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The role of G-quadruplex structures of LIGS-generated aptamers R1.2 and R1.3 in IgM specific recognition

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Abstract

Exploiting a variant of SELEX called “Ligand-Guided Selection” (LIGS), we recently identified two novel truncated G-rich aptamers, called R1.2 and R1.3, specific for membrane bound IgM (mIgM), the hallmark of B cells. Herein, the conformational behaviour of these aptamers has been analysed by multiple biophysical methods. In order to investigate their functional secondary structures, these studies have been carried out in pseudo-physiological buffers mimicking different cellular environments. Both aptamers proved to be highly polymorphic, folding into stable, unimolecular G-quadruplex structures in K⁺-rich buffers. In turn, in buffered solutions containing Na⁺/Mg²⁺ ions, R1.2 and R1.3 formed mainly duplex structures. Remarkably, these aptamers were able to effectively bind mIgM on B-cell lymphoma exclusively in the presence of potassium ions. These findings demonstrate the key role of G-quadruplex folding in the molecular recognition and efficient binding of R1.2 and R1.3 to mIgM expressed in lymphoma and leukemia cells, providing a precious rational basis for the design of effective aptamer-based biosensors potentially useful for the detection of cancer-relevant biomarkers.

Graphical abstract

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Keywords

Aptamers; membrane-bound IgM; G-quadruplex

INTRODUCTION

Nucleic acid-based aptamers are single-stranded DNA or RNA molecules that, adopting peculiar secondary structures, such as hairpins or internal stem-loops, bulges and G-quadruplexes, can recognize specific molecular targets with high affinity and selectivity.^{1–5} They are usually selected by an iterative methodology called Systematic Evolution of Ligands by Exponential enrichment (SELEX),¹ introduced in the early 90s independently by the research groups of Gold and Szostak.^{6–8} Using SELEX, several oligonucleotide aptamers were identified against different targets, emerging as promising candidate drugs and diagnostic tools. Among them, pegaptanib sodium, the active component of Macugen, was the first aptamer approved by FDA in 2004 for the treatment of age-related macular degeneration.^{9–11} Since the introduction of SELEX, significant advances have been made to improve the selection procedures of aptamers against complex targets,^{12,13} and suitable modifications of this method have been introduced to generate a large number of effective aptamers. For instance, cell-SELEX technology was introduced using whole cells for the screenings, thus allowing the direct generation of aptamers against cell-surface targets in their native conformation^{2,14,15} Recently, a variant of cell-SELEX, known as Ligand Guided Selection (LIGS), has been designed to identify highly specific aptamers against cell-membrane receptors in their native functional state using a secondary ligand able to outcompete and selectively elute specific aptamers from an evolved cell-SELEX library.^{12,16} By exploiting LIGS, a new aptamer was recently identified against membrane bound-immunoglobulin M (mIgM) expressed on B cells. Although this aptamer, termed R1, proved to recognize mIgM on target cells and efficiently compete with anti-IgM antibodies,¹⁶ its affinity was found to be too low for potential applications as a diagnostic tool for mIgM-expressing cells detection. R1 was thus truncated and, by systematic application of structure-activity relationship (SAR) studies, shorter variants were designed.¹⁷ These efforts produced two truncated aptamers: i) a 42-mer, named R1.2, of sequence d(CACTGGGTGGGGTTAGCGGGCGATTAGGGATCTTGAGTGGT), and ii) a 35-mer, indicated as R1.3, of sequence d(CACTGGGTGGGGTTAGCGGGCGATTAGGGATCTT),

obtained as a further truncation of R1.2. Both R1.2 and R1.3 were able to bind mIgM and showed improved affinity without any detectable loss in binding specificity.¹⁷ Furthermore, binding assays showed that R1.2 was able to recognize the soluble form of immunoglobulin M (sIgM) as well. The concentration of sIgM is 45-150 mg/dL in normal human serum,^{18,19} while overexpression of sIgM is observed during the early stages of infection.²⁰ On the other hand, mIgM is overexpressed only in B-cell leukemia and lymphoma.²¹⁻²³ Therefore, R1.2- and R1.3-based biosensors could be valid, effective tools for the rapid and sensitive detection of both sIgM and mIgM. Taking into account that the precise measurement of the amounts of specific immunoglobulins in serum may be dramatically helpful for the early diagnosis of several diseases, primarily cancer, a detailed analysis of the biophysical properties of R1.2 and R1.3 aptamers and their interaction with the target proteins – necessary prerequisite for the design of suitable aptamer-based biosensors – is highly demanded.

Herein, exploiting several spectroscopic techniques, i.e. CD, UV and NMR, along with gel electrophoresis, size exclusion chromatography and in silico prediction analysis, the conformational behaviour of R1.2 and R1.3 was studied in Na⁺- and K⁺-rich solutions, which mimic the extra- and intracellular media. In addition, a buffer containing both K⁺ and Na⁺ ions, in which the aptamers had been originally selected,¹⁷ was also studied for comparison. Then, the ability of R1.2 and R1.3 to bind mIgM expressed in human lymphoma cells, BJAB, was investigated by flow cytometry assays, exploiting the 3'-FAM-labeled analogues of R1.2 and R1.3 to obtain the binding affinity data. Thus, to ensure that the FAM fluorescent tag had no relevant effect on R1.2 and R1.3 solution behaviour, the conformational properties of the labelled oligomers were studied in comparison with the unmodified aptamers.

Overall, this study was aimed at evaluating the preferred secondary structures of R1.2 and R1.3 under different solution conditions, as well as their thermal stability. Particularly, the role of cations in regulating the aptamer polymorphism in solution was analysed. Interestingly, the binding affinity of R1.2 and R1.3 for mIgM was significantly enhanced in K⁺-rich solutions, suggesting that a G-quadruplex structuring is of crucial relevance in the aptamer-target recognition.

RESULTS AND DISCUSSION

Spectroscopic characterization and in silico prediction analysis of R1.2 and R1.3 foldings

R1.2 and R1.3 aptamers were identified by LIGS procedure against mIgM expressed on B cells in a previous work, and preliminary studies on their interaction with this protein were carried out in K⁺/Na⁺ buffer.¹⁷ Here, we investigated the conformational behaviour of these aptamers in the same buffer in which the binding with IgM was previously studied, containing both K⁺ and Na⁺ ions,¹⁷ as well as in other two media, containing essentially Na⁺ or K⁺, respectively mimicking the extracellular and intracellular environment.

The conformational behaviour of these anti-IgM aptamers was examined combining CD, CD-melting, UV and UV-melting data. In detail, all the spectroscopic data were acquired in three buffered solutions: i) a Na⁺-rich buffer (137 mM NaCl, 2.7 mM KCl, 10 mM

Na₂HPO₄, 1.8 mM KH₂PO₄, 5 mM MgCl₂, hereafter also referred as PBS/Mg²⁺ buffer), ii) a K⁺-rich buffer (10 mM Tris, 100 mM KCl, hereafter also referred as Tris/K⁺ buffer), and iii) a buffer containing both K⁺ and Na⁺ ions (hereafter also referred as K⁺/Na⁺ buffer), obtained by diluting 1:2 the salts contained in the PBS/Mg²⁺ buffer, so to reach 68.5 mM NaCl, 1.35 mM KCl, 5 mM Na₂HPO₄, 0.9 mM KH₂PO₄, 2.5 mM MgCl₂, and taking to 100 mM the KCl concentration,¹⁷ in order to evaluate the effects of the physiological cations on the aptamer conformations.

CD spectra of the aptamers at 1 μM concentration were recorded in the three buffered solutions. Both aptamers showed in all cases a negative band, with a minimum at 243 nm, and a positive band, with a maximum at about 265 nm, along with a broad shoulder extending up to ca. 305 nm (Figs. 1a,b). In particular, in Tris/K⁺ and K⁺/Na⁺ buffers – i.e. the buffers with the highest K⁺ content – the positive band showed an enhanced structuration degree, with a more marked CD signal than in the PBS/Mg²⁺ buffer (157 mM Na⁺, 5 mM Mg²⁺). Considering that both R1.2 and R1.3 contain 4 runs of at least 3 contiguous guanines, i.e. bear sequences with high propensity to form G-quadruplex structures, these spectral features suggest the presence of G-quadruplexes, mainly parallel, in solution.^{24–26} In all the analysed buffers the aptamers displayed an evident difference in the shoulder of the positive band, suggesting the coexistence of different conformations in solution: for R1.3 this shoulder, centered at about 290 nm, was more pronounced than in the case of R1.2, and plausibly due to the presence of hybrid and/or antiparallel G-quadruplexes in equilibrium with the parallel folding.^{24–26}

CD-melting experiments for R1.2 in PBS/Mg²⁺ buffer provided a sigmoidal behaviour with a T_{1/2} of 39 °C (Fig. 2a). The overlapped melting/annealing curves showed that the two profiles were superimposable, indicating that, under the used experimental conditions (heating/cooling rate: 1 °C/min), the denaturation/renaturation processes were completely reversible.

In parallel, CD-melting experiments were also carried out in Tris/K⁺ buffer to evaluate how diverse cations affect the conformational behaviour of the aptamer. The CD-melting curve showed a clear sigmoidal profile, with a T_{1/2} value of 50 °C (Fig. 2b), indicating the formation of a more thermally stable and more cooperatively melting structure than in PBS/Mg²⁺ buffer. On the other hand, the CD-annealing also showed a sigmoidal profile (Fig. 2b), but with a lower T_{1/2} value (T_{1/2} = 41 °C). Thus, differently from what observed in PBS/Mg²⁺ buffer, the CD-melting and annealing curves were not superimposable, indicating that the denaturation/renaturation processes were not reversible under these solution conditions, i.e. the structural transitions were slow relative to the steps of the temperature gradient (Fig. 2b).^{27,28} Finally, in K⁺/Na⁺ buffer the CD-melting experiments performed for R1.2 provided melting and annealing curves respectively with T_{1/2} values of 55 °C and 52 °C (Fig. 2c), i.e. higher values than those measured in Tris/K⁺ buffer, showing only scarce hysteresis (Fig. 2c).

After R1.2, also its shorter variant R1.3 was analysed by CD-melting experiments. In PBS/Mg²⁺ buffer, sigmoidal curves with only limited hysteresis were observed (Fig. 3a), characterized by T_{1/2} values of 43 and 42 °C (respectively for the melting and annealing

experiments), indicating the formation, under these conditions, of a more stable structure than in the case of R1.2. On the other hand, hysteresis phenomena and $T_{1/2}$ values similar to those of R1.2 were found for R1.3 in Tris/ K^+ and K^+/Na^+ buffered solutions (Figs. 3b,c and S1), also in these cases giving more cooperative transitions than when analysed in the PBS/ Mg^{2+} buffer.

CD spectra of both aptamers recorded during the melting and annealing experiments at 5 °C intervals, in the range 10-90 °C, are reported in Figs. S2-S3.

Furthermore, since the CD spectra of R1.3 (Fig. 1b) showed a marked, broad shoulder extending up to 305 nm in Tris/ K^+ and K^+/Na^+ buffers, we plotted the CD signal at 300 nm, extracted from the CD spectra recorded during the melting (Figs. S3c,e) and annealing (Figs. S3d,f) experiments, as a function of the temperature. At 300 nm, the contribution to the CD signal of parallel G-quadruplex structures should be fully negligible; thus allowing the exclusive monitoring of the unfolding/folding of the other G-quadruplex structures (plausibly hybrid and/or antiparallel) present in solution. Sigmoidal profiles were obtained (Fig. S4) with $T_{1/2}$ values in melting/annealing experiments of ca. 40/35 °C and 50/45 °C in Tris/ K^+ and K^+/Na^+ buffers respectively, proving that also non-parallel G-quadruplex foldings, though less stable than the parallel ones, were present in the K^+ -rich buffers.

Overall, these results suggested that the conformational behaviour of both R1.2 and R1.3 was strictly dependent on the used buffer. Indeed, different conformations were found for both aptamers in the three different solutions, with higher thermal stability in the K^+ -rich buffers than in the PBS/ Mg^{2+} buffer. Finally, comparing R1.2 and its truncated variant R1.3 in the same buffer, slight conformational differences were observed, essentially concerning the distribution among the various species in solution, but not their overall thermal stability.

To quantitatively estimate the composition of the mixtures of G-quadruplexes formed by R1.2 and R1.3 in K^+ -rich buffers in terms of parallel, hybrid and antiparallel structures, singular value decomposition (SVD) analysis was performed by exploiting the software recently developed by del Villar-Guerra et al.²⁴ Deconvolution analysis (Fig. S5) showed that the CD spectra of R1.2 and R1.3 in Tris/ K^+ and K^+/Na^+ buffers are produced by a mixture of parallel and hybrid conformations, with no contribution of antiparallel foldings. In detail, for R1.2 in both buffers (Figs. S5a,c) and R1.3 in K^+/Na^+ buffer (Fig. S5d) parallel foldings were slightly prevalent (53-58%) over the hybrid ones, while R1.3 in Tris/ K^+ buffer (Fig. S5b) consisted of essentially similar percentages of parallel and hybrid conformations.

Successively, R1.2 and R1.3 were analysed by UV spectroscopy, recording thermal difference spectra, UV-melting and UV-annealing. A thermal difference spectrum (TDS) is obtained by subtracting the UV spectrum registered at a temperature well above the $T_{1/2}$, when the oligonucleotide is fully denatured, from the one measured at a temperature well below the $T_{1/2}$, when the aptamer is fully structured.^{27,29} The TDS has a diagnostic profile for each type of nucleic acid conformation, allowing its unambiguous identification; in particular the TDS “fingerprint” of a G-quadruplex is featured by a marked negative band at 295 nm, due to the stacking interactions between G-tetrads.³⁰

TDS of R1.2 and R1.3 at 1 μM concentration showed a different behaviour depending on the used solution (Figs. 4 and S6). In PBS/Mg²⁺ buffer, the thermal difference spectra showed maxima at 268 nm for R1.2 and 269 nm for R1.3, with no detectable minima except the one at ca. 225 nm, typical of duplex structures (Figs. 4a,b).²⁹ Conversely, the TDS of both aptamers in Tris/K⁺ (Figs. 4c,d) and K⁺/Na⁺ (Figs. 4e,f) buffers showed maxima at ca. 273 nm, as well as a marked minimum at 295 nm, diagnostic of the presence of G-quadruplex structures.²⁹

UV analysis provides additional interesting data for structural studies on nucleic acids.³¹ In fact, UV-melting curves of duplex-structured nucleic acids recorded at 260 nm, i.e. at the maximum of their absorption, typically give sigmoidal profiles, with marked hyperchromic effects on increasing the temperature. In contrast, G-quadruplexes denaturation generally results in nice sigmoidal UV profiles when recorded at 295 nm, with small, but significant hypochromic effects observed on increasing the temperature.³⁰

R1.2 and R1.3 were thus studied by UV-melting and UV-annealing experiments in all the selected buffers. Considering that TDS of the two aptamers in PBS/Mg²⁺ did not show minima at 295 nm, in this buffer R1.2 and R1.3 were analysed following the absorbance changes at 260 nm on varying the temperature. Both UV-melting and UV-annealing curves for the two investigated systems had a sigmoidal profile, featured by a positive absorbance change on increasing the temperature (Figs. 5a,b, black lines), evidencing the presence of a duplex structure as main species in solution for both the aptamers in the PBS/Mg²⁺ buffer, in line with the TDS data. No significant hysteresis was found in this buffer recording the UV-annealing curves of R1.2 and R1.3 (Figs. 5a,b, blue lines). In contrast, the UV-melting curves monitored at 295 nm, in both Tris/K⁺ and K⁺/Na⁺ buffer, showed a sigmoidal decrease of the absorption on increasing the temperature (Figs. 5c-f, black lines), in agreement with the TDS data indicating G-quadruplex structures as the main species for both aptamers. In line with the CD data, a marked hysteresis emerged when the UV-annealing curves of both the aptamers were recorded in Tris/K⁺ buffer, whereas a reduced one was observed in K⁺/Na⁺ buffer. Analogously to the CD experiments, higher $T_{1/2}$ values were observed in the K⁺-rich buffers than in PBS/Mg²⁺ buffer, with the highest values found in K⁺/Na⁺ buffer, thus confirming the formation of more stable G-quadruplex structures under these conditions than in Tris/K⁺ buffer (Fig. 5).

A summary of the $T_{1/2}$ values determined by CD and UV-melting experiments is reported in Table 1. Taken together, the results obtained using the two different techniques were essentially in agreement, showing that the conformational behaviour of the aptamers was strongly dependent on the nature and concentration of the cations present in solution. Thus, in PBS/Mg²⁺ buffer, both the aptamers formed essentially duplex structures, with limitedly cooperative melting transitions; whereas in the K⁺-rich buffers two families of G-quadruplex structures were evidenced for both aptamers: the most abundant species were parallel G-quadruplexes (accounting for the bands with maximum at ca. 265 nm) with highly cooperative melting transitions and good thermal stability (about 50 °C in Tris/K⁺ and 55 °C in K⁺/Na⁺ buffer), accompanied by hybrid G-quadruplexes (accounting for the bands, here visible as shoulders, with maximum at about 290 nm). Kinetics of folding/unfolding were also dependent on the used buffer. Hysteresis phenomena were indeed observed only in the

K⁺-rich buffers, evidencing the slower formation kinetics of the G-quadruplexes than the duplex structures here investigated, also suggesting that the duplex structures formed in the PBS/Mg²⁺ buffer consisted of rapidly folding intramolecular hairpin constructs^{28,32} (see the following *in silico* analysis).

In silico prediction allowed us obtaining additional information on the aptamers foldings. In detail, duplex structures formed in PBS/Mg²⁺ buffer by both R1.2 and R1.3 were predicted using mfold (<http://www.bioinfo.rpi.edu/applications/mfold>). As shown in Fig. S7, the duplex structures formed by R1.2 and R1.3 were composed respectively by 3 and 2 short intramolecular constructs.

On the other hand, the most plausible models of G-quadruplex structures formed in K⁺-rich buffers were predicted by exploiting QGRS Mapper (<http://bioinformatics.ramapo.edu/QGRS/analyze.php>).³³ Taking into account the two highest scoring sequences as the output of QGRS Mapper (Fig. S8a), also considering that the main folding for both aptamers in K⁺-rich buffers was the parallel-type, well known to have all propeller loops and all the guanines in *anti* conformation, we obtained for the G-quadruplexes most probably formed by R1.2 and R1.3 the models represented in Figs. S8b,c. In addition, considering that long loops destabilize G-quadruplex structures,^{34,35} we can assume that the structures in Fig. S8b are plausible models for both the aptamers in the K⁺-rich buffers. As far as the hybrid-type conformations adopted by both aptamers are concerned, several models are plausible due to the different combinations of conformations that guanines involved in the G-tetrads can adopt, along with the different alternation of loop types in the structures, and therefore unambiguously defined schematic depictions cannot be obtained.

R1.2 and R1.3 3'-labeled with 6-carboxyfluorescein (FAM) – a tag typically used for a variety of fluorescence-based applications,^{17,36} and here exploited for flow cytometry experiments (see below) – were also analysed by CD and UV spectroscopy to evaluate if the presence of the fluorescent label somehow affected the aptamers folding. In particular, this study was performed only in the K⁺-rich buffers, where the aptamers form G-quadruplex structures. Indeed these conformations are typically very sensitive to the presence of aromatic polycyclic molecules, potentially able to give stacking interactions with external G-tetrads and thus to interfere with their folding.^{37–40} Remarkably, R1.2-FAM and R1.3-FAM aptamers showed CD spectra similar to those of unmodified R1.2 and R1.3, thus proving to display similar conformational behaviour as the natural oligonucleotides (Fig. S9). Moreover, the CD- and UV-melting curves of unmodified and FAM-labeled aptamers were essentially superimposable, with similar T_{1/2} values (Fig. S10). These results demonstrate that the presence of the fluorescein-derivatized tail attached at the 3'-end of the aptamers did not alter their overall conformational behaviour, and therefore these FAM-labeled oligomers can be exploited to determine the R1.2 and R1.3 affinity to their biological target (see below).

Native gel electrophoresis

In order to investigate the molecularity and aggregation propensities of the here studied unmodified and FAM-labeled aptamers, native gel electrophoresis experiments were carried out. R1.2, R1.3, R1.2-FAM and R1.3-FAM were analysed at 1 μM concentration in PBS/

Mg²⁺, Tris/K⁺ and K⁺/Na⁺ buffered solutions. A step ladder was used as the size marker, with 5 bp differences between adjacent DNA fragments, and the tel26 oligonucleotide, a 26-mer taken from the human telomere known to adopt a unimolecular G-quadruplex structure in solution,^{41,42} was exploited as a reference.

Considering the length and size of the investigated aptamers, the relative migration rate on the gel was as expected, i.e.: R1.3 > R1.3-FAM > R1.2 > R1.2-FAM (Figs. 6a,b). Furthermore, no difference was found for each aptamer on varying the buffer, evidencing that the compactness of the G-quadruplex structures adopted by R1.2 and R1.3 in both the K⁺-containing buffers was approximately the same found for the duplex structures formed by R1.2 and R1.3 in PBS/Mg²⁺ buffer. Considering that G-quadruplex structures typically migrate faster than duplexes of similar length, these results suggested that the flanking segments in the G-quadruplexes formed in the K⁺-containing buffers (Fig. S8b,c) significantly affect the compactness of the here investigated G-quadruplex structures.⁴³ The unmodified as well as the FAM-labeled aptamers showed a single band, analogously to tel26 (Figs. 6a,b), clear evidence that all the investigated aptamers were present in solution as single monomeric structures. Notably, the fluorescent tag – though clearly reducing the gel mobility of the oligonucleotides – neither affected the molecularity of R1.2 and R1.3 nor promoted the formation of aggregates, further corroborating the use of R1.2-FAM and R1.3-FAM as suitable models of R1.2 and R1.3 in studies with their molecular targets.

Size exclusion chromatography analysis

In order to further evaluate the number and nature of the species formed by R1.2 and R1.3 in solution, these aptamers were also studied by size exclusion chromatography (SE-HPLC), which is a particularly useful technique to investigate the molecularity and structural polymorphism of oligonucleotides.^{44,45} R1.2 and R1.3 were analysed at the same concentration used in the gel electrophoresis assays (1 μM) in PBS/Mg²⁺, Tris/K⁺ and K⁺/Na⁺ buffered solutions. Two unstructured polythymidylic oligonucleotides (T₄₂ and T₃₅) having the same length as R1.2 and R1.3, as well as tel26 were here used as reference oligonucleotides. The 26-mer tel26 is well known to fold in different G-quadruplex conformations, crucially depending on the nature of the cations in solution.^{41,42} Indeed, in the presence of Na⁺ ions, tel26 folds into a basket-type G-quadruplex; on the other hand, in K⁺-rich solutions, a hybrid-type G-quadruplex is observed.^{41,42} According to literature data, the analysis of tel26 by SE-HPLC showed only one peak (t_R = 9.41, 9.11 and 9.38 min in PBS/Mg²⁺, Tris/K⁺ and K⁺/Na⁺ buffers, respectively), consistently with the formation of single, unimolecular G-quadruplex structures in each buffer (Fig. S11).

In the case of R1.2 and R1.3 aptamers, only one peak was found in PBS/Mg²⁺ buffer with t_R = 8.62 and 8.94 min, respectively for R1.2 and R1.3 (Fig. S11a). In contrast, the presence of additional conformations coexisting with the predominant species was evidenced in Tris/K⁺ buffer, where the main peak with t_R = 8.52 min was accompanied by a small shoulder for R1.2 (Fig. S11b), whereas for R1.3 the main peak with t_R = 8.84 min was accompanied by a smaller peak at t_R = 8.41 min (relative peak area = 1.6%). In the K⁺/Na⁺ buffer, main peaks with t_R = 8.84 min for R1.2, and t_R = 9.12 min for R1.3, were observed. In addition, both aptamers chromatograms also showed an early peak (t_R = 7.23 min, relative area = 10.0%

for R1.2, and $t_R = 7.54$ min, relative area = 8.6% for R1.3), indicating the presence of different, less abundant species (Fig. S11c). In addition, T_{42} and T_{35} , having the same length as R1.2 and R1.3 respectively, but unable to give structured conformations, eluted faster than R1.2 and R1.3 in all the used buffers, thus confirming that the aptamers were stably folded into compact secondary structures.^{44,46}

Taken together, these data proved that R1.2 and R1.3 essentially adopted monomeric foldings in solution under the here studied conditions, with no propensity to form multimeric aggregates and different conformational features as a function of the buffer composition, in accordance with CD, UV spectroscopy and gel electrophoresis analyses.

NMR analysis

To get a deeper insight into the secondary structures adopted by R1.2 and R1.3 in the here used conditions, ^1H NMR experiments were also carried out. NMR samples were prepared at 0.1 mM concentration in PBS/ Mg^{2+} and Tris/ K^+ buffered solutions, in $\text{H}_2\text{O}/\text{D}_2\text{O}$ (9:1, v/v). Proton signals in the spectral region diagnostic of Hoogsteen-type hydrogen bonds (11-12 ppm) were observed in the ^1H NMR spectra of all the investigated systems, further demonstrating the formation of G-quadruplex structures (Fig. S12a).⁴⁷ However, no single, well-resolved signal appeared, but only broad envelopes were observed for all the imino resonances, evidencing that the aptamers folded into different G-quadruplex conformations equilibrating in a fast-to-moderate rate on the NMR time scale. Contrarily to the CD and UV data, R1.2 and R1.3 proved to fold into G-quadruplex structures also in PBS/ Mg^{2+} buffer. These different results can be explained considering that the NMR experiments were performed on samples 100-fold more concentrated than those typically analyzed with other spectroscopic techniques, and changes of DNA concentration of two orders of magnitude can significantly affect the conformational behaviour of oligonucleotides. In detail, high oligonucleotide concentrations (0.1 mM, in this case) can favour the formation of intermolecular structures. Therefore, in contrast to what observed at lower concentrations (1 μM), where intramolecular duplex structures are favoured, at 0.1 mM DNA concentration also intermolecular G-quadruplex structures are probably formed.

$\text{H}_2\text{O}/\text{D}_2\text{O}$ exchange experiments were also carried out. The same samples previously prepared in $\text{H}_2\text{O}/\text{D}_2\text{O}$ (9:1, v/v) buffers were lyophilized and then dissolved in 99.9% D_2O . In PBS/ Mg^{2+} buffer, no signal was detected in the imino region for both the aptamers, while a number of detectable resonances emerged from the broad envelopes in Tris/ K^+ buffer for both R1.2 and R1.3 (Fig. S12b), indicating that, in the structures formed in Tris/ K^+ buffer, some protons were indeed less exposed to the solvent, which is the case of imino protons inserted into internal G-tetrads. However, the absence of well-resolved ^1H NMR signals in the imino regions confirmed the general structural polymorphism of these G-quadruplex-forming aptamers, also preventing more detailed NMR investigations.

Flow cytometry assays

Once assured that the fluorescent tag had no relevant effect on the solution behaviour of R1.2 and R1.3, both in terms of preferred conformation and molecularity, the ability of these aptamers to bind mIgM expressed in human lymphoma cells, BJAB, was investigated by

flow cytometry assays using 3'-FAM-labeled analogues of both aptamers, under the same buffer conditions used in the biophysical characterization of the aptamer folding.

BJAB cells express a high number of copies of mIgM on their surface, which facilitate the evaluation of the aptamer binding affinity. It is well known that aptamers can adopt a bioactive conformation that is specifically recognized by the target, often stabilized by optimized salt conditions, which can also dramatically influence their binding.

Thus, we investigated the binding ability of FAM-labeled R1.2 and R1.3 against mIgM-expressing BJAB cells in the Na^+ - (PBS/ Mg^{2+}) and K^+ -rich (Tris/ K^+) buffers, mimicking the extracellular and intracellular environment, respectively. Interestingly, for both aptamers no binding was observed towards BJAB cells in PBS/ Mg^{2+} , where R1.2 and R1.3 mainly adopt duplex structures in solution. In contrast, both R1.2 and R1.3 tightly bound BJAB cells in Tris/ K^+ buffer (Figs. 7a-d), proving that the functional folding of these aptamers was stabilized in this buffer, favouring the formation of G-quadruplex structures. These results clearly indicate that R1.2 and R1.3 can recognize the binding site on mIgM only if structured in a stable unimolecular G-quadruplex folding.

We then determined the binding affinity of both R1.2 and R1.3 aptamers in the Tris/ K^+ buffer against mIgM-positive BJAB cells (Fig. 7, e-f), which were found to be 41 nM and 39 nM, respectively for R1.2 and R1.3. Specific binding was calculated by taking the difference between median fluorescence intensity of aptamer and random control. The Hill coefficient (n) and equilibrium dissociation constant (K_d) of aptamer-cell interaction were obtained by plotting specific binding against concentration and using Hill Slope on GraphPad Prism 7.04. For both aptamers, the Hill coefficients were <1 , indicating negative cooperativity. This is plausibly justified by the fact that, in the presence of several binding sites of mIgM on the cell surface, binding of the first aptamer molecules could actually shield some of the remaining protein binding sites.

Following the same procedure, the affinity constants for R1.2 and R1.3 in the buffer used for the selection of these aptamers (i.e. K^+/Na^+ buffer) proved to be ca. 43 nM and 138 nM, respectively;¹⁷ thus, under the studied conditions, R1.2 had essentially similar K_d values, but remarkably R1.3 showed a 3.5-fold increased affinity towards the target (Table S1). These data indicate that the buffer composition can play a crucial role in the formation of stable conformations for the aptamers, resulting in high affinity and thus more selective target recognition.

This observation significantly emphasizes on the effect of salt composition and concentration on aptamer secondary structure and bioactivity. In a SELEX library, each sequence is stabilized in a unique functional folding based on the salt concentrations of the chosen buffer, leading to variable affinities. When therapeutic applications are intended, physiological buffer conditions should be preferred for SELEX experiments, so to discover stabilized structures in physiological salt conditions. LIGS utilized in selecting these aptamers favours elution of low affinity aptamers by a secondary competitor. This low affinity could also be the consequence of unoptimized salt concentrations, disfavoring the functional folding of the studied aptamers. However, post-SELEX investigations of low

affinity aptamers allowed improving their affinities by either truncation or dimerization.^{17,48} Here, we showed that structurally optimized aptamers could display further enhanced performance by characterizing their functional folding, followed by cellular assays, thus allowing the determination of their main bioactive conformation and of the conditions ensuring their higher stability and higher binding affinities.

CONCLUSIONS

Structural elucidation of how aptamers fold in solution and bind to cell surface receptors is yet to be explored. There are few solved structures of aptamers bound to soluble proteins available in the literature.^{49,50} However, when the target of the studied aptamer is a receptor protein expressed on the cell surface, elucidation of the aptamer functional folding is nearly impossible. Here, we undertook a study to gain insight into the solution behaviour of two G-rich aptamers, termed R1.2 and R1.3, selected against mIgM expressed on BJAB cells. Using a combined approach involving several biophysical techniques and in silico prediction analysis, their conformational properties were investigated in different pseudo-physiological buffers, favouring either duplex or G-quadruplex structures. Conclusively, this study proved that both R1.2 and R1.3 are polymorphic aptamers, adopting mainly parallel, unimolecular G-quadruplex structures in K⁺-rich buffers, with specific features and thermal stability largely dependent on the solution conditions, as assessed by CD, CD-melting, UV, UV-melting, native PAGE and SE-HPLC analysis. Similar properties were displayed by their 3'-FAM-labeled analogues, here used in flow cytometry experiments to determine the ability of R1.2 and R1.3 to selectively recognize IgM proteins. Remarkably, affinity constants in the low nanomolar range were determined for both aptamers in the Tris/K⁺ buffer, while no binding with the protein was observed in the PBS/Mg²⁺-buffer. Under the latter conditions, the G-quadruplex structure of the aptamers is destabilized in favour of hairpin duplex-based architectures. On the other hand, the Tris/K⁺ buffer favours the G-quadruplex formation, indicating that both R1.2 and R1.3 fold into G-quadruplex structures in their bioactive conformations, which is necessary for a specific and high affinity recognition of mIgM expressed in B cells.

Studies on R1.2 and R1.3 variants are currently underway in our laboratories to search for specific changes able to freeze the conformational equilibria of their peculiar G-quadruplex structures. Additional data on the conformational behaviour of these aptamers are indeed crucial to elucidate their main features and further stabilize their functional folding, thus allowing the design of new analogues endowed with higher affinity, and consequently enhancing their potential in clinical and diagnostic applications.

METHODS

Materials.

The oligonucleotides R1.2, i.e. d(CACTGGGTGGGGTTAGCGGG CGATTTAGGGATCTTGAGTGGT), R1.3 i.e. d(CACTGGGTGGGGTTAGCGGG CGATTTAGGGATCTT), tel26 d[(TTAGGG)₄TT], T₃₅ and T₄₂ oligonucleotides were purchased from Biomers (Germany). The oligomers identity and purity were proved by MALDI-TOF mass spectrometry and high performance liquid chromatography (HPLC)

data, provided by the commercial supplier. The 3'-fluorescein-labeled aptamers were chemically synthesized by using FAM-labeled thymidine phosphoramidites as first monomers at the 3'-end using standard solid phase phosphoramidite chemistry, as previously reported.¹⁷ Gel Green[®] Nucleic Acid Stain was purchased from VWR.

Preparation of the oligonucleotide samples.

Purified and lyophilized oligonucleotides were dissolved in a defined volume of pure H₂O and their concentrations were determined by UV-vis spectroscopy in a 1 cm path length cuvette, measuring the absorbance at 260 nm (at 90 °C) and using the following molar extinction coefficients: $\epsilon_{260} = 462600 \text{ cm}^{-1} \text{ M}^{-1}$, $\epsilon_{260} = 382000 \text{ cm}^{-1} \text{ M}^{-1}$, $\epsilon_{260} = 294000 \text{ cm}^{-1} \text{ M}^{-1}$, $\epsilon_{260} = 304500 \text{ cm}^{-1} \text{ M}^{-1}$ and $\epsilon_{260} = 365400 \text{ cm}^{-1} \text{ M}^{-1}$ respectively for R1.2, R1.3, tel26, T₃₅ and T₄₂. UV spectra were recorded in the range 220-320 nm, with a medium response, a scanning speed of 100 nm/min and a 2.0 nm bandwidth and corrected by subtraction of the background scan with buffer. Solutions of both natural and fluorescently labeled aptamers at 10 μM concentration were prepared in PBS/Mg²⁺ (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 5 mM MgCl₂ pH 7.4) and Tris/K⁺ (10 mM Tris, 100 mM KCl, pH 7.4) buffered solutions. 1 μM working solutions were then prepared by diluting the 10 μM solutions in the PBS/Mg²⁺ and Tris/K⁺ buffers. In parallel, solutions of R1.2, R1.3, R1.2-FAM and R1.3-FAM at 10 μM concentration were also prepared by dilution of the respective stock solutions with a 550 μM KCl solution and, successively, 1 μM working solutions prepared by diluting the 10 μM solutions using the K⁺/Na⁺ buffer (100 mM KCl, 68.5 mM NaCl, 1.35 mM KCl, 5 mM Na₂HPO₄, 0.9 mM KH₂PO₄, 2.5 mM MgCl₂ pH 7.4), previously used for binding experiments.¹⁷ All the samples were annealed by taking them at 95 °C for 10 min, then slowly cooling to room temperature and finally keeping at 4 °C until subsequent use. Different annealing procedures, previously performed,¹⁷ i.e. heating the samples at 95°C for 10 min and maintaining them on ice for 1 h, did not affect the overall spectroscopic and chromatographic properties of the investigated aptamers (data not shown).

Circular dichroism (CD) spectroscopy.

CD spectra, CD-melting and CD-annealing were recorded on a Jasco J-715 spectropolarimeter equipped with a Peltier-type temperature control system (model PTC-348WI), using a quartz cuvette with a path length of 1 cm. Spectra were recorded in the range 220-320 nm with 2 s response, 100 nm/min scanning speed and 2.0 nm bandwidth. All the spectra were averaged over 3 scans and corrected by subtraction of the background scan with proper blank. The oligonucleotide sequences were analysed at 1 μM concentration in PBS/Mg²⁺, Tris/K⁺ and K⁺/Na⁺ buffered solutions. Thermal denaturation/renaturation curves were recorded following the CD signal at the maximum vs. the temperature in the range 10-90 °C, with heating/cooling rate of 1.0 °C/min. Normalized CD signal was calculated as $(\text{CD}_{\text{obs}} - \text{CD}_{\text{min}})/(\text{CD}_{\text{max}} - \text{CD}_{\text{min}})$ where CD_{obs} is the CD value at each temperature, CD_{max} and CD_{min} are the CD signals for the folded and unfolded oligonucleotide, respectively.

UV spectroscopy.

UV spectra, UV-melting and UV-annealing were obtained on a Cary 5000 UV-vis-NIR spectrophotometer equipped with a temperature controller system, using 1 cm path length cuvette. The UV characterization of the oligonucleotides was performed at 1 μ M concentration in PBS/Mg²⁺, Tris/K⁺ and K⁺/Na⁺ buffered solutions. The UV-vis spectra were recorded in the range 220-320 nm, using a scanning speed of 100 nm/min and corrected by subtraction of the background scan with proper blank. TDS were obtained by subtracting the UV spectra recorded at a temperature above the T_{1/2} (90 °C) from those measured at a temperature below the T_{1/2} (15 °C) ^{27, 29} The absorbance vs. temperature profiles were recorded by following the absorbance changes (at 295 nm or 260 nm) on varying the temperature in the range 15-80 °C, with heating/cooling rate of 1.0 °C/min. Normalized absorbance was calculated as (A_{obs} - A_{min})/(A_{max} - A_{min}), where A_{obs} is the absorbance at each temperature, A_{max} and A_{min} are the highest and the lowest absorbance recorded in each melting experiment, respectively.

Gel electrophoresis.

Oligonucleotide samples at 1 μ M concentration in PBS/Mg²⁺, Tris/K⁺ and K⁺/Na⁺ buffered solutions, together with a O'RangeRuler™ 5 bp DNA Ladder (ThermoFisher Scientific) were loaded and resolved on native 20% polyacrylamide (19:1 acrylamide:bisacrylamide) PAGE. TBE (Tris-Borate-EDTA, 1X) was used as running buffer. Samples were electrophoresed for 3 h at 100 V at room temperature, stained with GelGreen® Nucleic Acid Stain for 30 min and finally visualized by an UV transilluminator (BioRad ChemiDoc XRS).

Size exclusion chromatography.

SE-HPLC analyses were performed using an Agilent HPLC system, equipped with a UV/vis detector, on a Yarra 3 μ m analytical column (300 $\times$ 4.60 mm, Phenomenex), using a flow rate of 0.35 mL/min. The elution was monitored at λ = 254 nm. PBS/Mg²⁺, Tris/K⁺ and K⁺/Na⁺ buffered solutions (pH 6.8) were used as mobile phases. All the tested oligonucleotides were injected at 1 μ M concentration.

NMR experiments.

NMR data were collected on Bruker Avance III HD 400 MHz NMR spectrometer at 25 °C. NMR samples were prepared in 90% 10% H₂O D₂O or 99.9% D₂O at 0.1 mM oligonucleotide concentration. For experiments in D₂O, H₂O was replaced with D₂O by lyophilization and dissolution in 99.9% D₂O. NMR spectra were acquired with the use of the WATERGATE solvent suppression method. DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) was used as a reference to calibrate the chemical shifts, assuming that DSS resonates at 0 ppm. NMR spectra were processed and analysed with the use of Topspin 3.5 software.

Binding of R1.2 and R1.3 in PBS/Mg²⁺ and Tris/K⁺ buffers.

Specificity assays were performed for R1.2 and R1.3 in PBS/Mg²⁺ and Tris/K⁺ buffers at 4°C. The pH of all buffers was 7.4. The assays were performed by incubating 100 μ L of 2 μ M solution of R1.2 and R1.3 or random control in respective buffers with 2.0 $\times$ 10⁵ cells in 100 μ L of the specified buffer on ice for 45 min. After the incubation period, all samples

were washed twice with 2 mL of the same buffer. The cells were then reconstituted in 200 μ L of the buffer. Binding was analysed using FACSCalibur Flow Cytometer (Cytex Biosciences) by counting 5000 events. All the buffers were kept at a constant temperature of 4°C.

Determination of binding affinity of R1.2 and R1.3 in Tris/K⁺ buffer at 4 °C.

Binding affinity of R1.2 or R1.3 towards target cells was evaluated by using BJAB cells. 1500 μ M working solution of R1.2 or R1.3 was prepared by diluting the respective 10 μ M solution using the Tris/K⁺ buffer containing 10 mM Tris-HCl and 100 mM KCl in DNase and RNase free molecular biology grade water at a pH of 7.4. After 10 min at 95 °C, followed by 45 min on ice, the aptamer solutions of different concentrations were prepared by serial dilution. About 1.0×10^5 BJAB cells were suspended in 75 μ L of Cell Suspension Buffer composed of 10 mM Tris-HCl, 100 mM KCl, 100 mg/L tRNA and 1 g/L BSA in DNase and RNase free molecular biology grade water at pH 7.4. The cells were then incubated with 75 μ L of the aptamer solution of different concentration for 1 h at 25 °C, reaching the final volume of 150 μ L. After 1 h incubation, 2 mL of Tris/K⁺ buffer were added for washing, and the cells were then reconstituted in 200 μ L of the buffer. Aptamer binding towards BJAB cells was analysed with a FACSCalibur Flow Cytometer (Cytex Biosciences) by counting 5000 events for each concentration. The equilibrium dissociation constant (K_d) of aptamer-cell interaction was obtained by plotting the difference in median fluorescence intensity against concentration and specific binding was calculated using Hill slope on GraphPad Prism 7.04 (La Jolla, CA, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- (1). Stoltenburg R; Reinemann C; Strehlitz B SELEX-A (r)Evolutionary Method to Generate High-Affinity Nucleic Acid Ligands. *Biomol. Eng.* 2007, 24 (4), 381–403. 10.1016/j.bioeng.2007.06.001. [PubMed: 17627883]
- (2). Platella C; Riccardi C; Montesarchio D; Roviello GN; Musumeci D G-Quadruplex-Based Aptamers against Protein Targets in Therapy and Diagnostics. *Biochim. Biophys. Acta - Gen. Snbj.* 2017, 1861 (5), 1429–1447. 10.1016/j.bbagen.2016.11.027.
- (3). Bevilacqua PC; Ritchey LE; Su Z; Assmann SM Genome-Wide Analysis of RNA Secondary Structure. *Annu. Rev. Genet* 2016, 50, 235–266. 10.1146/annurev-genet-120215-035034. [PubMed: 27648642]
- (4). Jeddi I; Saiz L Three-Dimensional Modeling of Single Stranded DNA Hairpins for Aptamer-Based Biosensors. *Sci. Rep* 2017, 7, 1178 10.1038/s41598-017-01348-5. [PubMed: 28446765]
- (5). Leung KH; He HZ; He B; Zhong H I; Lin S; Wang YT; Ma DL; Leung CH Label-Free Luminescence Switch-on Detection of Hepatitis C Virus NS3 Helicase Activity Using a G-

Quadruplex-Selective Probe. *Chem. Sci* 2015, 6 (4), 2166–2171. 10.1039/c4sc03319a. [PubMed: 28808523]

- (6). Ellington AD; Szostak JW In Vitro Selection of RNA Molecules that Bind Specific Ligands. *Nature* 1990, 346 (1), 818–822. 10.1016/0021-9797(80)90501-9. [PubMed: 1697402]
- (7). Tuerk C; Gold L Systematic Evolution of Ligands by Exponential Enrichment: RNA Ligands to Bacteriophage T4. *Science* 1990, 249, 505–510. 10.1126/science.2200121. [PubMed: 2200121]
- (8). Ellington AD; Szostak JW Selection in Vitro of Single-Stranded DNA Molecules that Fold into Specific Ligand-Binding Structures. *Nature* 1992, 355 (6363), 850–852. 10.1038/355850a0. [PubMed: 1538766]
- (9). Ng EWM; Shima DT; Calias P; Cunningham ET; Guyer DR; Adamis AP Pegaptanib, a Targeted Anti-VEGF Aptamer for Ocular Vascular Disease. *Nat. Rev. Drug Discov* 2006, 5 (2), 123–132. 10.1038/nrd1955. [PubMed: 16518379]
- (10). Viores SA Pegaptanib in the Treatment of Wet, Age-Related Macular Degeneration. *Int. J. Nanomedicine* 2006, 1 (3), 263–268. [PubMed: 17717967]
- (11). Doggrell SA Pegaptanib: The First Antiangiogenic Agent Approved for Neovascular Macular Degeneration. *Expert Opin. Pharmacother* 2005, 6 (8), 1421–1423. 10.1517/14656566.6.8.1421. [PubMed: 16013991]
- (12). Mallikaratchy P Evolution of Complex Target SELEX to Identify Aptamers against Mammalian Cell-Surface Antigens. *Molecules* 2017, 22 (2), 1–12. 10.3390/molecules22020215.
- (13). Shamah SM; Healy JM; Cload ST Complex Target SELEX. 2008, 41 (1), 130–138. 10.1021/ar700142z
- (14). Shangguan D; Li Y; Tang Z; Cao ZC; Chen HW; Mallikaratchy P; Sefah K; Yang CJ; Tan W Aptamers Evolved from Live Cells as Effective Molecular Probes for Cancer Study. *Proc. Natl. Acad. Sci* 2006, 103 (32), 11838–11843. 10.1073/pnas.0602615103. [PubMed: 16873550]
- (15). Daniels DA; Chen H; Hicke BJ; Swiderek KM; Gold L A Tenascin-C Aptamer Identified by Tumor Cell SELEX: Systematic Evolution of Ligands by Exponential Enrichment. *Proc. Natl. Acad. Sci.* 2003, 100 (26), 15416–15421. 10.1073/pnas.2136683100. [PubMed: 14676325]
- (16). Zümrit HE; Ara MN; Fraile M; Maio G; Mallikaratchy P Ligand-Guided Selection of Target-Specific Aptamers: A Screening Technology for Identifying Specific Aptamers against Cell-Surface Proteins. *Nucleic Acid Ther.* 2016, 26 (3), 190–198. 10.1089/nat.2016.0611. [PubMed: 27148897]
- (17). Zumrut HE; Batool S; Van N; George S; Bhandari S; Mallikaratchy P Structural Optimization of an Aptamer Generated from Ligand-Guided Selection (LIGS) Resulted in High Affinity Variant toward mIgM Expressed on Burkitt's Lymphoma Cell Lines. *Biochim. Biophys. Acta - Gen. Subj* 2017, 1861 (7), 1825–1832. 10.1016/j.bbagen.2017.03.020. [PubMed: 28363693]
- (18). Furst DE Serum Immunoglobulins and Risk of Infection: How Low Can You Go? *Semin. Arthritis Rheum* 2009, 39 (1), 18–29. 10.1016/j.semarthrit.2008.05.002. [PubMed: 18620738]
- (19). Dal Porto JM; Gauld SB; Merrell KT; Mills D; Pugh-Bemard AE; Cambier J B Cell Antigen Receptor Signaling 101. *Mol. Immunol* 2004, 41 (6–7), 599–613. 10.1016/j.molimm.2004.04.008. [PubMed: 15219998]
- (20). Manson JJ; Mauri C; Ehrenstein MR Natural Serum IgM Maintains Immunological Homeostasis and Prevents Autoimmunity. *Springer Semin. Immunopathol* 2005, 26 (4), 425–432. 10.1007/s00281-004-0187-x. [PubMed: 15611856]
- (21). Eason AB; Sin S-H; Lin C; Damania B; Park S; Fedoriw Y; Bacchi CE; Dittmer DP Differential IgM Expression Distinguishes Two Types of Pediatric Burkitt Lymphoma in Mouse and Human. *Oncotarget* 2016, 7 (39), 63504–63513. 10.18632/oncotarget.11531. [PubMed: 27566574]
- (22). Li FJ; Kubagawa Y; McCollum MK; Wilson L; Motohashi T; Bertoli LF; Barton JC; Barnes S; Davis RS; Kubagawa H Enhanced Levels of Both the Membrane-Bound and Soluble Forms of IgM Fc Receptor (FcμR) in Patients with Chronic Lymphocytic Leukemia. *Blood* 2011, 118 (18), 4902–4909. 10.1182/blood-2011-04-350793. [PubMed: 21908424]
- (23). Gertz MA; Fonseca R; Rajkumar SV Waldenström's Macroglobulinemia. *Oncologist* 2000, 5 (1), 63–67. 10.1634/theoncologist.12-8-991. [PubMed: 10706651]

- (24). del Villar-Guerra R; Trent JO; Chaires JB G-Quadruplex Secondary Structure Obtained from Circular Dichroism Spectroscopy. *Angew. Chemie - Int. Ed* 2018, 57 (24), 7171–7175. 10.1002/anie.201709184.
- (25). del Villar-Guerra R; Gray RD; Chaires JB Characterization of Quadruplex DNA Structure by Circular Dichroism. *Curr. Protoc. Nucleic Acid Chem* 2017, 68, 17.8.1–17.8.16. 10.1002/cpnc.23. [PubMed: 28252181]
- (26). Randazzo A; Spada GP; Webba da Silva M Circular Dichroism of Quadruplex Structures. *Top. Curr. Chem* 2012, 330, 67–86. 10.1007/128_2012_331.
- (27). Mergny JL; Lacroix L UV Melting of G-Quadruplexes. *Curr. Protoc. Nucleic Acid Chem* 2009, 37 (1), 1–15. 10.1002/0471142700.nc1701s37.
- (28). Lane AN; Chaires JB; Gray RD; Trent JO Stability and Kinetics of G-Quadruplex Structures. *Nucleic Acids Res.* 2008, 36 (17), 5482–5515. 10.1093/nar/gkn517. [PubMed: 18718931]
- (29). Mergny JL; Li J; Lacroix L; Amrane S; Chaires JB Thermal Difference Spectra: A Specific Signature for Nucleic Acid Structures. *Nucleic Acids Res.* 2005, 33 (16), 1–6. 10.1093/nar/gni134. [PubMed: 15640442]
- (30). Malgowska M; Gudanis D; Teubert A; Dominiak G; Gdaniec Z How to Study G-Quadruplex Structures. *Biotechnologia* 2012, 93 (4), 381–390. 10.5114/bta.2012.46592.
- (31). Musumeci D; Rozza L; Merlino A; Paduano L; Marzo T; Massai L; Messori L; Montesarchio D Interaction of Anticancer Ru(III) Complexes with Single Stranded and Duplex DNA Model Systems. *Dalton Trans.* 2015, 44 (31), 13914–13925. 10.1039/c5dt01105a. [PubMed: 26154188]
- (32). Mergny JL; De Cian A; Ghelab A; Saccà B; Lacroix L Kinetics of Tetramolecular Quadruplexes. *Nucleic Acids Res.* 2005, 33 (1), 81–94. 10.1093/nar/gki148. [PubMed: 15642696]
- (33). Kikin O; D'Antonio L; Bagga PS QGRS Mapper: A Web-Based Server for Predicting G-Quadruplexes in Nucleotide Sequences. *Nucleic Acids Res.* 2006, 34 (Web Server issue), 676–682. 10.1093/nar/gkl253. [PubMed: 16452297]
- (34). Cheng M; Cheng Y; Hao J; Jia G; Zhou J; Mergny JL; Li C Loop Permutation Affects the Topology and Stability of G-Quadruplexes. *Nucleic Acids Res.* 2018, 46 (18), 9264–9275. 10.1093/nar/gky757. [PubMed: 30184167]
- (35). Guédin A; Gros J; Alberti P; Mergny JL How Long Is Too Long? Effects of Loop Size on G-Quadruplex Stability. *Nucleic Acids Res.* 2010, 38 (21), 7858–7868. 10.1093/nar/gkq639. [PubMed: 20660477]
- (36). Musumeci D; Platella C; Riccardi C; Moccia F; Montesarchio D Fluorescence Sensing Using DNA Aptamers in Cancer Research and Clinical Diagnostics. *Cancers* 2017, 9 (12), 1–43. 10.3390/cancers9120174.
- (37). Sengupta A; Ganguly A; Chowdhury S Promise of G-Quadruplex Structure Binding Ligands as Epigenetic Modifiers with Anti-Cancer Effects. *Molecules* 2019, 24 (3), 582 10.3390/molecules24030582.
- (38). Asamitsu S; Obata S; Yu Z; Bando T; Sugiyama H Recent Progress of Targeted G-Quadruplex-Preferred Ligands Toward Cancer Therapy. *Molecules* 2019, 24 (3), 429 10.3390/molecules24030429.
- (39). Ruggiero E; Richter SN G-Quadruplexes and G-Quadruplex Ligands: Targets and Tools in Antiviral Therapy. *Nucleic Acids Res.* 2018, 46 (7), 3270–3283. 10.1093/nar/gky187. [PubMed: 29554280]
- (40). Musumeci D; Amato J; Randazzo A; Novellino E; Giancola C; Montesarchio D; Pagano B G-Quadruplex on Oligo Affinity Support (G4-OAS): An Easy Affinity Chromatography-Based Assay for the Screening of G-Quadruplex Ligands. *Anal. Chem* 2014, 86 (9), 4126–4130. 10.1021/ac500444m. [PubMed: 24725064]
- (41). Petraccone L; Spink C; Trent JO; Garbett NC; Mekmaysy CS; Giancola C; Chaires JB Structure and Stability of Higher-Order Human Telomeric Quadruplexes. *J. Am. Chem. Soc.* 2011, 133 (51), 20951–20961. 10.1021/ja209192a. [PubMed: 22082001]
- (42). Ambrus A; Chen D; Dai J; Bialis T; Jones RA; Yang D Human Telomeric Sequence Forms a Hybrid-Type Intramolecular G-Quadruplex Structure with Mixed Parallel/Antiparallel Strands in Potassium Solution. *Nucleic Acids Res.* 2006, 34 (9), 2723–2735. 10.1093/nar/gk1348. [PubMed: 16714449]

- (43). Niermann M; Bolten M; Eimer W Optimization of the Hydrodynamic Bead Model for the Analysis of DNA Conformations in Solution. *J. Phys. Chem. B* 1999, 103 (45), 10065–10074. 10.1021/jp9921831.
- (44). Largy E; Mergny JL Shape Matters: Size-Exclusion HPLC for the Study of Nucleic Acid Structural Polymorphism. *Nucleic Acids Res.* 2014, 42 (19), 1–15. 10.1093/nar/gku751. [PubMed: 24376271]
- (45). Riccardi C; Musumeci D; Russo Krauss I; Piccolo M; Irace C; Paduano L; Montesarchio D Exploring the Conformational Behaviour and Aggregation Properties of Lipid-Conjugated AS1411 Aptamers. *Int. J. Biol. Macromol* 2018, 118 (Part B), 1384–1399. 10.1016/j.ijbiomac.2018.06.137. [PubMed: 30170359]
- (46). Benito S; Ferrer A; Benabou S; Aviñó A; Eritja R; Gargallo R Evaluation of the Effect of Polymorphism on G-Quadruplex-Ligand Interaction by Means of Spectroscopic and Chromatographic Techniques. *Spectrochim. Acta - Part A Mol. Biomol. Spectrosc* 2018, 196, 185–195. 10.1016/j.saa.2018.02.006.
- (47). Adrian M; Heddi B; Phan AT NMR Spectroscopy of G-Quadruplexes. *Methods* 2012, 57 (1), 11–24. 10.1016/j.ymeth.2012.05.003. [PubMed: 22633887]
- (48). Batool S; Argyropoulos KV; Azad R; Okeoma P; Zumrut H; Bhandari S; Dekhang R; Mallikaratchy PR Dimerization of an Aptamer Generated from Ligand-Guided Selection (LIGS) Yields a High Affinity Scaffold against B-Cells. *Biochim. Biophys. Acta - Gen. Subj* 2019, 1863 (1), 232–240. 10.1016/j.bbagen.2018.10.006. [PubMed: 30342154]
- (49). Hermann T; Patel DJ Adaptive Recognition by Nucleic Acid Aptamers. *Science* 2008, 287 (5454), 820–825. 10.1126/science.287.5454.820.
- (50). Patel DJ Structural Analysis of Nucleic Acid Aptamers. *Curr. Opin. Chem. Biol* 1997, 1 (1), 32–46. 10.1016/S1367-5931(97)80106-8. [PubMed: 9667843]

Figure 1.

CD spectra recorded for **a)** R1.2 and **b)** R1.3 aptamers both at 1 μ M concentration in the PBS/Mg²⁺, Tris/K⁺ and K⁺/Na⁺ buffered solutions (black, red and blue lines respectively).

Figure 2. Overlapped CD-melting (black lines) and CD-annealing (blue lines) curves of R1.2 at 1 μ M concentration in **a)** PBS/ Mg^{2+} , **b)** Tris/ K^{+} , **c)** K^{+} / Na^{+} buffered solutions, recorded respectively at 267, 266 and 265 nm (heating/cooling rate: 1 $^{\circ}$ C/min), and related $T_{1/2}$ values.

Figure 3. Overlapped CD-melting (black lines) and CD-annealing (blue lines) curves of R1.3 at 1 μ M concentration in **a)** PBS/ Mg^{2+} , **b)** Tris/ K^{+} , **c)** K^{+} / Na^{+} buffered solutions, recorded respectively at 266, 265 and 265 nm (heating/cooling rate: 1 $^{\circ}$ C/min), and related $T_{1/2}$ values.

Figure 4. Thermal difference spectra of R1.2 (left panels) and R1.3 (right panels) at 1 μM concentration in PBS/ Mg^{2+} (a-b), Tris/ K^+ (c-d), and K^+/Na^+ (e-f) buffered solutions.

Figure 5.

Overlapped UV-melting (black lines) and UV-annealing (blue lines) of R1.2 (left panels) and R1.3 (right panels) both at 1 μ M concentration in PBS/Mg²⁺ (a-b), Tris/K⁺ (c-d), and K⁺/Na⁺ (e-f) buffered solutions, recorded at 260 or 295 nm (heating/cooling rate: 1 °C/min), and related $T_{1/2}$ values.

Figure 6.

Native PAGE experiments. Oligonucleotide samples were loaded at 1 μ M concentration and resolved on 20% native PAGE, **a)** Lane L: 5 bp DNA ladder. Lane 1: R1.2 in PBS/Mg²⁺ buffer. Lane 2: R1.2 in Tris/K⁺ buffer. Lane 3: R1.2 in K⁺/Na⁺ buffer. Lane 4: R1.3 in PBS/Mg²⁺ buffer. Lane 5: R1.3 in Tris/K⁺ buffer. Lane 6: R1.3 in K⁺/Na⁺ buffer. Lane 7: tel26 in Tris/K⁺ buffer, **b)** Lane L: 5 bp DNA ladder. Lane 1: R1.2-FAM in PBS/Mg²⁺ buffer. Lane 2: R1.2-FAM in Tris/K⁺ buffer. Lane 3: R1.2-FAM in K⁺/Na⁺ buffer. Lane 4: R1.3-FAM in PBS/Mg²⁺ buffer. Lane 5: R1.3-FAM in Tris/K⁺ buffer. Lane 6: R1.3-FAM in K⁺/Na⁺ buffer. Lane 7: tel26 in Tris/K⁺ buffer. FAM = 6-carboxyfluorescein.

Figure 7. Evaluation of binding with BJAB cells of R1.2 (a) and R1.3 (b) in Tris/K⁺ buffer and R1.2 (c) and R1.3 (d) in PBS/Mg²⁺. Binding affinity analysis of R1.2 (e) and R1.3 (f) in Tris/K⁺ buffer against BJAB cells with their respective Hill coefficient (n) and equilibrium dissociation constant (K_d).

Table 1.

$T_{1/2}$ values ($^{\circ}\text{C}$) of the CD- and UV-melting transitions for R1.2 and R1.3 in PBS/ Mg^{2+} , Tris/ K^{+} and $\text{K}^{+}/\text{Na}^{+}$ buffers; in parenthesis, the wavelengths (nm) at which these experiments have been carried out.

		PBS/ Mg^{2+}		Tris/ K^{+}		$\text{K}^{+}/\text{Na}^{+}$	
		R1.2	R1.3	R1.2	R1.3	R1.2	R1.3
$T_{1/2}$ ($^{\circ}\text{C}$) ± 1 (by CD spectroscopy)	Melting curve	39 (267)	43 (267)	50 (266)	50 (265) 40 (300)	55 (265)	56 (265) 50 (300)
$T_{1/2}$ ($^{\circ}\text{C}$) ± 1 (by UV spectroscopy)	Melting curve	35 (260)	45 (260)	48 (295)	47 (295)	52 (295)	51 (295)