cells in the artery wall for a significant period of time following the scraping procedure.[52] It is believed that such cell permeabilization in the artery wall probably occurs by a mechanism similar to that which allows rapid cytosolic entry of antisense oligos during scrape delivery of cultured cells.[43] The key point here is that while effective delivery into the proper subcellular compartment may be achieved in AVI BioPharma's particular *in vivo* application, nonetheless, this does not imply that effective cytosol/nuclear delivery will also occur equally well in other *in vivo* applications where the cells are not mechanically perturbed.

Still another increasingly popular antisense application which provides evidence for effective cytosol/nuclear delivery *in vivo* is the successful use of Morpholinos for generating morphants (i.e., antisense-mediated morphological changes which mimic mutational changes) in early-stage embryos of sea urchins, frogs, and zebra fish.[9,27] Again, appearances may be deceiving in regard to delivery, at least in the case of zebrafish. This is because the antisense oligos are injected into the zebrafish eggs before or very shortly after fertilization, at which time the normal permeability barriers between embryonic cells have not yet formed. Only somewhat after the 32-cell stage in zebrafish embryos do the normal permeability barriers begin to form between cells of the developing embryo.[53,54] Thus, when antisense oligos are injected into such eggs or embryos just beginning to undergo cell divisions, those antisense oligos have full access to all cells of the organism, and will reside and function within all those cells through multiple cell divisions.[16] Preliminary results from collaborators at the University of Oregon suggest that when antisense oligos are instead injected into the yolk or the vascular compartment of later stage embryos wherein the normal cellular permeability barriers have formed, those oligos fail to generate the expected phenotypic changes, suggesting a lack of reliable *in vivo* delivery.

Further evidence suggesting that Morpholino oligos do not readily cross cell membranes *in vivo* comes from studies with frog eggs/embryos, whose cells exhibit normal permeability barriers immediately after the first cell division. In this system, when fluorescent-tagged Morpholinos are injected into one cell of a 2-cell stage frog blastomere, the oligos remain only in the direct descendents of that injected cell (one side

of the developing embryo), at least through the free-swimming tadpole state (stage 43). The same basic result was also found in a functional test wherein the Morpholino was targeted against a stably-integrated transgene expressing green fluorescent protein.[16]

Thus, while many scientists in the antisense field contend that antisense oligos readily enter the proper subcellular compartment of many or all cells *in vivo*, many remain skeptical of most claims for antisense activity *in vivo*, at least in those cases where no delivery mechanism is apparent and no delivery component was used to achieve entry into the cytosol/nuclear compartment of the cells.

There are a number of important reasons for continued skepticism concerning *in vivo* delivery of bare antisense oligos:

1. One would expect that cells *in vivo* should not be significantly more permeable than cells in culture to large polar molecules such as antisense oligos, and this expectation appears to be supported by multiple studies with vertebrate embryos. For instance, preliminary studies indicate that a Morpholino oligo which generates a distinctive phenotypic change (fluorescent blood) when injected into early-stage zebrafish embryos fails to generate that same phenotype when injected into later-stage embryos wherein normal permeability barriers have formed. Further, Morpholino oligos injected into one cell of a 2-cell stage frog blastomere remain on one side of the frog embryo through the free-swimming tadpole stage.[16]

2. Early attempts to use 'naked' antisense oligos to inhibit virus infections in mice and other model organisms, where a definitive answer would be expected if the oligos were effective and high levels of *in vivo* delivery occurred, appear to have been relatively ineffective.

3. In most reports of *in vivo* antisense activity, S-DNAs were utilized, which are well known to generate multiple non-antisense effects due to their actions in the extracellular medium and at cell surfaces.[55,56] It is noteworthy that several of those non-antisense effects have been shown to be sequence dependent – which can lead to erroneous conclusions even when apparently valid control oligos are used in the experiments.

4. Experiments targeting cellular genes in whole animals inherently entail many more uncertainties than targeting defined marker genes transfected into cultured cells, and results *in vivo* are often indirect and more open to interpretation. These greater uncertainties and less direct results in animal studies raise the chances that positive results are in reality just normal statistical fluctuations, flaws in experimental design, or any of a host of other problems common to complex experimental systems.

5. It appears unlikely that all of the major pharmaceutical companies, who together invested billions of dollars in antisense research in the late 1980s and early 1990s, would have abandoned (in the late 1990s) their attempts to develop antisense therapeutics if they had been able to repeat the claimed *in vivo* successes of the small biotech antisense companies.

Notwithstanding this somewhat pessimistic assessment, I would like to conclude this section with two positive statements regarding *in vivo* delivery:

First, I believe that achieving safe and effective delivery of antisense oligos into the cytosol/nuclear compartment of a wide variety of cell types *in vivo* probably constitutes the last major challenge which must be met in order for antisense oligos to fulfill their great promise of safe and effective therapeutics for a broad range of viral diseases, cancers, possibly autoimmune diseases, and a host of other currently intractable disease states.

Second, based on recent advances by several research groups including GeneTools, it appears likely that this delivery challenge will be met soon, perhaps within the coming five years.

Applications

Research tools

PNAs have been used for an unusually wide variety of novel research applications. Probably because of their exceptionally high binding affinity and unusually flexible backbones, PNAs have proven particularly useful for forming triplexes with DNA and RNA. With duplex DNA targets, in low salt conditions (to destabilize the DNA/DNA duplex) an all-pyrimidine PNA can invade a targeted purine or pyrimidine stretch of duplex DNA to form a PNA/DNA/PNA triplex and a single-stranded displacement loop comprising the pyrimidine-containing strand of DNA.[17] For the case of single-stranded, all-purine RNA targets, PNAs also can form ultra-stable PNA/RNA/PNA triplexes which have the unique capability of blocking translocation of ribosomes – even in downstream amino acid-coding regions of a targeted mRNA. Thus, for these rare target sequences, such specially-designed PNAs can be effective in a region of the mRNA which is normally only targetable by modifier-type antisense structural types.[6]

Because of their exceptionally high binding affinity, PNAs also excel in targeting inherently short RNA sequences, such as the short RNA sequence exposed in the telomerase enzyme.[57,58]

A particularly promising exploitation of high affinity PNAs is their use as antisense antibacterials, where the limited porosity of bacterial cell walls largely preclude use of antisense oligos longer than about 12 to 14 bases. A major advance in this application was recently reported wherein addition of a short cell wall-permeabilizing peptide to a 12-mer PNA dramatically increased the PNAs antibacterial efficacy.[59]

As noted in earlier sections, PNAs are also attractive because of their compatibility with peptide synthesis conditions. This allows one to synthesize a PNA, and then while the PNA is still protected and on its synthesis resin, amino acids are added stepwise to form peptide adducts suitable for enhancing delivery into cells[35,60] or for other applications such as diagnostics.

PNAs have been used in a novel extra-cellular therapeutics application wherein an antibody/PNA adduct is used to bind to cancer-specific antigens on the surface of cancer cells *in vivo*. This is followed by addition of a second complementary PNA to which is attached a suitable radioisotope. The isotope-carrying PNA then rapidly pairs to its complementary PNA linked to the antibody bound to the cancer cells. This process effectively concentrates the isotope in proximity to the cancer cells – thereby substantially increasing the damage to the cancer cells and decreasing damage to the non-cancer cells.[61]

PNAs also have been investigated for use in a variety of experimental systems for detecting and quantitating human or animal genetic sequences.[62–66]

In contrast to the wide-ranging applications of PNAs, so far Morpholinos have been used primarily for classical antisense applications in complex systems. Such applications include correcting splicing errors in pre-mRNAs in cultured cells[44,45] and in extra-corporal treatment of cells from thallasemic patients.[67]

Morpholinos are often used for classic antisense inhibition of targeted mRNAs in cultured cells when both high efficacy and high specificity are desired.[68–70] In this context, a Morpholino targeted against the C-myc mRNA is in Phase 3 clinical trials for prevention of restenosis following balloon angioplasty.[50] In this application it is likely that delivery is achieved *in vivo* via scrape delivery into cells of the artery wall during the angioplasty procedure.

Perhaps the most demanding application of Morpholinos is in developmental biology.[9,27,71–74] For this application it is necessary to inject a very small volume of a high concentration of oligo into eggs or early-stage embryos[9] or to electroporate oligos into specific tissues in later-stage embryos.[74] Obviously, oligos for this application must be very soluble in water (i.e., multi-millimolar). Such oligos also must exhibit extremely high sequence specificity and have negligible toxicity and negligible non-antisense effects. The need for exquisite specificity is because within a brief period of time (a few days) the rapidly developing organism expresses most of its entire set of genes and so the antisense oligo must efficiently inhibit its targeted mRNA without significantly affecting any of the very large number of other mRNA species which are present at one time or another during embryogenesis (but mostly absent from terminally differentiated cells). Developmental biology applications also require that the oligos be stable in biological systems for long periods of time. To the best of our knowledge, to date Morpholinos are the only antisense type shown to work predictably, specifically and without toxicity in this very demanding application.

A particularly interesting application in developmental biology is the use of Morpholinos to selectively target zygotic RNAs without concomitant inhibition of maternal RNAs coded by the same gene.[75] This is achieved by targeting intron/exon splice junctions, which are present in the newly transcribed zygotic pre-mRNAs, but absent from the already-spliced maternal mRNAs.

Another valuable strategy which can be utilized in studies with frog eggs is to inject the test Morpholino oligo into only one cell of a 2-cell stage blastomere. The other cell of the blastomere is either not injected or is injected with a control Morpholino oligo. In the course of development, all cells on one side of the embryo come from the injected cell and all cells from the other side of the embryo come from the non-injected or control-injected cell. It has been shown that in this manner one side of the embryo serves as an excellent control for the other side because Morpholino oligos in cells of one side of the embryo cannot pass over to cells of the other side of the embryo.[16]

Clinical diagnostics

For the past few decades, it has appeared to many scientists involved in nucleic acid research that nucleic acid probes should be greatly superior to antibodies

for detecting infectious diseases in the clinic. This is because nucleic acid probes (or other probes) can have higher binding affinities than antibodies as well as greater specificity than even monoclonal antibodies. Most importantly, generating a probe specific for a selected genetic sequence (analyte strand) is much simpler, faster, and more reliable than generating a corresponding pathogen-specific antibody. In spite of these apparent advantages, after several decades of development, probe diagnostics have only made minor inroads into the clinical diagnostics arena, with antibody-based diagnostics still enjoying nearly complete dominance.

There are three principal challenges which may be responsible for classic probe diagnostics having failed to gain a significant foothold in the clinic: 1. Stringency. Probes generally require precise control over stringency (i.e., salt, denaturant, temperature) during probe/target pairing, giving false positives if stringency is too low and false negatives if stringency is too high. This raises the specter of poor reliability or reproducability, which can be catastrophic in the context of clinical diagnostics.

2. Speed. For samples containing low concentrations of analyte strands, for example ~600 virus particles per ml of blood, which corresponds to about 1 attomolar, pairing of probe to its complementary analyte strand typically requires many hours, or a relatively complex time-consuming, expensive, error-prone pre-amplification of a key portion of the analyte strand by polymerase chain reaction (PCR) or some analogous target amplification procedure.

3. Sensitivity. The concentration of analyte strands (e.g., viral genetic sequences) in clinical samples are often in the zeptomolar (10^{-21}) or attomolar (10^{-18}) range and probe diagnostic methods for detecting these very low analyte concentrations generally require complicated time-consuming and expensive pre-amplification of a portion of the analyte strand (such as by PCR) or use of complicated and expensive detection equipment not appropriate for a clinical setting.

One major exception to the limited sensitivity of probe diagnostics systems is the Branched DNA system developed at Chiron and now widely used in the clinic for quantitation of HIV.[76] Still, even the Branched DNA system is slow, complicated, and expensive compared to most antibody-based diagnostics.

High-affinity non-ionic probes, such as PNAs and Morpholinos, are well-suited to overcoming these many challenges currently impeding the use of probe diagnostics in most clinical applications.

With respect to stringency, the reason precise control of stringency is important with standard nucleic acid probes is that under low stringency conditions (i.e., too much salt or too little denaturant or too low of a temperature) the target sequence of the analyte strand, complementary to the probe, is largely unavailable for pairing to the probe because of extensive secondary structure in the analyte strand. Conversely, when the stringency is too high (i.e., too little salt or too much denaturant or too high of a temperature) the probe is unable to stably bind to its target sequence in the analyte strand.

In contrast to the case for standard ionic probes (e.g., DNA and RNA), when using a non-ionic probe and pairing in salt-free water, essentially all secondary structure in the analyte strand is disrupted due to electrostatic repulsion between backbones, allowing full access of the probe to its target sequence in the analyte strand. Further, because the probe has no backbone charge, these same salt-free conditions have little or no impact on the probe/target pairing (see Figure 8 and Reference 1). Thus, by using a non-ionic probe (PNA or Morpholino) the challenge of precisely controlling stringency can be disposed of simply by carrying out the pairing step in salt-free water at a temperature anywhere in a fairly large range (ambient to about 50 °C). As a consequence, use of non-ionic probes can significantly simplify the diagnostic system. More important, it can also greatly reduce the chance of false negatives and false positives.

With respect to speed, a method has been developed which allows very rapid pairing (seconds to a few minutes) between a probe and its target sequence in an analyte strand, even when that analyte strand is present at extremely low concentrations (zeptomolar) in a biological sample.[77] In one embodiment of that method a surface (e.g., microbeads or a porous frit) is derivatized with both a weakly-basic oligoamine (effective pKa ~6) and a non-ionic probe. When a biological sample buffered at about pH 5 (to assure ionization of the oligoamine) is contacted with that oligoamine/probe surface, all polyanionic nucleic acids, including any analyte strands, are adsorbed in seconds to that oligoamine/probe surface via electrostatic bonds between the cationic oligoamines and the anionic nucleic acids. The surface is then washed with salt-free water to disrupt secondary structures in the analyte strand and allow pairing between surface-bound probes and analyte strands held in immediate

proximity to the probe due to electrostatic bonding to the interspersed oligoamines. This probe/target pairing is generally complete within a couple of minutes, even when the analyte strand was originally present at extremely low concentrations. The surface is next washed with pH 8 buffer to deionize the weakly basic oligoamines and thereby terminate the electrostatic bonding between the surface-bound oligoamines and nucleic acids. Washing the surface with pH 8 buffer effects removal of all nucleic acids except analyte strands still linked to the surface via Watson/Crick bonds between the surface-bound probes and target sequences of the analyte strands.

To summarize this rapid-pairing strategy, biological sample buffered at pH 5 is contacted with the oligoamine/probe surface to capture all nucleic acids. The surface is then washed with water to allow pairing between the probe and target sequences within any analyte strands. Several minutes later the surface is washed with pH 8 buffer to remove all non-analyte strands. By this means, pairing which would normally take many hours is achieved near quantitatively in several minutes.

In regard to sensitivity, in the research laboratory low-copy-number analyte strands are routinely detected either by selectively pre-amplifying a portion of the analyte strand (such as by PCR) or by highly amplifying a signal associated with the probe – where said amplification is generally complicated, labor intensive, and expensive. While these complexities and costs may be acceptable in a research setting, I believe they constitute a major impediment to use of probe diagnostics in most clinical applications.

Thus, it appears that the last major challenge in developing probe diagnostics suitable for the clinic is the development of a direct detection scheme which is capable of reliably detecting as few as about ten to a hundred analyte strands in a 1–5 ml biological sample, and which is also fast, simple, and cheap. While such a simple high-sensitivity direct detection capability appears not to be available at this time, GENE TOOLS is embarking on development of a direct detection method using novel structures and a unique scheme which exploits the special properties of non-ionic probes. This detection scheme actually comprises two key aspects, as follows: 1) a component effective to provide a very large signal (tens of thousands of fluorophores) per analyte strand; and, 2) a mechanism for dramatically reducing the typical level of background signal.

In light of past progress and expected upcoming developments, it is possible that probe diagnostics will finally win a major share of the clinical diagnostics market in the foreseeable future. I further believe that because of the compelling advantages afforded by non-ionic probes, the probe components in those clinical diagnostics will almost surely have non-ionic backbones.

Therapeutics

It should be appreciated that both PNAs and Morpholinos are relatively new structural types, with the currently preferred embodiments only about a decade old. Furthermore, the rate of their development was substantially slowed during much of the past decade because until recently most of the economic resources available in the antisense field from government, the pharmaceutical industry, and investors have been focused on the seriously flawed S-DNAs.

In spite of the above factors, *in vivo* studies with bare PNAs and bare Morpholinos (i.e., oligos with no added delivery component) are now being undertaken by a number of groups – and results from at least some of these studies suggest that I might be mistaken in my belief that a delivery component will generally be required for effective delivery of antisense oligos into the cytosol/nuclear compartment of cells of living animals.

One study providing support for effectiveness of bare PNAs *in vivo* utilized a PNA targeted against the neurotensin receptor 1 and another PNA targeted against the opioid mu receptor. These bare PNAs were injected into the periaqueductal gray region in brains of rats. Each PNA produced the physiological response expected if it had down regulated its targeted mRNA in neuronal cells, and subsequent biochemical assessment of the brains of the treated rats indicated that the concentrations of the protein products coded by the targeted mRNAs were significantly reduced, as would be expected from an antisense mechanism.[78]

In regard to *in vivo* use of bare Morpholinos, AVI BioPharma, owner of the patents covering Morpholinos, is aggressively pursuing the development of a number of different Morpholino therapeutics. To date, none of these prospective Morpholino therapeutics contain a delivery component. In spite of this, AVI BioPharma and their multiple collaborating groups have reported positive results from studies in a variety of different animal species and in humans for Mor-

pholinos targeted against the mRNAs of a number of different genes.[79–82]

If the foregoing results are valid, and not just special cases of exceptional cell permeabilities in such sites as the brain and liver, then in light of the impressive properties of these advanced antisense structural types, it seems possible that in the future safe and effective antisense therapeutics (Morpholinos and/or PNAs) will be introduced for the treatment of a wide range of diseases.

Conversely, if the *in vivo* results with bare PNAs and bare Morpholinos prove to be invalid or merely special cases, then the introduction of a broad range of antisense therapeutics will likely be delayed until after safe and effective delivery components are developed.

Editor's note: As the inventor and commercial producer of Morpholinos, Dr. Summerton has disclosed his financial interest in Morpholino technology. Also, Dr. Summerton wishes to state that his first-hand experience with PNAs has been limited to experimental comparisons between Morpholinos, PNAs, 2′O-methyl RNAs, and S-DNAs with respect to properties expected to be important for diagnostic applications and for therapeutic applications against viral diseases and cancers.

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