12-1-2012

G-Ruption: The Third International Meeting On G-Quadruplex And G-Assembly

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Published in final edited form as:
Biochimie. 2012 December ; 94(12): 2475–2483. doi:10.1016/j.biochi.2012.09.002.

G-ruption: The third international meeting on G-quadruplex and G-assembly

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Abstract

A three and a half day conference focusing on nucleic acid structures called G-quadruplexes (G4s) and other guanine-based assemblies was held in Sorrento, Italy (June 28–July 1, 2011) and featured 35 invited talks and over 89 posters. The G-quadruplex field continues to expand at an explosive rate with the emergence of new connections to biology, chemistry, physics, and nanotechnology. Following the trend established by the previous two international G4 meetings, the conference touched upon all these areas and facilitated productive exchanges of ideas between researchers from all over the world.

Keywords

G-quadruplex DNA; G-assembly; Therapeutics; Nanotechnology; Biology

1. Introduction

In late June 2011 and beneath the shadow of Mt. Vesuvius arose an outburst of lively presentations on roles for G-quadruplexes (G4s) in fields spanning physical chemistry to biology (Fig. 1). Once the subject of a small number of researchers, G4s are now widely recognized as the non-canonical DNA or RNA structures formed from stacks of G-quartets, which themselves comprise four guanines connected *via* Hoogsteen hydrogen bonds and stabilized by a monovalent metal cation. Interest in G-quadruplexes has grown dramatically over the past decade because they have unique properties that may be leveraged for fields ranging from nanofabrication to therapeutic medicine. The biophysical toolbox used to study G4 structures is expanding to include new techniques such as single-molecule optical tweezers, Raman spectroscopy, and ion mobility mass spectrometry. These, together with advances in NMR and X-ray structural studies, are enabling a clearer view of G4 topologies, aiding the development of rules for predicting different G4 structures. Simple one-quadruplex models are being replaced by more physiologically relevant multi-quadruplex systems, which allow investigation of interactions among G4s. New quadruplex specific

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ligands are being developed, some with promising anticancer properties and selectivity not only for G4 *vs.* duplex DNA but also for specific G4 folds. In addition, these ligands are finding applications as probes for G4s in biological settings, and ascatalysts for G4 formation in nanotechnology applications. G-quadruplexes and their assemblies are being used as sensitive probes for monovalent metal cations, and as promising materials with mechanical, electrical and optical properties useful for application in nanotechnology. From the perspective of natural biology, the emergence of strong evidence in recent years for the existence of G4s within living cells has caused a shift in the focus of questions from *whether* G4s influence biology to *how*, *where*, *when*, and with *what* functional consequences? Here we review some of the many exciting findings discussed at the meeting.

2. Discussion

The conference started with the plenary lecture by J. Brad Chaires (University of Louisville, USA) titled *Structure and stability of human telomeric DNA*. It captured the history of discoveries leading to our current understanding of G4 structures, and raised discussion of four “myths” in the quadruplex community. These included the ideas that 1) G4 melting is a simple two-state process, 2) Polyethylene glycol (PEG) drives G4 folding by acting as a crowding agent, 3) H-bonding and π–π stacking between nucleotides are primary driving forces for G4 folding, and 4) All G-quadruplexes are essentially similar structures. Chaires focused on the first two myths, but noted for the last two that cation binding is actually the primary driver of G4 folding and that the precise differences among G4-forming sequences can impact G4 folds significantly.

Myth 1

A thorough understanding of G4 folding and stability requires determining thermodynamic parameters, which can be extracted from melting data only if the process is truly reversible (i.e. a two-state equilibrium between folded and unfolded species without any intermediates). Melting can be efficiently monitored using UV–vis spectroscopy at 295 nm [1]. The reversibility of the melting process can be determined using the *two wavelength test* [2] or the *singular value decomposition* (*SVD*) *method* [3]. In the former method absorbances at two wavelengths are plotted against each other; a straight line signifies a true two-state equilibrium, whereas deviation from linearity indicates the presence of at least one intermediate. For SVD, a three-dimensional matrix is constructed using wavelength, temperature and system response. Analysis of this matrix yields the number of species that participate in a melting process. The application of SVD was demonstrated using $dTTG_3(TTAG_3)_3A$ whose melting was determined to proceed through a single intermediate. As a result, a simple two-state model was not applicable in this case and a significantly more complicated model had to be used in order to extract thermodynamic parameters for this system.

Myth 2

There are remarkable differences in macromolecule concentrations typically encountered within cells (~400 mg ml^{-1}) compared with *in vitro* studies (~1 mg ml^{-1}). Compelling evidence exists that this difference is of great importance to G4 formation. Researchers commonly use molar concentrations of PEG, ethanol, methanol, or acetonitrile to mimic the crowded cellular environment. The belief that these chemicals impact G4 folding simply by functioning as crowding agents was the most controversial part of the talk. A true crowding agent should lead to a structure with smaller volume, e.g. *via* dimer formation, surface binding, etc. Four tandem copies of the human telomeric DNA repeat form parallel-stranded G4 and at least four other folding topologies depending on buffer conditions and flanking residues [4]. These topologies could be converted to the parallel fold upon addition of high

amounts of PEG or various other cosolutes, as clearly demonstrated by Brahim Heddi (from the laboratory of Anh Tuân Phan, Nanyang Technological University, Singapore) who presented the first NMR structure of $dTAG_3(T\text{-}TAG_3)_3$ obtained in K^+ buffer in the presence of 40% (v/v) of PEG 200 [5]. A parallel-stranded quadruplex core of this structure is remarkably similar to the famous Parkinson's crystal structure of $dAG_3(TTAG_3)_3$ in K^+ conditions (PDB ID: 1KF1) [6]. Yet parallel-stranded G4 has a higher hydrodynamic radius as compared to other G4 topologies, indicating PEG is not acting simply as a crowding agent. Both Chaires and Heddi suggested based on their data that addition of PEG leads to dehydration of the quadruplex, most probably because PEG binds to the G4 grooves and subsequently releases water, which in turn triggers the observed conformational switch. In contrast, the cellular environment is not dehydrating and is crowded by large biological molecules. To better mimic physiological conditions R. Hänsel, (Poster 32, *Questioning the molecular crowding effect on structure and stability of human telomeric G-repeat sequences*, Goethe University, Germany) used bovine serum albumin [7], *Xenopus laevis* egg extract, or 40% of the macromolecular polysaccharide Ficoll 70 [8], but these macromolecules did not lead to the conformational switch induced by PEG in human telomeric DNA. In contrast, 20% Ficoll 400 has been shown to induce a partial structural conformational switch or to cooperate with 20% PEG 200 to induce a full conformational switch to parallel-stranded G4 in human telomeric DNA [5]. In conclusion, Chaires suggested using the word *dehydrating* rather than *crowding* in relation to PEG or ethanol. Although not everyone was convinced that this is a critical distinction, all agreed that the debate would help focus efforts to understand how particular conditions influence the stability of different G4 folds.

An important question about physiologically relevant G4 structures (parallel, antiparallel or hybrid) of the human telomere, was addressed in the talk of Michaela Vorlickova (Academy of Sciences of the Czech Republic, Czech Republic). Based on spectroscopic and structural evidence, it is commonly believed that human telomeric DNA in dilute K^+ solution exists in a hybrid conformation, while in Na^+ rich environment the structure is antiparallel [9]. However, Vorlickova observed that the structural transition from the Na^+ to K^+ form is fast (seconds after K^+ addition) and proceeds in a non-cooperative manner (with nearly linear changes in CD signal upon addition of K^+) suggesting that the starting antiparallel fold is mainly preserved [10]. Of note, the structural transition between two different folds is usually slow (hours, days) and displays non-linear spectroscopic changes indicative of cooperativity. Vorlickova used Raman spectroscopy, which can detect subtle differences in syn- vs. anti-conformation of guanines, to support her hypothesis. When low concentration of human telomeric DNA in Na^+ was titrated with K^+, the Raman signature of the G4 did not change, suggesting that the same folding topologies exist in both buffers. In contrast, Raman and CD data indicated increases in guanines with *anti*-conformation of glycosidic bonds (indicative of parallel G4 topology) when the concentration of human telomeric DNA was increased. In general, the exact conformation of human telomeric DNA appears to be very sensitive to the number of TTAGGG repeats and nucleotides flanking the G-runs and, in fact, relates back to *Myth* 4 of Brad Chaires's presentation. Most interestingly, the conformation of the human telomere DNA quadruplex depends on DNA concentration [10]. This is why distinctly different quadruplex structures were detected for $AG_3(TTAG_3)_3$ by X-ray, nuclear magnetic resonance and circular dichroism spectroscopy.

The most common mode of ligand binding to the quadruplex core is assumed to be end stacking. However, literature examples also include groove-binding, loop-binding and intercalation. As TMPyP4 is an excellent (although not selective) G4 stabilizer, its mode of binding is of particular interest especially for nanomaterial application. Kotlyar's group demonstrated experimentally that TMPyP4 could intercalate into long G-wires if metal cation is absent [11]. Rosa Di Felice (CNR institute Nanoscience, Italy) presented theoretical work using classical molecular dynamics simulations and time dependent density

functional theory calculation of TMPyP4 intercalation into parallel G4s of varying length. The work clearly demonstrated that TMPyP4 is a viable intercalator so long as the quadruplex has more than 8 tetrads, potassium ion is absent, and the G4 motif is capped either by another porphyrin or long empty terminal G4 segment [12]. Porphyrin intercalation leads to higher flexibility of G4 structure as a result of larger inter-tetrad stacking distances even in the areas removed from porphyrin intercalation sites.

Although information is rapidly emerging concerning modes of interaction between G4s and small molecule ligands, information about G4 interactions with proteins is relatively sparse. Thus it was exciting to learn of new studies involving the thrombin binding aptamer (TBA; 5′-GGTTGGTGTGGTTGG-3′), which was developed as potent inhibitor of thrombin, a pro-coagulant factor of critical clinical importance. Previous structural studies of a classical TBA left uncertainties concerning its mode of interaction with thrombin [13]. Naoki Sugimoto (Konan University, Japan) demonstrated that thrombin and potassium use different mechanisms to stabilize G4 formation distinguished by specific binding between thrombin and thymine bases within quadruplex loops. Furthermore, water –which may be of limited availability under crowded macromolecular conditions – is required for the binding of thrombin to TBA [14]. Interestingly, water may be more available in the relatively dilute environment of the bloodstream, where thrombin principally functions, consistent with the biological relevance of these findings. Irene Russo Krauss (Università degli Studi di Napoli Federico II, Italy) designed a modified TBA aptamer, mTBA, containing a chain polarity inversion site and with higher affinity for thrombin, albeit with less inhibitory activity. A high resolution X-ray structure revealed selective binding of mTBA to a key region of thrombin, exosite I, and also provided an explanation for the reduced inhibitory activity because mTBA did not fully block the fibrinogen-interacting His71 side chain [15]. A better understanding of key interactions between thrombin and TBA should help in the further design of new generations of anti-thrombin aptamers.

With the advances in the G4 field, new methods of studying quadruplex structures emerge. Mass spectrometry (MS) has been an invaluable technique in the G4 field for determining the strand stoichiometry of different quadruplex conformations. More recently, sophisticated MS techniques have proven useful for obtaining information about the actual structures present in complex mixtures, and Valérie Gabelica (University of Liege, Belgium) is a world leader in developing these techniques. Valérie demonstrated the ability of ion-mobility MS to discern a mixture of parallel and antiparallel dimeric structures formed by well-studied $(TAGGGT)_2$, with the mixture slowly converting to the parallel structure over time [16]. In the ion mobility cell, different conformations of G4 display different mobilities due to variations in their collision cross section, allowing parallel structures to be differentiated from antiparallel ones. Importantly, the data obtained by ion mobility MS correlated with those obtained in solution by CD, indicating that solution structures were conserved in the gas phase. However, the different conformations did show variability in their propensity for loss of ammonium ions and collapse of their structure in the gas phase under conditions of increasing bias voltage, with antiparallel G4s being less stable. The study demonstrated that this tendency can be exploited to gain information about G4 topology and stability.

Laser-tweezers based single-molecule techniques are gaining popularity in the G4 field. Their advantages include great sensitivity to biomolecular population dynamics (individual subpopulations with abundance as low as ~2% could be identified in heterogeneous samples), ability to measure mechanical stability of G4 structures, and capacity to follow relatively fast processes. In one approach, the DNA is tethered between the two optically trapped beads, force is applied between the two beads, and the DNA is stretched. Two parameters are measured, the contour length change and the rupture force, characteristic values of which are used to identify specific DNA folds (including different G4 structures).

Soma Dhakal (from the laboratory of Hanbin Mao, Kent State University, USA) reviewed his laboratory's previous studies of forces required to unwind the intramolecular G4 and *i*-motif structures formed by individual DNA strands from the the insulin-linked polymorphic region (ILPR) sequences found within a critical transcriptional regulatory region upstream from the promoter of the human insulin gene [17,18]. Furthermore, he provided compelling evidence that using force-induced folding/unfolding within the context of a duplex DNA fragment from the ILPR region, either G4 or *i*-motif structures formed readily under appropriate buffer conditions (100 mM KCl, pH 7.5 for G4 and 100 mM LiCl, pH 5.5 for *i*-motif). In contrast, *i*-motif and G4 are mutually exclusive when conditions are favorable for the formation of both structures (100 mM KCl, pH 5.5) possibly due to steric constraints [19]. Knowledge of G4 (and *i*-motif) formation and stability in the context of duplex DNA sheds light on potential biological roles for G4 in the regulation of insulin gene expression and on quadruplex stability under conditions where competition with the DNA duplex exists.

It is well known that folding of intramolecular G4 is fast, whereas the association of tetrastranded structures (such as $[dTG_5T]_4$) is slow and often leads to formation of kinetically trapped metastable intermediates. Rui Moriyama (from the laboratory of Atsushi Maruyama, Kyushu University, Japan) developed a cationic comb-type copolymer poly($_L$-lysine)-*graft*-dextran (PLL-*g*-Dex) which consists of cationic poly($_L$-lysine) backbone (<20 wt%) decorated with hydrophilic dextran (>80 wt%). PLL-*g*-Dex was shown to facilitate association (1000-fold) and dissociation (6-fold) of dTG_4T [20] as well as to accelerate DNA hybridization in a mixture of dTG_4T/dTG_5T [21], thus acting as a catalyst *via* lowering of the activation barrier for quadruplex folding. In addition, PLL-*g*-Dex was shown to accelerate the exchange of one strand of a tetrastranded G4 for an external single strand and to facilitate the conversion of metastable G4s containing mixtures of different strands to stable homomeric G4s, all of which may occur through a triplex intermediate. Moriyama suggested that PLL-*g*-Dex acts as a chaperone for G4 folding by reducing repulsion between individual strands, and could be used to manufacture nanotechnological devices requiring fast assembly of DNA strands. Demonstration of this catalysis in G4s with longer stacks of G-quartets would be useful in this regard.

Bioinformatic analyses have shown that there are approximately 375,000 sequences with the potential to form intramolecular G4s across the human genome [22,23], leaving little doubt that these non-canonical DNA structures impact biological processes. Elucidating the nature of biological functions of G4 is a hot topic of current research, and several talks at the conference presented exciting data illustrating the wide variety of *in vivo* processes that involve G4s. It has been demonstrated that the 5′ untranslated region (UTR) of several mRNAs can harbor G4 structures which usually function to repress translation [24]. Jean-Denis Beaudoin (Université de Sherbrooke, Canada) presented bioinformatic evidence for the existence of ~4000 potential quadruplex-forming sequences in 3′ UTRs of the human transcriptome. Experimentally, he demonstrated that representative candidate sequence in the 3′ UTR of the lipoprotein receptor-related protein 5 (LRP5) gene indeed forms G4 *in vitro*. Intriguingly, the presence of this structure increased gene expression of a reporter construct *in vivo*, possibly due to enhanced use of an upstream polyadenylation signal, producing more abundant and shorter transcripts than expected. Another biological function for G4s that has been postulated for many decades is as a protective cap at chromosomal telomeres. Brad Johnson (University of Pennsylvania, USA) presented the first solid evidence for such a protective function at yeast telomeres, at least when other forms of telomere capping are defective. His team demonstrated that introduction of any of three different G4-stabilizing proteins, or treatment with a G4-stabilizing ligand or inactivation of a G4 unwinding helicase, restored the growth of yeast which suffered from defective telomere capping due to a mutation in the telomeric protein Cdc13 [25].

Johnson's lab has also contributed to genome-wide analysis of G4 formation. The team has analyzed changes in transcriptional patterns by microarray in both yeast and human cells with mutated forms of G4-unwinding proteins [26,27], as well as senescent human cells and cells treated with G4-stabilizing ligands. In all cases, preferentially altered transcription of genes that contain potential quadruplex-forming sequences was observed. An inventive method for identifying G4-forming sites throughout the genome was presented by Raphaël Rodriguez (from the Jackson and Balasubramanian laboratories University of Cambridge, UK). Treatment of human cells with the G4-binding small molecule pyridostatin resulted in double-strand breaks throughout the genome, evidenced by the formation of foci of the DNA damage response protein γH2AX, and phosphorylation of the kinase DNA-PK. Rodriguez performed chromatin immunoprecipitation sequencing (ChIP-SEQ) analysis after pyridostatin treatment using an antibody to the γH2AX protein to directly determine the locations of the damage foci, and found an enrichment of these foci in genomic regions with high G4-forming potential [28].

Richard Sinden (Florida Institute of Technology, USA) discussed experiments leveraging the power of *E. coli* genetics to 1) address factors regulating *in vivo* the balance between duplex and several different G4 forms of DNA and 2) identify factors that impact mutagenesis within sequences with the potential to form G4s. R-loop formation *via* transcription templated by the C-rich strand, but not negative supercoiling, was sufficient to shift the equilibrium toward G4 formation. Lagging strand replication of the G-rich strand led to a higher frequency of deletions than did leading strand replication. Inactivation of certain combinations of DNA helicases (e.g. RecQ and RecG) augmented deletions related to the lagging strand replication defect, whereas deletion of others had little impact (DinG) or even inhibited deletions (Hfq). Overall, the findings (together with findings reported by A. Nicholas and K. Paeschke, see below) suggest that the propensity for G-quadruplex formation and the impact of G4s on genome stability depend on the details of the particular G4 forming sequence, the context in which it forms, and the helicases with which it interacts.

Zheng Tan (Chinese Academy of Sciences, China) reviewed his recently-published studies indicating that formation of intramolecular G4 at the 3′ end of the human telomeric single-stranded overhang can inhibit telomere extension by telomerase as well as by the recombination-based Alternative Lengthening of Telomerase (ALT) pathway [29]. Furthermore, his laboratory used telomere repeat DNA affinity chromatography to identify a novel protein that preferentially binds and stabilizes the single-stranded form of the telomere overhang and which stimulates extension by telomerase *in vitro*. This protein, TTIP (telomere-telomerase interacting protein), is an isoform of hnRNPA2, and its role in telomere maintenance is supported by its coexpression in tissues with high telomerase activity, its localization at telomeres, and by the telomere lengthening that follows its overexpression in cultured cells. In a different set of studies presented by Karina Porter (from the laboratory of Tracy Bryan, Children's Medical Research Institute, Australia), evidence was provided that not all G4 conformations actually inhibit telomere extension by telomerase. Bryan's group had previously demonstrated that parallel tetramolecular G4s are a favored substrate for extension by *Tetrahymena* telomerase [30]. Noting the conservation of key residue required for this activity, the team tested *in vitro* if this might also be true for human telomerase. Indeed, applying several approaches, including using strontium to assemble a particularly stable tetramolecular substrate, Porter nicely demonstrated that the human enzyme robustly extended several parallel stranded G4 substrates. Given the apparent capacity of human telomeres to fold into different G4 conformations, and that various small molecule ligands may have differential abilities to target and stabilize these conformations, efforts to develop anti-telomerase chemotherapies will need to keep these considerations in mind.

In the yeast genome, a significant subset of potential G4-forming sites is bound by the helicase Pif1, as presented by Katrin Paeschke (Zakian laboratory, Princeton University, USA). Paeschke presented her recently-published work demonstrating that Pif1 promotes replication through these G4s [31], as well as more recent work characterizing the *in vitro* G4 binding capabilities of the Pif1 protein. Pif1 has remarkably high binding affinity and specificity for G4 DNA, apparently many times higher than other known G4 helicases such as Sgs1 or WRN. The role of Pif1 in unwinding G4s and hence preventing instability of a G-rich human minisatellite sequence (CEB1) inserted in the yeast genome had previously been demonstrated by the laboratory of Alain Nicolas (Université Pierre et Marie Curie, France) [32]. At this conference, Nicolas presented further characterization of the mechanism of G4-induced instability of CEB1. Consistent with a role for Pif1 in facilitating replication of G-rich sequences, Nicolas's group was able to detect X-shaped DNA structures on 2D gels, indicative of replicative stalling within the CEB1 sequence. Unexpectedly, instability occurs only if it is the leading strand template that is G-rich, leading to a model in which G4 formation blocks progression of polymerase on the leading but not the lagging strand [33].

Guanine-based structures are an attractive material for semiconductors, nanoelectronics and bionanotechnology because guanines have the lowest ionization potential among the four DNA bases (allowing for efficient charge migration) and their arrangement in quadruplex or other extended structures allows for an excellent **π**-overlap and improved conductivity as compared to canonical duplex DNA. In addition, G4-based structures are resistant to heat, denaturation and DNase, and guanines are amenable to facile chemical modification. Gian Piero Spada (Universita di Bologna, Italy) described methods by which lipophilic derivatives of guanosine (LipoG) can be induced to switch between G-quartet arrangements and linear polymers (G-ribbons) of potentially unlimited length [34]. Several external stimuli strongly and reversibly shift the equilibrium between the two forms, including variation in alkali metal concentrations, exposure to light in the case of a LipoG possessing a photoactive unit at C8, or changes in solvent polarity in the case of a LipoG coupled to terthiophene. The last case is of particular interest because polythiophenes are excellent semiconductors. A related set of studies was presented by Irena Drevensek Olenik (J. Stefan Institute, Slovenia) who examined surface assemblies of LipoG derivatives on water or in Langmuir–Blodgett films. In comparison to other nucleosides, guanosine-based molecules had uniquely low critical surface pressures and better reversibility of surface area changes under varying surface pressures [35]. Furthermore, the types of surface assemblies could be adjusted by varying the number of alkanoyl tails. These approaches support the use of guanine derivatives in the controlled and flexible assembly of nanomaterials.

Irit Lubitz (from the laboratory of Alexander Kotlyar, Tel Aviv University, Israel) presented her work on self-assembly of a conjugate between Ag or Au nanoparticles (NP) and tetramolecular G4-wires of adjustable length modified at both ends with phosphorothioate groups that could be anchored securely into NPs [36,37]. Using this strategy, a variety of intricate arrangements could be achieved in a highly controlled fashion. As proof of principle, two or three Ag–NPs were connected together *via* a G-wire [37]; furthermore, stable G4-coated Au–NP were prepared and arranged into flower shapes [36]. NP–G4 conjugates could find application in detection and assembly techniques that are based on reliable attachment of the NPs to metal surfaces (for example to measure DNA conductivity) or could be utilized for laser photothermal therapy and diagnostics.

A practical approach to G4 applications in nanotechnology was presented by Wolfgang Fritzsche (Institute of Photonic Technology, Germany). His lab utilizes DNA nanostructures for preparation of biosensors using electrical field-based integration techniques. The binding of G4-specific ligands to G4-based nanostructures positioned within electrode gaps changes the resistance in the surface region, which could be easily measured and used as a sensor of

ligand presence. Another type of sensor consists of a noble metal plasmonic nanoparticle modified with G4 DNA. Binding of a G4-specific ligand to the particle surface changes the surface plasmon resonance, thus signifying interaction. While DNA-based nanomaterials are becoming widely spread, their integration into existing nanodevices is rather challenging but holds great promise for the future manufacturing of optoelectronic devices.

Liliya Yatsunyk (Swarthmore College, USA and Université de Bordeaux, France) described a novel approach for harnessing the stability of G4 structures in the assembly of nucleic acid-based nanomaterials. The inherently low specificity of G–G pairing, along with the slow kinetics of tetramolecular quadruplex assembly, limits the potential to easily form defined nanostructures. Dr. Yatsunyk cleverly circumvented these obstacles by extending a central G-quadruplex core with three parallel DNA duplexes each assembled *via* unique patterns of base pairing. These hybrid structures were formed in a highly specific manner with rapid kinetics and used to build one-dimensional nanowires. The self-assembling hybrid structures potentially could be derivatized with a variety of modifiers to generate novel probes, structural scaffolds, and nanomachines.

Jean-Louis Mergny (Université de Bordeaux, France) treated the meeting participants to a sampling of his laboratory's 2011 vintage (a very good year!). Highlights included the following. 1) Ongoing efforts to understand how primary sequence dictates G4 assembly and stability, including >10-fold enhancement in tetramolecular formation rates *via* 8-methyl-2′-deoxyguanosine modification of the first guanine within a G-run [38], demonstration that mirror-images of natural G4s can form from L-DNA [39], and a reminder that the bioinformatic algorithms commonly used to predict intra-molecular G4-forming potential are neither fully sensitive (e.g. a loop may be longer than 21–30 nt, provided the other loops are short) nor specific (e.g. runs of cytosines in intramolecular loops can be inhibitory) [40]. 2) Analyses of the binding mode and selectivity of small molecule ligands for G4s. A fluorescence-based assay of thiazole orange displacement from different G4 and duplex targets was used to test fifteen different ligands, and although some showed good selectivity *vs.* duplex DNA, none showed dramatic preferences for particular G4 folds [41]. Furthermore, SELEX was used to identify optimal targets of a highly G4-selective pyridocarboxamide ligand, but only a minority of the selected aptamers had clear G4-forming potential [42], cautioning assumptions about the absolute specificity of small molecule ligands especially in the context of the binding sites available in the whole genome. 3) Synthesis and characterization of novel G4 ligands, including a family of 2,4,6-triarylpyridines, and demonstration that a compound's capacity to stabilize different G4s correlates with its ability to stabilize independent of whether K^+ or Na^+ is the ambient cation. In addition, efficient G4 stabilizers generally demonstrate significant cytotoxicity [43]. The Mergny laboratory thus continues as a major epicenter for progress in the area.

As quadruplex-based DNA makes its way into the world of nanomaterials, intrinsic optical properties of guanines and G4-based structures are of great interest. Pascale Changenet-Barret (Laboratoire Francis Perrin, France) presented a combined experimental and theoretical study of the fluorescent properties of long G-wires (~800 tetrads) in comparison to monomeric chromophore dGMP [44]. The average fluorescence lifetime of G-wires is about 120 times longer than that of the monomer and is influenced by the type of cation. The major emitting species in G4 wires are exciton states, which are significantly delocalized compared with duplexes due to structural rigidity of the G4-core. Future work in this area will focus on understanding the effects of quadruplex length and the type of cation on fluorescent properties of G4 structures as well as mechanisms of excitation transport. This work could lead to improved design of guanine wires with desired optical properties for applications in optoelectronic devices.

Progress and development in the G4 field would benefit greatly from reliable methods for detection of G4 structures *in vivo*. In principal, photodetection could be achieved using the fluorescence of the quadruplex itself or the fluorescence of an interacting ligand. In the former case the use of unmodified guanines as fluorescent probes would provide the easiest and most physiologically relevant approach toward quadruplex detection but is hampered by the low quantum yield of quadruplexes as was demonstrated by Changenet-Barret above. To overcome this, the laboratory of Nathan Luedtke (University of Zürich, Switzerland) added the following substituents to the 8-position of 2′-deoxyguanosines using phosphoramidite chemistry: 2-pyridyl (2PyG [45]), 2-phenylethenyl (StG), or 2-(pyrid-4-yl)-ethenyl (4-PVG [46]). Luedtke demonstrated that these modified guanosines are highly sensitive and reliable fluorescent probes for G4 detection at concentrations as low as 250 pM. These probes could be incorporated into G4 structures with minimal perturbation of G4 fold and stability as was also demonstrated by Anh Tuân Phan who used different modification at the 8-position (bromo-, methoxy-, amino-, and oxo-) [47]. 2PyG, StG, and 4-PVG have high molar extinction coefficients and high quantum yields that are not quenched upon their incorporation into G4 structures. Impressively, StG has 10–100-fold higher quantum yield than *any* reported guanosine derivative in DNA. The fluorescence properties of these modified guanosines are highly sensitive to the local structural environment. Luedtke demonstrated that higher fluorescence intensities result from the energy transfer reactions within G4s as compared to the same DNAs when unfolded or in duplex structures. DNA with labeled guanosines will be useful for understanding factors impacting the equilibrium between G4 (and possibly *i*-motif) and duplex DNA.

Luedtke also discussed possible approaches to G4 detection *in vivo* using G4-specific fluorescent ligands. Zn(II) phtalocyanine, ZnPc, is the best photo behaved molecule, whose cellular permeability can be substantially improved by its modification with guanidinium [48]. Therefore ZnPc is a promising platform for further ligand modification where both the metal and aromatic system can be adjusted. Although the metal has little effect on affinity and specificity of the ligand, it does have a substantial effect on dark toxicity and on phototoxicity, which may be useful in the development of anti-cancer drugs. Another ligand that might prove useful for *in vivo* detection of G4 structures is thiazole orange reported recently by Kotlyar's group to provide efficient and selective staining of long G4 wire structures (and also triplexes) over single stranded and duplex DNA [49].

Vyacheslav Filichev (Institute of Fundamental Sciences, Massey University, New Zealand) and Erik Pedersen (University of Southern Denmark, Denmark) use intercalating nucleic acids (INA) and twisted intercalating nucleic acids (TINA) to modulate the properties of G4-forming oligonucleotides in order to achieve therapeutically desirable effects in several biological systems. If inserted between runs of guanines, both INA [(R)-1-O-(1-pyrenylmethyl)glycerol] and TINA [(R)-1-O-[4-(1-pyrenylethynyl)-phenylmethyl] glycerol] can enhance triplex-forming potential of G-rich sequences leading to modulation of gene expression *via* sequence-specific targeting [50,51]. This strategy was used to modify an oligonucleotide targeted to the KRAS promoter, and downregulate transcription of this oncogene [50]. Conversely, placement of the pyrene moieties at the ends of runs of guanines can *stabilize* G4s *via* stacking interactions. The resulting G4-forming oligonucleotides can be introduced into cells to act as "G4-decoys", sequestering transcription factors that would normally bind to the corresponding genomic quadruplexes. This strategy was applied to mimic G4s that act as transcriptional repressors in the HRAS promoter and resulted in downregulation of HRAS transcription and inhibition of proliferation of prostate and bladder cancer cell lines [52,53]. Both destabilization [51] and stabilization [54] of G4s by INAs and TINAs have also been used to target sequences corresponding to HIV-1 DNA, resulting in reduced intracellular replication of the virus [54].

Jussara Amato (Università degli Studi di Napoli Federico II, Italy) described another way of regulating gene expression through the use of peptide nucleic acid (PNA) probes that target a G4-centered structure without disrupting the core quadruplex scaffold [55]. She and her colleagues targeted a sequence immediately upstream of the c-Kit oncogene promoter that is unique in the human genome. This sequence forms a distinctive parallel intramolecular fold in the presence of K^+ and NH_4^+, raising the possibility that it could be targeted in a highly selective fashion. Several PNA probes designed to bind the stem and loop regions were identified. These probes can either bind and stabilize the G4 fold, or cause its unwinding, depending on the stabilizing cation. Thus it may be possible to use PNA or perhaps other ligands to selectively target G4s and regulate gene expression.

The high stability of G4 nucleic acid structures and their ability to selectively recognize small molecules or cations suggest potential uses for these molecules in biosensing applications. The laboratory of Shigeori Takenaka (Kyushu Institute of Technology, Japan) has been exploiting the cation-binding abilities of G4s to develop molecular probes for cellular cations, particularly potassium [56]. At this conference, Takenaka presented his group's progress in taking this technology from an *in vitro* context into cells. A TBA sequence conjugated with a peptide chain containing fluorescent dyes at its termini shows a change in fluorescence upon G4 formation that is highly specific for K^+ [57]. Upon introduction into human cells in culture, however, such G-rich sequences are transported to the nucleolus and cause cellular toxicity. The clever addition of a nucleolar export sequence to the probe circumvented this problem and allowed measurement of intracellular K^+ concentrations; controls in the presence of known potassium-lowering drugs validated the technique.

The laboratory of Janez Plavec (National Institute of Chemistry, Slovenia) is well known for their use of ^{15}N NMR in elucidating structural diversity of G4s and dynamics of cation movements in the central cavity of G4 DNA by using $^{15}NH_4^+$ in place of K^+. Plavec presented new insights into (dTG_4T) [58] and $d(G_3CT_4G_3C)_2$ [59] structures. One of the simplest quadruplex systems, $(dTG_4T)_4$ and its uridine-substituted analogs formed quadruplexes with 4 G-tetrads, 4 G- and 1 T-tetrad (at the 5′ end) or a head-to-head dimer, each with unique cation dynamics as a function of the precise oligonucleotide sequence and the nature of the stabilizing cation. A bimolecular quadruplex formed by $d(G_3CT_4G_3C)_2$ is stabilized by one ammonium ion and two water molecules (and not by three cations), contrary to the common belief that cations are required for G-tetrad stabilization. Both the ammonium and water are in fast exchange with bulk solvent indicating that this G4 structure is highly flexible and able to undergo partial opening. Thus cation movement is directly correlated with quadruplex dynamics and structure, and this type of knowledge will be crucial for understanding biological functions of quadruplexes.

The search for novel and selective small molecule G4 ligands constituted a major part of the work presented at this meeting. Ligands were designed to serve as potential G4-binding pharmacological agents (targeting oncogenes, telomeric DNA or messenger RNA) or probes for detection of G4 existence and function in biological settings (see Luedtke, above). Gary Parkinson's team from the University of London in UK, in collaboration with that of Stephen Neidle, focuses on obtaining high resolution structures of ligands bound to telomeric G4s by X-ray crystallography, in order to inform the design of potential anti-cancer drugs with improved selectivity and affinity. At the time of the conference, the team had solved the structures of 12 ligand-G4 complexes, all of which contained parallel-stranded G4 topology. Parkinson reported the structure of naphthalene diimide ligands bound to human telomeric G4. The ligands stacked over the terminal 3′ G-quartet surfaces, with extensive π–π contacts, leading to a 1:1 G4:ligand stoichiometry [60]. Ligand side-

chains were positioned within the G4 grooves, making extensive contacts with the DNA phosphate groups. Combination of π–π stacking and groove binding is consistent with a high level of specificity of naphthalene diimide ligands for G4 *vs.* duplex DNA and suggests strategies for further improvements in affinity and selectivity of ligands, specifically by targeting grooves.

Other applications of G4-specific ligands for quadruplex separation or as structural switches were discussed by Ta-Chau Chang (National Taiwan University, Taiwan) who reported synthesis and characterization of 3,6-Bis(1-methyl-4-vinylpyridinium)-9-(12′-bromododecyl)carbazole (BMVC-12C-Br) [61]. This molecule is a derivative of BMVC, a previously reported G4 stabilizer and fluorescent probe. Similar to BMVC, BMVC-12C-Br fluoresces weakly in its free state, but its fluorescence increases by at least one order of magnitude upon binding to G4. BMVC-12C-Br is amphiphilic and could be used to form a water/oil emulsion. Dr. Chang demonstrated a novel method based on emulsion-induced filtration with the aid of BMVC-12C-Br to identify and separate G4 structures from duplex DNA and parallel G4 structures from antiparallel or hybrid forms. He also discussed how fluorescence imaging microscopy can be used for cancer diagnosis due to differential accumulation of BMVC and its analogs in cancer (preferentially in the nucleus and mitochondria) compared with normal (in the lysosomes) cells [62]. In addition, Dr. Chang demonstrated that combining PEG unit or *n*-ethylene glycol with known G4 stabilizers leads to new molecules capable of inducing controlled structural switches between different G4 topologies and increasing the melting temperature of human telomeres by more than 45 °C. This work provides further evidence that the local water structure plays key role in inducing conformational change of human telomere [63].

Marie-Paule Teulade-Fichou (Curie Institute, France) presented a comprehensive overview of G4 ligand development and started her talk with a rather philosophical question: What is a perfect G4 ligand? She summarized lessons learned from the study of three successful ligand series developed by her laboratory, including quinacridines, bisquinolinium compounds, and metal complexes of terpyridine. Quinacridines with trinaphthylene-like polyaromatic cores are excellent G4 stabilizers but have poor to moderate selectivity [64]. They were used to determine the separate contributions to G4 stabilization of π–π core stacking and side-chain interactions. Interestingly, a quinacridine ligand without side-arms, TrisQ, displayed the best stabilizing ability and selectivity suggesting that the side arms, while potentially important for establishing some G4 interactions, could also participate in binding to duplex DNA, thus lowering specificity. On the other hand, bisquinolinium compounds with a core of either pyridine dicarboxamide (e.g. 360A) or of phenanthrolinedicarboxamide (e.g. PhenDC3), are highly selective DNA and RNA G4 ligands. Teulade-Fichou demonstrated the ability of these ligands to interact with G4 RNA *in vitro* and in cellular environments leading to the inhibition of gene expression, which correlated with the stability of RNA quadruplex [40,65].

Currently, the gold standard ligand for G4 specificity and affinity is telomestatin, but the labor-intensive nature of its synthesis precludes its wide application. Teulade-Fichou reported on a synthesis of a telomestatin analog, TOxaPy, which binds exclusively to the antiparallel G4 conformation of human telomeric DNA in Na^+ buffers and does not bind to its K^+-induced parallel fold [3]. TOxaPy is the first reported ligand that displays such highly desired specificity for a particular G4 conformation. Interestingly, N-methyl mesoporphryin IX displays preferential selectivity complementary to that of TOxaPy. This ligand recognizes parallel telomeric G4 but not its antiparallel conformation, as was demonstrated by J. Nicoludis (Poster 53, *Interactions of Human and Yeast Telomeric G-quadruplex with N-methylated porphyrins*, Swarthmore College, USA). Ligands with broad selectivity for G4 DNA could be used for general G4 detection, but to understand the roles of G4 structures in

biology and to target particular G4 structures for therapy (e.g. in cancer) will require ligands with very narrow selectivity for specific G4 folds.

The closing lecture was delivered by Anh Tuân Phan who summarized the current state of knowledge of DNA and RNA G4 architectures and stability both for individual quadruplexes and their higher order assemblies. Phan reported on his lab's newest NMR structural investigations of G-rich DNA sequences capable of inhibiting HIV-1 integrase [66,67]. HIV integrase is responsible for inserting viral DNA into the genome of human cells, which is required for the viral life cycle. Examined sequences adopt dimeric structures involving two intramolecular parallel-stranded quadruplexes stacked *via* a 5′-5′ interface. Dimer formation was confirmed by NMR, electrophoretic mobility, UV–vis melting or by the observation that addition of 1–3 thymines to the 5′ but not to 3′ end of DNA sequences diminished (or abolished) dimer formation. Phan proposed a new and simple analysis of concentration dependent melting curves (assuming melting is reversible) in order to obtain reliable information about strand stoichiometry [66].

Although structural information for G-rich DNA oligonucleotides with four guanine stretches that form a single quadruplex is abundant, less is known for longer repeat sequences due to their substantial structural heterogeneity and complexity. Phan's laboratory investigated sequences with 5–7 TTAGGG repeats and asked questions about the dominant structural fold under conditions when the number of guanine repeats exceeds that required for a single G4 formation, but is insufficient for the formation of two G4s. $d(TTAGGG)_{5–7}$, for example, could form a quadruplex with hybrid (3 + 1) topology and a 9–21 nt propeller loop [68]. The long loop was shown to diminish G4 stability, but could potentially be used as a telomere-specific recognition element in drug development, and its targeting by a complementary DNA oligonucleotide was demonstrated. Once more than 8 guanine repeats are present and more than one G4 structure is possible, the questions become *whether* individual G4s interact with each other, *how* they interact, and *what* is the effect of their interaction on the overall DNA stability. Viktor Viglasky (P.J. Safarik University, Slovakia) attempted to answer these questions using studies of 4–12 tandem repeats occurring in telomeres or in the insulin-linked polymorphic region *via* UV and CD spectroscopy, and *via* temperature-gradient gel electrophoresis. Interestingly, tandem G4s on the same DNA strand do show signs of interaction but rather than stabilizing one another, may in fact encourage one another to melt, at least under conditions of low ionic strength and in the absence of high molecular crowding [69].

Current knowledge of G4 RNA stability and physiological topology is limited. So far, all reported G4 RNAs adopt propeller-type parallel-stranded geometry regardless of buffer conditions, possibly due to the preference of RNA for *anti*-glycosidic conformation. There is a stunning similarity between the parallel DNA and RNA G4 topologies. High resolution structural data exist only for human telomeric RNA (TERRA) consisting of two repeats. Phan's laboratory investigated physiologically relevant long TERRA sequences (up to 96 nt) *via* NMR and RNase T1 cleavage assays and suggested that they form "beads-on-a-string" structures, where each bead consists of parallel G4 structures [70]. Adjacent G-quadruplexes may interact *via* 5′-5′stacking, as was observed in a previous high resolution NMR structure of 10 nt TERRA sequence lacking flanking residues at the 5′ end [71].

3. Concluding remarks

Just as G4s are proving themselves useful in the construction of nanomaterials, it is not surprising that nature also appears to have appropriated these structures for various uses. Although findings presented at the conference or published recently strongly indicate an impact of G4 structures on biological processes including DNA replication and

transcription, mRNA splicing and translation, DNA recombination and repair, and telomere function, many fundamental questions remain. Among these are 1) To what extent are G4s essentially pathologic structures that interfere with the normal functions of nucleic acids or rather are regulatory elements subserving positive functional roles? 2) Do G-quadruplexes form only transiently or are they stable structures that exist at high levels within the context of flanking duplex DNA? 3) How is G4 formation regulated by chromatin, and in turn how does G4 formation impact chromatin structure? 4) Do G4 structures have intrinsic activities (e.g. by impacting supercoiling, chromatin compaction or the exposure of ssDNA), or are most G4 functions mediated by the binding of proteins or other factors? and 5) Are different subclasses of G4s targeted by distinct subsets of proteins? Answers to these questions are likely to be as complex as they are exciting, and will rely on improved and more direct methods for detection of G4s *in vivo*. Furthermore, the promising approaches to using G-quadruplexes as diagnostic and therapeutic tools that have emerged recently demand more detailed understanding of how G4s impact natural biology, so that particular G-quadruplexes and biological processes can be targeted selectively. The recent inroads made in the design of G4-selective small molecule ligands and in understanding the structural bases for G4 folds and their interactions with such ligands and with proteins will be invaluable in the future investigations of G4 biology and in the design of selective therapeutic agents. Finally, impressive advances are occurring in the application of G4 structures and assemblies to nanotechnology. Construction of functional G4-based nanodevices with desired mechanical, optical, and electronic properties is only possible if G4 assemblies can be made with high control over the speed of their formation and their final structure. This control may be achieved *via* combination of G4s with other nanomaterials, such as metal nanoparticles or DNA origami. It seems likely that G4-based structures will find new applications in subfields of biology, chemistry, physics, and engineering, and we look forward to hearing about these developments at the next G4 meeting scheduled to be held in Singapore in 2013.

Acknowledgments

The authors would like to thank the organizers A. Randazzo, C. Giancola, L. Petraccone, B. Pagano, C. A. Mattia for such a motivating and inspiring meeting and all the presenters for their high quality talks and for the comments on the manuscript. Finally, we would also like to thank University of Naples “Federico II”, University of Salerno, TA Instrument, Simadzu and COST MP0802 action coordinated by Lea Spindler for making this conference possible. We apologize to those whose work could not be included owing to space limitations.

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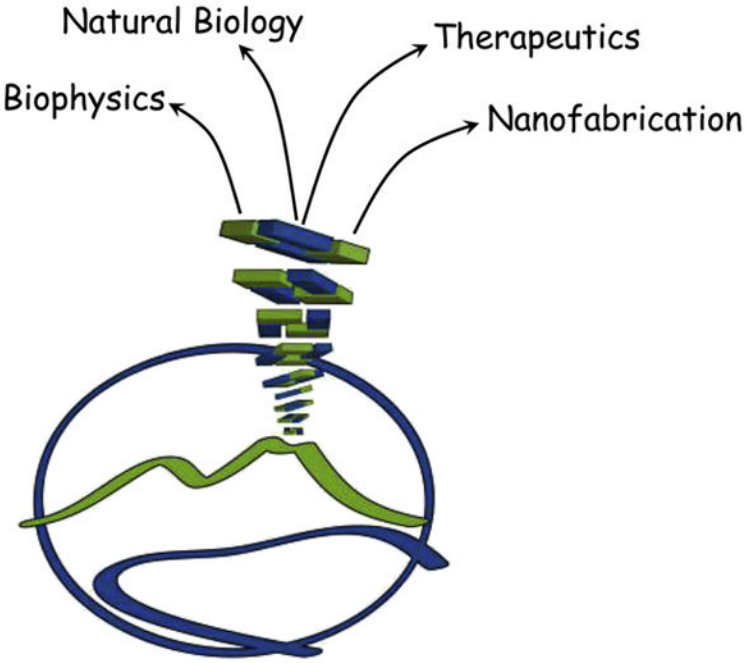

Fig. 1.
G-quadruplex based approaches are making major contributions to diverse fields of investigation.