G-Quadruplex on Oligo Affinity Support (G4-OAS): An Easy Affinity Chromatography-Based Assay for the Screening of G-Quadruplex Ligands

ARTICLE *in* ANALYTICAL CHEMISTRY · APRIL 2014

Impact Factor: 5.64 · DOI: 10.1021/ac500444m · Source: PubMed

CITATIONS
5

READS
81

7 AUTHORS, INCLUDING:

Domenica Musumeci
University of Naples Federico II
71 PUBLICATIONS **402** CITATIONS
SEE PROFILE

Jussara Amato
University of Naples Federico II
74 PUBLICATIONS **374** CITATIONS
SEE PROFILE

Daniela Montesarchio
University of Naples Federico II
114 PUBLICATIONS **1,190** CITATIONS
SEE PROFILE

Bruno Pagano
University of Naples Federico II
56 PUBLICATIONS **850** CITATIONS
SEE PROFILE

analytical chemistry

Letter

pubs.acs.org/ac

G-Quadruplex on Oligo Affinity Support (G4-OAS): An Easy Affinity Chromatography-Based Assay for the Screening of G-Quadruplex Ligands

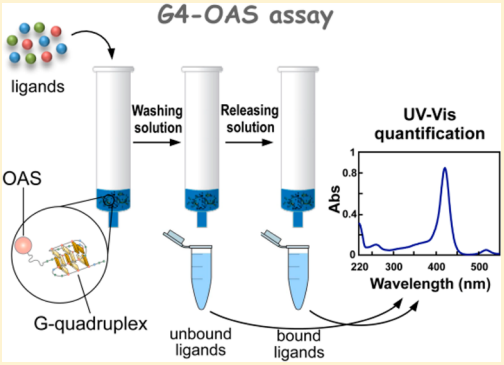
Domenica Musumeci,[†] Jussara Amato,[‡] Antonio Randazzo,[‡] Ettore Novellino,[‡] Concetta Giancola,[‡] Daniela Montesarchio,*[,†] and Bruno Pagano*[,‡]

[†]Department of Chemical Sciences, University of Naples Federico II, via Cintia 4, I-80126 Napoli, Italy

[‡]Department of Pharmacy, University of Naples Federico II, via D. Montesano 49, I-80131 Napoli, Italy

Supporting Information

ABSTRACT: A simple, cheap, and highly reproducible affinity chromatography-based method has been developed for the screening of G-quadruplex binders. The tested compounds were flowed through a polystyrene resin functionalized with an oligonucleotide able to form, in proper conditions, a G-quadruplex structure. Upon cation-induced control of the folding/unfolding processes of the immobilized G-quadruplex-forming sequence, small molecules specifically interacting with the oligonucleotide structure were first captured and then released depending on the used working solution. This protocol, first optimized for different kinds of known G-quadruplex ligands and then applied to a set of putative ligands, has allowed one to fully reuse the same functionalized resin batch, recycled for several tens of experiments without loss in efficiency and reproducibility.



The discovery of small organic molecules efficiently recognizing G-quadruplex (G4) DNA is one of the "hottest" research fields in the study of these unusual nucleic acids structures, particularly motivated by the need for effective anticancer agents.[1] In fact, G4 structures in telomeres and oncogene promoters have been recognized as key elements in the regulation of cancer cell proliferation.[2] Thus, considerable efforts are currently devoted to the design of novel compounds able to target them. This exceptional impulse for the search of molecules efficiently recognizing peculiar G4 structures is producing a huge number of putative ligands,[3] for which fast and reliable screening methodologies are urgently required.

Several methods can be employed to select G4 ligands, such as fluorescence titration, G4-fluorescent intercalator displacement (G4-FID), fluorescence resonance energy transfer (FRET)-based melting, nuclear magnetic resonance (NMR), isothermal titration calorimetry (ITC), surface plasmon resonance (SPR), or electrospray ionization mass spectrometry (ESI-MS) assays.[4] Although usually effective, all these methods rely on an expensive apparatus, requiring specific expertise, and/or special ligand properties (e.g., fluorescence). For instance, ESI-MS analysis of small molecule−nucleic acid interactions requires specific buffer conditions that can dramatically influence the G4 conformations.[4] Other assays, like G4-FID and FRET, may generate false positives and/or false negatives, due to interactions (e.g., fluorescence enhancement or quenching) between the used fluorophores and the tested G4-binders, leading to inaccurate evaluation of the ligand affinity.[5] Furthermore, ITC, though allowing a deep insight into the energetic aspects of the interactions between G4 and ligands, is not suitable for extensive and quick screenings.[6] Therefore, novel analytical methods for the identification of ligands efficiently targeting G4 DNA are strongly called for, especially in those cases in which current methods are not reliable or easily applicable.

Affinity chromatography is a powerful method for probing small molecule−biomacromolecule interactions, which relies on simple and efficient assays by immobilizing either of them on a solid support, also allowing screenings of large libraries.[7] This technique is largely applied to select bioactive compounds from mixtures as, for example, is the case of aptamers obtained through SELEX methodology.[8] A mirror concept has also been profitably exploited to capture proteins from cell extracts by taking advantage of G4-forming aptamers linked to target surfaces analyzed through MALDI-TOF experiments[9] or electrochemical methods.[10] Indeed, a large number of analytical applications has been realized on the basis of immobilized G-quadruplex-forming oligonucleotides also for sensitive small molecules or cations detection.[11]

With the aim of developing innovative affinity chromatography methods enabling a rapid and efficient identification of G4-specific ligands, here we propose a simple protocol based on a G4-functionalized resin in which the capture of the ligand (and its successive release) is achieved upon control of the G4-

Received: January 31, 2014
Accepted: April 11, 2014
Published: April 11, 2014

   dx.doi.org/10.1021/ac500444m | *Anal. Chem.* 2014, 86, 4126−4130

folding/unfolding processes.[12] To realize this binding assay, we selected the Oligo Affinity Support (OAS, Figure 1a), a

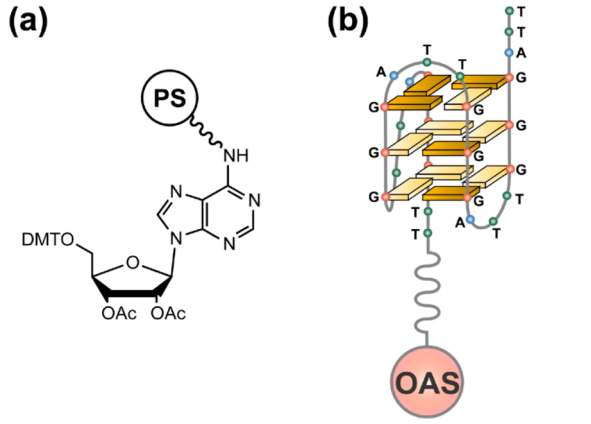


Figure 1. Schematic representation of (a) the OAS resin (for details, see Supporting Information) and (b) the OAS resin functionalized with the oligonucleotide sequence tel_{26} folded in the G4 conformation presumably adopted in a K^+-containing solution.

commercially available polystyrenic resin prefunctionalized with a 4,4′-dimethoxytriphenylmethyl (DMT)-protected adenosine through the adenine exocyclic amino group.[13] On this resin, developed for the affinity chromatography of oligonucleotides, a G4-forming sequence was hence assembled using routine protocols based on standard phosphoramidite monomers. As an intrinsic feature of the OAS-based solid phase synthesis, the final aq. ammonia treatment removed all the protecting groups without detaching the oligomer from the solid support (Supporting Information, Figure S1). The 26-mer human telomeric DNA sequence $^{5'}(TTAGGG)_4TT^{3'}$ (hereafter referred to as tel_{26}) was chosen as a model G4-forming oligomer to demonstrate the feasibility of the method. The functionalized OAS resin, hereafter referred to as tel_{26}-OAS (Figure 1b), was then suspended in a K^+-containing solution and subjected to an annealing procedure to promote the G4 folding of the immobilized tel_{26}. In our experimental setup, schematically depicted in Figure 2, the putative ligands were flowed through the tel_{26}-functionalized resin; small molecules specifically binding the G4 structure were thus captured on the solid support, while compounds having low-to-null affinity were eluted with the washing solution, as determined by UV

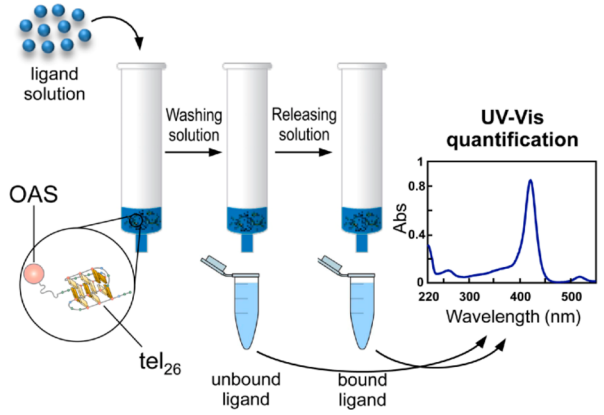


Figure 2. Schematic representation of the G4-OAS assay for the evaluation of putative G4 binders.

measurements. The binding was confirmed by inducing G4 denaturation, which released the bound ligands in solution and, taking advantage of the reversibility of the G4 folding process, ensured a full reusability of the functionalized resin.

This protocol was tested first on a number of small molecules known for their ability to recognize G4 structures with different affinity. In detail, the following ligands, encompassing different G4-binding modes, from $\pi-\pi$ stacking with terminal guanine tetrads to groove interactions, were analyzed: the cationic porphyrin 5,10,15,20-tetrakis(1-methyl-4-pyridinio)porphyrin (TMPyP4),[14] thiazole orange (TO),[15] two acridine derivatives [3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl]acridinium methosulfate (RHPS4) and acridine-9-carboxylic acid (9-Acr-COOH)],[16] and two pyrrole-containing polyamide analogues (netropsin and distamycin)[5,17] (Supporting Information, Figure S2 and Table S1).

For each ligand, we first verified the absence of unspecific binding on the nude support, leaving a 60 μM ligand solution in contact with the OAS resin (5 mg) and then performing exhaustive washings with different solutions. By UV monitoring of the eluates,[18] we observed that the use of 100 mM KCl/15% DMSO (dimethyl sulfoxide) as the washing solution allowed one to almost quantitatively remove all the tested ligands from the nude OAS resin (Figure 3a). Valuably, small percentages of

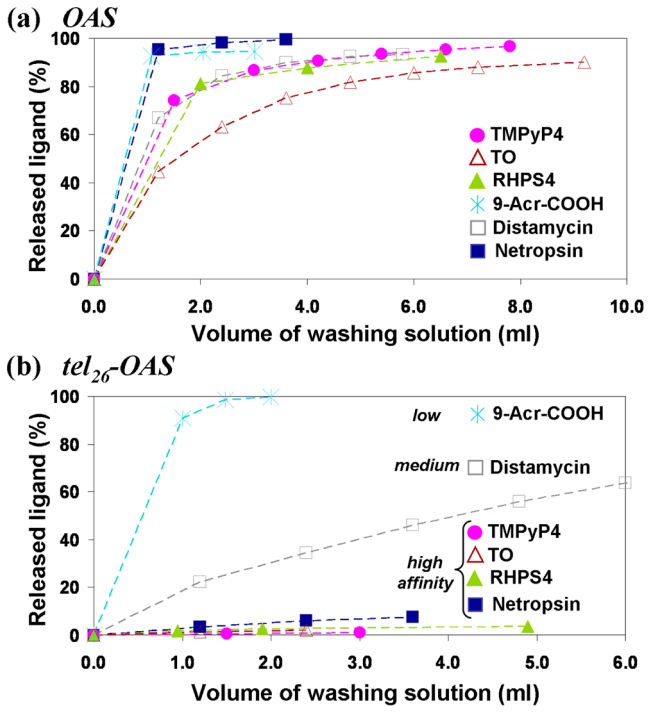


Figure 3. Amount of the released ligands, expressed as percentage of the quantity loaded (a) on nude OAS and (b) on tel_{26}-OAS, as a function of the washing solution volume (100 mM KCl/15% DMSO). Maximum error associated with the percentages determination has been calculated to be within ±2%.

DMSO, previously proved to be well tolerated by a G4 structure,[19] were introduced in the working solutions to ensure a good solubility of the ligands. Once one found the best conditions to minimize unspecific binding events, these were chosen for the washing steps in the binding assays. Next, each ligand was tested in its ability to bind the tel_{26}-OAS. The amount of G4-bound ligand was determined through UV

dx.doi.org/10.1021/ac500444m | Anal. Chem. 2014, 86, 4126−4130

quantification by subtracting the unbound ligand eluted upon washing from the initially loaded amount. In these analyses, TMPyP4, TO, RHPS4 and netropsin were efficiently retained (93−99% of the loaded amount) by tel_{26}-OAS, distamycin was partially retained (28%), while 9-Acr-COOH was completely released (Figure 3b and Supporting Information, Table S4). Notably, this retention order reflects the affinity trend of these ligands for the human telomeric G4,[14−17] thus confirming the general reliability of the method.

With the aim of developing a protocol based on a fully reusable resin, several solutions, inducing G4 unfolding, were analyzed in their ability to determine a fast and quantitative recovery of the ligands bound to tel_{26}-OAS. These tests, allowing one also to confirm the binding data for the G4 ligands, proved that optimal conditions were achieved using a releasing solution containing Ca^{2+} ions and 15% DMSO or, in the case of TO and RHPS4, pure DMSO. The efficiency of these solutions was evaluated by monitoring the amount of ligand released upon treatment of tel_{26}-OAS with increasing elution volumes (Supporting Information, Figure S7). The values for the amount of bound ligands thus measured were in general agreement with those determined by difference, analyzing the unbound ligands (Supporting Information, Table S4).

The system optimized for well-known G4 ligands was then applied to the compounds showed in Figure S3 (Supporting Information). These molecules have been selected as G4 binders by means of virtual screening procedures using the sequence $^{5'}$TGGGGT$^{3'}$ (compounds **1**−**6**) or the human telomeric sequence (compounds **7**−**8**) as working models.[19,20] With the indication of being promising ligands, although not fully characterized in their interaction with human telomeric G4, here they have been considered as ideal systems for our "physical screening" assay. First, for these compounds, we verified the absence of unspecific binding on nude OAS (Figure 4a). All these species were recovered in very high percentages (87%−95%) by eluting the resin with the washing solution (100 mM KCl/15% DMSO), with the sole exception of compound **7**. Its recovery from the polystyrenic support was indeed only partial (67%), probably due to unspecific interactions with OAS, associated also with solubility problems in the working solution.

The results of the binding assays of ligands **1**−**8** on tel_{26}-OAS resin are shown in Figure 4b. Compounds **1**, **3**, **4**, and **8** were released upon standard washings of the resin in 87%, 92%, 89%, and 79%, respectively, thus showing very low affinity for the human telomeric G4. On the other hand, **2**, **5**, **6**, and **7** showed from medium to good affinity for tel_{26}, being released in 17%, 37%, 10%, and 14%, respectively, as calculated by UV analysis (Supporting Information, Table S5).[21] In these binding assays, the percentages of the G4-bound ligand, calculated by difference from the unbound ligand recovered with the washing solution, were consistently in good agreement, with the sole exception of compound **7**,[22] with those obtained by direct UV measurements of the eluates of tel_{26}-OAS treated with the appropriate releasing solution (Supporting Information, Table S5).

In order to further reduce the unspecific binding of compound **7** to the OAS resin, as well as to improve its solubility, we repeated the binding assay on OAS and tel_{26}-OAS resins also testing 20% DMSO solutions. Under these conditions, the improved solubility of the ligand allowed its 98% recovery from the nude resin (vs 67% obtained with the

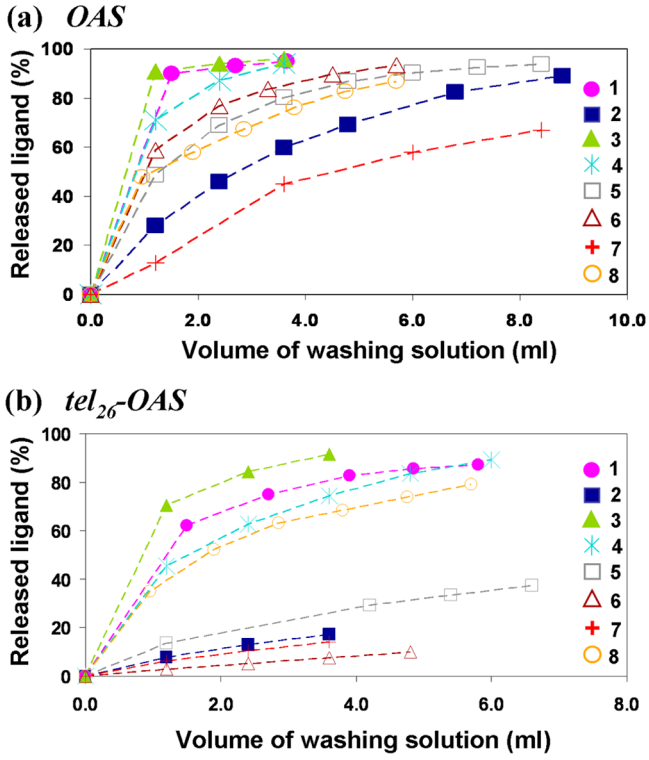


Figure 4. Amount of the released ligands, expressed as percentage of the quantity loaded (a) on nude OAS and (b) on tel_{26}-OAS, as a function of the washing solution volume (100 mM KCl/15% DMSO). Maximum error associated with the percentages determination has been calculated to be within ±2%.

15% DMSO solution) and its almost complete recovery from the G4-functionalized support (96% obtained with the washing and then the releasing solution in sequence, Supporting Information, Figure S8). On this basis, **7** can be considered as a medium affinity ligand for the telomeric G4, better than distamycin or **5**, which at 20% DMSO did not bind tel_{26}-OAS. It is worth noting that DMSO is able to reduce the unspecific interactions of the ligands with the polystyrenic support but also their binding affinity to G4.[23] Overall, the use of the 15% DMSO solution proved to be the best compromise for the binding assays in terms of minimization of unspecific interactions with the polystyrene support and solubility of the ligands, in turn not sensibly affecting the conformational stability of the G4 on the support[19] and ensuring a good affinity of the ligands for the G4.

To verify the G4-binding specificity of the ligands, a control resin carrying a 26-mer scrambled sequence (scr_{26}, Supporting Information), unable to form a G4 structure, was prepared and used for the binding assays using the above-described procedure. For all the tested ligands, selected among the high-to-medium affinity G4 binders (RHPS4, netropsin, **2**, **6**), results similar to those obtained on the nude OAS were observed (Supporting Information, Figure S9). In addition, a model duplex (ds-OAS), obtained by hybridizing the scr_{26}-OAS with the complementary 24-mer $^{5'}$TCACACACACACACA-CACACACTT$^{3'}$ ($compl_{24}$), was also analyzed in order to verify the selectivity of the tested ligands for the G-quadruplex with respect to a duplex structure. Binding assays on ds-OAS were first carried out with netropsin and RHPS4, known to bind also to double stranded DNA,[16a,24] and here used as positive controls. Successively, ligands **2**, **6**, and **7**, which resulted to be

dx.doi.org/10.1021/ac500444m | Anal. Chem. 2014, 86, 4126−4130

the best binders for tel_{26}-OAS,[25] were incubated with the OAS-functionalized duplex to evaluate their G4 vs. duplex discrimination ability. In these assays, the three compounds showed lower affinity for ds-OAS than tel_{26}-OAS (Supporting Information, Figure S12). Therefore, the control assays, carried out either on a single strand or on a duplex immobilized on OAS resin, confirmed that the high-affinity binding of the best ligands to tel_{26}-OAS is ascribable to a specific G4 recognition. Noteworthy, ligands **2** and **7** showed a very good capacity to discriminate between duplex and G4 structures within our experimental setup. In order to test the potential extension of this screening to small libraries of ligands, the G4-OAS binding assay was finally performed by analyzing a mixture of ligands on tel_{26}-OAS resin (Figure 5).

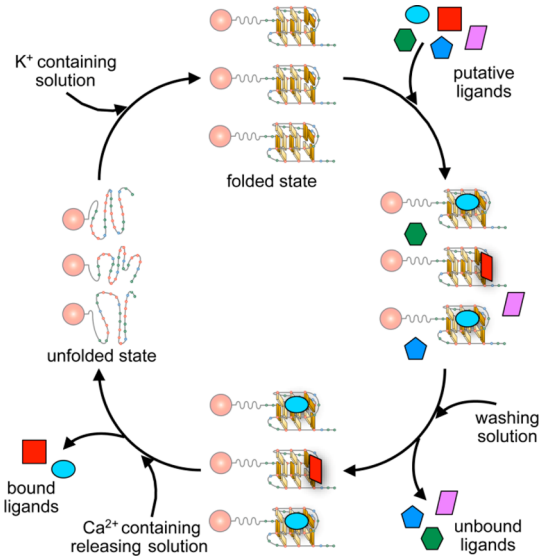


Figure 5. Schematic representation of the G4-OAS assay for the identification of G4 ligands from mixtures.

Three ligands, showing different affinity for G4 structures, were selected. In particular, we chose two ligands, netropsin and TO, with medium and good affinity, respectively, and one, 9-Acr-COOH, with very low affinity. By using the optimized protocol for the binding assay on tel_{26}-OAS resin, netropsin and TO were initially fully retained on the resin and 9-Acr-COOH was immediately released, when eluting with the K^+-containing washing solution. Next, netropsin could be selectively released upon treatments with the Ca^{2+}-containing releasing solution, while TO, still bound to tel_{26}-OAS, was finally recovered only upon washings with 100% DMSO (Supporting Information, Figure S12). This experiment shows that the here described protocol can be profitably exploited not only to identify novel G4 binders from mixtures but also to comparatively evaluate their binding strength, qualitatively selecting low/medium/high affinity G4 ligands.

In conclusion, we have developed a simple, cheap, and highly reproducible analytical assay for the identification of G4 ligands, only requiring a solid phase-immobilized oligonucleotide, easily synthesized on a modified but commercially available solid support, and UV−vis analyses. Remarkably, we were able to reuse the same tel_{26}-OAS resin batch for tens of experiments cyclically performed, without loss in efficiency and reproducibility. In this study, we have demonstrated that the here proposed system, which could be easily automated, can be employed for the screening of libraries of small molecules for the selection of new G4 ligands to be tested for their biological activity. Experiments are currently underway aimed at extending this protocol to OAS resins functionalized with other biologically relevant G4-forming sequences, to evaluate the ligands specific recognition ability of distinct G4 topologies.

■ ASSOCIATED CONTENT

Supporting Information

Preparation and details of the binding assays, experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: daniela.montesarchio@unina.it.
*E-mail: bruno.pagano@unina.it.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the "Italian Association for Cancer Research" (MFAG 11947).

■ REFERENCES

(1) Collie, G. W.; Parkinson, G. N. *Chem. Soc. Rev.* **2011**, *40*, 5867−5892.
(2) (a) Neidle, S. *FEBS J.* **2010**, *277*, 1118−1125. (b) Balasubramanian, S.; Hurley, L. H.; Neidle, S. *Nat. Rev. Drug Discovery* **2011**, *10*, 261−275.
(3) Monchaud, D.; Teulade-Fichou, M. P. *Org. Biomol. Chem.* **2008**, *6*, 627−636.
(4) (a) Murat, P.; Singh, Y.; Defrancq, E. *Chem. Soc. Rev.* **2011**, *40*, 5293−5307. (b) Pagano, B.; Cosconati, S.; Gabelica, V.; Petraccone, L.; De Tito, S.; Marinelli, L.; La Pietra, V.; di Leva, F. S.; Lauri, I.; Trotta, R.; Novellino, E.; Giancola, C.; Randazzo, A. *Curr. Pharm. Des.* **2012**, *18*, 1880−1899.
(5) Giancola, C.; Pagano, B. *Top. Curr. Chem.* **2013**, *330*, 211−242.
(6) Pagano, B.; Mattia, C. A.; Giancola, C. *Int. J. Mol. Sci.* **2009**, *10*, 2935−2957.
(7) Tian, R.; Xu, S.; Lei, X.; Jin, W.; Ye, M.; Zou, H. *TrAC, Trends Anal. Chem.* **2005**, *24*, 810−825.
(8) Mairal, T.; Ozalp, V. C.; Lozano Sanchez, P.; Mir, M.; Katakis, I.; O'Sullivan, C. K. *Anal. Bioanal. Chem.* **2008**, *390*, 989−1007.
(9) (a) Zhang, T.; Zhang, H.; Wang, Y.; McGown, L. B. *Anal. Bioanal. Chem.* **2012**, *404*, 1867−1876. (b) Connor, A. C.; Frederick, K. A.; Morgan, E. J.; McGown, L. B. *J. Am. Chem. Soc.* **2006**, *128*, 4986−4991. (c) Dick, L. W., Jr.; McGown, L. B. *Anal. Chem.* **2004**, *76*, 3037−3041.
(10) Sun, A.; Qi, Q.; Wang, X.; Bie, P. *Biosens. Bioelectron.* **2014**, *57*, 16−21.
(11) (a) Lin, Z.; Li, X.; Kraatz, H. B. *Anal. Chem.* **2011**, *83*, 6896−6901. (b) Lin, D.; Wu, J.; Yan, F.; Deng, S.; Ju, H. *Anal. Chem.* **2011**, *83*, 5214−5221. (c) Sharon, E.; Freeman, R.; Willner, I. *Anal. Chem.* **2010**, *82*, 7073−7077.
(12) To the best of our knowledge, a similar concept has been previously applied only to a biotinylated G4-forming aptamer immobilized on a streptavidin-modified monolithic column for protein detection, in Zhao, Q.; Li, X. F.; Le, X. C. *Anal. Chem.* **2008**, *80*, 3915−3920.

(13) This kind of functionalization, suitably modified to obtain bonds cleavable on demand, was exploited also to prepare nucleoside analogs libraries, in (a) de Champdoré, M.; De Napoli, L.; Di Fabio, G.; Messere, A.; Montesarchio, D.; Piccialli, G. *Chem. Commun.* **2001**, 2598−2599. (b) De Napoli, L.; Di Fabio, G.; D'Onofrio, J.; Montesarchio, D. *Synlett* **2004**, *11*, 1975−1979.
(14) (a) Arora, A.; Maiti, S. *J. Phys. Chem. B* **2008**, *112*, 8151−8159. (b) Martino, L.; Pagano, B.; Fotticchia, I.; Neidle, S.; Giancola, C. *J. Phys. Chem. B* **2009**, *113*, 14779−14786. (c) Wei, C.; Jia, G.; Zhou, J.; Han, G.; Li, C. *Phys. Chem. Chem. Phys.* **2009**, *11*, 4025−4032.
(15) Allain, C.; Monchaud, D.; Teulade-Fichou, M. P. *J. Am. Chem. Soc.* **2006**, *128*, 11890−11893.
(16) (a) Cheng, M. K.; Modi, C.; Cookson, J. C.; Hutchinson, I.; Heald, R. A.; McCarroll, A. J.; Missailidis, S.; Tanious, F.; Wilson, W. D.; Mergny, J. L.; Laughton, C. A.; Stevens, M. F. *J. Med. Chem.* **2008**, *51*, 963−975. (b) Ferreira, R.; Aviñó, A.; Mazzini, S.; Eritja, R. *Molecules* **2012**, *17*, 7067−7082.
(17) Pagano, B.; Virno, A.; Mattia, C. A.; Mayol, L.; Randazzo, A.; Giancola, C. *Biochimie* **2008**, *90*, 1224−1232.
(18) For ligands with low UV molar extinction coefficient, quantification of released ligand could, in some cases, be obtained also by means of fluorescence or, more generally, by ESI-MS analysis.
(19) Cosconati, S.; Marinelli, L.; Trotta, R.; Virno, A.; Mayol, L.; Novellino, E.; Olson, A. J.; Randazzo, A. *J. Am. Chem. Soc.* **2009**, *131*, 16336−16337.
(20) (a) Cosconati, S.; Rizzo, A.; Trotta, R.; Pagano, B.; Iachettini, S.; De Tito, S.; Lauri, I.; Fotticchia, I.; Giustiniano, M.; Marinelli, L.; Giancola, C.; Novellino, E.; Biroccio, A.; Randazzo, A. *J. Med. Chem.* **2012**, *55*, 9785−9792. (b) Di Leva, F. S.; Zizza, P.; Cingolani, C.; D'Angelo, C.; Pagano, B.; Amato, J.; Salvati, E.; Sissi, C.; Pinato, O.; Marinelli, L.; Cavalli, A.; Cosconati, S.; Novellino, E.; Randazzo, A.; Biroccio, A. *J. Med. Chem.* **2013**, *56*, 9646−9654.
(21) In agreement with this experimental model, showing their human telomeric G4 recognition, compounds **5**, **6**, and **7** have been proven to induce selective DNA damage at telomeric level; see ref 20.
(22) Actually, the evaluation of the unbound amount of **7** (14%) was underestimated due to the not negligible unspecific retention (ca. 30%) of the ligand on the nude resin.
(23) For example, in the case of distamycin, the ligand was retained by the tel_{26}-OAS for 80% in a totally aqueous solution, for 28% in a 15% DMSO solution, and completely released with 20% DMSO (see Supporting Information).
(24) (a) Ren, J.; Chaires, J. B. *Biochemistry* **1999**, *38*, 16067−16075. (b) Pagano, B.; Fotticchia, I.; De Tito, S.; Mattia, C. A.; Mayol, L.; Novellino, E.; Randazzo, A.; Giancola, C. *J. Nucleic Acids* **2010**, *2010*, 247137.
(25) On the basis of the here described binding assays, also **5** should be included among the best G4 binders identified among the investigated ligands (see Figure 4); this compound was not considered in the control tests on ds-OAS, being already shown to recognize dsDNA also; see ref 20a.