Fluorescent Oligonucleotide Probes for Screening High-Affinity Nucleobase Surrogates

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Abstract: Double-helical oligonucleotide probes featuring a single-nucleotide gap opposed by one of the canonical nucleobases and flanked by the fluorescent nucleobase analogue pyrrolocytosine have been synthesized and titrated with Pd^{II} chelates of dipicolinamide and its N^2,N^6-dialkylated derivatives. The fluorometric titrations revealed greatly increased affinity of the Pd^{II} chelates for the nucleobases opposing the gap compared to the respective free nucleotides in solution. Owing to the constrained environment of the single-nucleotide gap, the relative stabilities of the various Pd^{II} mediated base pairs were also significantly different from those previously reported at monomer level.

Metal ion mediated base pairing has drawn continuing attention since the late 1990s,[1] mainly because of potential applications in DNA nanotechnology, sensors for metal ions and expanding the genetic alphabet.[2] In recent years, the possibility of employing metallated oligonucleotides as high-affinity probes for native nucleic acid sequences has also been explored.[3] While sufficient affinity can be achieved with many metal complexes,[4] discrimination between the canonical nucleobases is a much more demanding task that can only be tackled through extensive screening.

NMR titrations have identified a number of metal chelates exhibiting a high affinity for and, in some cases, a modest selectivity between nucleobases.[5, 6] Interpretation of these results is, however, complicated by the fact that studies carried out at monomer level fail to take into account the steric constraints imposed by the base stack of a double-helical nucleic acid. For example, to reduce steric crowding, the partners of a metal ion mediated base may assume a perpendicular conformation that is not easily accommodated within the base stack.[7] In other words, formation of a stable metal ion mediated base pair at monomer level does not necessarily translate into increased hybridization affinity at oligonucleotide level.

Reliable determination of the base pairing pattern of an artificial nucleobase surrogate typically involves synthesis of the respective nucleoside, its conversion into a phosphoramidite building block, incorporation into an oligonucleotide and, finally, hybridization studies with the modified oligonucleotide and unmodified counterparts. Such a procedure is very laborious and

imposes a severe bottleneck for screening large numbers of nucleobase surrogates. A simpler method, combining the relative ease and high throughput of monomer studies with the predictive power of oligonucleotide studies, would be desirable.

In this paper we present a novel method for screening potential high-affinity nucleobase surrogates based on double-helical oligonucleotide probes featuring a single-nucleotide gap flanked by a fluorescent nucleoside analogue (Scheme 1). A similar method has recently been described to identify intercalators showing specific affinity for abasic sites in DNA.[8] In our study, fluorometric titrations of the oligonucleotide probes with Pd^{II} chelates of dipicolinamide and its N^2,N^6-dialkylated derivatives revealed base pairing preferences significantly different from those observed at monomer level, in terms of both absolute and relative stability of the various Pd^{II} mediated base pairs.

< Scheme 1 >

Primary and secondary structures of the oligonucleotide probes used in this study (ON1a, ON1c, ON1g and ON1t) are presented in Fig. 1A. The double hairpin structure was selected to ensure strong and concentration-independent hybridization of the stem regions. Furthermore, in a probe consisting of a single oligonucleotide the constituent strands of the double-helical regions are by definition present in strictly equimolar concentrations, eliminating the possibility of free single-stranded oligonucleotides in the samples. The double-helical stems were separated by a single unpaired residue (A, C, G or T in ON1a, ON1c, ON1g and ON1t, respectively) and the single-nucleotide gap flanked by the fluorescent nucleobase analogue pyrrolocytosine (pyrrolo-C). Pyrrolo-C emits at a wavelength where unmodified nucleic acids are transparent (approximately 450 nm) and this emission is quenched on stacking with neighboring bases.[9, 10] Binding of aromatic molecules in the gap should, hence, be detectable fluorometrically. While increase in intensity or shift in the wavelength of the fluorescence emission on binding of the nucleobase surrogate would admittedly allow more reliable detection, the assay described in the present study has the advantage of using oligonucleotide probes readily synthesized from commercially available building blocks and not requiring any derivatization of the nucleobase surrogates.

< Figure 1 >

Fluorescence emission intensities of oligonucleotide probes ON1a, ON1c, ON1g and ON1t were recorded over a range of 420 – 480 nm in the presence of various (0 – 5 µM) concentrations of Pd^{II} chelates of dipicolinamide (1a), N^2,N^6-dimethyldipicolinamide (1b) and N^2,N^6-diisopropyldipicolinamide (1c) (Fig. 1B). Concentration of the oligonucleotide probes was 3.0 µM and the excitation wavelength 350 nm. The pH of the samples was 7.4 (20 mM cacodylate buffer) and the ionic strength 50 mM (adjusted with $NaClO_4$). Prior to analysis, each sample was heated to 90 °C and then allowed to cool to 25 °C.

Emission spectra of the oligonucleotide probes were similar to the one previously reported for pyrrolo-C,[9] exhibiting a broad band with a maximum at 450 nm. In all cases, emission decreased on increasing chelate concentration, consistent with binding of the chelate to the gap of the oligonucleotide probe and stacking with the pyrrolo-C residue (Scheme 1). In contrast, no change in emission was observed on titration with the uncomplexed ligands 1a, 1b and 1c or with canonical ribonucleosides, even at titrant concentrations as high as 0.10 M (data not shown). The spectra of ON1a at various concentrations of 1a-Pd are presented as an illustrative example in Fig. 2 (all spectra

are presented in the supporting information). Emission intensities at 450 nm, recorded as averages of three measurements, were used for construction of the titration curves.

< Figure 2 >

Figure 3 depicts normalized fluorescence emissions of the oligonucleotide probes ON1a, ON1c, ON1g and ON1t as a function of concentration of the Pd^{II} chelates 1a-Pd, 1b-Pd and 1c-Pd. As discussed above, fluorescence was quenched by increasing concentration of the Pd^{II} chelate with all combinations of the oligonucleotide probe and the Pd^{II} chelate. In the case of 1a-Pd, saturation was observed at chelate concentrations exceeding the oligonucleotide concentration. In other words, one equivalent of 1a-Pd was enough to decrease the fluorescence emission almost to the saturation level, suggesting that each of the oligonucleotide probes presents a single high-affinity binding site for the Pd^{II} chelates. In all likelihood this binding site is the single-nucleotide gap. With the sterically more demanding 1b-Pd and 1c-Pd, deviation from linearity was less pronounced but nonetheless detectable in most cases. The dependence of the normalized fluorescence intensities on the chelate concentration may be expressed by Eqn. (1)

$$\frac{I}{I_0} = 1 + (I_\infty - 1)\frac{c(chelate)}{K_d + c(chelate)} \qquad (1)$$

where $I_¥$ is fluorescence intensity at infinite chelate concentration and K_d dissociation constant of the complex formed by the metal chelate and the oligonucleotide probe. The values of these parameters were obtained by non-linear least squares fitting of the experimental data to Eqn. (1). The dissociation constants were further converted into the respective stability constants, presented in Table 1. In addition to Pd^{II}, several other metal ions (Cu^{II}, Ag^{I}, Ni^{II}, Zn^{II}, Cd^{II} and Hg^{II}) were screened for their ability to mediate base pairing between 1a, 1b or 1c and the canonical nucleobases. The affinities of the Cu^{II} chelates were approximately an order of magnitude lower than those of the respective Pd^{II} chelates and the affinities of the other metal chelates too low to be determined reliably by the present method (data not shown).

< Figure 3 >

< Table 1 >

The present data, together with those obtained previously by NMR titrations[6] allow comparison of the base pairing behavior of Pd^{II} chelates 1a-Pd, 1b-Pd and 1c-Pd at monomer level and within the base stack of a double-helical oligonucleotide (Fig. 4). All of the Pd^{II} mediated base pairs studied were stabilized in the oligonucleotide environment, presumably by base stacking interactions. On the other hand, the free ligands showed no affinity for the gap of the oligonucleotide probes, underlining the importance of the bridging metal ion for efficient binding. In all likelihood, the binding mode established for 1a-Pd, 1b-Pd and 1c-Pd at the monomer level by NMR titrations[6] is retained at the oligonucleotide level. Accordingly, the least bulky chelate 1a-Pd would bind to N1 and the bulkier chelates 1b-Pd and 1c-Pd to N7 of purine residues (the pyrimidine binding site is always N3). In each case, base stacking and possibly hydrophobic interactions provide extra stabilization.

< Figure 4 >

While considerable destabilization of the Pd^{II} mediated base pairs by sterically demanding substituents on the amide nitrogens was observed with the oligonucleotide probes, the effect was much less clear in the NMR titrations carried out at monomer level. In fact, the bulkiest chelate 1c-Pd exhibited the highest affinity for adenine, cytosine and uracil. The sterically more constrained environment of the single-nucleotide gap of the oligonucleotide probes compared to bulk solvent appears the most likely explanation for this difference. In solution, steric clash caused by the bulky alkyl substituents may be alleviated by rotation about the N-Pd bond. Within a double-helical oligonucleotide, such rotation will inevitably disrupt base stacking, negating some of the stabilization. In the present study, however, the net effect of the oligonucleotide environment was stabilizing even with the bulkiest chelate 1c-Pd.

Owing to the steric requirements of the single-nucleotide gap, the base pairing pattern of a metal chelate could be different with the oligonucleotide probes and with free nucleotides in solution. Restricted rotation about the N-Pd bond could lead to improved discrimination between the canonical nucleobases in the former case. The Pd^{II} chelates employed in the present study indeed exhibited different base pairing preferences at oligomer and monomer levels, but the selectivity of base pairing was actually higher in the latter case. Nevertheless, the results obtained with the oligonucleotide probes are probably more representative of the base pairing pattern of these Pd^{II} chelates when incorporated into a double-helical oligonucleotide.

Experimental Section

Mass spectra were recorded on a Bruker Daltonics microTOF-Q ESI mass spectrometer and UV spectra on a PerkinElmer Lambda 35 UV/Vis spectrophotometer. Freshly distilled triethylamine was used for preparation of the HPLC elution buffers. The other chemicals were commercial products and were used as received.

The oligonucleotide probes ON1a, ON1c, ON1g and ON1t were assembled on a CPG-supported succinyl linker using an ABI 3400 DNA/RNA synthesizer and conventional phosphoramidite strategy. Cleavage of the linker and removal of the base and phosphate protections were accomplished by treatment with 33% aq. NH_3 at 55 °C for 16 h. The oligonucleotides were purified by RP HPLC on a Hypersil ODS C18 column (250 ´ 4.6 mm, 5 µm) eluting with a mixture of MeCN (linear gradient from 5 to 35% over 15 min) in 50 mM aq. triethylammonium acetate. The flow rate was 1.0 mL min^{-1} and the detection wavelength 350 nm (excitation wavelength of the fluorescent pyrrolo-C residue). To prevent hybridization of the oligonucleotides during purification, the column was thermostated to 70 °C. The identities of the purified oligonucleotides were verified by ESI-MS and the concentrations determined UV spectrophotometrically using molar absorptivities calculated by an implementation of the nearest-neighbors method.[11]

Fluorescence emission spectra were recorded on a Cary Eclipse fluorescence spectrophotometer between 420 and 480 nm, the excitation wavelength being 350 nm. Concentration of the fluorescent oligonucleotide probes was kept constant at 3.0 µM and concentration of the metal chelates varied from 0 to 5.0 µM. Before acquiring the spectra, each sample was heated to 90 °C and then allowed to cool to 25 °C. The titration curves were constructed by plotting the emission at 450 nm (average of three measurements) as a function of the chelate concentration.

Acknowledgements

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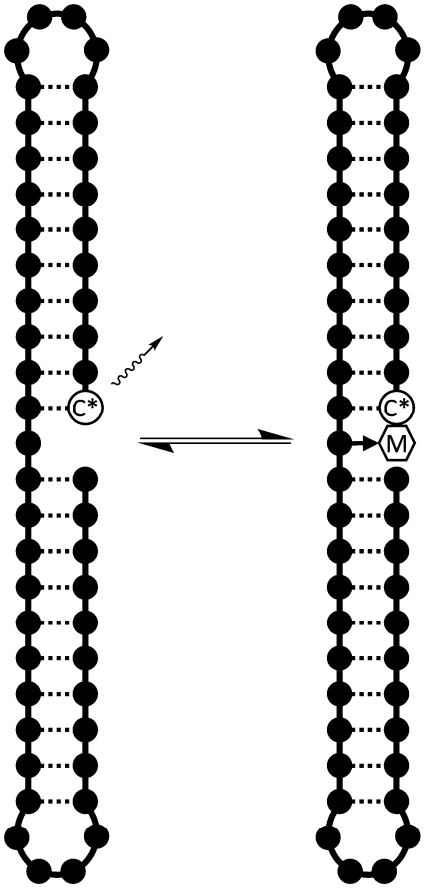


Scheme 1. Binding of a metal chelate in the single-nucleotide gap of a fluorescent oligonucleotide quenches the emission.

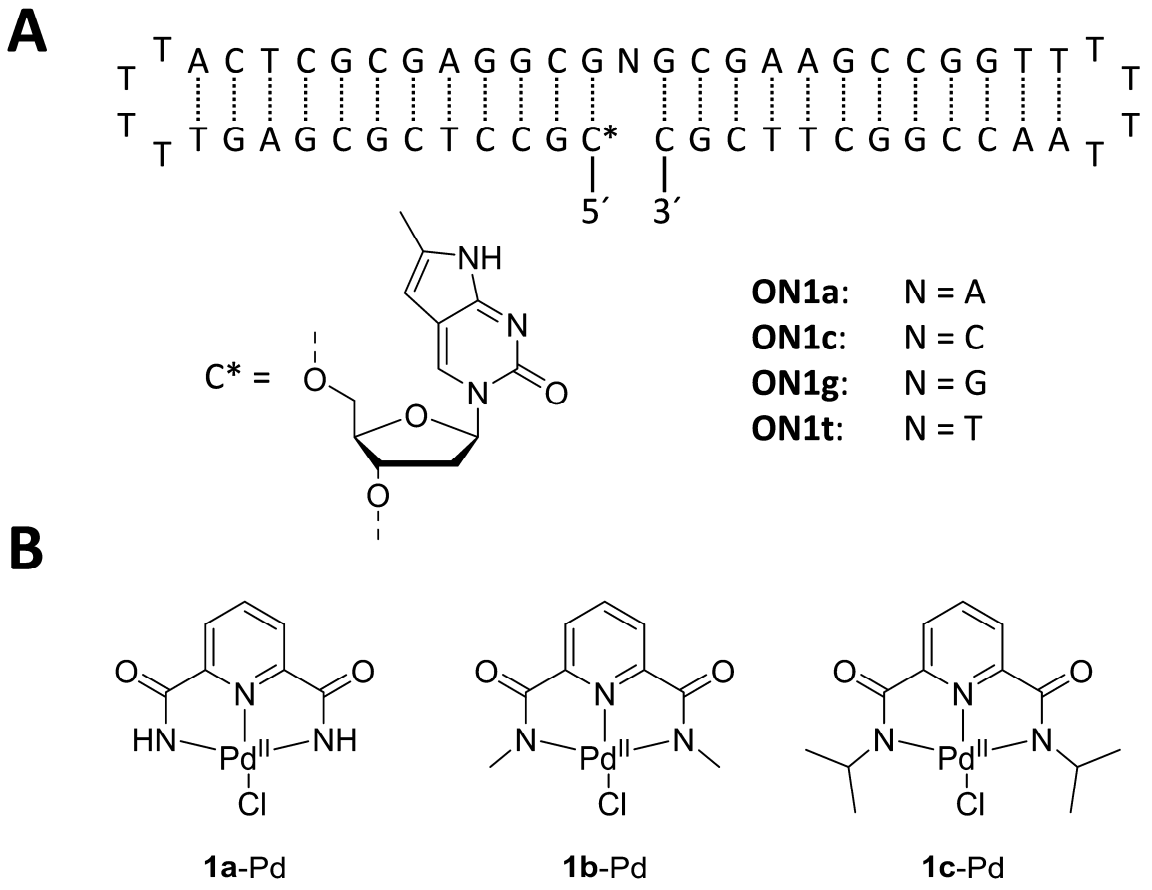


Figure 1. Structures of A) the fluorescent oligonucleotide probes and B) the metal chelates employed.

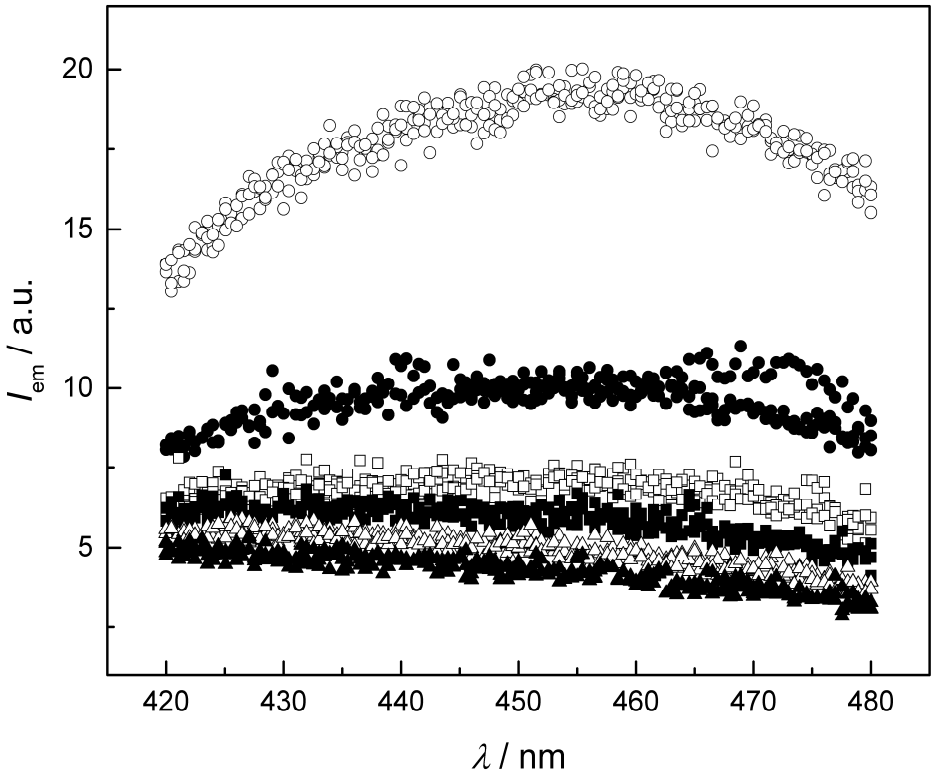


Figure 2. Emission spectra of oligonucleotide probe ON1a at 0 (™), 0.33 (˜), 0.67 (£), 1.0 (¢), 2.0 (r) and 5.0 (p) µM 1a-Pd; [ON1a] = 3.0 µM; pH = 7.4 (20 mM cacodylate buffer); I($NaClO_4$) = 50 mM; T = 25 °C; I_{ex} = 350 nm.

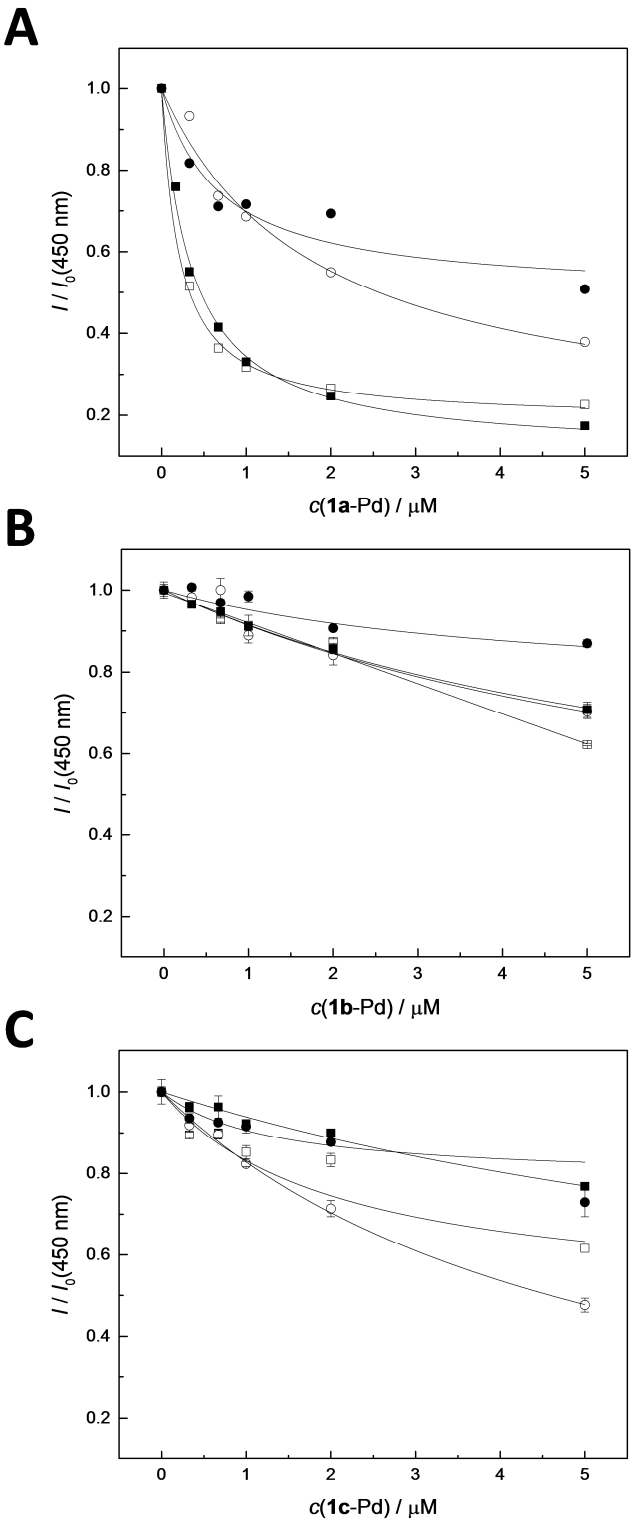


Figure 3. Normalized fluorescence emission intensities of oligonucleotide probes ON1a (£), ON1c (™), ON1g (¢) and ON1t (˜) as a function of concentration of the Pd^{II} chelates A) 1a-Pd, B) 1b-Pd and C) 1c-Pd; [oligonucleotides] = 3.0 μM; pH = 7.4 (20 mM cacodylate buffer); $I(NaClO_4)$ = 50 mM; T = 25 °C. The error bars represent standard deviations between three independent measurements.

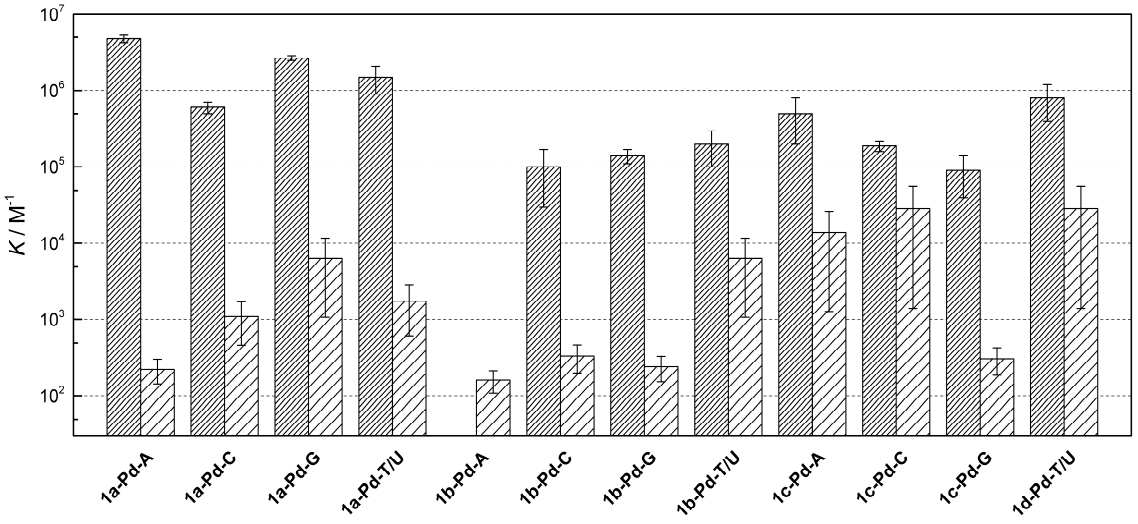


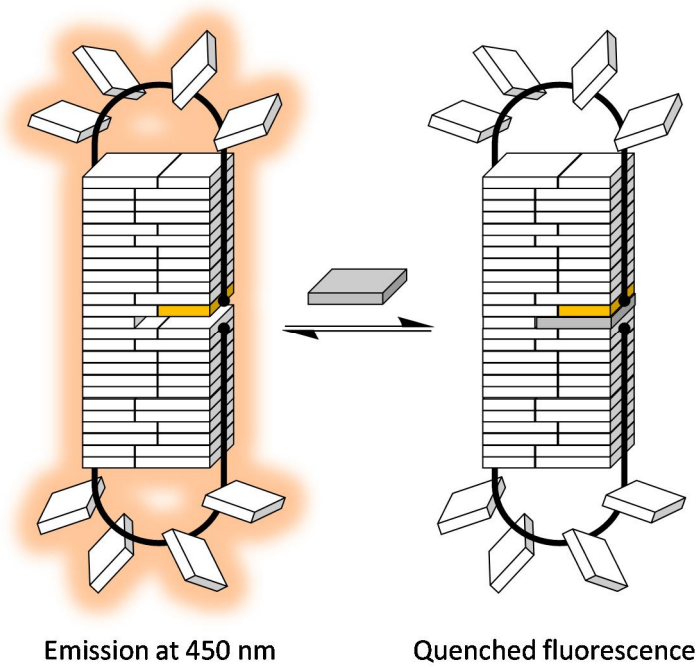
Figure 4. Stability constants for Pd^{II} mediated base pairs between 1a, 1b and 1c and the canonical nucleobases in the oligonucleotide probes (densely hashed columns) and in solution (sparsely hashed columns); pH = 7.4 (20 mM cacodylate buffer) / 7.2 (120 mM phosphate buffer); T = 25 °C. The error bars represent the standard deviations of the non-linear least-squares fits of the experimental data to Eqn. (1).

Table 1. Stability constants of the ternary complexes formed by the metal chelates 1a-Pd, 1b-Pd and 1c-Pd with the oligonucleotide probes ON1a, ON1c, ON1g and ON1t; pH = 7.4 (20 mM cacodylate buffer); $I(NaClO_4)$ = 50 mM; T = 25 °C.

Probe	K / 10^5 M^{-1} [b]		
	1a-Pd	1b-Pd	1c-Pd
ON1a	48 ± 5	n/a[a]	5 ± 3
ON1c	6 ± 1	1.0 ± 0.7	1.9 ± 0.3
ON1g	27 ± 2	1.4 ± 0.3	0.9 ± 0.5
ON1t	15 ± 6	2 ± 1	8 ± 4

[a] Could not be determined reliably.

[b] The error limits represent the standard deviations of the non-linear least-squares fits of the experimental data to Eqn. (1).



Titrations of novel oligonucleotide probes featuring a single-nucleotide gap opposed by one of the canonical nucleobases and flanked by a fluorescent nucleobase analogue with Pd^{II} chelates of dipicolinamide and its N^2,N^6-dialkylated derivatives have revealed base pairing preferences significantly different from those observed in solution, in terms of both absolute and relative stability of the various Pd^{II} mediated base pairs.

Keywords: base pair; palladium; chelate; oligonucleotide; fluorescence