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FISHING G- QUADRUPLEXES IN SOLUTION WITH A PERYLENE DIIMIDE DERIVATIVE LABELED WITH BIOTINS

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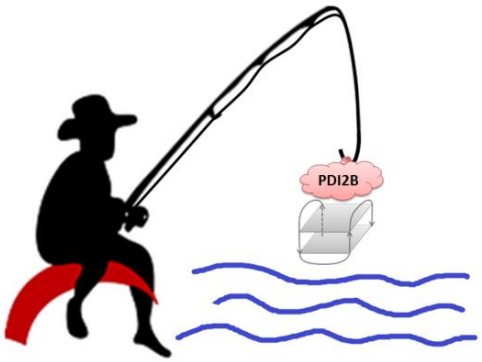
A new fluorescent, non-cytotoxic perylene diimide derivative with two biotins at the *peri* position, **PDI2B**, has been synthesized. This molecule is able to interact selectively with G-quadruplexes with scarce or no affinity towards single- or double-stranded DNA. These features have enabled us to design a simple, effective, safe, cheap, and selective method for fishing G-quadruplex structures in solution by use of **PDI2B** and streptavidin coated magnetic beads. The new cyclic method reported leads to the recovery of more than 80% of G-quadruplex structures from solution, even in the presence of an excess of single stranded or duplex DNA as competitors. Moreover, **PDI2B** is a G4 ligand that can display higher thermal stabilization and greater affinity for 2- over 3-tetrad quadruplexes, which constitutes a novel type of behavior.



G-quadruplexes (G4s) are non-canonical secondary DNA or RNA structures formed by guanine-rich stretches. Four guanines can interact by Hoogsteen hydrogen bonds forming a planar *G-tetrad;* two or more G-tetrads become stabilized by stacking interactions and by the presence of monovalent cations in the central channel.[1] The resulting folded structure is highly polymorphic.[2] The large number of potential G4 forming sequences spread throughout the genome[3] renders G4 an attracting research field, as these structures have been reported to be involved in a wide range of biological processes such as telomere maintenance,[4] genome stability, regulation of oncogene expression, replication,

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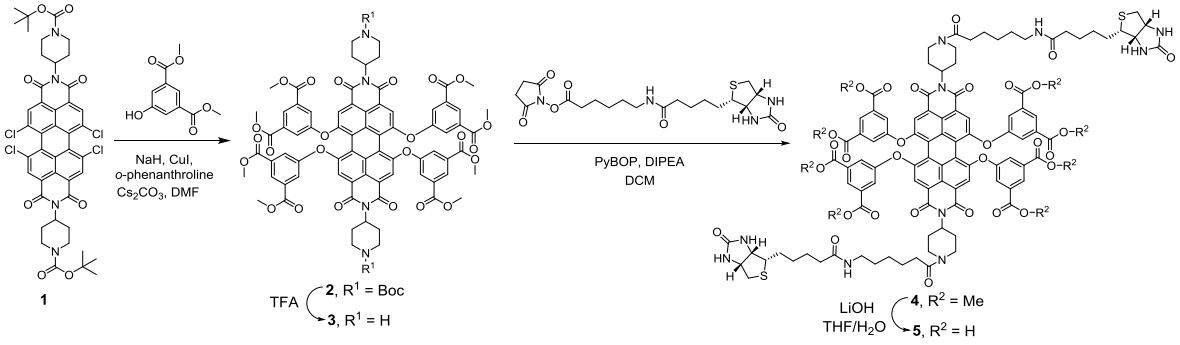
translation, etc.[5] Therefore, the development of a new molecule capable of interacting with these structures and catching them selectively in solution could contribute significantly to achieve progress in the G4 research field.

Perylene diimide (PDI) derivatives are very versatile molecules, extensively used as dyes in car industry, photovoltaic devices development, labeling of biomolecules, supramolecular assemblies and as catalysts as well. [6] PDIs with five condensed aromatic rings in their structure are regarded as potential G-quadruplex ligands that offer the advantage of allowing suitable polar chains to be added to the aromatic core structure to increase solubility and/or promote G4 groove binding. Actually, a number of PDI derivatives have been shown to interact with G-quadruplex.[7] A classical example is the widely studied PIPER (N,N'-bis-(2-(1-piperidino)ethyl)-3,4,9,10-perylene tetracarboxylic acid diimide), which was described for the first time in 1998 as a G4 binder. [8] PIPER can bind to G-quadruplexes by π- π stacking with the 3' terminal G-tetrad and is able to induce the formation of the G-quadruplex structure.[9]

Therefore, we took advantage of these features to synthesize the molecule **5**, (*N*,*N*'-bis-{4-[1-oxo-6-biotinamidohexyl]piperidin-4-yl}-1,6,7,12-tetrakis[3,5-bis(hydroxycarbonyl)phenoxy]perylene-3,4,9,10-tetracarboxylic diimide), which is referred as **PDI2B** (Scheme 1). Starting from a *N*-Boc protected bispiperidinyl derivative of a tetrachloroperylenetetracarboxylic diimide, we have synthesized the octaester perylenediimide by an improved method from a previously known example[10] that was used for the synthesis of water-soluble perylenediimide-cored dendrimers. [11] Thus, microwave irradiation of a mixture of *N*-Boc bispiperidinyl tetrachloroperylenetetracarboxylic diimide **1** and dimethyl-5-hydroxyisophthlate under copper-catalyzed cross-coupling Ullmann reaction conditions gave the intermediate **2**, which was then *N*-Boc deprotected under acid treatment to give **3**. The diamine **3** was coupled to a commercial biotine derivative to give **4** and the octaester was hydrolyzed under basic conditions to get **5**, hereafter denoted as **PDI2B** (See Figs. S1 - S28).



Scheme 1. Synthesis of **5** (*N*,*N*'-bis-{4-[1-oxo-6-biotinamidohexyl]piperidin-4-yl}-1,6,7,12-tetrakis[3,5-bis(hydroxycarbonyl)phenoxy]perylene-3,4,9,10-tetracarboxylic diimide) which is referred to as **PDI2B**.

In view that **PDI2B** is a fluorescent molecule with 64 % quantum yield and 6.57 ± 0.03 ns lifetime under our working conditions, its potential application as a fluorescent probe to target G-quadruplex

structures has been evaluated. No changes in the **PDI2B** fluorescence lifetime were observed in the presence of different DNA G-quadruplexes (Fig. S31) although a quenching effect is observed when **PDI2B** is titrated against G4 DNA. Interestingly, the quenching effect with 2-tetrad quadruplexes (Bom17 and TBA) is greater than with 3-tetrad quadruplexes (Tel22 and c-myc), whereas this quenching effect is almost negligible –within the experimental error- with the duplex, single strand and i-motif samples, revealing high selectivity of **PDI2B** towards G-quadruplexes (Figure 1A). These results suggest selectivity of **PDI2B** for G4 structures over other DNA conformations as well as higher affinity of **PDI2B** towards 2-tetrad quadruplexes than over 3-tetrad quadruplexes.

Moreover, the FRET melting experiments reveal thermal stabilization of G-quadruplex structures by **PDI2B** to different extents (the larger the ΔT_m, the higher the observed stabilization) as a function of the number of G-tetrads (Figure 1B). Most of the G4 ligands exhibiting some selectivity for one quadruplex type over another prefer the parallel G4 conformation. [13] With **PDI2B** greater stabilization is observed for the 2-tetrad G-quadruplexes (FBom17T and FTBAT) than for the 3-tetrad quadruplexes (F21T, F21RT, F25CebT, F21CTAT and FmycT), whereas no thermal stabilisation is observed for the intramolecular duplex DNA, FdXT, where F and T stand for the Fam (F) and Tamra (T) fluorophores. To the best of our knowledge, **PDI2B** is the first G4 ligand reported with higher thermal stabilization towards 2-tetrad than over 3-tetrad G-quadruplexes; this behaviour has not been described hitherto: the radar plots of other known G4 ligands such as Braco19, TmPyP4, 360A, Phen-DC3, TrisQ and pyridostatin reported by De Rache *et al*. [12] indicate greater thermal stabilization of the 3-tetrad human telomeric G-quadruplex (F21T), in sharp contrast with the profile of **PDI2B**.

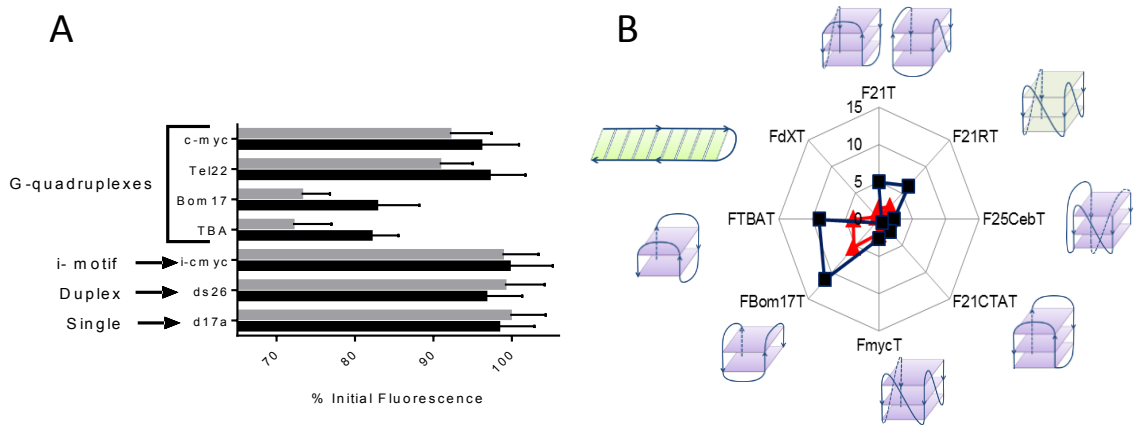


Figure 1. A) Percentage of **PDI2B** initial fluorescence in the presence of a (■)3-fold or (■) 10-fold excess of DNA in several conformations: single strand, duplex, i-motif and 2-tetrad (TBA and Bom17) and 3-tetrad (c-myc and Tel22) G-quadruplexes. C_{PDI2B} = 2 µM and T = 25 °C. B) ΔTm Radar Plot of several oligonucleotides in the presence of **PDI2B** (▲) 5 µM and (■) 10 µM. C_{Oligo} = 0.2 µM. I = 0.11 M (90 mM LiCl, 10 mM KCl, 10 mM LiCaC) and pH = 7.2 except for i-motif (pH = 5.8).

To properly assess the affinity of **PDI2B** with the different G4 structures, we have studied the interaction between **PDI2B** and *Bombyx* telomeric DNA (Bom17) and the human telomeric DNA (Tel22), as representative examples of 2- and 3-tetrad quadruplexes, respectively. ITC titrations of both G-

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quadruplex structures (P) with increasing amounts of **PDI2B** (D) have been conducted. The thermograms reveal a unique binding mode (Fig. S32). The thermodynamic parameters of the interaction of **PDI2B** with Bom17 and Tel22 are collected in Table 1. In light of these data, it can be concluded that the binding is entropically driven for both systems. **PDI2B** displays 4-fold greater affinity constant, K_a, towards 2-tetrads G4 than towards 3-tetrads G4. It should be noted that no binding was observed with calf thymus double-stranded DNA, confirming that **PDI2B** is selective for G-quadruplexes. The binding constants obtained with G4 are the same order of magnitude than those previously reported.[14] Thermal stabilization is one of the notable effects of stabilizing G4 ligands; nevertheless, the obtaining of a high affinity constant is not a guarantee for high ΔT_m.[15] As to the **PDI2B** compound, it is possible to correlate higher affinity and quenching effect with enhancement of thermal stability (Figure 1).

Table 1. Thermodynamic parameters for binding of **PDI2B** to Tel22 and Bom17 at I = 0.11 M (90 mM LiCl, 10 mM KCl, 10 mM LiCaC), pH = 7.2 and T = 25˚C determined using an "Independent sites" model.

	10^{-5} K_a, M^{-1}	ΔH, kJ/mol	ΔS, J/mol·K
PDI2B/Bom17	3.00 ± 0.02	-1.9 ± 0.1	98.3
PDI2B/Tel22	0.75 ± 0.02	-4.3 ± 0.2	79.1

On the other hand, no CD changes were observed in the molar ellipticity of Bom17 and Tel22 in the presence of different amounts of **PDI2B** (Fig. S33), suggesting that **PDI2B** does not induce any significant conformational change in the G-quadruplexes conformations.

These findings are supported by MD simulations, which have allowed us to outline an atomistic model of the binding mode of **PDI2B** with both Bom17 and Tel22 G-quadruplexes. The equilibrium structures (Figs. S34 and S35), show that the binding can be described as host-guest interaction. In particular, a molecular recognition process occurs along the MD trajectory (Figs. S36 and S37), followed by important structural changes. In detail, **PDI2B** is inserted into a DNA pocket of complementary shape, and the two structures are held together by noncovalent interaction.

Selected interatomic distances related to H-bond formation or cleavage and to typical stacking interactions are reported in Figure 2 as a function of the simulation time. Concerning the **PDI2B/**Bom17 system, at about 20 ns **PDBI2** fastly approaches the oligonucleotide, placing the "elbow" shaped group, (formed by folding one biotin arm) into a cavity formed between two thymine residues of the loop previously joined by H-bonding between H3 and O4 (Figure 2A, top left structure, black line). While this part of the molecule remains fixed (Figure 2A, green line), the other biotin arm is oriented towards the G-quadruplex, interacting weakly with different DNA residues during the MD (Figure 2A, top right structure, red line).

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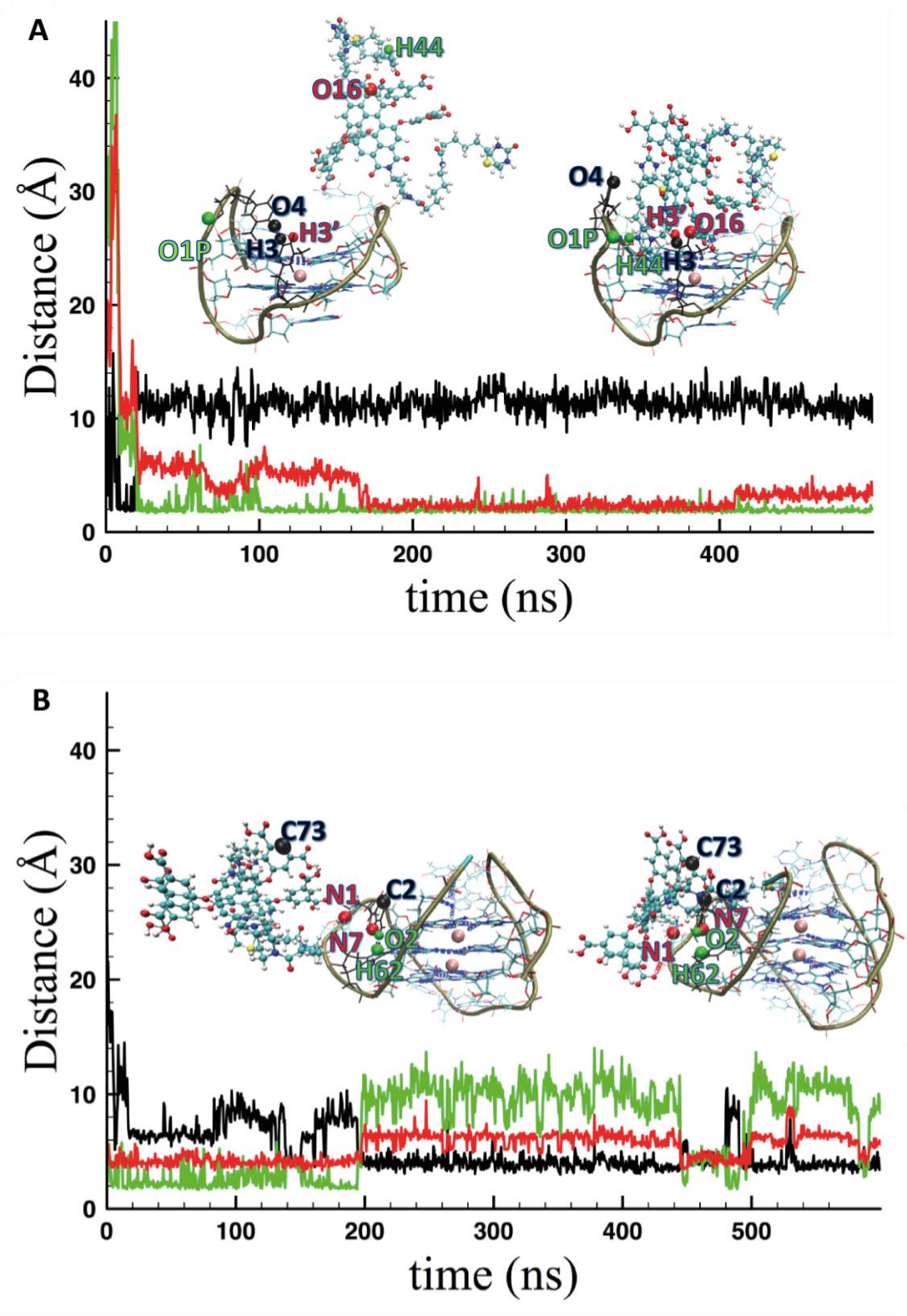

Figure 2. Selected interatomic distances of A) **PDI2B**/Bom17 system: H-bond breakage between T7/H3' and T12/04 (**black**); H-bond formation between T7/H3' and **PDI2B**/O16 (**red**); H-bond formation between T13/H3 and T12/O1P (**green**) and B) **PDI2B**/Tel22 system: Stacking distance between **PDI2B**/C73 and A9/C2 (**black**); increasing distance between T8/N1 and A9/N7 (**red**); H-bond cleavage between T7/O2 and A9/H62 (**green**).

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On the other hand, the binding mechanism of **PDI2B** with Tel22 is somewhat different. **PDI2B** approaches fastly the G-quadruplex structure and, at about 200 ns, one biotin arm of **PDI2B** is inserted within the external GGGTAA loop, previously closed by a H-bond between the thymine 7 and adenine 9 bases (Figure 2B, top left structure, green). After the movement of adenine 9 (Figure 2B, red), one of the 5-hydroxyisophtalic acid "bay groups" interacts by π-π stacking with adenine 9 (Figure 2B, black). Lastly, additional H-bond interactions occur between the biotin group and thymine and adenine bases in the GGGTTAGGG loop (Figure 2B, top right). Also in this case the second biotin arm exhibits more flexible behaviour and continuously changes its position. In particular, it can either be folded over the central part of the organic compound (Figure S35A) or extended on the other side of the loop, close to the other biotin arm, generating a structure in which the G-quadruplex loop is embraced by the two biotin arms (Figure S35B). Additional data and structural description of the final structures for **PDI2B**/Bom17 and **PDBI2B**/Tel22 systems are reported in the supporting information (Figures S38-S41 and as supporting material video files PDI2B_Tel22.mpg and PDI2B_Bom17.mpg).

Since **PDI2B** is able to interact with G-quadruplexes displaying no affinity towards other DNA structures such as single- or double-stranded DNA, this molecule could be very useful for the recovery of G-quadruplexes structures in solution by means of streptavidin-coated magnetic beads. Actually, protein-coated magnetic beads are becoming increasingly popular for the recovery and purification of RNA/DNA. For example, magnetic beads have been employed recently in the detection of G4 structures in human genomic DNA by inmmobilizating the hf2 antibody.[16] The well-known high affinity of streptavidin coated magnetic beads with biotin (10^{19}) has enabled us to use this molecule with two biotin residues in its structure to selectively catch G-quadruplex molecules in solution. A method based in the use of a cytotoxic pyridostatin derivative (IC_{50}=13.4 μM in HT1080 cells) consisting in a single step for recovery, has been previously described.[17] Our method, designed to recover large amounts of G-quadruplex structures in solution and implemented for the first time in this work, makes use of a cyclic methodology depicted in Figure 3A. The method makes no use of urea or heating for DNA recovery since it can be achieved by removal of K^+ from the solution and, more importantly, **PDI2B** is much less cytotoxic (IC_{50} > 100 μM) (Fig. S42) than pyridostatin (IC_{50}=2.5 μM)[18] in HeLa cell line. This absence of cytotoxicity constitutes an important advantage in terms of safety for its potential routine use in laboratory tasks. In addition, if pulling down G4 from cellular extracts were feasible, then the absence of cytoxicity would be quite a favourable issue to maintain the cellular integrity.

Taking advantage of the fluorescence of **PDI2B**, we have corroborated by fluorescence measurements that at least 97% of **PDI2B** remains attached to the streptavidin coated magnetic beads after 5 min of incubation. Then the **PDI2B**-streptavidin coated magnetic beads were incubated for another 5 min with a solution of DNA oligonucleotide and the amount of DNA in the supernatant was determined by absorbance measurements. Afterwards, the DNA-**PDI2B**-streptavidin coated magnetic beads were washed twice with warm water and the process was repeated for several cycles. As observed in Figure 3B, single- and double- stranded DNAs are not retained to the **PDI2B**-streptavidin coated magnetic

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beads, since almost all the DNA remains in solution. By contrast, G4 structures are caught by this procedure even though multiple cycles are required, 12 cycles to recover 90% with 2-tetrads G4 (Bom17 and TBA) and 14 cycles to reach 80% with 3-tetrads G4 (Tel22), an effect that can be easily related to the higher affinity constant of **PDI2B** for Bom17 than for Tel22.

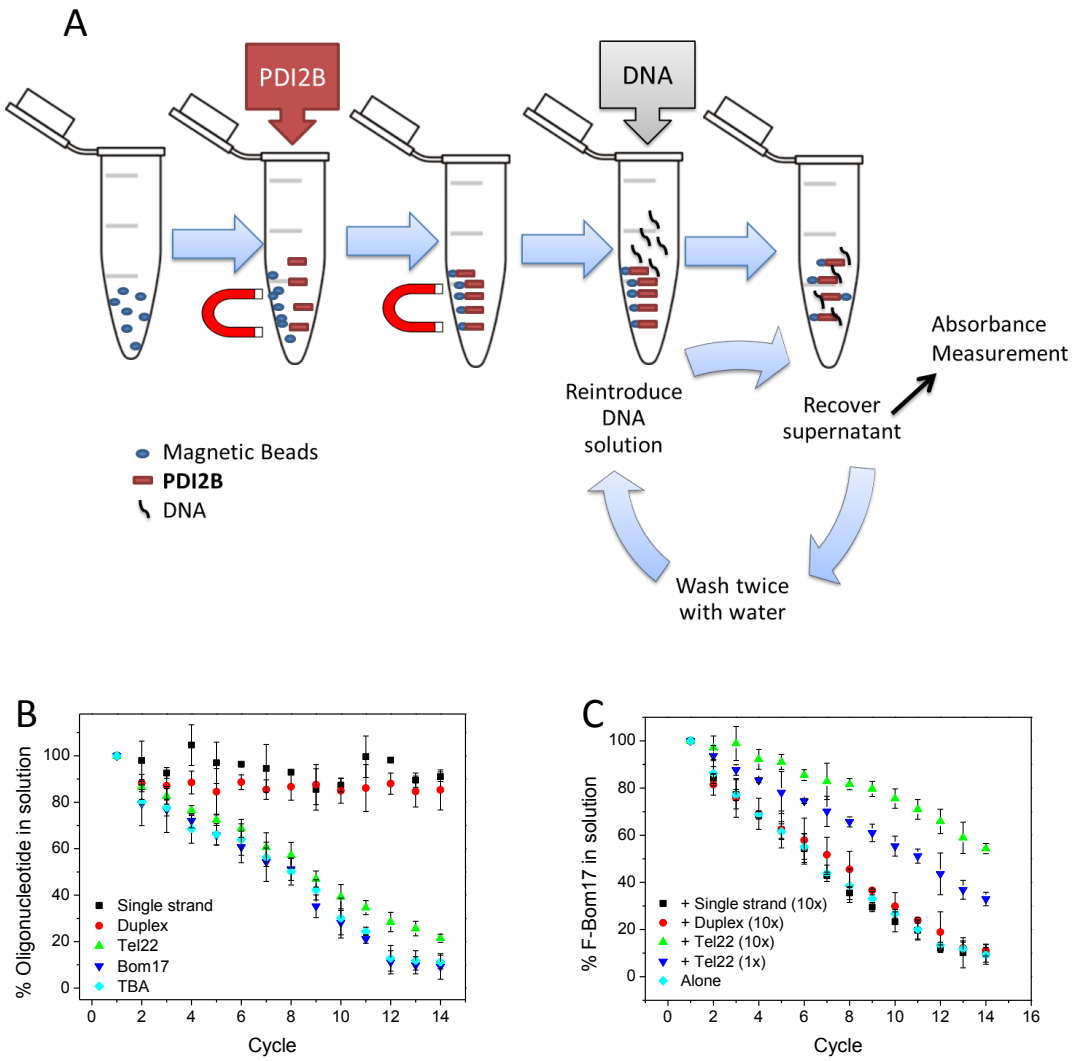

Figure 3. A) Experimental design for the fishing of DNA G-quadruplex structures in solution by means of streptavidin coated magnetic beads. B) Fraction of oligonucleotide remaining in solution after each cycle. C) Fishing of labelled Bom17 with FAM (F-Bom17) followed by fluorescence measurements. Plot of the fraction of F-Bom17 remaining in solution in the presence of unlabeled competitors: single stranded DNA (10×), duplex (10×), or Tel22 quadruplex either in equimolar amounts (1x) or with 10-fold excess (10x).

To unambiguously confirm the selective pulling down of G4 structures, some competition fishing experiments were performed with a labelled oligonucleotide (F-Bom17), both alone and in mixtures containing unlabeled oligonucleotides acting as potential competitors. Figure 3C shows that when F-Bom17 is incubated with 10-fold excess of single stranded DNA or duplex, there are no changes in the recovery of F-Bom17 compared to in the absence of competitor. On the other hand, when the pulling-down of the labelled Bom17 is conducted in the presence of an equimolar amount of non-labelled Tel22

the uptake efficiency is (roughly) 70%, whereas in the absence of competitor the efficiency rose to 90%. By contrast, with 10-fold excess of Tel22 competitor, this efficiency is about 50%.

To conclude, the efficacy of the G4 pull down by **PDI2B** is notable since with only 1 μM PDI2B, approximately 10% of Tel22 can be recovered in a unique step and up to 80% by using the presented cyclic methodology, even in the presence of single or double DNA competitors (see Figures 3B and 3C). By contrast, 3 μM of the pyridostatin derivative is used to caugh 10% of Tel22. [17] Therefore, our procedure is effective, cheap (only involves warm water and the magnetic beads are recycled) and safe (not cytotoxic); by contrast, it takes somewhat, as it requires a limited number of cycles.

In conclusion, a new perylene diimide derivative, **PDI2B**, able to selectively interact with G-quadruplex structures has been synthesized. The presence of the biotin residues at the *peri* positions as well as the absence of cytotoxicity has enabled us to design a new, effective and safe method to selectively pull down G-quadruplex structures in solution even in the presence of other DNA structures as competitors.

Acknowledgements

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