ANNA 2024

Advances in Noncanonical Nucleic Acids:

Book of Abstracts

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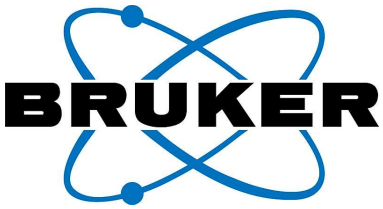

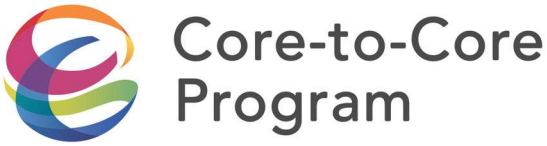

PROGRAMME

Thursday, October 24th, 2024

10:00	**Bus transfer to Portorož**
12:00 – 13:00	**Lunch,** *Grand Hotel Portorož*
13:00 -	**Registration**

Afternoon session *Chair: Daniela Montesarchio*

13:20 – 13:30	**Opening remarks,** *Janez Plavec, Head of NMR centre*
13:30 – 14:00	Dongsheng Liu, *Tsinghua University, Beijing*
14:00 – 14:30	Akimitsu Okamoto, *University of Tokyo*
14:30 – 15:00	P.I. Pradeepkumar, *Indian Institute of Technology Bombay*
15:00 – 15:30	Jussara Amato, *University of Naples Federico II*
15:30 – 16:00	**Coffee break**
16:00 – 16:30	Antonio Randazzo, *University of Naples Federico II*
16:30 – 17:00	Claudia Sissi, *University of Padova*
17:00 – 17:30	Viktor Víglaský, *P. J. Šafarik University, Košice*
17:30 – 18:00	Hanyang Yu, *Nanjing University*
18:00 – 18:30	Maja Marušič, *National Institute of Chemistry, Ljubljana*

19:00	**Dinner,** *Marina Portorož*

Friday, October 25th, 2024

Morning session *Chair: Naoki Sugimoto*

9:00 – 9:30	Stephen Neidle, *University College London*
9:30 – 10:00	Rakesh Veedu, *Murdoch University, Perth*
10:00 – 10:30	Liliya Yatsunyk, *Swarthmore College*
10:30 – 11:00	**Coffee break & Poster session**
11:00 – 11:30	Jean-Louis Mergny, *Ecole Polytechnique, Palaiseau*
11:30 – 12:00	Lukáš Trantírek, *CEITEC, Brno*
12:00 – 12:30	Chiara Platella, *University of Naples Federico II*
12:30 – 13:30	**Lunch**, *Grand Hotel Portorož*

Afternoon session *Chair: Antonio Randazzo*

13:30 – 14:00	Shigeori Takenaka, *Kyushu Institute of Technology, Kitakyushu*
14:00 – 14:30	Hisae Tateishi-Karimata, *FIBER, Kobe*
14:30 – 15:00	Emanuela Ruggiero, *University of Padova*
15:00 – 15:30	Konstantinos Tripsianes, *CEITEC, Brno*
15:30 – 16:00	**Coffee break & Poster session**
16:00 – 16:30	Thomas Carell, *Ludwig Maximilian University of Munich*
16:30 – 17:00	Chuanzheng Zhou, *Nankai University, Tianjin*
17:00 – 17:30	Marko Trajkovski, *National Institute of Chemistry, Ljubljana*
19:00	**Dinner,** *Hiša Torkla, Korte*

Saturday, October 26th, 2024

Morning session *Chair: Claudia Sissi*

9:00 – 9:30	Zhen Xi, *Nankai University, Tianjin*
9:30 – 10:00	Jurij Lah, *University of Ljubljana*
10:00 – 10:30	Roberto Improta, *National Research Council, Naples*
10:30 – 11:00	**Coffee break & Poster session**
11:00 – 11:30	Sara N. Richter, *University of Padova*
11:30 – 12:00	Masayasu Kuwahara, *Nihon University, Tokyo*
12:00 – 12:30	Shuntaro Takahashi, *FIBER, Kobe*
12:30 – 13:30	**Lunch**, *Grand Hotel Portorož*
14:00	**Bus transfer to Ljubljana**

INVITED LECTURES

DNA Supramolecular Hydrogels

Dongsheng Liu

Department of Chemistry, Tsinghua University, Beijing, China

Based on the excellent rigidity of DNA duplexes, we designed and prepared kinds of pure DNA supramolecular hydrogels, which possess an “all-rigid” molecular network. Based on experimental results and theoretical studies, we also demonstrate these hydrogels have extraordinary permeability of macromolecules such as proteins. Due to their supramolecular nature, these hydrogels also retain extraordinary self-healing and fast-responding thixotropic properties, which make them injectable and writable.

Holding all these advantages, the DNA supramolecular hydrogels are excellent mimics of extra-cellular matrix (ECM). We will show its application in server spinal-cord injury repairing, where implanted stem cells only respond to in-situ signals generated by the injured animals.

Through DNA sequence design, we enabled the univariant mechanical strength of materials based on DNA supramolecular hydrogel platform and demonstrate that “Univariate mechanical strength does not influence neural progenitor cell fate in 3D matrix” for the first time.

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Cell Regulation by Oligonucleotide Aggregation Control

Kunihiko Morihiro, Akimitsu Okamoto

Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, Japan

I. Assembly of hairpin DNA/RNA[1,2]

We have developed a hairpin DNA assembly technology that enables cancer-selective immune activation to induce cytotoxicity. The designed artificial DNA hairpins assemble into long nicked double-stranded DNA triggered by intracellular microRNA-21 (miR-21), which is overexpressed in various types of cancer cells. The products from the hairpin DNA assembly selectively kill miR-21-abundant cancer cells in vitro and in vivo based on innate immune activation. Our approach is the first to allow selective oncolysis derived from intracellular DNA self-assembly, providing a powerful therapeutic modality to treat cancer.

We also designed and synthesized an oncolytic RNA hairpin pair that was selectively cytotoxic toward cancer cells expressing abundant oncogenic microRNA-21 (miR-21). Although the structure of each hairpin RNA was thermodynamically metastable, catalytic miR-21 input triggered it open to generate a long nicked dsRNA. We demonstrated that RNA hairpin pair functioned as a cytotoxic amplifier of information in the presence of miR-21 in various cancer cells and tumor-bearing mice. This work represents the first example of the use of short RNA molecules as build-up-type anticancer agents that are triggered by an oncogenic miRNA.

II. Catalytic hairpin assembly[3]

DNA decoys inhibit cellular transcription factors and are expected to be among the nucleic acid drugs used to downregulate the transcription process. To reduce undesired decoy function in normal cells, we adopted catalytic hairpin assembly (CHA) to produce a DNA duplex from a hairpin DNA pair in response to miR-21. We designed the DNA hairpin pairs to form a DNA decoy that binds to NF-κB, whose overexpression is related to many diseases, including cancer. The transformation of the DNA hairpin pair to the NF-κB DNA decoy was catalyzed by miR-21, which is expressed in various types of cancers. Intracellular CHA progression and the inhibitory effect against NF-κB were observed only in miR-21 overexpressing cancer cells. The intracellular miR-21-catalyzed production of the NF-κB DNA decoy has the potential to reduce side effects on normal cells, thereby strengthening the therapeutic profile of the CHA-decoy system.

References:

1. Morihiro, K.; Osumi, H.; Morita, S.; Hattori, T.; Baba, M.; Harada, N.; Ohashi, R.; Okamoto, A. *J. Am. Chem. Soc.*, **145**, 135-142 (2023).
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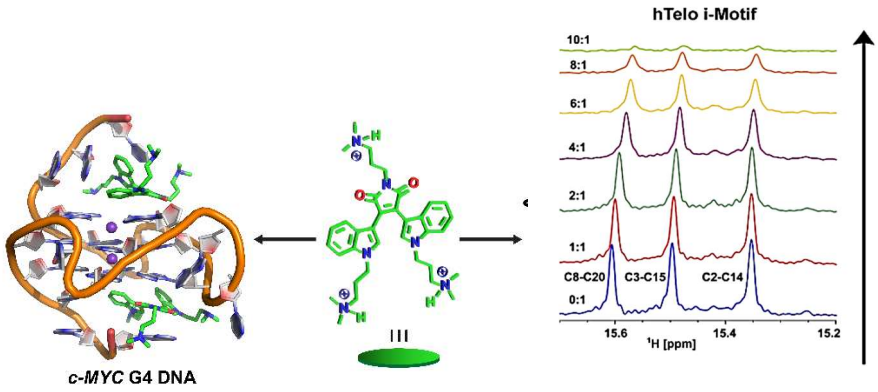
Acknowledgements: This work was supported by JST ACT-X (JPMJAX191I to KM), JSPS KAKENHI (19K15408 and 20H04698 to KM, 23H00317 to KM and AO, 23K17969 to AO), and AMED Grant (23ak0101194h0001 to KM, JP22ym0126805j0001 to AO).

Stabilization of G-quadruplex and Destabilization of i-Motif DNA Structures by Designer Molecules

Satendra Kumar[1], Rajesh Kumar Reddy Sannapureddi[2], Sruthi Sudhakar[1], Ramanathan Rajesh[1], Bharathwaj Sathyamoorthy[2], P. I. Pradeepkumar[1]

[1]*Department of Chemistry, Indian Institute of Technology Bombay, Powai, Mumbai, India*
[2]*Department of Chemistry, Indian Institute of Science Education and Research, Bhopal, India*

G-quadruplex (G4) and i-Motif (iM) structures are targeted by small molecule ligands to modulate their biological functions.[1-3] We previously reported that bisindolylmaleimide-based (**BIM**) ligands stabilize the parallel G4 structures of the *c-MYC* and *c-KIT* oncogenes. NMR titration studies indicate that the lead ligand **BIM-Pr1** forms a 2:1 complex with *c-MYC* G4 DNA, engaging both the 5' and 3' quartets, a finding further supported by molecular modeling and MD simulations.[4] Additionally, biophysical and modeling studies demonstrate that these ligands destabilize several iM structures, leading to conformational unfolding.[5] Given that the formation of G4 and iM structures are predominantly mutually exclusive, our results have implications for the development of G4/iM interacting ligands for biological and sensing applications.



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Unlocking the potential of peptides to target G-quadruplex structures

Jussara Amato

Department of Pharmacy, University of Naples Federico II, Italy

G-quadruplexes (G4s) are non-canonical DNA/RNA secondary structures formed within guanine-rich strands that play important roles in various biological processes, including gene regulation, telomere maintenance, DNA replication, and RNA translation.[1] The biological functions and formation of these structures are strictly controlled by several proteins that bind and stabilize or resolve them.[1] Many G4-binding proteins feature an arginine and glycine-rich motif known as the RGG or RG-rich motif.[2]

Here, we employed a combination of several biophysical methods to provide valuable insights into the interaction of a peptide containing an RGG motif shared by numerous G4-binding proteins (NIQI)[2] and of various peptides derived from the Rap1 protein[3] with biologically relevant DNA G4s having different structural topologies.[4,5] We also shed light on the key amino acids involved in the binding process. Our findings contribute to lay the basis for the development of a new class of peptide-based G4 ligands as an alternative to small molecules.[4,5]

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Acknowledgments: This work was supported by the Italian Association for Cancer Research (IG 24590) and PRIN PNRR 2022 (P20223RKJ7).

Unveiling the biological effects of DNA G-quadruplex ligands through multi-omics data integration

Francesca Romano, Carolina Persico, Anna Di Porzio, Nunzia Iaccarino, Antonio Randazzo

Department of Pharmacy, University of Naples Federico II, Italy

The double helix is the canonical structure of DNA, but its conformation is highly dynamic and can adopt alternative secondary structures, including G-quadruplexes (G4s). G4s are prevalent in regulatory regions of the genome, such as oncogene promoters and telomeres, and have emerged as promising targets for therapeutic intervention.

Several studies have demonstrated the potential of G4 ligands as anti-cancer agents. However, to translate these ligands into effective drugs, a deeper understanding of their mode of action is essential.

To address this challenge, we conducted a multi-omics study that integrates transcriptomic, proteomic, and metabolomic analyses to elucidate the mechanisms of G4-interacting agents. Through this approach, we aim to map the intricate network of interactions that G4 ligands engage with and how these interactions translate into functional outcomes within the cell. By doing so, we hope to identify key molecular players and pathways that mediate the anti-cancer effects of G4 ligands.

Here, we present our very preliminary findings from the transcriptomic, proteomic, and metabolomic analyses of cells treated with a variety of G4 ligands. Our results suggest that structurally similar G4 ligands can exhibit distinct modes of action, indicating that even subtle variations in ligand structure can lead to vastly different biological outcomes. This observation underscores the complexity of targeting G-quadruplexes for therapeutic purposes and highlights the need for a more nuanced understanding of G4 biology to guide drug development efforts.

Non-canonical secondary structures at alternative sequences

Michele Ghezzo, Beatrice Moras, Claudia Sissi

Department of Pharmaceutical and Pharmacological Chemistry, University of Padova, Padova Italy

The polymorphism of nucleic acids is likely the most fascinating feature they present. In living organism, it can work as an efficient and tunable regulatory mechanism thus attracting the interest of medicinal chemists to exploit it as a tool or as a target for drug design. As well, the structural plasticity of nuclei acids is frequently used for the design of smart materials and devices.

In the last three decades the interest for the different structures that these biomolecules can assumed exponentially grown. Nevertheless, some new (and some old) intriguing sequences can still hide new features worth to be explored.

Here we will focus on the structural equilibria that can occur at C-rich sequences.

We will discuss on how these sequences behave in different experimental conditions. In particular, we will comment on how smooth changes in the chemistry of nucleic acids or in their sequence composition might drive different folding and properties.

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Acknowledgements: AIRC (grant IG 2021 - ID26474, PI CS), and European Union-Next GenerationEU (PNRR M4 C2-Investimento 1.4- CN00000041)

15 years of life with non-canonical structures

Viktor Víglaský, Lukáš Trizna, Diana Pitková

Department of Biochemistry, Faculty of Science, P.J. Šafárik University, Košice, Slovakia

There is no doubt that the non-canonical motifs formed from nucleic acids play a very important role in the regulation of key cellular processes. The position of the individual bases in each non-canonical motif must be in a suitable position to allow them to be paired, either by Watson-Crick or Hoogsteen pairing. In case of G-quadruplexes and I-motifs it is exclusively a pairing between guanines and cytosines, respectively. However, they need either the presence of potassium or a slightly acidic pH to stabilize. Other non-canonical motifs utilize various combinations of these pairings. Our pioneering work was to impose some perturbation on the base pairing in the G4 structure by using platinum derivatives wchich have preferential affinity for the bond at the C7 position in guanine, preventing the formation of the C3-C7 bond[1]. Recently, we have designed a universal smart system in which any non-canonical structure can be spontaneously formed even without a chemical change in the sequence. The system also mimics plasmids[2].

Another challenge was the problems of multimerization of G-quadruplexes as well as their arrangement in tandem. Our most important results have been published, but many uncertainties remain that have not yet been published[3,4]. For example, some G-quadruplexes multimerize, and a single point mutation in a region completely different from the G-rich region is sufficient for these sequences to lose the ability to multimerize. Sometimes under dehydrating conditions we have observed the opposite effect.

In our laboratory, we were among the first groups to start a series of analyses of G-rich regions in the genomes of viruses, such as Papilloma, Ebola and HIV viruses[5-8].

We did not omit analyses of the interaction of G-quadruplexes with different ligands either. For example, we have shown that thiazole orange in complex with G4 exhibits a unique CD profile in the visible region of the spectrum[8].

Only recently we have offered an alternative view of the representation of nucleic acids based on their sequence, which made it possible to find also new unusual structures[9]. In parallel with the work of prof. Cech we predicted that sequences of the (UG)n type are able to adopt left-handed G4, even in the case of DNA under dehydrating conditions, but in addition we showed that the d(AG)n sequence forms a very similar motif to the tetrahelical structure of VK[10].

We are currently working on dynamic hydrogels in which we can modulate their thermodynamic properties using a combination of specific ligands developed in our laboratories.

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Functional Xeno-Nucleic Acids

Hanyang Yu

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Nucleic acids can fold into distinct tertiary structures with binding affinities and catalytic activities, and thus offer versatile molecular tools to chemistry and biomedicine. However, functional DNAs and RNAs are inherently constrained due to the susceptibility to nuclease degradation and a limited repertoire of chemical functionality. Xeno-nucleic acids (XNAs) are synthetic genetic polymers with superior biological stability and enriched chemical diversity and provide promising modalities for various biomedical applications.

We primarily focus on threose nucleic acid (TNA), which contains a noncanonical tetrose moiety and is thus refractory to nuclease digestion. We leverage *in vitro* selection to identify TNA aptamers towards therapeutic target proteins, and catalytic TNAs (TNAzymes) with RNA endonuclease and RNA ligase activities. Specifically, an RNA-cleaving TNAzyme discriminates single point mutation within the substrate, and induces allele-specific gene silencing in living cells.[1] An RNA ligase TNAzyme introduces a noncanonical 2'-5' phosphodiester linkage in the product, and can thus modulate RNA function.[2] An *in vitro* selected c-Myc-binding TNA aptamer can be assembled into a heterobifunctional PROTAC to mediate targeted protein degradation.[3] We also develop a chemoenzymatic method to install various chemical groups site-specifically onto DNA, and identify chemically modified DNAzyme variants that exhibit substantially enhanced catalytic activities and reduced metal ion dependence.[4] These studies underscore the importance of chemical modification in delivering functional nucleic acids with appealing properties.

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Learning shapes and numbers through the G-tract length

Urša Štefan[1], Vaclav Brazda[2], Janez Plavec[1,3], Maja Marušič[3]

[1]*Faculty of Chemistry and Chemical Technology, University of Ljubljana, Slovenia*
[2]*Institute of Biophysics of the Czech Academy of Sciences, Brno, Czech Republic*
[3]*Slovenian NMR Centre, National Institute of Chemistry, Ljubljana, Slovenia*

The diverse and dynamic structure of DNA at the local level is integral to the regulation of vital physiological processes. We offer an analysis of G-quadruplex formation in sequences comprising five or six guanine residues, which have been identified through the investigation of the gapless human genome and are associated with genes linked to cancer and neurodegenerative diseases1. We undertook a comprehensive analysis of the impact of G-tract and loop elongation using a combination of NMR spectroscopy, CD spectroscopy, and polyacrylamide gel electrophoresis2. Although both types of elongation result in structural polymorphism, we were able to determine the topologies of four out of the eight examined sequences. One of these sequences contributes to a very scarce selection of currently known intramolecular four G-quartet structures in potassium solution and displays a remarkably stable G-quadruplex core. Our findings indicate that the examined sequences are incompatible with five or six G-quartet structures with propeller loops. However, it is possible that other loop types may be a viable alternative. Finally, we present a novel approach to specific G-quadruplex targeting that could be implemented in structures with more than four G-quartets.

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Targeting Quadruplex DNA: Chemistry to Clinical Trials

Stephen Neidle

The School of Pharmacy, University College London, UK

31 years ago I submitted a research proposal to the Cancer Research Campaign in the UK to target telomeric DNA with a series of anthraquinone-based compounds. The concept was to exploit the emerging structural data on four-stranded telomeric quadruplex (G4) DNA structures, in the knowledge that telomeric-like sequences were also known to occur at sites of genomic instability in, for example, acute myeloid leukaemia and retinoblastoma. The perceived uncertainty in the knowledge then of G4 structures was cited as a problem. For example, the apparent disagreement between crystallographic and NMR structures of the *Oxytricha* quadruplex was considered as a hindrance to progress, and further work in the field by my laboratory "was not advisable". We subsequently showed that the previous crystal structure was incorrect and that there was no discrepancy with the NMR studies.

Undeterred, we established a fruitful collaboration with Laurence Hurley, who had devised an effective assay for telomerase, the enzyme complex that maintains telomere integrity in cancer cells, by synthesizing telomeric TTAGGG repeats. We suggested that stabilizing an intramolecular G4 comprising four repeats, would inhibit the enzyme and perhaps elicit an anticancer response. This was then demonstrated by us. We subsequently improved the G4 and telomerase selectivity by designing a trisubstituted acridine compound BRACO-19, which showed effective telomerase inhibition and telomere uncapping in vivo, which accompanied anticancer activity in a xenograft model. However, the anticancer potency of BRACO-19 and optimized analogues was insufficient to warrant further development of the series.

We have since refined this approach into an anticancer strategy with the design (using phenotypic screening and structure-based methods) of a novel quadruplex-specific chemotype based on a tetrasubstituted naphthalene diimide core. A lead compound, QN-302 has emerged from a combined structural, medicinal chemistry and pharmacology program at UCL. QN-302 is a potent and selective quadruplex binder and has single-digit nM potency against a range of cancer cell lines, notably pancreatic cancer ones (including those with gemcitabine chemoresistance). Transcriptional profiling has revealed that the major genes down regulated by the drug also play significant roles in the development and metastasis of this hard-to-treat disease. QN-302 has good bioavailability and has demonstrated significant *in vivo* activity against several pancreatic cancer models. QN-302 was granted Orphan Drug Status for pancreatic cancer in 2023 by the USA Food and Drug Administration (FDA). Following scale-up, toxicological and formulation studies it was subsequently granted clearance as an Investigational New Drug (IND) by the FDA for Phase 1 dose-escalation clinical trials in August 2023. These started in November 2023. Early indications from the trial suggest that the drug is well-tolerated and can slow disease progression.

Novel thiomorpholino oligonucleotides (TMOs) as a robust next generation platform for spice switching antisense therapies

Rakesh N Veedu[1,2], Marvin H Caruthers[2,3]

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[2]*ProGenis Pharmaceuticals, Perth, Australia*
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Synthetic nucleic acid therapeutics including antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs) continue to demonstrate their potential in RNA-targeting drug development, and several ASO drugs have been approved recently for clinical use. One successful ASO-based therapeutic approach is the modulation of splicing by targeting pre-messenger RNA (pre-mRNA) in the nucleus. Multiple FDA-approved ASO drugs use this strategy, including Exondys51, Vyondys53, Amondys45, and Viltepso to treat Duchenne muscular dystrophy (DMD); and Spinraza to treat spinal muscular atrophy (SMA). Phosphorodiamidate morpholino oligomer (PMO) chemistry currently utilized for DMD drugs has significant limitations as PMOs show rapid kidney clearance and poor cellular uptake that leads to high and costly dosages. Therefore, it is crucial to develop next-generation splice-switching oligonucleotide chemistries with improved efficacy, safety, and biodistribution.

We have recently developed and investigated the potential of novel thiomorpholino oligonucleotides (TMOs) to induce splice switching. We synthesized various TMOs and evaluated their efficacy to induce exon skipping in a Duchenne muscular dystrophy (DMD) *in vitro* model using H2K mdx mouse myotubes [1], and also, we have evaluated the efficacy in *mdx* mice *in vivo*. Our experiments demonstrated that TMOs can efficiently internalize and induce excellent exon 23 skipping potency compared with a conventional PMO control and other widely used charged nucleotide analogs. Based on the present study, we propose that TMOs represent a new, promising class of nucleic acid analogs for future RNA targeting oligonucleotide therapeutic development.

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Formation of left-handed G4s and their interactions with a traditional G4 ligand, N-methylmesoporphyrin IX

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The Left-handed G-quadruplexes (LHG4) belong to a class of recently discovered non-canonical DNA structures under the larger umbrella of G-quadruplex DNAs (G4s). Biological relevance of these structures and their ability to be targeted with classical G4 ligands are underexplored. Two motifs, named *Motif1* and *Motif2*, with the 12-nt sequences d[G(TGG)$_3$TG] and d[(GGT)$_3$GTG], respectively, have been identified to fold into LHG4 structures (1,2). We aimed to determine whether, for a specific gene within the human genome, *Motif1* and *Motif2* fold into putative LHG4s. We identified SLC2A1 sequence which consists of the LHG4 *Motif1* connected to a RHG4 motif with the sequence d[(TGG)$_4$] by an adenine. We aimed to determine whether *Motif1* maintains its left-handed characteristics within SLC2A1 upon addition of nucleotides in the 5'- and 3'-direction from its genomic context and upon interaction with a well-established G4 binder, NMM (N-methylmesoporphyrin IX). We also studied the double motif sequences *Motif1-Motif1*, *Motif1-Motif2*, *Motif2-Motif1*, and *Motif2-Motif2*, for their ability to handle 3'- and 5' nucleotide additions. We employed biophysical and X-ray structural studies to address these questions. Our results indicate that each sequence under investigation adopts a two-subunit, four tetrads either fully LH or hybrid LH/RHG4 structures. Additions of 5'-G or 3'-G is NOT tolerated in any of the sequences, but additions of other nucleotides is sometimes tolerated. NMM binds to all the sequences but likely only to either already existing RH subunit in the hybrid LH/RHG4 structures or converts one of the subunits to the RH fold before binding. Our work deepens our understanding of left-handed G4 structures and especially their propensity to form in vivo.

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Quadruplexes are (even more) everywhere!

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We are developing tools to study the folding and polymorphism of unusual nucleic acid structures [1] and to understand duplex-quadruplex competition [2]. This allowed us to discover new DNA folds [3] and demonstrate that DNA i-motif levels are overwhelmingly depleted in living human cells [4]. G-quadruplexes (G4s), which are often found in aptamer sequences, are involved in key biological processes, including transcription [5], and can contribute to tumorogenesis (for a review[6]). We applied the G4Hunter algorithm [7] for the prediction of G4 propensity to a variety of genomes, including cancer genomes. We found that G4s modulate the production of somatic somatic variants (SVs) in cancers [8]. This enrichment was only observed in regions demonstrated to form G4s in cells rather than in regions with a sequence compatible with G4 formation but without confirmed G4 formation.

In parallel, we analyzed the B-MYB oncogene as abnormal expression of B-MYB has been identified in various cancers, including lung cancer, and is associated with poor prognosis[9]. We found that the B-MYB gene promoter contains several G/C rich motifs compatible with G4 formation. We investigated and validated the existence of G4 structures in the promoter region of B-MYB, first *in vitro* using a combination of bioinformatics, biophysical, and biochemical methods, then in cell with G4access method recently developed by JC Andrau and colleagues [10].

So far, a number of G4 ligands have been developed for potential therapeutic applications in human pathologies, including cancer and infectious diseases. When the biological effects of G4 ligands are studied, the analysis is often limited to nucleic acid targets. However, recent evidence indicates that G4 ligands may target other cellular components and processes such as lysosomes, autophagy and mitochondria. G4 ligands haven an impact on lysosome biology and autophagic flux, as well as on the transcriptional regulation of lysosomal genes [11].

At the RNA level, we observed that sequences containing a succession of short GG blocks or uneven G-tract lengths unable to form three-tetrad G4s (GG motifs), are overwhelmingly more frequent than canonical motifs involving multiple GGG blocks. We performed a systematic analysis and experimental evaluation of a number of biologically important RNA regions involving RNA GG motifs. We show that most of these motifs do not form stable intramolecular G4s but need to dimerize to form stable G4 structures. The strong tendency of RNA GG motif G4s to associate may participate in RNA-based aggregation under conditions of cellular stress. Finally, we used computational tools to predict non-canonical nucleic acid structures in the lncRNA transcriptome [12]. We found that the majority of the predicted G4s form highly stable G4 structures *in vitro*, and identified previously unreported G4s in biologically important lncRNAs. In contrast, none of the potential i-motif sequences were able to form iM structures, consistent with the idea that RNA is nearly unable to adopt this fold. Unexpectedly, these C-rich sequences instead form Z-RNA structures, which have not been previously observed in regions containing cytosine repeats and represent an interesting and underexplored target for protein–RNA interactions.

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G-quadruplex-based inhibitor of tyrosine kinase receptor

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The dysfunction of the FGF/FGF receptor (FGFR) signaling axis is implicated in a range of human diseases, including congenital craniosynostosis, various dwarfism syndromes, chronic kidney disease (CKD), obesity, insulin resistance, and numerous tumors. Current therapeutic strategies to address aberrant FGF/FGFR signaling primarily rely on small-molecule compounds that target the ATP binding site within the intracellular tyrosine kinase domain of FGFR, known as tyrosine kinase inhibitors (TKIs). However, TKIs generally exhibit low selectivity for specific FGFR variants, frequently affecting other FGFR variants and unrelated receptor tyrosine kinase family members. As a result, the therapeutic use of TKIs is associated with significant side effects, highlighting the need for alternative treatment strategies.

The lack of selectivity in TKIs stems from the high structural similarity of ATP binding sites across the receptor tyrosine kinase family. To address this fundamental limitation, we aimed to leverage SELEX technology to develop a DNA aptamer that targets the extracellular domain of FGFR, which shows greater structural diversity among receptor tyrosine kinase family members. Through this approach, we successfully identified a G-quadruplex-based aptamer that specifically interacts with a single variant of the FGF receptor, effectively inhibiting its downstream signaling pathways. I will discuss the potential for developing a second-generation DNA aptamer with enhanced biological properties.

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When G-quadruplex-forming aptamers meet small-molecule G-quadruplex ligands

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The high interest in G-quadruplex structures is well motivated by the main roles played by natural, genomic G-quadruplexes as targets for small-molecule anticancer drugs, but increasing attention has been devoted also to *ad hoc* synthesized oligonucleotides forming G-quadruplex structures. Indeed, over the past two decades, numerous G-quadruplex-forming oligonucleotides have been discovered for specific therapeutic applications as aptamers, i.e. nucleic acid molecules forming peculiar structures capable of selectively binding cellular targets[1]. Notably, the applications of nucleic acid aptamers are not only restricted to their use as drugs, but they are also intensively studied as carriers for delivering drugs into cancer cells[2].

In this context, I will here present two different applications of G-quadruplex-forming aptamers in their combination with small-molecule G-quadruplex ligands.

In the first case study, *N*-acetylgalactosamine (GalNAc)-functionalized aptamers have been evaluated by biophysical techniques and biological assays as carriers for the selective delivery of previously identified synthetic and natural anticancer G-quadruplex ligands[3-6] to hepatocellular carcinoma cells[7].

In the second case, the thrombin binding aptamer TBA_{15} has been modified to incorporate both a well-known small-molecule G-quadruplex ligand, i.e. a naphthalene diimide[8], and a 3-hydroxypropylphosphate group at either its 5'- and/or 3'-end so to develop effective and low-toxic anticoagulant agents[9]. Molecular dynamics-based analyses unveiled the structural features determining the higher thermal stability and nuclease resistance, as well as the higher anticoagulant activity due to thrombin recognition, for some of these aptamers compared to the others and the parent TBA_{15}[10].

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Naphthalene diimide carrying ß-Cyclodextrin as novel G-quadruplex binder

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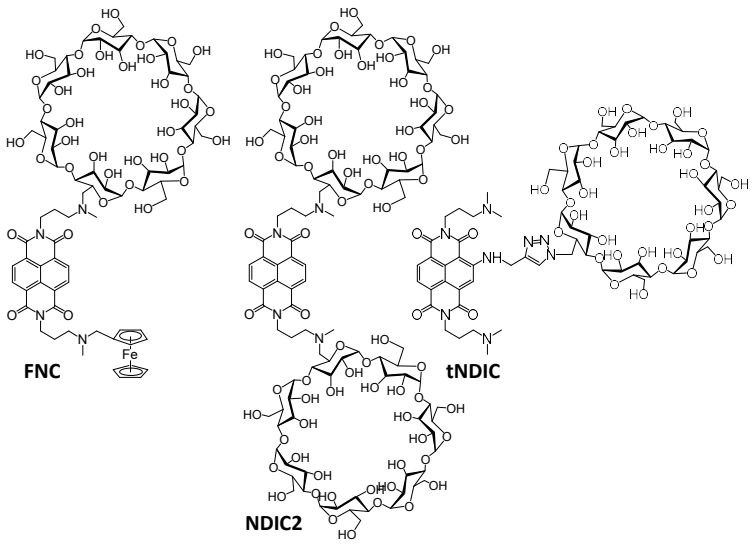
The authors have established a method for electrochemical detection of genes using FND derivatives in which ferrocene is introduced at both substituent ends of naphthalene diimide (NDI), as a threading intercalator.[1] Through these studies, we have designed and synthesized FNC in which ferrocene and □-cyclodextrin (ßCD) are introduced into NDI. Since ßCD can encapsulate ferrocene, the ferrocene in the FNC is simultaneously encapsulated by ßCD present in the FNC. This reduces the electrochemical response of ferrocene of FNC. However, when FNC intercalates with DNA duplex in a threading fashion, the ferrocene moiety is released from the ßCD and the electrochemical response is restored. This has enabled electrochemical detection of DNA duplex in a homogeneous system.[2,3]

On the other hand, it was also found that FND can bind strongly to G-quadruplex (G4).[4,5] Using this, we found that telomere G4 elongated by telomerase can be detected electrochemically. Since telomerase activity is associated with cancer, an electrochemical diagnostic method for cancer was realized.[6]

NDIC2, which has ßCDs at both ends instead of ferrocene of FND, sterically inhibits stitched intercalation into DNA duplex, but does not inhibit the stacking interaction of the NDI moiety with the G-quartet plane for G4. In addition, ßCD is known to encapsulate nucleobases. Therefore, if the NDI moiety of NDIC2 binds to the G-quartet plane by stacking and at the same time ßCD encapsulates the nucleobase protruding from the loop, it is expected that high complex stabilization ability can be realized. The experimental results show that NDIC2 can bind strongly to parallel-type G4 of RNA.[7]

As a new attempt of ßCD induced NDI, tri-substituted NDI with ßCD, tNDIC was synthesized by introducing ßCD at the naphthalene moiety of NDI. This ligand was found to bind strongly to parallel-type G4, especially this ligand was found to bind strongly to genomic RNA G4 of SERS-CoV-2.

Finally, the results obtained here suggested that molecular design of a combination of NDI and ßCD may be developed into a new G4 ligand.



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Nearest-neighbor parameters with AI beyond experiments: Prediction of RNA secondary structures containing mismatches

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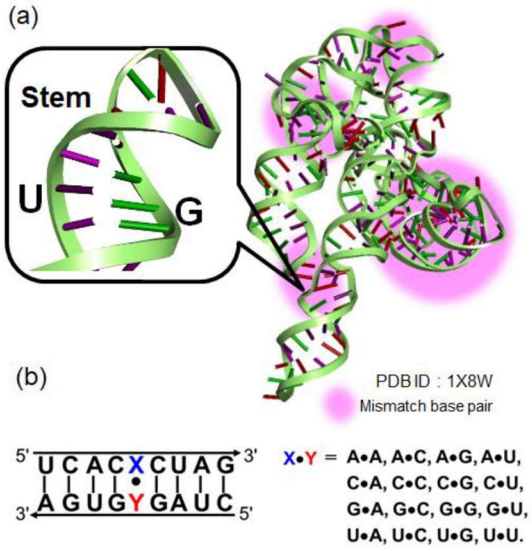
The stability of RNA structures in cells is important for predicting RNA functions. Intracellular crowded environments affect significantly structure and stability of nucleic acids.1 Living cells contain various organelles, cytoskeletons, and soluble and insoluble biomolecules, both of low molecular weight. Biomolecules occupy a significant portion of the cellular volume, accounting for up to 40%, resulting in crowded and intricate intracellular environments referred to as the molecular crowding effect. In order to predict the RNA structures, we have developed nearest neighbor parameters for RNA/RNA duplexes consisting with Watson-Crick base pairs under the molecular crowding contditions.2 However, it is essential to accurately predict the stability of not only Watson-Crick base pairs, but also Non-Watson-Crick base pairs base pair because functional RNAs contain mismatches (Figure 1a). Here, we evaluated the thermodynamic parameters of duplexes with single mismatched and fully matched base pairs in dilute and crowded conditions (Figure 1b). Mismatched duplexes were destabilized with the increasing order of pyrimidine•pyrimidine < purine•pyrimidine < purine•purine under the crowding condition, with the exception of A•G mismatch. We proposed a method for predicting individual contribution of the 12 mismatches to the stability of RNA that provided a basis for an increasingly accurate prediction of intracellular RNA secondary structure. In the presentation we will discuss the results of using pseudo-cellular systems and AI to predict RNA structure in the intracellular environments.



(a) Schematic image of the mismatch in functional RNA (Group I intron). (b) Schematic representation of RNA duplexes formed by 5'-rUCACXCUAG-3'and 5'-rCUAGYGUGA-3'sed in this study. "X"and "Y"denote positions of base pairs and mismatches shown

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Quadruplex DNA structures as regulatory elements in the immediate early gene promoters of HSV-1

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Non-canonical DNA structures, such as G-quadruplexes (G4s) and i-motifs (iMs), are genetic elements that form transiently in the cell and regulate specific cellular mechanisms, not only in human cells but also in the genomes of prokaryotes, plants and microorganisms, including viruses.[1] The development of antibodies that are capable of specifically recognizing these structures, namely the BG4 for G4s and the iMab for iMs, has boosted the research in the field, showing that the two structures might form independently and have different biological roles.[2]

The human herpes simplex virus type 1 (HSV-1) is a double-stranded DNA virus particularly enriched in guanines and cytosines (~70%), which contributes to its high propensity to form quadruplexes. Indeed, a genome-wide bioinformatics investigation showed an impressive abundance in all herpesviruses of putative G4-forming sequences, mainly located in regulatory (promoters) and repeated genomic regions.[3] We demonstrated that the promoter regions of the immediate early (IE) genes, which are the major regulators of the viral life cycle of HSV-1 and other α-herpesviruses, are enriched in highly stable and conserved G4s. We previously reported that each promoter contains multiple, adjacent G4s, ranging from four to fifteen structures, the stabilization of which results in the inhibition of viral transcription.[4]

To further explore the structural dynamism of the IE genes, we here investigated iM formation on the C-rich strand.

We employed a combined experimental approach using circular dichroism, thermal difference spectroscopy and bromine footprinting to assess the folding of the selected sequences *in vitro* and confirmed the folding of HSV-1 IE iMs. Interestingly, among all tested sequences complementary to G4s, only one sequence per gene was folded into iM, suggesting that G4s and iMs might have different roles. Subsequently, the folding state of both G4s and iMs was monitored *in cells* through CUT&Tag analysis with BG4 and iMab, at different times post-infection, and correlated the results with the transcription levels of each gene. We observed that the G4 enrichment was generally higher than iM, likely due to the abundance of G4s, and that it increased with the infection progression and with increased gene transcription. In contrast, iMs were mostly present at the start of the infection, while their enrichment decreased at longer times of infection.

In conclusion, our findings provide the first evidence for the existence of folded G4s and iMs within the HSV-1 genome in the chromatin context, where they have different abundance and possibly distinct roles. This supports the hypothesis that the dynamic interplay of these structures is key in regulating the HSV-1 life cycle.

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RECQ4 conformational flexibility modulates distinct functions and coacervation with G-quadruplexes

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Helicases are ubiquitous molecular motor proteins known to catalyse duplex DNA unwinding in an ATP-dependent fashion. A subset of DNA helicases is also known to unwind non-canonical secondary DNA structures, called G-quadruplexes (G4s), which induce genome instability if left unresolved. RECQ4 protein is a helicase implicated in G4 metabolism, which contains an intrinsically disordered region (IDR) at the N-terminus of its sequence.

We have characterized a positively charged polyelectrolyte within the IDR of RECQ4, which we termed the RECQ4-specific motif (RSM; 40 amino acids), because it is conserved among various RECQ4 homologues. The combination of biochemical, biophysical, and optical methods, has allowed us to decipher how RSM is able to switch between phases, conformational states and binding partners.

The structural, kinetic, and thermodynamic profile of the RSM interactions revealed distinct modes of binding depending on the partner. On one hand, RSM remains highly flexible in polyelectrolyte interactions with nucleic acids. The disordered state in these complexes minimizes the entropic penalty of binding, resulting in relatively strong but non-specific interactions. On the other hand, RSM forms a stable helix when it interacts with replication protein A (RPA), the major protein that binds to single-stranded DNA in eukaryotic cells. The induced folding comes at an entropic cost, resulting in an interaction characterized by high specificity but low affinity. NMR competition experiments show that the two binding modes are mutually exclusive.

Interestingly, when the RSM binds to G4 structures, liquid-liquid phase separation (LLPS) occurs. By integrating equilibrium and kinetic binding data in a global numerical model, a three-step mechanism of charge-driven coacervation is deduced. The oppositely charged RSM and G4 molecules form a complex in solution that follows a rapid nucleation-growth mechanism leading to a dynamic equilibrium between dilute and condensed phases. Additional experiments confirmed the exchange of RSM and G4 between solution and droplets.

The dynamic nature of coacervation and the ability to explore the two extremes of the structural continuum of complexes in selecting partners (RPA or DNA) suggest a regulatory role for the RSM to enable diverse RECQ4 functions in DNA replication and DNA repair in general.

The second dimension of the genetic code

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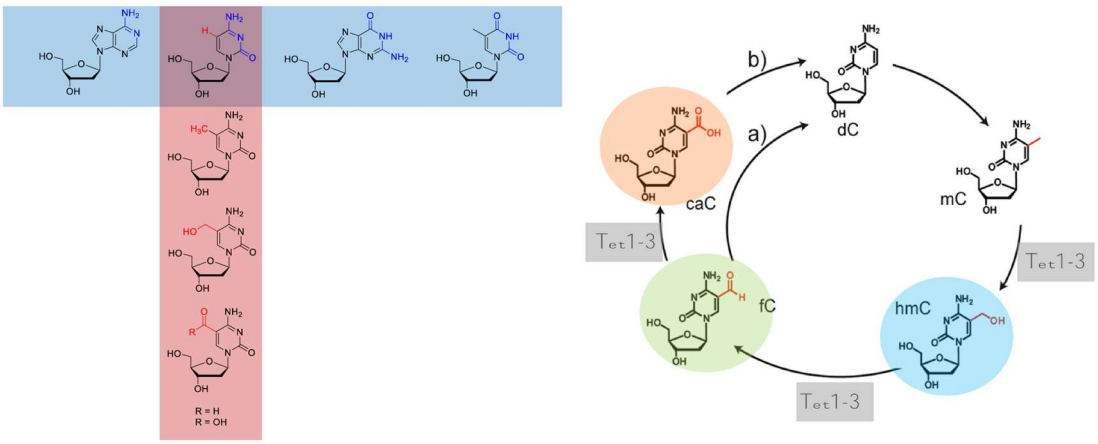
DNA stores genetic information in the form of the sequence of the four canonical bases dA, dC, dG and dT. DNA contains in addition epigenetic information, which is established by the four modified cytidine bases 5-methylcytidine (mdC), 5-hydroxymethylcytidine (hmdC), 5-formylcytidine (fdC) and 5-carboxycytidine (cadC) (Fig. 1, left).[1] Additionally, 5-hydroxymethyluridine may play a role as well. These bases are generated by Tet enzymes. The position and the kind of modified dC-base at a specific position in the genome establishes an unknown 2nd code in our genetic system (Fig. 1, left). Setting and erasing of these epigenetic bases controls the complete development process starting from an omnipotent stem cell and ending with an adult specialized cell (Fig. 1, right). I am going to discuss results about the function and the distribution of the new epigenetic bases hmdC, fdC and cadC in the genome.[2] I am showing how metabolic states influence the chemistry by setting and erasing these modified bases. Synthetic routes to these new bases will be discussed that enable the preparation of oligonucleotides containing these bases embedded. Particularly, isotope dilution and isotope tracing mass spectrometry was used us to understand the chemistry that occurs on these bases in the genome.[3] Interesting is the fact that base excision repair seems to play a central role during erasure of the bases and again mass spectrometry helped to quantify the repair processes involved epigenetics.[4] Finally, I am dicussing potential präbiotic origins of modified bases.[5]



Illustrations of the 2nd orthogonal code that is present in DNA (left) and proposal of how the epigenetic bases are interconverted to establish dynamic changes of the epigenetic code during cellular development (right).

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Base excision repair of DNA-embedded uridine (rU) by uracil DNA glycosylase

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Uracil DNA glycosylase (UNG), the first base excision repair (BER) enzyme to be discovered (by Nobel laureate Tomas Lindahl in 1974), catalyzes hydrolysis of the glycosidic bond of 2'-deoxyuridine (dU) ribonucleotide embedded in DNA, releasing uracil and generating a deoxyribose apurinic/apyrimidinic (AP) site. Subsequently, apurinic/apyrimidinic endonuclease (APE1) cleaves the phosphodiester backbone immediately 5' to the AP site, creating a nick that is repaired by polymerase ß and ligase to restore intact double-stranded (ds) DNA. In addition to playing an essential role in this error-free DNA repair pathway, UNG is also involved in error-prone DNA repair in B cells to generate antibody diversification and in host defenses against viral infection.

Ribonucleotides (including rA, rG, rC and rU) misincorporation is another common form of DNA damage. Ribonucleotide excision repair (RER) initiated by RNase H2 is the predominant pathway for ribonucleotide removal from genomic DNA. Although UNG can process dU analogues such as 1-(ß-D-2'-deoxy threo-pentofuranosil)uracil, the enzyme is often assumed to be unable to recognize and process rU because rU is a natural component of RNA. Herein we report that rU embedded in DNA can be repaired by UNG. Using synthesized oligonucleotides and UNG produced in *Escherichia coli*, we showed that UNG catalyzes uracil removal from rU embedded in DNA but exhibits no activity toward rU in RNA. Biochemical and crystallography studies revealed that the 2'-OH of rU is well accommodated by UNG and is directly involved in catalyzing N-glycosidic bond hydrolysis. The product generated by uracil removal from rU by UNG can be processed by downstream BER enzymes. In addition, we developed an new method to quantitatively analysis of the rU embedded in DNA, and based the method, we demonstrated that UNG also contributed to the removal of rU in living cells.

Our discovery that UNG shows rU-removal activity provides an alternative mechanism by which UNG can carry out its diverse biological functions. Deciphering the linkage between the rU-removal activity of UNG and its role in other biological processes and diseases is a necessary but challenging task.

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Crossroads of (divalent) cations and (dimeric) DNA structures

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Short (tandem) repeats are highly mutable genomic elements that play pivotal roles in both normal physiological processes and the development of neurodegenerative diseases. Although the mechanisms underlying their expansion and contraction remain elusive, the prevalence of short tandem repeat-derived sequences in the human genome suggests their fundamental biological importance.

Emerging evidence highlight the intricate relationship between repetitive sequences and the formation of non-canonical DNA structures, such as G-quadruplexes and i-motifs. These structures deviate from the canonical B-DNA helix, exhibiting unique hydrogen-bonding patterns and nucleobase stacking geometries. G-quadruplexes and i-motifs, enriched in guanine- and cytosine-rich regions, respectively, have been implicated in various cellular processes, including DNA transcription and gene expression.[1] Their formation is influenced by environmental factors, such as ion concentration and pH, underscoring their potential roles as biosensors. Yet, repetitive sequences that are neither guanine- nor cytosine-rich may also be susceptible to the nature and concentration of cations in solution.

NMR spectroscopy has been instrumental in elucidating the structural details of repeat-containing oligonucleotides and their propensity to adopt non-canonical conformations. By studying these molecules in solution, we can gain insights into the fundamental mechanisms governing DNA folding and its modulation by changes in (micro)environments. Our presentation will show our recent NMR-based investigations of short tandem repeat polymorphisms, focusing on their propensity to form dimeric DNA structures in the presence of divalent cations.[2] Notably, solution-state NMR appears as one of the most informative biophysical techniques for deciphering dimeric DNA structures formed in the presence of divalent cations, thus offering valuable insights into the complexity and diversity of DNA folding.

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Genome Therapy: A New Approach for Tumor Growth Inhibition

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By central dogma, chromatin DNA was spatial-temporally regulated at multiple levels, including chromatin unfolding, DNA transcription, post-transcription, mRNA translation, post-translation. Accordingly, gene regulation tools at multiple levels were also discovered and artificially exploited for different biological studies and gene therapy applications. With the deep knowledge of the evolution and progression of complex diseases such as cancers, single target based gene therapy has met with great challenges in reducing side-effect and drug resistance. The fast development of novel gene delivery methods and gene regulation technologies moved gene therapy from single gene causing illnesses to multiple gene-associated disorders in a more personalized, precise, safe and efficient manner. To find an efficient therapy solution, the strategies of mimicking chromatin DNA to precisely regulate gene expression through combining various gene regulation tools at different levels as an integrative toolbox are promising to combat complex diseases in the near natural way. In this way, a number of gene regulation tools could be rationally integrated as a smart toolbox and loaded into chromatin-like payloads to mimic the chromosome-mediated gene decoding process for disease therapy. Therefore, we here termed this artificial chromosome-like gene network regulation at multiple levels with different tools simultaneously as genome therapy. In this talk, we will discuss our efforts towards multiple gene regulations for antitumor efficacy with branch-PCR assembled gene nanovector mimicking chromatin-like activity.

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Thermodynamics of i-motif formation

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Although DNA is most stable in the duplex form, it can adopt other structures that occur in certain contexts. Widely known are G-quadruplexes, which are formed from guanine-rich sequences, and i-motifs, which arise from the cytosine-rich complementary strands. In i-motif structures, two parallel duplexes are intercalated in an antiparallel manner and stabilized by three hydrogen bonds between each unprotonated and protonated cytosine (1). Although i-motif formation is favored *in vitro* in slightly acidic solutions, some studies suggest that this conformation can also occur *in vivo* (2).

The formation of i-motif structures from cytosine-rich DNA fragments *in vitro* is usually considered a two-state process. Therefore, the formation of intermediates along the folding pathway is not well understood. The present work addresses this issue by thermodynamic analysis of isothermal titration calorimetry (ITC) data, which predicts temperature-pH conformational stability phase space (phase diagram) of cytosine-rich DNA fragments. The predicted species are consistent with those observed by NMR spectroscopy, linking the thermodynamic driving forces of species formation to their structural features. The phase space of conformational stability of i-motifs appears to be of a complexity comparable to that of G-quadruplexes, RNA or proteins.

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The quest for the ultimate DNA fluorescent probe: the contribution of Quantum Mechanical calculations.

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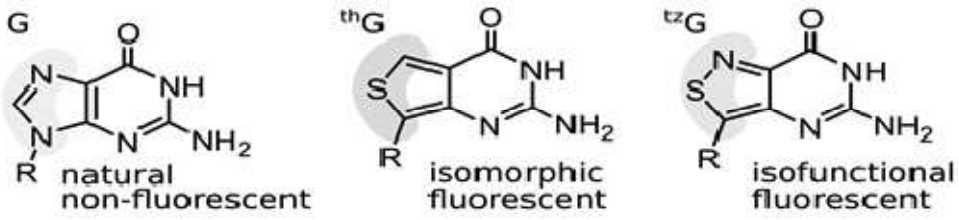
Fluorescence-based experiments are ideal for monitoring biomolecule dynamics and interactions with high spatial and temporal resolutions. Nucleic Acids suffer from the very low fluorescence quantum yield of their natural nucleosides, so that many efforts have been devoted to developing fluorescent nucleoside analogues (FNAs) to report locally on DNA structure and interactions. FNAs should ideally (i) replace natural nucleosides without any modification of DNA structure and function, (ii) keep high brightness, (iii) exhibit spectroscopic properties sensitive to the environment and ultimately (iv) reveal quantitative, local information on DNA structure and its interactions with biomolecules. [8] An important advance has been recently achieved with the development of the thieno-guanosine analogues thG and ^{tz}G (Fig. 1), which perfectly replace G in DNA duplexes, being truly isomorphic, while keeping high quantum yields environmental sensitivity.[1-7] In order to to validate thG and ^{tz}G as fluorescent mimic of guanosine, it is however necessary to associate any change in their photophysical properties with a well-defined electronic structure and conformation of the probe and its nearest interacting neighbors. In this contribution we shall discuss the role of Quantum Mechanical calculations to achieve such a picture, based on calculations on systems of different size and complexity, from the isolated FNA in solution, to double and quadruple helices.



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Genome-wide mapping of G-quadruplexes in Mycobacterium tuberculosis genome: new avenues for drug discovery

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Mycobacterium tuberculosis (*Mtb*), the causative agent of tuberculosis (TB), remains a global health crisis, ranking as the second leading cause of death worldwide after COVID-19. The emergence of drug-resistant strains, prolonged treatment regimens, and the absence of an effective vaccine underscore the urgent need for novel targets and therapeutic approaches. G-quadruplexes (G4s) present a promising alternative target, particularly given *Mtb's* high GC content (65.6%) and the prediction of over 10,000 G4 motifs in its genome.

This study aimed to assess G4 formation genome-wide in *Mtb* cells under two physiological conditions: exponential growth and oxidative stress, the latter mimicking *Mtb* internalization in host macrophages. We employed two genome-wide techniques: Chromatin Immunoprecipitation sequencing (ChIP-seq) and Cleavage Under Targets and Tagmentation (CUT&Tag), the latter being applied to a bacterial genome for the first time.

Both techniques were optimized for the *Mtb* genome. While ChIP-seq presented challenges due to a low signal-to-noise ratio, CUT&Tag offered several advantages, including streamlined experimental processes and reduced sequencing depth requirements. We validated the CUT&Tag protocol using an antibody targeting the β-subunit of the RNA polymerase complex, observing a correlation between actively transcribed genes and transcription complex occupancy.

Subsequent G4 mapping using the BG4 antibody revealed that G4 motifs in *Mtb* are predominantly located in gene coding sequences and mainly formed by two-layered G4s. This finding contrasts with the tendency of G4s to form in promoters in eukaryotes, despite *Mtb's* high GC content. Notably, G4 motif abundance increased under oxidative stress. Integration of RNA-seq with CUT&Tag data demonstrated that G4s are associated with reduced gene transcription, thereby affecting gene expression levels.

This study marks the first successful application of CUT&Tag to a bacterial genome, proving its value in studying bacterial chromatin and transcriptional dynamics. Our findings unveil unique features and potential functions of the G4 landscape in *Mtb*, suggesting that G4s may play a role in *Mtb* survival within macrophages. These insights open new avenues for understanding *Mtb* pathogenesis and developing novel therapeutic strategies.

Fluorescent binders with enhanced selectivity for G-quadruplex topologies

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Guanine quadruplexes (G4s) form various topologies, such as parallel, antiparallel, hybrid 1, and hybrid 2, depending on the base sequence and environment. Thioflavin T (ThT), known as a fluorescent stain for amyloid, is one of the G4 binders and can fluorescently stain G4s. G4s have several binding sites, and G4 binders bind in end-stacking mode, groove-binding mode, and intercalation mode. While ThT is said to bind mainly in end-stacking mode, simulations suggest that the three-dimensional overlap between the G-quartet and the methyl group at the N3 position differs depending on the topology. In fact, if the methyl group is replaced with a larger substituent, the relative fluorescence intensity of a parallel G4 with a relatively small overlap is greater than that of an antiparallel G4 with a relatively large overlap[1,2]. In addition, introducing an aminoalkyl group to the N3 position allows the introduction of various substituents via amide bonds, etc. This opens up the possibility of creating ThT derivatives with higher topology selectivity. Furthermore, ThT derivatives modified with ligand molecules or chelators may be useful as target-specific fluorescent probes[3–5].

We have previously demonstrated that conjugates with protein ligands such as desthiobiotin and cortisol exhibit concentration-dependent fluorescence response in the presence of target proteins. This is presumably because the ligand moiety fits into the cavity, which is the binding site of the target protein, restricting the rotation of the dihedral angle between the benzothiazole ring and the dimethylaminobenzene ring in ThT. Conjugates with ethylenediaminetetraacetic acid (EDTA), a chelator of divalent metal ions, showed increased or decreased fluorescence response in the presence of G4, depending on the type and concentration of the divalent metal ion. This may be because the configuration of the conjugate when the divalent metal ion is coordinated brings about differences in the binding state between the ThT moiety and G4. It is relatively difficult to clearly distinguish the difference in G4 topology by binding small molecule compounds. Depending on the conditions such as salt concentration and pH, the binding of compounds may induce a specific topological structure. In addition, there are probes designed to target the difference in loop sequence, but the binding of compounds may affect the structure formation. In this presentation, we will discuss chemical modifications that were effective in recognizing the topology.

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Nearest-neighbor parameters meet AI: Functional prediction of DNAzyme

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Nucleic acids (DNA and RNA) dynamically fold and unfold to exert their functions in cells. These folding and unfolding behaviors are also basis for various technical applications. To understand the biological mechanism of nucleic acid function and design active materials using nucleic acids, the prediction method for each function from the sequence information of DNA and RNA is required. As these functions based on base pairing such as Watson-Crick base pairs, the stability of forming base pairing is fundamental information to establish the functional prediction of DNA and RNA. Nearest neighbor (NN) model is the most successful method to predict the Gibbs free energy (ΔG^{o}_{37}) of the formation of duplexes such as DNA/DNA, RNA/RNA, and RNA/DNA.[1-3] Thus, the NN model can be used as a dataset to predict the nucleic acids function based on the duplex stability, although such functional prediction has been not developed yet.

In this study, we focused on the nuclease reaction of DNAzyme and ribozyme (here mainly used RNA-cleaving 10-23 DNAzyme). These enzymatic reactions were based on the ΔG^{o}_{37} of the formation of duplex with a target strand as well as the secondary structure of the enzyme strand. As the enzymatic activity depends on Mg^{2+} concentrations and molecular crowding conditions, we improved the NN model to segmentate the thermodynamic parameters of each NN base pair into those affected by cation and cosolulte concentrations. As a result, we could extend the NN model available in different Mg^{2+} concentrations and various molecular crowding conditions with different water activity, dielectric constants, and excluded volume effect. By using the updated NN parameters and enzymatic activity in different Mg^{2+} concentrations and various molecular crowding as a database, we characterized the relationship between the sequence information and enzymatic activity with AI techniques. Our results suggest that NN parameters supported to efficiently and accurately predict the enzymatic reaction, which highlights the advantage of NN parameters for predicting the functions of nucleic acids from the sequence information.

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POSTER PRESENTATIONS

G-quadruplex formation after dsDNA oxidation is dependent on lesion position

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Excessive production of reactive oxygen species within the cell results in oxidative damage to DNA, with guanine being the most vulnerable to oxidation among the four major nucleobases, due to its low redox potential. This susceptibility increases in nucleotide sequences where multiple guanine nucleotides are positioned in succession, forming G-tracts. These guanine-rich sequences are commonly located in promoters and telomeres, where they can fold into tetrahelical structures known as G-quadruplexes. The fundamental unit of a G-quadruplex is the G-quartet, a planar structure composed of four guanine moieties, hydrogen-bonded through Hoogsteen base-pairing[1].

Guanine oxidation yields 8-oxoguanine, which is initially repaired *via* excision from DNA, leading to AP site formation. Since oxidation products have altered hydrogen bonding properties, oxidative damage in guanine-rich DNA can trigger structural rearrangements, potentially impacting key cellular processes, such as replication and transcription[2,3,4].

We incorporated oxidation products of guanine into a double-stranded guanine-rich DNA construct in a position-specific manner. By utilizing NMR spectroscopy and complementary biophysical methods, we observed the effect of oxidation on helix dissociation and possible G-quadruplex formation. Oxidized G-quadruplexes mitigated the damage *via* partial or complete exclusion of the damaged G-tract. However, in most cases, the thermodynamically preferred equilibrium structure is the double helix. Using two-dimensional NMR methods, we determined the topological specifics of the oxidized G-quadruplexes and evaluated the possibility of utilizing machine learning software to predict the structure of novel DNA sequences containing oxidized lesions.

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Drug-like iron chelators/G-quadruplex binders as synergic dual targeting agents

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Iron plays critical roles in many different cellular processes, and its homeostasis is closely linked to many different physiological pathways, including cell growth and cell cycle progression[1]. The most recent anticancer treatment strategies focus on either depleting the cells with an appropriate chelator or increasing its loading by administering iron complexes to induce ferroptosis. Indeed, low iron levels prevent cell proliferation, whereas high iron levels cause reactive oxygen species (ROS) to be generated, inducing the damage of guanine nucleobases in G-quadruplex (G4) structures and leading to genome instability.

In this study, we demonstrated that dual-targeting compounds with synergistic anticancer activity can be obtained by appropriately designing a molecular chimera that embodies structural requirements for both iron chelation and G4 binding. G4s are noncanonical nucleic acid secondary structures formed by guanine-rich DNA or RNA sequences[2], which are involved in cellular processes such as regulation of oncogene expression and telomere maintenance. Evidence shows that G4-binding ligands can lead to apoptosis and autophagy in cancer cells[3]. We designed and synthesized a library of putative dual-targeting agents and evaluated them through biophysical and biological experiments. To assess the ability of these compounds to bind and stabilize G4s, we performed circular dichroism (CD) melting experiments. Compound **16** emerged as the most promising of the series, so its G4-binding properties were further investigated via microscale thermophoresis (MST) and nuclear magnetic resonance (NMR) experiments. The results of biophysical and biological experiments allowed us to identify compound **16** as a lead candidate and a pharmacological tool able to chelate iron, stabilize G4s in human leukemia Jurkat cells and, worthy of note, to localize in the cell nucleus, thus acting as an intrinsically fluorescent nuclear tracer for labile iron pool.

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Structural characterization of let-7 RNA family and the Lin28 protein

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MicroRNAs (miRNAs) are small non-coding RNA molecules that can regulate gene expression by destabilizing target mRNAs or inhibiting their translation.[1,2] Due to their importance in numerous cellular processes, miRNA biogenesis is a highly regulated, multistep process. Although the general mechanisms of miRNA biogenesis are well understood, many regulatory factors - particularly those involving structural elements like the apical loop in precursor miRNAs (pre-), remain poorly characterized.[3] The let-7 miRNA family is one of the most critical groups for cancer incidence and progression, regulating key oncogenic pathways across various tumors, and acting as essential tumor suppressors that control differentiation and growth. Its dysregulation is linked to numerous abnormal physiological processes, making the study of its regulatory mechanisms particularly significant.[4] While let-7 family members share similar mature regions, their apical loop sequences are divergent, highlighting the importance of understanding how these variations impact miRNA processing.[3] By utilizing NMR spectroscopy, we aim to characterize full-length precursor and truncated RNA constructs to gain high-resolution structural insight into key regulatory elements. Our initial NMR data provide a strong foundation for structure elucidation, evident from good dispersion of the signals in 2D ^{1}H-^{1}H NOESY and 2D ^{13}C-^{1}H HSQC spectra. Additionally, we will investigate the role of Lin28, a protein known to inhibit let-7 biogenesis, in RNA binding and processing. We believe, this project will reveal many missing factors in let-7 family regulation important to be accounted for during a potential therapeutic intervention.

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NMR spectroscopy evaluation of complexes formed by known G-quadruplex ligands and I-Motif

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I-motif is a non-canonical DNA structure formed in C-rich strand, held together by hemi-protonated and intercalated cytosine base pairs[1], representing a clear example of the versatility of DNA. Despite their acidic pH requirements for hemi-protonation, their *in vivo* existence has been established[2,3,4], suggesting a role in cell regulation and maintenance, complementary to the most studied G-quadruplex. For pharmaceutical purposes, several ligands are known to bind G-quadruplex with high affinity, while a few ligands are known to bind I-motif selectively[5].

In our work, by NMR spectroscopy, we evaluated the complexes formed by known G-quadruplex ligands and an intramolecular I-motif, formed by a 15-mer single-stranded DNA sequence, named C21T3, whose structural features were previously solved[6]. All the cytosine residues are involved in the I-motif core and are connected through two TTT loops on the upper side of the I-motif and one TTT loop at the bottom. The selected G-quadruplex ligands were TmPyP4, Mitoxantrone, Braco19 and PhenDC3. It turned out that all the ligands destabilized the I-motif model as revealed by the decrease of the i-Motif protons signals. However, ligands showed different destabilization modes: while increasing the concentration of PhenDC3, Braco19 and Mitoxantrone caused a linear broadening of the C21T3 NMR signals, TmPyP4 did not induce a linear broadening of the i-Motif signals. In line with this observation, we then focused on the characterization of the C21T3: TmPyP4 complex. NMR titrations showed that the broadening and the up-field shifting of the signals corresponding to the ligand protons provided evidence for the binding occurring.

The analysis of 1H NMR spectra of the DNA upon titration with TmPyP4 revealed that the ligand destabilizes preferentially the upper side of the I-motif. 2D NOESY experiments confirmed that the NMR signals of the DNA protons in this region are the most perturbed upon complex formation.

The analysis of the cross-peak disappearance in the 2D NOESY spectrum of the complex compared to the 2D NOESY spectrum of isolated C21T3 provides the structural characterization of C21T3 I-motif in the complex.

In conclusion, from the analysis of the intermolecular cross-peaks between the C21T3 protons and the TmPyP4 ones, we proposed the two most probable binding sites causing the preferential destabilization of the T12-T14 loop compared to the T3-T5 one, which both resides in the upper side of I-motif.

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NMR study of the cation-dependent RNA structural switch

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Long-noncoding RNA (lncRNA) REG1CP was shown to promote cancer cell proliferation and tumorigenicity by activating the REG3A gene in colorectal cancer. The mechanism involves the lncRNA acting as a two-site address code which necessitates an RNA: DNA triplex formation (anchoring REG1CP) at one end and a structure-dependent binding and recruitment of helicase FANCJ to the REG3A locus on the other end[1]. FANCJ is supposed to facilitate transcription of REG3A by unwinding the duplex, thus enhancing DNA accessibility within the REG3A promoter and activating transcription which is normally repressed. A G-rich sequence within REG1CP was proposed to fold into a non-canonical structure called G-quadruplex (G4)[1]. The G4 structure in REG1CP is supposed to be recognized by the helicase FANCJ, which is a known G4 binder[2].

Due to potentially high importance of G-quadruplex formation in REG1CP for regulation of REG3A oncogene, we initiated an NMR structural study of a 30-nt oligoribonucleotide (ORN) derived from the G-rich region of REG1CP to gain insight into its structural properties in solution. Surprisingly, we observed that the G-rich sequence forms two mutually exclusive structures; a canonical hairpin (HP) and a noncanonical G4. The position of the equilibrium depends on external factors such as cations. In solution with K^+ ions, the G4 represents the major form while the HP is the minor structure. On the other hand, the HP is formed in the absence of cations, and in the presence of Li^+, Na^+ or Mg^{2+} cations. The HP structure can further multimerize under certain conditions via the single-stranded overhang at the 3' end of the sequence. By careful optimization of sequence length, composition and selection of solution conditions, we were able to obtain samples of good quality which enabled resonance assignment and a deeper structural study with NMR. The RNA switch found in REG1CP offers a mechanistic model that can affect the functionality of this lncRNA in response to external stimuli, which may dictate which protein partners are recruited and bound by the structured lncRNA scaffold.

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Evidence of a stable RNA G-triplex structure in the SARS-CoV-2 genome

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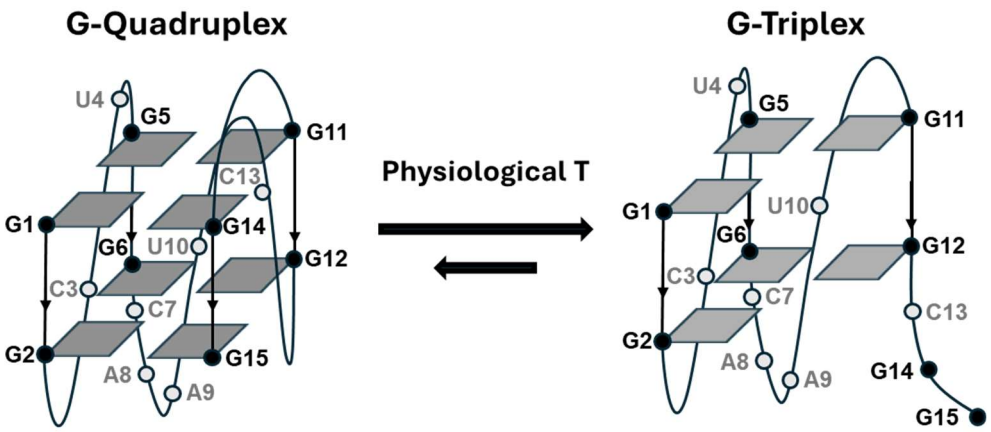
RG1 (5'-GGCUGGCAAUGGCGG-3') is an RNA G-quadruplex-forming sequence within the SARS-CoV-2 genome that has been proposed as a potential therapeutic target for COVID-19.[1,2] However, its thermodynamic stability and folding mechanism have not been thoroughly explored. In this study, we comprehensively characterized the stability and unfolding mechanism of RG1 using a wide range of spectroscopic, calorimetric, and computational techniques. Notably, our results indicate that RG1 undergoes a complex unfolding process involving an intermediate, rather than a simple two-state transition. A detailed analysis of the unfolding mechanism revealed that, at physiological temperature, the most populated species is the intermediate, rather than the RG1 G-quadruplex conformation as previously assumed (Figure 1). Our data are consistent with the intermediate species being a two G-triads RNA triplex, which could serve as a complementary therapeutic target for COVID-19. Furthermore, the observed ability of RG1 to adopt two alternative non-canonical nucleic acid structures could be the basis of a more complex regulatory switch, as previously proposed for DNA c-MYC promoter,[3] potentially involved in the dynamic regulation of viral replication or gene expression. Our findings open new avenues for further studies on the presence and biological role of RNA G-triplexes *in vivo*.



Schematic representation of the RG1 G-quadruplex and G-triplex conformations in equilibrium at physiological temperature.

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Reevaluation of quadruplex propensity of Aptamer

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Aptamers are short, single-stranded DNA or RNA sequences that can fold into unique three-dimensional structures capable of binding specifically to target molecules with high affinity, similar to antibodies. A unique structural feature of a number of aptamers is their ability to adopt a G-quadruplex fold, a four-stranded structure formed by DNA or RNA sequences rich in guanine. While G4 formation has already been proposed or demonstrated for some aptamers, we wanted to investigate how frequently a quadruplex-prone motif would emerge from a SELEX process. To this aim, we investigated quadruplex candidate sequences found in the UTexas Aptamer Database, currently the largest in terms of the number of aptamer sequences. This curated database includes over 1400 aptamer sequences extracted from 400 publications spanning several decades (1990–2022). We therefore analyzed G-quadruplex and i-motif propensity of these 1400 sequences. While no likely i-motif forming candidate motif was found, nearly 1/4 and 1/6 of all DNA and RNA aptamers, respectively, were predicted to form G4 structures. Interestingly, we found many motifs for which G4 formation was not reported or suspected: out of 309 sequences containing a potential stable G4 motif, the word "quadruplex" appeared only for 53 of them (17%), and we experimentally confirmed G4 formation for all sequences experimentally tested. These observations argue for a significant reevaluation of G4 propensity among aptamer sequences.

This work has been conducted in the sustainability period of the project SYMBIT No. CZ.02.1.01/0.0/0.0/15_003/0000477 as its follow-up activity.

Complete analysis of Inverted repeats and G-quadruplexes in the gapless assembly of human chromosome Y

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Recent advances in sequencing methods have led to major progress in the gapless assemblies of the human genome, including the elusive Y chromosome. This accomplishment closes a significant knowledge gap. Prior efforts were hampered by challenges in sequencing repetitive DNA structures such as direct and inverted repeats. In our study, we applied the G4Hunter algorithm to analyze the presence of G-quadruplex forming sequences (G4s) and the Palindrome analyzer to detect short, inverted repeats (IRs) within the current human reference genome (GRCh38) and the new telomere-to-telomere (T2T) Y chromosome assemblies. This analysis served a dual purpose: identifying the location of potential G4s within the genomes and exploring their association with functionally annotated sequences. Compared to GRCh38, the T2T assembly exhibited a significantly higher prevalence of both G-quadruplex forming sequences and inverted repeats. Notably, these G4s were abundantly located around precursor RNA, exons, genes, and within protein binding sites. Inverted repeats are located abundantly around exons and mobile elements, and, unexpectedly, also within gene annotations. This remarkable co-occurrence of G4s and IRs with these critical regulatory regions suggests their role in fundamental DNA regulation processes. Our findings indicate that the current human reference genome significantly underestimated the number of G4s and IRs, potentially overlooking their crucial regulatory roles and significant bio-medicinal potential. G4s are known to play an important part in gene regulation, while inverted repeats serve as precursors for the formation of cruciform DNA, which can act as functional epitopes in various biological processes.

References: There are no references included as the article is currently undergoing the review process. Once the review is completed, references will be added accordingly.

Acknowledgements: This work has been conducted in the sustainability period of the project SYMBIT No. CZ.02.1.01/0.0/0.0/15_003/0000477 as its follow-up activity.

Proto-nucleotides as a part of Proto-RNA in origin of life

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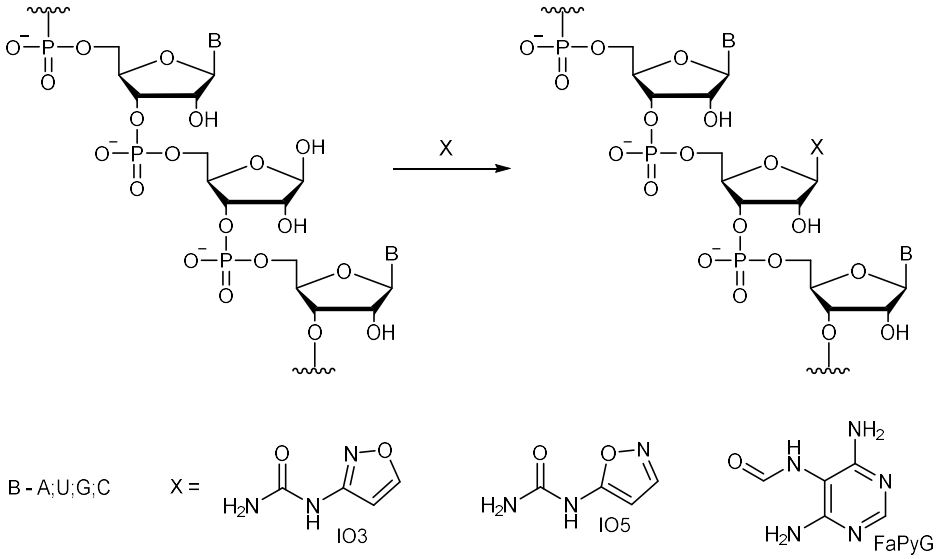
The RNA World hypothesis tells us that RNA as a bio molecule able to store information and catalyze its own replication, played a central role in the early evolution of life[1,2]. This hypothesis suggests that RNA is a product of chemical evolution rather than a product of abiogenesis, that is canonical RNA came from a precursor called proto–RNA. It is possible that at the beginning of chemical evolution, simpler and more available molecules preceded all three structural components of RNA. The point is that an unlimited number of proto-RNA intermediates may have existed during the development of canonical RNA[3]. All of this gives a promising mechanism to explain the evolutionary transition of a proto–RNA to canonical RNA. Under prebiotic conditions N–isoxazolyl–ureas[4,5] and formamidopyrimidines can be regioselectively attached to abasic sites[6] of oligonucleotides via glycosylation.



The abasic RNA can be loaded under prebiotically plausible conditions with the proto-nucleosides. In addition, all of this proto-nucleobases could have been part of such a system to stabilize the intramolecular bonds of the macromolecules through hydrogen bonds. The plausibility of the proto-nucleobases as a component of a potential proto-RNA, the base pairing and physicochemical properties of proto-loaded RNA it is an important step forward in the study of the origin of life.

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6-(2,4-Dichlorobenzoyl)-7-(2,4-dichlorophenyl)indolo[3,4-*jk*]phenanthridin-5(4*H*)-one as a specific DNA binder recognizing two different G-quadruplex topologies

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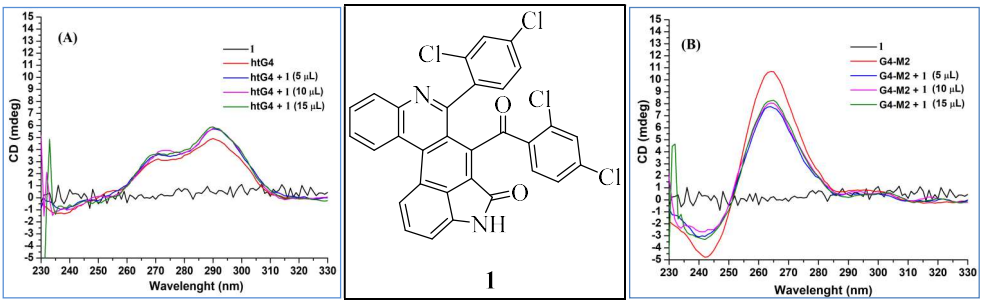
Targeting DNA is an efficacious cancer therapy[1] and elaboration of some novel tools for controlling the oncogene expression represents a potent route to treat cancer. In particular, modulation of the non-canonical DNA conformations, G-quadruplexes (G4s)[2] by specific ligands may crucially influence some driver genes in cancer[3], by this providing remarkable therapeutic benefits. We have recently found that the title compound **1**, which features a fused core containing the phenanthridine[4] and indolinone[5] anticancer pharmacophores, exhibits good antiproliferative properties on Glioblastoma LN229 and Lung Carcinoma NCI-H460 cells[6]. We report herein the results of binding studies of **1** with two G4 DNA sequences: an extended four-repeat hybrid-type human telomeric htG4[7] and M2 parallel G4[8], by using circular dichroism (CD) spectroscopy.



The tested ligand 1: chemical structure and CD titration spectra of htG4 (A) and M2 G4 (B) with varying amounts of 1 in 100 mM KPi (pH 7.4) and KCl.

The results of CD titration experiments strongly suggest that **1** may selectively stabilize G4 over duplex DNA[6] and also discriminate amongst the two investigated G4s. Especially, in the case of htG4 the dose-dependent increase in the intensity in the positive band at 290 nm demonstrates that **1** interacts with G4 stacking by intercalating with the G-tetrads, thus inducing overall stabilization (Fig. 1, (A)). Contrarily, in the case of M2 G4, exhibiting all strands in parallel orientation, the step-vice addition of **1** caused a dose-dependent decrease in the intensity of G4's characteristic bands: a major positive band at 265 nm and a negative band at 243 nm (Fig. 1, (B)). The signal reduction is likely a result of the expected end-stacking interactions of **1** with the sequence's G-tetrads, which are anticipated to disrupt the structure of the G4 sequence.[10]

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CGAG-rich oligonucleotides found in the promoter region of the neurodevelopmental regulator AUTS2 gene can form structurally distinct non-canonical hairpins, which may potentially play a role in its regulation

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The AUTS2 gene plays a crucial role in brain development by regulating neuron numbers, promoting the growth of axons and dendrites, and guiding neuronal migration. Importantly, the AUTS2 protein can be expressed as two major isoforms, a long and a short isoform, with their ratios significantly varying at different stages of brain development[1]. The expression of the two isoforms is highly regulated and misregulations in their expression have been correlated to developmental delay and intellectual disability[2]. It is still unknown how the expression of the long and short isoforms is regulated on the molecular level. We have identified a CGAG-rich region comprising a putative protein binding site (PPBS), d(AGCGAAAGCACGAA), found in the promoter region of the AUTS2 gene near the transcription start site of the long isoform[3]. Since we suspected that the CGAG-rich oligonucleotides are structurally polymorphic we decided to focus or structural studies on three truncated variants, with lengths between 32 and 38 residues, found in the CGAG-rich region. Using basic DNA folding prediction software and data from the literature we concluded that CGAG-rich oligonucleotides will most likely form structurally polymorphic hairpins which will contain many different non-Watson-Crick base pairs[4,5]. We extensively characterized the three variants by utilizing NMR spectroscopy[3]. We confirmed that all three variants form thermally stable non-canonical hairpins which are characterized by structurally conserved stem regions dominated by G:C and sheared G:A base pairs. The different number of CGAG repeats contained in each variant caused differences in the composition of nucleotides that made up the stem and loop regions. Consequently, the major structural differences between the variants were observed in the loop regions while the topology of the stem was similar in all three variants. Since the PPBS is contained in the loop regions of all three variants, which are structurally very distinct, these structures could be potentially biologically relevant in regulating the AUTS2 gene. Additionally, the information we provide on the change of the structural landscape of the CGAG-rich oligonucleotides based on the different number of CGAG repeats surrounding the PPBS site is also important in the context of potential regulation of gene expression.

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Unraveling the effect of binding of the RG-rich peptides to G-quadruplex structures by NMR

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Interactions between proteins and nucleic acids are crucial for the regulation of many cellular pathways. However, exact mechanisms at the atomic level are often still poorly understood due to difficulties *in vitro* mimicking of intracellular conditions that are needed for breakthrough structural studies.

One example of such important biological interactions are the ones between non-canonical nucleic acid secondary structures called G-quadruplexes and the arginine/glycine-rich (RGG/RG) domains of DNA/RNA binding proteins.[1] G-quadruplexes are structurally diverse and capable of performing a broad range of cellular functions, most notably regulation of gene expression, which may be facilitated by the binding of various DNA or RNA processing proteins. Nucleolin, a multifunctional nucleolar protein, contains an intrinsically disordered C-terminal RG/RGG-rich domain. It plays a role in various cellular functions and is also capable of G-quadruplex binding.[2]

We investigated the interaction between the nucleolin-derived RG/RGG-rich peptides and two different G-quadruplexes, one being a well-studied anti-parallel TBA G-quadruplex and the other parallel M2 G-quadruplex. We also assessed binding of RG-rich peptides by the G-quadruplexes formed by oligonucleotide with four d(G_4C_2) hexanucleotide repeats, that are characteristic for the gene C9orf72 and the onset of ALS neurodegenerative disease.[3] We show that the investigated binding has a moderate strength and that the binding is influenced even by the smallest differences in the amino acid sequence of RG/RGG-peptides, while a specific amino acid sequence may be responsible for the major contribution towards the binding affinity. Folding of the oligonucleotide into the G-quadruplex during temperature annealing is also potentially affected by the presence of the peptides, resulting in altered G-quadruplex topology. Our results may become of greater interest considering the importance of the investigated interaction for the development of ALS and FTD diseases.

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Unraveling RNA G-quadruplex structures in the 5'-UTR of the *RANKL* gene: Implications for osteoporosis regulation

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Osteoporosis, a bone disease marked by increased bone resorption, is closely linked to elevated RANKL expression levels[1]. Understanding the molecular mechanisms that regulate RANKL expression could unlock new therapeutic strategies. One such mechanism involves the formation of G-quadruplexes—four-stranded nucleic acid structures known to influence gene expression at both DNA and RNA levels[2].

We identified a 20-nucleotide G-rich sequence in the 5'-UTR of the *RANKL* gene. Previous studies using DNA constructs of the wild-type sequence (RANwt; 5'-GGGGAGGGAGCGGGAGAGGG) revealed the formation of two distinct G-quadruplex topologies[3,4]. Expanding on this work, we explored RNA constructs derived from the rRANwt sequence. We observe significant signal overlap, indicating the formation of multiple conformations. By introducing G-to-U modifications, we isolated four different RNA G-quadruplex structures: alongside a 'regular' three-layered parallel form, we identified two G-quadruplexes containing one two-nucleotide bulge and one structure featuring two concurrently present bulges.

Contrary to expectations, observed G-quadruplex structures displayed similar thermal stabilities and were present in the rRANwt construct in varying populations. Furthermore, we demonstrated that this G-rich sequence affects the expression of a luciferase reporter gene, pointing to a potential regulatory mechanism of *RANKL* gene expression through RNA G-quadruplex formation in its 5'-UTR.

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Interaction of BPBA ligand with G-quadruplex DNA: Insights from NMR and molecular modeling studies

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G-quadruplexes (G4s) are highly polymorphic noncanonical DNA/RNA secondary structures formed by stacking of at least two G-tetrads, a planar association of four guanines connected by intervening loops.[1] Low-molecular-weight compounds affecting nucleic acid conformational equilibria by preferentially binding to a given form could represent a promising strategy for therapeutic applications.[2,3] Recently, a new hit compound, namely BPBA, was found to bind both telomeric repeat-containing RNA (TERRA) G4 and several DNA G4s derived from oncogene promoters. Biological assays showed that BPBA is endowed with a preferential cytotoxic effect on osteosarcoma cancer cells, where it induces a DNA damage response at the telomere level.[4]

Since BPBA induced significant perturbations in both imino and aromatic protons of the 1D ^{1}H NMR spectra of the G4, here we selected the *c-Kit2* G4/BPBA complex as the promising system for a detailed structural characterization. Different 2D NMR experiments were conducted on the complex in a 1:2 G4/ligand ratio, allowing us to acquire new molecular coordinates for *c-Kit2* G4 in the presence of the drug and subsequently determine the G4 3D structure. Based on experimental NMR restraints, molecular docking and subsequent molecular dynamics simulations were performed. In agreement with NMR results, computational analysis revealed that BPBA acts as an end-stacker G4 ligand. In particular, it shows a preference for binding to the 3' external G-tetrad, with a well-defined favored binding pose. Conversely, at the 5' G-tetrad, BPBA explores several different orientations and binding poses, suggesting a greater ligand mobility and an overall lower affinity for this binding site. Shedding light on the structural requirements for the interaction of this hit compound with G4 structures will enable us to optimize the molecular scaffold of BPBA, designing new, improved derivatives, thus paving the way for a new class of ligands with improved pharmacological properties.

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Assessing the Influence of Cytosine Methylation on G-Quadruplex Formation

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DNA cytosine methylation is a well-characterized epigenetic modification that regulates gene expression without altering the DNA sequence itself[1]. Recently, increasing attention has been directed towards the intricate interplay between cytosine methylation and non-canonical DNA structures, such as G-quadruplexes (G4s)[2]. The formation of G4 structures within G-rich regulatory regions, such as promoters, can alter DNA methylation patterns, thereby influencing gene expression and leading to changes in the transcriptome[3]. Although significant progress has been made in understanding G4 structure and stability, the precise impact of cytosine methylation on G4 formation remains unclear and warrants further investigation.

In this study, we examined the impact of introducing C5-methylated cytosines (C^m) at specific CpG sites within the well-characterized bcl2Mid G4, located in the GC-rich region upstream of the P1 promoter - an important site for interactions with transcription factors that regulate BCL2 expression. The bcl2Mid G4 adopts a 3+1 hybrid topology with one propeller-type loop and two lateral loops[4]. By individually substituting cytosine residues with C^m in the loops, we assess the resulting changes in G4 structure and thermodynamic stability.

We found that introduction of C^m causes localized structural alterations in the major bcl2Mid G4. Cytosine methylation modulates G4 structure in a sequence-dependent way, highlighting its potential role in altering the structural landscape of G4s. These insights have important implications for epigenetic regulation and cancer biology.

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Characterizing the structural features of mt-tRNA derived fragments

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The mitochondrial tRNA derived fragments (mt-tRFs) are a group of highly conserved non-coding RNAs that are broadly gaining attention in the scientific community due to their role as critical regulators of homeostasis, cancer cell viability, tumorigenesis, ribosome biogenesis, chromatin remodeling, translational and retrotransposon regulation, intergenerational inheritance, and viral replication[1]. More specifically, these cleavage products of mature or pre-tRNAs have been observed to be aberrantly dysregulated in several cancers, and actively participate in cell proliferation, apoptosis and invasive metastasis in different malignant human tumors[2,3]. Advancements in state-of-the-art research techniques over the past decade have identified clinically relevant tRFs as potential biomarkers for cancer diagnosis and prognosis. However, this remarkable world of tRFs remains underexplored due to lack of any structural information. Given the significance of these critical regulatory molecules, a detailed understanding of the process of biogenesis, maturation and mechanism of action can be considered as a crucial advancement in the field of tRNA biology. Therefore, to bridge the gap between their function and mechanism of action we have aimed to achieve critical insight into their structural dynamics and conformational states at atomic level of resolution utilizing NMR spectroscopy.

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Characterization of structural features of $d(G_4C_2)_n$ sequences related to ALS and FTD

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Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are two neurodegenerative diseases with devastating consequences. Most frequent genetic cause of ALS and FTD is a large increase in the number of $d(G_4C_2)$ repeats located within the non-coding region of *C9orf72* gene[1]. Although the mechanism of these diseases remains unclear, it was proposed that non-canonical structures including G-quadruplexes formed by extensive repeats of $d(G_4C_2)$ drive the pathology by limiting the normal functioning of genes and cellular processes.

Here, we studied the folding of DNA oligonucleotides containing the guanine-rich repeats $d(G_4C_2)$, $d(G_4C_2G_4)$, $d(G_4C_2)_2$, $d(G_4C_2)_3$ and $d(G_4C_2)_4$. Our previous studies utilizing dynamic light scattering (DLS) and atomic force microscopy (AFM) indicated the formation of aggregates with highly variable lengths for the sequences $d(G_4C_2)_2$, $d(G_4C_2)_3$ and $d(G_4C_2)_4$ ranging from 2 nm to over 80 nm[2].

To better understand the fundamental folding of $d(G_4C_2)$ repeats we extended the number of studied sequences by adding two DNA sequences of intermediate length. By conducting nuclear magnetic resonance (NMR) studies we investigated the basic G-quadruplex fold and what hinders its further stacking into long aggregates. Since the NMR spectra suggested the presence of complex G-quadruplex structures, we employed complementary methods such as circular dichroism (CD) and polyacrylamide gel electrophoresis (PAGE) to further explore the structural features and gather additional information that could help us identify the fundamental folding patterns of $d(G_4C_2)_n$ sequences. Our findings demonstrate that these sequences adopt distinct G-quadruplex conformations stabilized by K^+ ions[3]. In solutions, we observed monomeric G-quadruplex units as well as higher-order structures formed through stacking of single G-quadruplex units.

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Complex Biophysical and Computational Analyses of G-Quadruplex Ligands: The Porphyrin Stacks Back

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G-Quadruplexes (GQs) are non-canonical nucleic acid structures formed by stacking G-quartets, stabilized by monovalent cations such as K^+ and Na^+. These structures can form in guanine-rich genomic regions like telomeres, oncogene promoters, and replication initiation sites, where they regulate key biological processes such as gene expression and cellular senescence[1]. In cancer, GQs are particularly relevant, as their stabilization can interfere with telomerase, an enzyme overexpressed in more than 90% of cancer cells, as well as inhibit the expression of oncogenes like c-Myc, making them attractive therapeutic targets[2]. The stabilization of GQs by small molecule ligands, such as porphyrins, acridines, and carbazole derivatives, has been widely explored for therapeutic purposes. Among these, meso-tetrakis-(N-methyl-4-pyridyl) porphyrin (TMPyP4) has been extensively studied for its ability to stabilize GQs but displayed a limited selectivity[3]. However, a significant driving force behind research in this field is the desire to develop new ligands that demonstrate enhanced selectivity and binding ability. In this study[4], molecular docking and molecular dynamics simulations were employed to guide the synthesis of novel porphyrin derivatives with potential GQ-stabilizing activity. A number of computational models based on GQs derived from human telomeric sequences and the NHEIII1 region of the c-Myc gene (with both parallel and hybrid topologies) were constructed and their interaction with a number of novel porphyrin derivatives evaluated. On the basis of the results obtained, a selection was made of the most promising derivatives, which were then subjected to synthesis. Among these, a 5,10,15,20-tetrakis-4-pyridylporphyrin derivative with amidomethyl substituents (PL7) demonstrated strong binding affinity and selectivity for parallel GQs. PL7/GQ interaction was characterized by NMR spectroscopy (^{1}HNMR, NOESY, ROESY) and mass spectrometry (MS/MS). The selectivity between single-stranded DNA and GQs and between the various topologies of the latter was evaluated through spectroscopic studies (UV and CD). Preliminary biological assays on breast cancer (MCF-7) cells indicated promising cytotoxicity for these porphyrin derivatives, highlighting their potential as selective GQ-stabilizing agents for anti-cancer therapies. This study confirmed the strong affinity of porphyrin ligands for parallel GQs, a topology favored under molecular crowding in the cellular environment. PL7, with its improved selectivity and given its ease of synthesis, could be considered a good alternative to TMPyP4 in GQ-targeting studies.

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M08s-1 duplex/quadruplex anti-thrombin aptamer: structural features determining its high efficiency

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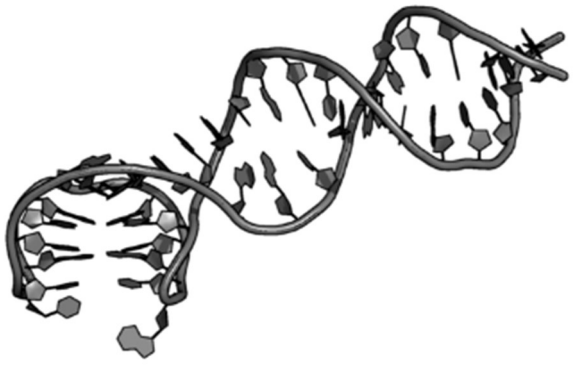
DNA or RNA aptamers have garnered clinical interest as anticoagulants for their ability to be therapeutically regulated, either by controlling their circulating half-life or reversing their function with an antidote. Indeed, aptamers targeting several members of the coagulation pathway have been developed and progressed into clinical trials[1]. During the last decades, particular attention has been paid to the study of anticoagulant aptamers that are able to recognize human α-thrombin[2], the coagulation factor that maintains blood hemostasis by balancing procoagulant and anticoagulant actions. The first aptamer to be isolated to thrombin is TBA, a 15mer oligonucleotide that adopts an antiparallel G-quadruplex structure[3]. A mixed structure, formed by both a duplex and a quadruplex domain, is instead adopted by 26mer NU172, the only anti-thrombin aptamer evaluated in Phase II of clinical trials[4]. Recently, some of us selected, by using the MACE-SELEX approach, a new anti-thrombin DNA aptamer having a guanine-rich sequence of 43 nucleotides and showing an enhanced anticoagulant activity with respect to both TBA and NU172[5].

The X-ray structure of the complex between thrombin and a truncated form of M08s-1 (named M08s-1_41mer, Figure 1), which provides a reasonable explanation of the improved properties of this oligonucleotide, is here presented. The study of the interaction between M08s-1 and thrombin has been complemented by surface plasmon resonance and circular dichroism experiments. Furthermore, ^{1}H-NMR and electrophoretic analyses have been performed in order to investigate the conformational behavior of this aptamer in the free state[6]. Details will be discussed at the Meeting.



Cartoon representation of the duplex-quadruplex structure of M08s-1_41mer as found in the crystal structure of its complex with thrombin (PDB code 8BW5).

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Self-assembly of d(G_4C_2) sequences: from G-quadruplexes to liquid crystalline phases

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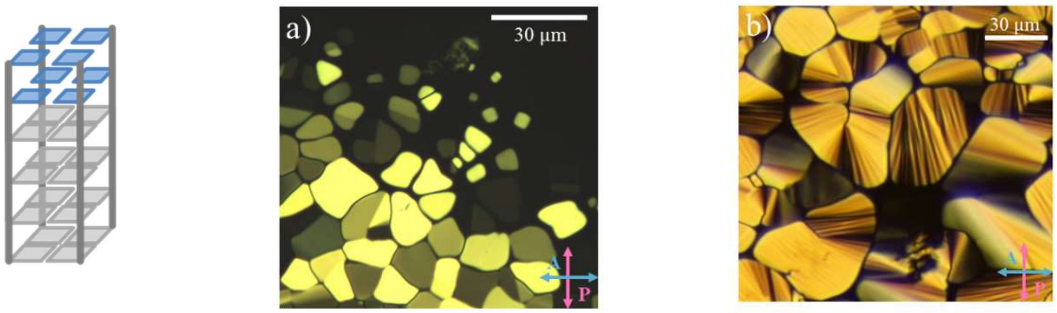
Guanine-rich DNA sequences self-assemble into highly stable fourfold helical structures known as G-quadruplexes. We studied quadruplex formation of sequences d$(G_4C_2)_n$ with n = 1, 2, 3 and 4. These sequences are associated with some fatal neurological disorders, especially amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD).[1]

We used dynamic light scattering to measure diffusion coefficients and resolve the length of G-quadruplex aggregates in solution. We found that all sequences assemble into longer structures than previously reported.[2,3] The d(G_4C_2) sequence formed long quadruplex stacks with lengths beyond 80 nm. The d$(G_4C_2)_2$ formed a relatively short stacked dimeric quadruplex, while d$(G_4C_2)_3$ and d$(G_4C_2)_4$ formed multimers corresponding to 6 or 7 stacked intramolecular quadruplexes. Due to their polyelectrolyte nature, concentration dependence of d$(G_4C_2)_n$ sequences was also studied. In the concentration range from 0.1 mM do 2 mM the diffusion coefficients decreased due to stacking of quadruplexes. Above the 2 mM d$(G_4C_2)_n$ concentration, however, the solution dynamics of quadruplexes notably speeds-up and diffusion coefficients increased. This behaviour is typical for DNA and other polyelectrolyte solutions.

In highly concentrated aqueous solutions (c > 50 mM) all sequences showed extensive orientational ordering of G-quadruplex structures and the formation of columnar liquid crystalline (LC) phases. Polarisation optical microscopy revealed a wide range of LC textures (Fig. 1), ranging from those previously observed for short DNA duplexes[4] to some specific to DNA quadruplexes. Moreover, a connection between different LC phases and the length of aggregates was established. This is a feature typical for chromonic LC systems, where the LC formation is controlled not only by temperature but also by solution concentration and the extent of stacking interactions.



Self-assembly of d(G4C2): the basic unit is a tetrameric parallel G-quadruplex (left), stacking of these quadruplexes leads to long columnar aggregates that orientationally order into liquid crystalline phases with different textures at lower (center) and higher concentrations (right).

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Unravelling G-quadruplex-membrane interactions: a biophysical study

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The interaction between nucleic acids and membranes is widely used in medicine for gene therapy and vaccine technology. Moreover, this type of interaction is also involved in gene replication for both prokaryotes and eucaryotes [1]. It is generally accepted that the negatively charged phosphate backbone of nucleic acids can interact with polar headgroups of membranes in the presence of divalent cations [2]. However, previous studies mainly focused on the interaction between canonical DNA structures and phospholipids, leaving a gap in knowledge regarding the interaction between non-canonical DNA structures and biological membranes. Among non-canonical DNA structures, G-quadruplexes are of particular interest due to their biological role and therapeutic applications [3].

In this work, we investigated the interaction of both DNA and RNA G-quadruplexes with two different model membranes. Additionally, the impact of topology was evaluated, employing Tel23 and cMyc sequences as models for hybrid and parallel topology, respectively. On the other hand, we used liposomes composed of pure phosphatidylcholine lipids to mimic a zwitterionic membrane, while a mixture of phosphatidylcholine and phosphatidylglycerol in an 80/20 molar ratio was employed to mimic a negatively charged membrane.

We used several biophysical techniques including differential scanning calorimetry, isothermal titration calorimetry, fluorescence spectroscopy, circular dichroism spectroscopy and confocal fluorescence microscopy.

Our findings show that G-quadruplex structures preferentially interact with membranes in the gel phase, while membranes preferentially interact with parallel G-quadruplex topologies. This study paves the way for elucidating the molecular mechanisms of these interactions and exploring their potential therapeutic applications.

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Recent Advances in Artificial Circular Nanosystem with Non-canonical DNA Motifs

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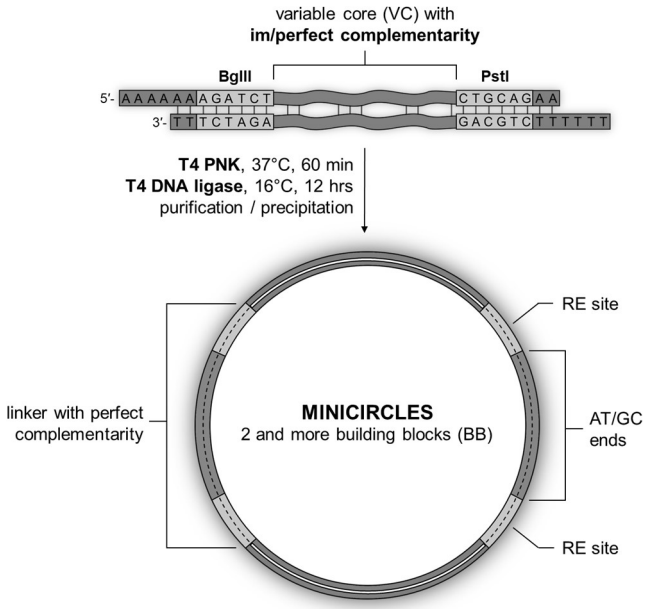
The ability of a DNA molecule to form non-canonical motifs such as Z-DNA, G-quadruplexes, triplexes, i-motifs, three and four-way junctions is now well established. The 3D structure of a non-canonical motif depends on several factors such as nucleotide sequence, ion concentration, presence of specific ligands or pH. There is still great interest in analyzing the relationship between occurrence of (un)folded non-canonical motifs and their impact on biological processes. New findings on this topic are still emerging[1,2]. Non-canonical structures have become a promising tool for nanotechnology applications in addition to their important biological role3.

An efficient method for preparing circular nanosystems based on DNA molecules has recently been demonstrated. This method allows the insertion of a non-B-forming DNA strand between the standard dsDNA linkers. Furthermore, the design of the minicircles is fully modifiable, allowing any motif to be incorporated into the overall structure4. We are coming up with new knowledge about these nanostructures and other novel variants with potential uses.



Design and preparation scheme of artificial DNA-based minicircles

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Intercalated Cytosine Structures: Stability and Formation in Telomeric DNA

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Recent research employing immunoprecipitation methods has demonstrated that C-rich regions of genomic DNA adopt intercalated cytosine structures, referred to as i-motifs, within the nucleus of the cell[1,2]. Tandem repeats of d(TAACCC), commonly localized in telomeric DNA regions, have been shown to form inter- or intramolecular i-motifs under mildly acidic conditions that allow for hemiprotonation of cytosines[3]. Furthermore, some i-motifs have also been shown to form at physiological pH levels[4]. I-motifs are most abundant in genes upregulated in the G0/G1 phase of the cell cycle and like G-quadruplexes are believed to be important regulatory elements controlling gene transcription, telomeric elongation and DNA replication[1]. To further understand the formation of intra- and intermolecular i-motifs and their dependence on C-tract length, we synthesized sequences similar to the aforementioned telomeric sequence with varying lengths of C-tracts.

By modulating the length of C-tracts, we could distinctly observe a balance between intermolecular and intramolecular i-motif formations using native polyacrylamide gel electrophoresis. Longer C-tract sequences favored intermolecular i-motif formation, while shorter ones lead toward formation of intramolecular structures. Interestingly, UV and NMR thermal denaturation assays revealed that i-motifs gain thermodynamic stability as the C-tract lengthens. Most compellingly, UV melting experiments showcase near-physiological melting points, with sequences containing four consecutive cytosines stabilized at a remarkable 39.41°C, while telomeric tandem repeats exhibit apparent melting temperature of 33.38 °C. Our findings suggest a deeper link between cytosine arrangement and the dynamic, adaptable nature of telomeric DNA structures and reveal the need for further exploration of sequence-structure relationship for efficient targeting of i-motifs in stem cell and cancer research.

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Trizna, Lukaš

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Zhou, Chuanzheng