

Two sides of the coin: affinity and specificity of nucleic acid interactions

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During the past decade, synthetic nucleobase oligomers have found wide use in biochemical sciences, biotechnology and molecular medicine, both as research and/or diagnostic tools and as therapeutics. Numerous applications of common and modified oligonucleotides and oligonucleotide mimics rely on their ability to sequence-specifically recognize nucleic acid targets (DNA or RNA) by forming duplexes or triplexes. In general, these applications would benefit significantly from enhanced binding affinities of nucleobase oligomers in the formation of various secondary structures. However, for high-affinity probes, the selectivity of sequence recognition must also be improved to avoid undesirable associations with mismatched DNA and RNA sites. Here, we review recent progress in understanding the molecular mechanisms of nucleic acid interactions and the development of new high-affinity plus high-specificity oligonucleotides and their mimics, with particular emphasis on peptide nucleic acids.

Affinity and specificity are two major characteristics of any nucleic acid-based assay that determine its efficiency. Generally, sequence specificity and binding affinity of DNA–DNA, DNA–RNA and/or RNA–RNA interactions negatively correlate (anticorrelate) with each other [1], except for some special cases, such as oligonucleotide aptamers [2,3]. This anticorrelation means that, as affinity for the chosen target sequence increases, the likelihood of association with closely related but nontarget sequences also increases. At first, such reasoning might sound odd given that, in many cases of biomolecular interactions, these characteristics correlate positively: high-affinity binding leads to higher selectivity of the ligand–target association [4–6]. This logic also appears to contradict the workability of hybridization, affinity capture, sequencing and amplification techniques, which are based on nucleic acid interactions. How could all these methods work if the formation of nucleic acid duplexes and triplexes necessary for their functioning (Figure 1) were either weak or non-specific? But in reality, there is no contradiction here: to be successful, these methods do not always require sequence recognition with single-mismatch sensitivity. Indeed, the target sites and corresponding probes are normally long enough to give a very low probability of encountering the wrong sites with one (or two) mismatch(es).

Thus, many techniques that involve DNA and/or RNA

recognition via complementary pairing do not require high sequence-specificity for probe–target binding. However, in other techniques, this requirement is essential, especially given the current trend in basic and applied research to study entire genomes and/or essentially unprocessed samples. In these cases, the ability of single-stranded nucleobase oligomers to form exclusively specific complexes could be of significant importance because certain techniques are unsuccessful owing to the poor specificity of particular probes (or non-optimal conditions for their specificity). Most biotechnological and medicinal applications of nucleic acids and their derivatives would also benefit from probes with enhanced binding affinities. However, enhanced affinity of a modified nucleobase oligomer must be accompanied by a corresponding increase in sequence specificity, which would avoid decreases in the selective targeting of specific sites owing to the tight binding of a modified oligonucleotide to undesired sequences [1]. Therefore, the correct experimental setting and probe design need to be chosen to obtain adequate specificity.

It should be noted that the scope of this review covers mostly the *in vitro* studies and only considers the secondary structures of nucleic acids. Tertiary interactions, which play a major role in the 3D rearrangement or folding of pre-formed DNA and RNA secondary structures, are not discussed. In addition, we focus on protein-free interactions rather than on techniques such as RecA-assisted hybridization.

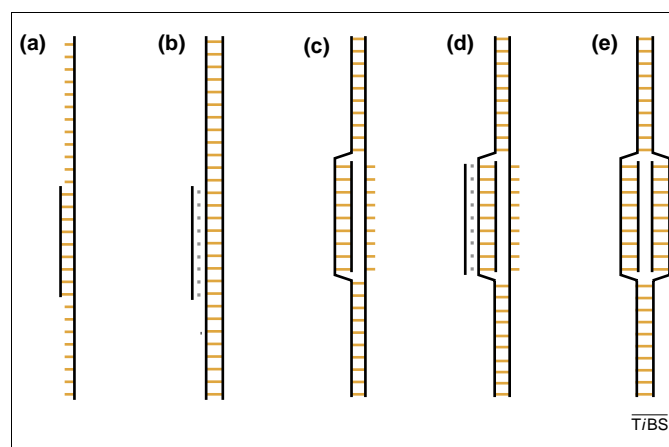


Figure 1. Common secondary structures of nucleic acids formed between a nucleobase oligomer and a DNA or RNA target, including (a) duplex, (b) triplex, (c) invasion duplex, (d) invasion triplex and (e) double-duplex-invasion complexes. Watson–Crick and Hoogsteen-type base pairs are shown as strokes and dots, respectively.

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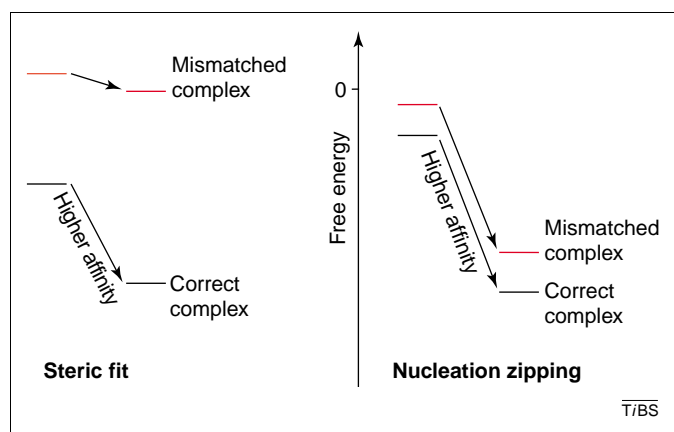


Figure 2. Correlative and anticorrelative relationships between affinity and specificity for two major modes of biomolecular interaction. If biochemical recognition is directed by steric fit, higher binding affinity is almost always accompanied by higher recognition specificities (positive correlation) because the larger free-energy gap between correct and mismatched complexes is usually seen in this case for high-affinity binding. Thus, most mismatched complexes will be unstable in this case. By contrast, negative correlation (anticorrelation) between affinity and specificity is intrinsic in the nucleation-zipping mode of recognition. The free-energy gap between correct and mismatched complexes is not substantially affected by an increase in affinity so that the mismatched complexes will also be stable in the latter case.

What is so special about nucleic acid interactions?

Several biomolecular interactions involving complex biomolecules have been described by using the 3D concept of shape complementarity (or steric fit, both static and induced) [3–8]. This mode of biochemical recognition enables both high specificity and high affinity to be achieved. In these cases, the correlation between affinity and specificity – the ‘more is always better’ principle – is supported by theoretical analyses and can be applied to most drug discovery efforts, including the selection of aptamer oligonucleotides [4,6].

The shape-complementarity concept means that biochemical recognition in enzyme–substrate, antigen–antibody and aptamer–small-molecule complexes is provided by a precise steric fit between surface regions of the binding ligand and target (including mutual, conformational fine-tuning). Ligand binding to closely related but mismatched targets therefore results in significant energetic penalties. Indeed, a mismatched complex lacks many local interactions along the large, misfit contact area. Thus, the free-energy loss for incorrect pairing is comparable to the free-energy gain in correct pairing, conforming the affinity–specificity correlation and, essentially, zero-tolerance of mismatches even for high-affinity binding [4,6] (Figure 2, left panel).

However, such harmony is not universally valid for regular nucleic acid interactions. The reason for this lies in a totally different mode of recognition – the 1D nucleation-zipping mechanism – exhibited by DNA and RNA oligomers in the formation of duplexes or triplexes [9–19]. Two primary modes of biomolecular interactions – steric fit and nucleation-zipping – differ dramatically in terms of affinity–specificity relationships. By analogy, static and induced steric fits between pairs of biomolecules can be viewed as ‘lock and key’ or ‘hand in glove’ models, respectively [6]. The slightest change in the shape of a key

will totally impair its function and a glove will not fit a hand at all if one finger is bigger than required.

By contrast, a strong zip with one irregular or missing link can still be fastened via bypassing this small mismatched part. This analogy neatly describes the formation of the secondary structure of nucleic acids. These are formed by flexible linear oligomers comprising combinations (base pairs or base triads) of four different structural units (A, G, C and T or U nucleobases). Consequently, these structures are able to readily accommodate mismatched nucleobases for duplex formation (Figure 3). A single mismatched unit results in a relatively small and essentially constant energetic penalty. Indeed, in nucleic acid interactions, the free-energy gap ($\delta\Delta G$) between the correct and mismatched complexes lies in the range of 3–5 kcal mol⁻¹ [20–24]. Thus, free-energy loss owing to nonspecific binding of nucleobase oligomers cannot be substantially changed by an increase in binding affinity (Figure 2, right panel): ‘more’ isn’t always ‘better’ in nucleic acid recognition.

For proteins, there is also one notable exception to the ‘more is always better’ rule for affinity–specificity relationships. A special class of DNA-binding proteins called zinc fingers can be constructed from modular building blocks, each recognizing a specific DNA subsite. It was found that polydactyl zinc-finger proteins with the lowest affinities show the best specificities to single-base mismatches [25,26]. This is not surprising given that linearly arranged DNA-recognition domains function here, in a way shown in Figure 3.

The understanding of this fundamental distinction of nucleic acid interactions leads to reasonable hypotheses on how to improve DNA/RNA-binding affinity and recognition selectivity simultaneously, thus facilitating the rational design of sequence-specific high-affinity nucleobase probes and/or ligands. Examples of these and implications for future developments are given in the following sections.

Equilibrium conditions: structured oligonucleotides as site-selective high-affinity ligands

With few exceptions, stronger binding is advantageous over the formation of unstable DNA–DNA or DNA–RNA complexes that are occasionally required for rapid turnover of nucleic acids (e.g. in target-dependent multiple-probe transformation or ligand-directed target modification [27–29]). However, as described earlier, similar increases in the equilibrium stability constants for both matched and mismatched binding is generally expected for regular oligonucleotides in response to any stabilizing factor (e.g. increasing salt concentration and/or length of interacting oligomers). This should result in a gradual decrease of nucleic acid specificity with increase in binding affinity because a narrower window of experimental conditions will exist for effective discrimination of matched versus mismatched complexes.

A theoretical study of duplex and triplex modes of nucleic acid interactions with linear oligonucleotides showed that a wide range of conditions could be found for long oligomers where a high affinity to the correct target co-exists with a low affinity to multiple-mismatch targets [1]. This study proved, however, that, for isolated

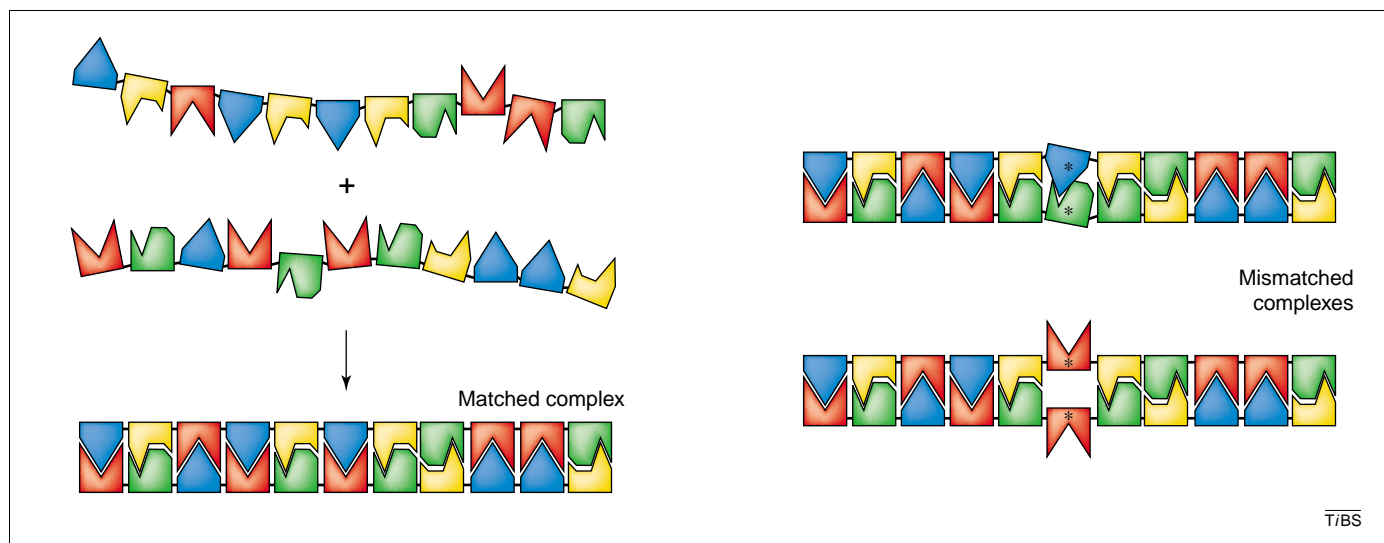


Figure 3. Nucleic acid duplex formation showing the complementary recognition mechanism of nucleation-zipping. Here, each interacting unit (oligomer) is a 1D elastic array of four primary elements that selectively bind another oligomer in only two possible pairs. As a result, a strong and specific complex is formed between the two corresponding oligomers. However, several incorrect complexes could also be formed. Single mismatches can be relatively stable because the complete duplex is still assembled with the mismatched pair (marked by asterisks). The same principle applies to triplexes.

mismatches and higher stability-constant values, incorrect equilibrium binding takes place as efficiently as correct binding because the formation of singly mismatched complexes is not associated with a sufficiently large free-energy loss. Thus, particular experimental conditions must be very carefully chosen to ensure both affinity and specificity. In addition, special consideration must be given to the design of high-affinity nucleic acid ligands to preserve reliably their selectivity for the correct targets. One way of achieving these goals is to use specially constructed oligonucleotide ligands with a pre-arranged secondary structure, rather than linear oligomers. The following sections discuss several strategies aimed at achieving these goals, starting with a simplified thermodynamic analysis of optimizing experimental conditions, which, in several cases, serves as the key to resolving the affinity–specificity dilemma.

Stringency clamping

Figure 4a shows typical equilibrium dependencies associated with environmental parameters, such as temperature, pH or salt concentration, for duplex and triplex binding of nucleic acids with oligonucleotides, analogs and mimics. Equivalent binding of an oligonucleotide ligand, and the like, to the complementary target is shifted to different parameter values compared with singly mismatched targets. This shift is determined by the free-energy difference between perfect and imperfect nucleobase pairing, which is usually small (see earlier). As a result, there is a limited range of parameter values under which the correct complex is formed and incorrect complexes are not formed.

Figure 4b presents the graph (from Figure 4c) in a similar way as the dependence of the yield of correct and incorrect complexes on binding affinity of an oligonucleotide ligand. Here, a higher affinity represents larger binding constants observed at the corresponding parameter values. A window of conditions can be seen where

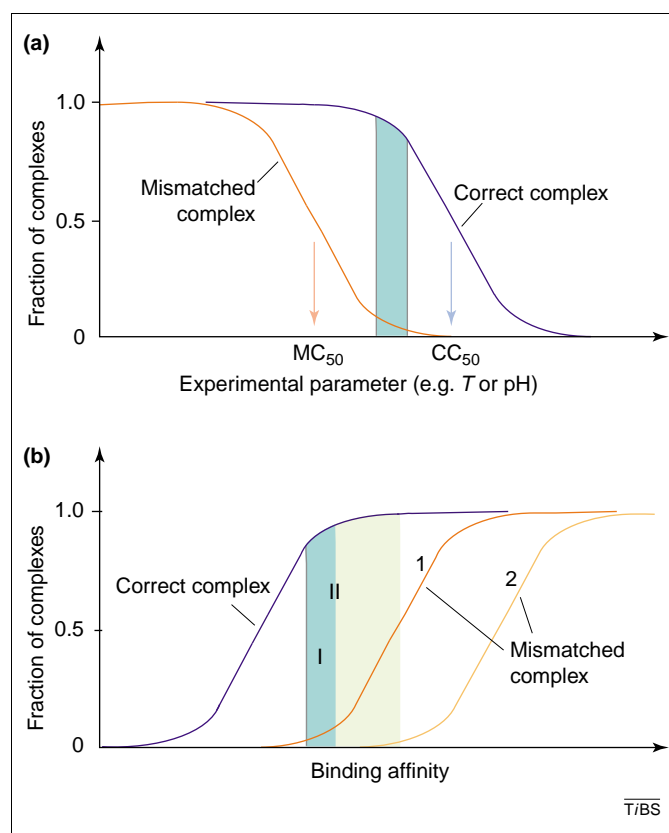


Figure 4. Equilibrium binding of a nucleobase oligomer to correct and mismatched DNA or RNA targets as a function of (a) experimental conditions or (b) their binding affinity. The window of optimal conditions for the selective formation of correct complexes (Curve 2) relative to correct ones significantly widens the range of parameter values that are most suitable for specific recognition [window II (light and dark green) is larger than window I (dark green only)]. Blue and red arrows in (a) indicate the parameter values CC_{50} and MC_{50} , where the half-yield of correct and mismatched complexes are obtained, respectively.

high affinity meets high specificity. Beyond this window, an increase in affinity results in complete loss of specificity. Because this window of optimal conditions is normally quite narrow, finding a way of its widening by shifting the mismatched curve(s) farther outward would be highly desirable (Figure 4b).

The most straightforward but still complicated way to approach this is to increase the match–mismatch energy difference (discussed in the next section). An elegant strategy called stringency clamping was proposed by Roberts and Crothers for DNA triplexes [30] and later generalized by Kramer, Libchaber and colleagues for other nucleic acid complexes, including duplexes [31]. The idea is based on specially designed oligonucleotide ligands capable of forming an alternative structure that is weaker than the complex with a correct target but stronger than the complex with an incorrect target (Figure 5). This structure acts as a stringency clamp by interfering strongly with mismatched binding. Examples of stringency clamps are hairpin-like ligands for duplex formation, and imperfect duplexes for triplex-forming oligonucleotides (Figure 5a).

Figure 5b shows how stringency-clamping strategies can widen the window of optimal conditions for affinity and specificity. In thermal optimization for target binding, unstructured ligands have an optimal temperature range

of ΔT , whereas a structured ligand has a much wider range ($\Delta T'$), which is also shifted to lower temperatures where the correct complex is more stable. Structured ligands therefore distinguish mismatches in a broader range of experimental conditions and provide a higher affinity compared with unstructured ligands. The stringency-clamping approach has been successfully applied to triplex affinity capture of a DNA target [30] and for the fluorescent detection of nucleic acids by molecular beacons [31].

Ligand pre-organization, multiple recognition and topological stabilization

Oligonucleotides with a pre-arranged secondary structure give higher binding affinities and an increase in match–mismatch free-energy difference. Hairpin-like ligands capable of forming both Watson–Crick and Hoogsteen bonds with a single-stranded target (Figure 6a) were first proposed by the research groups of H el ene and Kool [32–34]. Oligonucleotide pre-organization into a clamping construct that partly resembles its structure within a final triple-stranded complex provides a substantial entropic advantage to complex formation [34], significantly increasing binding affinity. Furthermore, oligonucleotide clamps discriminate mismatched sites twice – in the Watson–Crick pairs and in the Hoogsteen pairs (Figure 6a). Therefore, better specificity can be achieved using this mode of recognition compared with linear oligonucleotides [35].

Kool and colleagues went a step further by introducing circular ligands (Figure 6b), thus totally closing the loop and gaining an extra entropic advantage [35,36]. It was found that DNA minicircles bind their single-stranded DNA and RNA targets tighter than similar unclosed hairpin-like ligands. Indeed, circular oligonucleotides are among the most stable and sequence-selective DNA/RNA-binding ligands known to date. They provide a match–mismatch energy difference of up to $\sim 10 \text{ kcal mol}^{-1}$; that is, two-to-three times more than that with standard oligonucleotides. A bicyclic oligonucleotide ligand has been described that has an exceptionally strong affinity (nearly ten orders of magnitude higher than a regular DNA complement) and the highest level of discrimination (the binding constants for singly mismatched targets are almost eight orders of magnitude lower) [37].

DNA minicircles – termed padlock and earring ligands – can be assembled directly on targeted nucleic acids by post-binding ligation [38–42] (Figure 6c). This type of approach represents a robust solution to the affinity–specificity dilemma. Here, high-sequence specificity is achieved by the multiple-coincidence recognition scheme, and by interference of mismatches with the ligation process. In addition, high complex-stability is achieved by additional topological stabilization via ligand–target concatenation [41]. One design that yields maximal stability and sequence specificity is based on pre-opening of the DNA double helix at a designated position by bispeptide nucleic acid (bis-PNA) oligomers (Figure 6c; see next section), thus making the target DNA strand(s) locally accessible for Watson–Crick recognition [40–44].

In the final structure (earring), a segment of circular oligonucleotide is threaded sequence-specifically between complementary strands of double-stranded DNA (dsDNA).

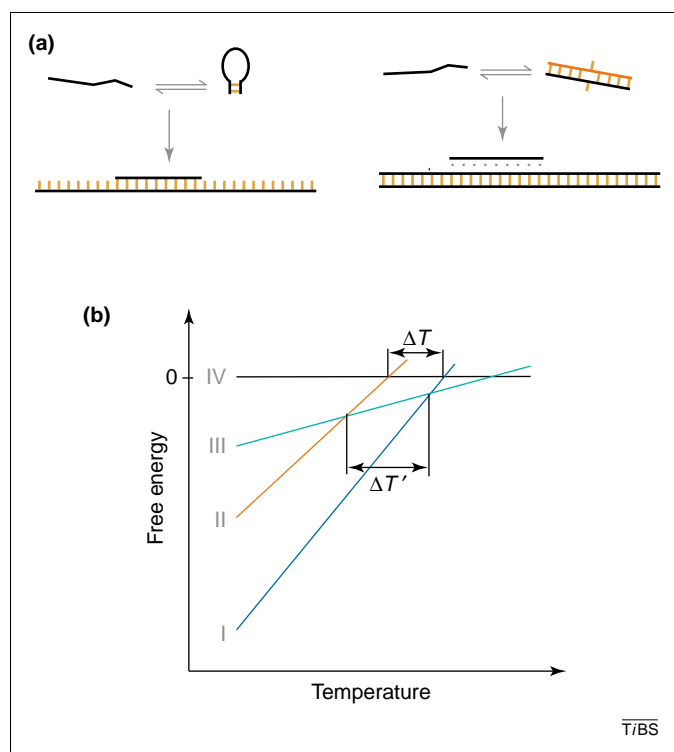


Figure 5. A stringency-clamping approach based on the enhanced specificity of structured nucleic acid ligands. (a) Stringency clamp for a duplex-forming oligonucleotide with a weak hairpin-like intramolecular structure (left), and a triplex-forming oligonucleotide clamped by an imperfect intermolecular duplex (right) (other stringency clamps are also possible). (b) The thermodynamic basis of the stringency-clamping effect (adapted, with permission, from Ref. [31]). Straight lines indicate the free energy of a hypothetically structured ligand (curve III) and its complexes with correct (curve I) and mismatched (curve II) nucleic acid targets. Each graph has been plotted as a function of temperature ($\Delta G = \Delta H - T\Delta S$). Curve IV corresponds to an unstructured ligand where $\Delta G = 0$. ΔG , ΔH and ΔS correspond to the free-energy, enthalpy and entropy changes; ΔT and $\Delta T'$ correspond to optimal specificity ranges of temperature.

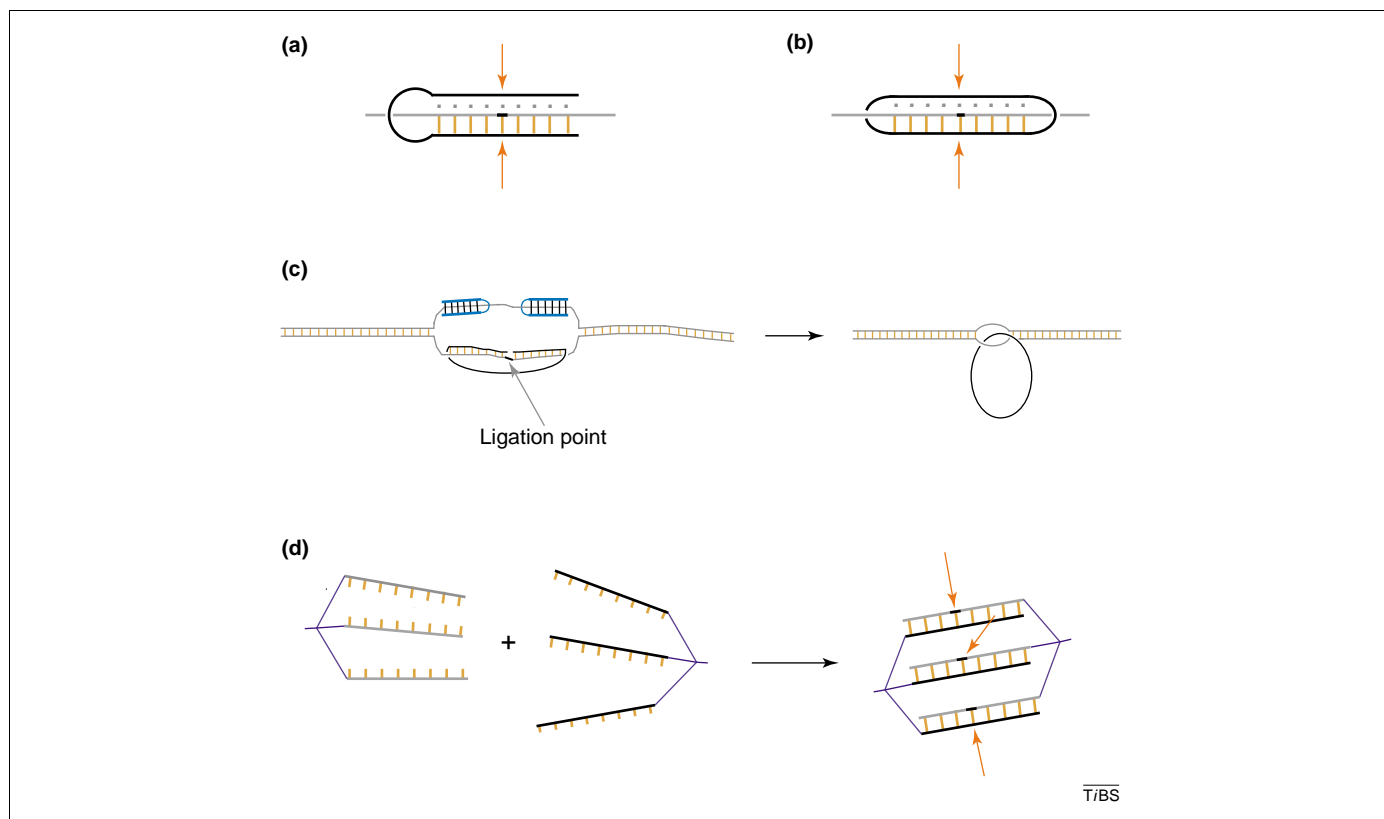


Figure 6. Ligand pre-organization, multiple recognition, and topological stabilization as solutions to the nucleic acid affinity–specificity problem. (a) Hairpin-like and (b) circular triplex-forming oligonucleotides. (c) Formation of an earring-like structure facilitated by PNA openers (blue). This structure has a true topological linkage because the target connection with a circular probe cannot be broken without melting of the target DNA. (d) Interaction of branched (Ψ -shaped) complementary oligonucleotides. Red arrows indicate possible mismatched nucleobases, which will be double- or triple-checked in each case.

Therefore, in this construction, an oligonucleotide ligand is firmly fastened to the chosen dsDNA site and cannot move along the targeted DNA unless strand separation in the flanking sequences occurs. The use of PNA openers as independent recognition elements for proper preorganization of the targeted site significantly enhances sequence selectivity for binding of the circularized oligonucleotide ligand owing to the entire five-step, ‘bipartite-plus-tripartite’ recognition (two PNA oligomers, two oligonucleotide arms plus DNA ligase) [45,46]. Alternatively, the energy of DNA supercoiling can be used as a driving force for the local opening of dsDNA and subsequent formation of earrings on closed circular dsDNAs without any engagement of PNA or other openers [47].

A multiple-coincidence strategy of DNA and/or RNA recognition has been shown to result in very stable and highly specific nucleic acid targeting with the use of tethered bipartite probes or separate, but cooperatively interacting oligonucleotide ligands [48,49]. Finally, non-linear branched oligonucleotides – Y/ Ψ -shaped molecules and dendrimers – have been used as high-affinity duplex- or triplex-forming nucleic acid ligands [50–54]. Here, complex stability is significantly increased by the linking of multiple individual recognition domains (Figure 6d). These branched ligands should have increased specificity for mutual recognition owing to multiple discrimination of the same mismatch by several different branches (as has been shown for dendritic structures; M.S. Shchepinov, pers. commun.). Furthermore, dendrimer ligands also show high specificity binding to common nucleic acid

targets [54], and their complexes are more kinetically stable than those of separate branches [53].

Oligonucleotide dendrimers can be considered as an intermediate step between linear nucleic acid ligands and aptamers [2,3]. The research groups of Mirkin and Letsinger recently developed gold nanoparticles that are heavily functionalized with oligonucleotides [55,56]. These supramolecular constructs take the design of 3D DNA-based probes several steps further than simple branched structures: they have an extremely high binding affinity and sequence specificity, especially when combined with a traditional stringency factor such as temperature or ionic-strength variation.

Another example of a high-affinity oligonucleotide with a pre-arranged secondary structure is locked nucleic acid (LNA) – a conformationally constrained RNA analog [57,58]. (For chemical structures of various nucleobase oligomers discussed in this article, including LNA and PNA, see Figure 1 in Ref. [57].) In LNA, the reduced conformational flexibility of an internally bridged ribose increases the local organization of the sugar-phosphate backbone towards the duplex structure, resulting in improved LNA affinity for complementary DNA and RNA sequences. Some studies reported the superior sequence-specificity of LNA oligomers compared with regular oligonucleotides; however, no consistent or systematic data are currently available to derive any definite conclusions, and the design optimization of LNA-based probes must be done for adequate mismatch discrimination [59,60].

Non-equilibrium conditions: PNA binding to dsDNA

Peptide nucleic acid is a DNA or RNA synthetic mimic, in which nucleobases are attached to a protein-like (polyamide) uncharged backbone, yielding a chimeric pseudo-peptide-nucleic acid chemical structure [61–65]. Owing partly to the electric neutrality of the PNA backbone, mixed-base PNA oligomers form very stable duplexes with DNA and RNA (Figure 1a), with higher sequence specificity at equilibrium than regular oligonucleotides [66–68]. Similar to the LNA, the high sensitivity of PNA to mismatches in PNA–DNA and PNA–RNA duplexes could be explained by structural pre-organization of PNA oligomers [69,70] and/or larger local distortion of mismatched duplex structures compared with other nucleobase oligomers [71].

A strong binding affinity together with the higher sequence specificity of PNA results in a superior activity of antisense PNAs compared with some oligonucleotide derivatives, such as morpholino- and 2'-O-methoxyethyl-modified derivatives [72–74]. Nevertheless, similar to regular oligonucleotides, the equilibrium mismatch-discriminative ability of mixed-base PNAs rapidly declines with increasing length PNA oligomers [1,71,75] because, in this case, sequence specificity also anticorrelates with an increase in affinity. It is worth noting that 8–15mer PNA probes carrying all four nucleobases show good selectivity for single-stranded DNA targets during PNA-based affinity electrophoresis [24] and on PNA microarrays [76–78], whereas longer, 16mer mixed-base PNAs exhibit some problems with sequence specificity [79] (≥ 15 mer probes are required for targeting unique sites in entire genomes). However, in non-equilibrium PNA binding to dsDNA, two different types of PNA oligomers have shown both high affinity and high specificity.

Pyrimidine PNAs

By analogy with pyrimidine oligonucleotides, pyrimidine PNAs were originally thought to bind dsDNA through the formation of ordinary triplexes (Figure 1b). Yet, in reality, something totally different occurs. Two identical PNA oligomers bind the designated homopurine dsDNA sites via strand displacement by forming a new type of complex called an invasion triplex or the P-loop (Figure 1d). This unusual mode of ligand–DNA interaction is characterized by an exceptionally high binding affinity [80]. The reason for this is that the $(\text{PNA})_2$ –DNA triplex is more stable than the canonical $(\text{DNA})_3$ triplex owing to the lack of electrostatic repulsion between the participating DNA and PNA strands and the additional H-bonds, water bridges and van der Waals interactions between the DNA and PNA backbones [81].

PNA clamps or bis-PNAs are used to further enhance strand-invasion efficiency. Here, a pair of pyrimidine-PNA oligomers with mirror-symmetrical sequences is covalently linked via a flexible linker [82]. The linkage of two PNA oligomers enables one PNA strand to be arranged for Watson–Crick binding and the other PNA strand for pH-independent Hoogsteen binding. As a result, binding of ≥ 10 mer pyrimidine PNAs to dsDNA is virtually irreversible under normal conditions; PNA would therefore be expected to show poor sequence specificity. However, in

contrast to this theory, a wide-range of conditions has been found under which high sequence-specificity of PNA–DNA complex formation co-exists with high binding affinity of PNA to dsDNA [14,83–85].

These findings led to the assumption that the elementary steps of pyrimidine-PNA binding to dsDNA are different from the binding of regular oligonucleotides to PNAs of mixed purine–pyrimidine composition in the formation of duplexes and triplexes. The two-step mechanism of pyrimidine-PNA interaction with complementary purine sites on dsDNA was originally suggested by Demidov *et al.* [14] and later proved by further studies [85,86]. According to this mechanism, a reversible, search-stage transient formation of an unstable Hoogsteen PNA– $(\text{DNA})_2$ triplex occurs, followed by an irreversible locking-stage stable formation of a final P-loop complex (Figure 7a), thus enabling PNA oligomers to reach both high specificity and high affinity at non-equilibrium.

Pseudocomplementary PNAs

Similar reasoning can be applied to a new kind of strand-invading PNA, the so-called pseudocomplementary PNAs (pcPNAs), which were recently introduced towards sequence-universal targeting of dsDNA [15,87–89]. Pseudocomplementarity means that two specific derivatives of a paired purine and pyrimidine are structurally altered in such a way that they do not match each other but are capable of a stable Watson–Crick-type pairing with their natural nucleobase counterparts. Therefore, in addition to ordinary guanines and cytosines, pcPNAs carry 2,6-diaminopurines and 2-thiouracils as the pseudocomplementary substitutes for adenine and thymine, respectively [90].

Consequently, modified nucleobases significantly destabilize pcPNA–pcPNA duplexes formed by two mutually complementary mixed-base PNA oligomers but do not affect or stabilize the binding of each pcPNA oligomer to its DNA complement. Thus, two pcPNA oligomers invade highly selectively only the complementary dsDNA site with mixed sequences of purines and pyrimidines by forming very stable double-duplex invasion complexes (Figure 1e). These properties of pcPNAs make them unique and promising ligands capable of denying access of DNA-binding proteins to dsDNA [87,88,91,92] and/or facilitating essentially sequence-universal recognition of dsDNA by oligonucleotides [93].

Figure 7b shows a possible two-step binding process for pcPNA invasion into a DNA duplex. Similar to invasion triplexes, transient formation of an unstable intermediate structure (the invasion duplex) is responsible for the high sequence-specificity of pcPNAs, whereas stable formation of the final structure – the double-duplex invasion complex – is responsible for the high binding affinity of pcPNA. Indeed, the lifetime of this final complex under normal conditions is very long [15,88]. Therefore, pcPNA binding to dsDNA can also be considered as virtually irreversible, even under conditions where its dissociation is thermodynamically favorable (e.g. after removal of free pcPNAs from solution). This form of kinetic stabilization is similar to that of PNA–DNA invasion triplexes [14] or regular DNA–DNA duplexes [94]. Indeed, the energetics

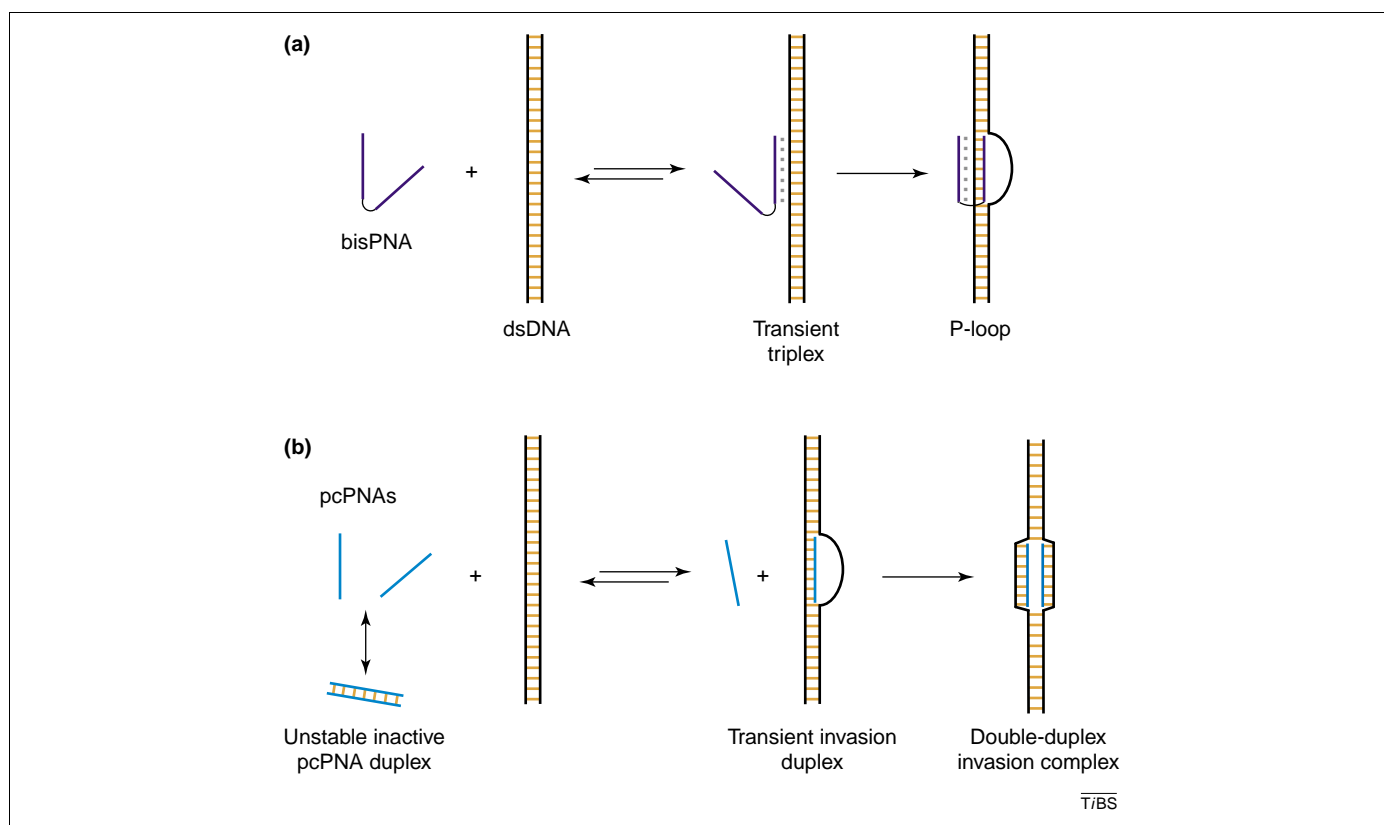


Figure 7. Strand-invasion PNA binding to duplex DNA. This is a two-step process, starting with formation of a transient complex followed by formation of a final complex. **(a)** Triples-invasion complex (P-loop), which is formed by a pyrimidine bis-PNA clamp consisting of identical or similar PNA oligomers connected by a flexible linker. **(b)** Double-duplex invasion complex formed by a pair of mixed-base pseudocomplementary PNAs (pcPNAs).

of the basic steps of these processes look similar, with loss of one base-pair per separation of one link occurring in all cases [15]. Therefore, the dissociation of different complexes at every step is unfavorable until the complete separation of bound oligomers.

It is worth noting that the formation of weak pcPNA pairs, which are incapable of strand invasion because paired pcPNA-oligomers effectively clamp each other, should enhance sequence-specificity of the quasi-equilibrium stage via the stringency-clamping effect (see earlier). Therefore, this effect also contributes to the selectivity of pcPNA–dsDNA complex formation.

Specificity of PNA–DNA interactions

Strand-invasion PNA binding to dsDNA involves an unusual type of biomolecular interaction in which the corresponding participants recognize each other at non-equilibrium. This new principle of biochemical recognition – the two-step binding process for complexes of pyrimidine-PNAs with dsDNA [14,95] – makes possible a kinetic correlation between high recognition specificity and very high binding affinity [95–98]. However, such a conclusion cannot be taken for granted: experimental conditions exist where only poor PNA specificity is observed [1,85]. This is because the kinetic parameter values that describe the two-step binding should be self-consistent. Only then can equilibrium discrimination take place at non-equilibrium, enabling PNAs to bind the nucleic acids tightly and virtually irreversibly, and, at the

same time, efficiently distinguish complementary from mismatched sequences in the course of PNA–DNA complex formation.

The selectivity of the two-step binding process can be described by the idea of kinetic specificity. Kinetic specificity can be characterized as a ratio of the occupancy rates of matched and mismatched targets at times that are short compared with the characteristic time of the locking process, but long compared with the characteristic time of the binding process. However, the idea of kinetic specificity for the one-step binding reaction of common oligonucleotide interactions is meaningless. By contrast, the idea of equilibrium binding affinity for virtually irreversible locking becomes meaningless (affinity is infinitely high); instantaneous occupancies of matched and mismatched sites must be considered instead [1,14,96,97].

Concluding remarks

As a rule, the various applications of oligonucleotides and other nucleobase oligomers in basic and applied research rely on their ability to recognize targeted nucleic acids, DNA or RNA, sequence-specifically via noncovalent interactions by forming duplexes or triplexes. A quadruplex mode of recognition has recently attracted attention as a promising way of targeting nucleobase probes and ligands to certain nucleic acid targets [99]. Covalent nucleic acid modifications at designated sites are also possible with the use of chemically reactive oligonucleotides [100].

Nucleic acid complexes are widely used in biochemical sciences, biotechnology and molecular medicine, and corresponding techniques would benefit significantly from nucleobase oligomers with enhanced binding affinities. However, consideration must be given to the anti-correlation effect between nucleic acid affinity and specificity, which can be elucidated from the nucleation-zipping model of duplex or triplex formation and has been proven by theoretical analysis [1]. Therefore, improvements in the affinity of nucleic acid ligand–target binding must be accompanied by a corresponding increase in the discriminating selectivity of matched versus mismatched complexes.

Three approaches aimed at achieving this goal have been discussed here. The first is to improve the affinity of nucleic acid probe–ligand binding by raising a free-energy penalty for a single mismatch; circular oligonucleotides and dendrimers represent useful examples. The second is to widen the window of conditions where equilibrium mismatch discrimination might occur for complete binding to the correct target; the stringency-clamping strategy represents a good example. The third is to use high-affinity ligands at non-equilibrium that gives, for example, kinetically controlled selectivity against single-mismatch binding (as for PNA).

In principle, common oligonucleotides and their analogs are imperfect candidates for sequence-specific recognition of nucleic acids because an increase in their binding affinity is often accompanied by a loss of target specificity (unless special precautions are taken). However, specific modifications could provide exceptions to this rule, as exemplified by LNA and PNA. In this regard, a better understanding of how the increased binding affinities of PNA- or LNA-oligomers do not compromise their high sequence specificity at equilibrium is required. This understanding will facilitate the rational search for new high-affinity probes. Nevertheless, the lack of high specificity oligonucleotides for binding nucleic acids does not preclude their use in other applications, including probe hybridization, PCR amplification and antisense strategy, because these methods do not require a high specificity of recognition. Moreover, certain approaches exist that overcome this disadvantage of nucleobase oligomers in both stable and selective targeting of DNA and RNA.

In summary, a variety of structured oligonucleotides with different designs have the ability to bind specific nucleic acid targets strongly at equilibrium. Furthermore, the two-step mode of PNA binding to dsDNA exemplifies an additional direction for selective high-affinity recognition of nucleic acids at non-equilibrium. These examples represent elegant solutions to the general affinity–specificity problem in the nucleic acid field. Other promising developments aimed at solving this problem can be expected in the near future.

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