Development of metal complexes for specific targeting G-quadruplexes and the application studies

He, Lei

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**DEVELOPMENT OF METAL COMPLEXES FOR
SPECIFIC TARGETING G-QUADRUPLEXES AND
THE APPLICATION STUDIES**

HE LEI

SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

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GQ & THE APPLICATION STUDIES**

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Table of Contents

<i>Acknowledgements</i>	I
<i>Table of Contents</i>	III
<i>Abstract</i>	VI
<i>List of acronyms and abbreviations</i>	IX
CHAPTER 1. Introduction	1
1.1 The Nature of G-quadruplex	2
1.1.1 The General Structures and Topologies of G-quadruplexes	2
1.1.2 Functionalities of G-quadruplexes	5
1.2 Binders of G-quadruplexes	10
1.2.1 Biomolecules	11
1.2.2 Organic Compounds	11
1.2.3 Metal Complexes	14
1.3 Probes for G-quadruplexes	19
1.3.1 GQ-specific Antibody	20
1.3.2 Organic Dyes	21
1.3.3 Luminescent Metal Complexes	22
1.3.4 Combination of GQ Recognition Moiety and Luminescent Emission Molecule	22
1.4 The Motivation and Significance of the Study	24
1.5 References	26
CHAPTER 2. Octahedral Ruthenium Complexes that Selectively Stabilize G- quadruplexes	34
2.1 Introduction	35
2.2 Results and Discussion	37
2.2.1 Synthesis of Novel Octahedral Ruthenium Complexes	37
2.2.2 Ru1 Selectively Stabilizes and Binds to GQ Structures	40
2.2.3 Ru1 Distinguishes the Topology of G-quadruplexes	43

2.2.4 The Significance of Two Ammonia Moieties on z-Axis	45
2.2.5 Molecular Docking of Ru1 on Different GQ Structures	47
2.2.6 Potency Against Telomerase and Cytotoxicity Towards Cancer Cells	48
2.3 Conclusion.....	50
2.4 Experimental section	51
2.5 References	57
<i>CHAPTER 3. Aza-bridged Bisphenanthrolinyl Pt(II) Complexes: Efficient Stabilization and Topological Selectivity of Telomeric G-quadruplexes</i>	<i>60</i>
3.1 Introduction	61
3.2 Results and Discussion.....	63
3.2.1 Synthesis of [Pt(bpa)] ²⁺ Complexes with Planar Structure	63
3.2.2 High Efficiency of Pt Complexes on Stabilizing Both DNA and RNA GQs....	65
3.2.3 Stabilizing Preferences of Pt Complexes Toward Antiparallel G-quadruplex Topology.....	68
3.2.4 Molecular Docking of Pt Complexes on Different GQ Structures	69
3.2.5 Effects of the Alkyl Side Chains on Distinguishing DNA Structures	71
3.2.6 Potential Cytotoxicity Towards Cancer Cells.....	72
3.3 Conclusion.....	73
3.4 Experimental Section	73
3.5 References	78
<i>CHAPTER 4. [Pt(bpa)]²⁺ Complex: a GQ ‘Light-switch’ for Visualizing and Tracking the Folding Dynamics of RNA GQ in Live Cells</i>	<i>82</i>
4.1 Introduction	83
4.2 Results and discussion.....	84
4.2.1 Synthesis and Characterization of [Pt(bpa)] ²⁺ Complexes	84
4.2.2 [Pt(bpa)] ²⁺ Selectively Stabilize and “Lighted-Up” by GQ Structures	86
4.2.3 Production of a Triplet-state Emission in [Pt(bpa)] ²⁺ upon Binding onto GQ ..	90
4.2.4 Contribution Factors for the Luminescence Turn-On of [Pt(bpa)] ²⁺ by GQ	92

4.2.5 Imaging and Tracking the Un/folding Dynamics of GQ in Live Cells by Using [Pt(bpa)] ²⁺	97
4.3 Conclusion.....	99
4.4 Experimental section	100
4.5 References	104
<i>CHAPTER 5. G-quadruplex Nanowires Direct the Efficiency and Selectivity of Electrocatalytic CO₂ Reduction</i>	106
5.1 Introduction	107
5.2 Results and discussion.....	108
5.2.1 Preparation and Characterization of GQ Nanowire.....	108
5.2.2 Electroreduction of CO ₂ over a Co ^{II} Pc-tsGQwire Decorated Electrode	111
5.2.3 GQ Nanowire Steer the Efficiency and Selectivity of CO ₂ Electroreduction .	113
5.2.4 Electron Conductivity of DNA Structures and Improved Orientation of Metal Complexes Contributed to CO ₂ Electroreduction	117
5.2.5 GQ Nanowire as a Universal Host Scaffold for Various Metal Complexes to Improve Their Performance in CO ₂ Electroreduction	121
5.3 Conclusion.....	123
5.4 Experimental Section	124
5.5 References	128
<i>Summary and Recommendations</i>	131

Abstract

The non-canonical G-quadruplex (GQ) structure are constructed by the assembly of guanine-rich DNA/RNA sequences. Similar to the canonical double helix structure; GQ not only involve in a plethora of significant biological processes and represent potentially important target in drug development, but also exhibit some technological functionalities. Metal complexes with extensive aromatic ligands could bind GQ with high specificity and demonstrate unique features in biomedicine, biotechnology and nanotechnology. In this thesis, several series of metal complexes were developed as binders/stabilizers to target specific GQ topology in various cancerous cell lines. The complexes express excellent anticancer potency and GQ-dependent luminescence for intracellular tracking of RNA GQ. The unique binding and electronic communication between metal complexes and GQ nanowires were applied to the electrochemical reduction of CO₂. Unprecedented high efficiency and selectivity of metal-dependent CO₂ reduction was realized on GQ nanowire decorated electrodes.

Chapter 1 reviewed the biological and nanotechnological functionalities of GQs and summarized the binders currently reported for realizing GQ stabilization and imagination. Biomolecules, some organic compounds and inorganic complexes as GQ binders were elaborated in this chapter. The chapter disclosed the significance of using metal complexes for GQ-targeting anticancer therapy by comparing the merits and drawbacks of each type of GQ binders.

Chapter 2 reported a series of noncanonical octahedral Ru(II) complexes, containing an x-y planar tetradentate and two *trans*-z-axis ammonia ligands. These compounds realized efficient stabilization on G-quadruplexes *via* π -stacking between the aromatic planar ligands and external G-tetrad layer. In addition, two ammonia group on z-axis

displayed unique interaction within GQ ion channel, which bestowed both excellent binding against duplex DNA and remarkable selectivity to antiparallel GQ topologies.

Chapter 3 reported the design of two platinum complexes with the planar configuration as GQ stabilizers by using an *aza*-bridged *bis*-(1,10-phenanthroline-2-yl)amine (bpa) derivatives as coordination ligands. These two phenanthrolines moieties in bpa could enter into a flat plane prior binding onto nucleic acids, and therefore endow the two Pt complexes with a remarkable stabilization effect on DNA/RNA GQs. The introduction of the alkyl tail from *aza* linkage enabled the complexes to distinguish GQ structures in view of the topological folding structures and improved their binding resistance to duplex DNA.

In Chapter 4, a series of $[\text{Pt}(\text{bpa})]^{2+}$ complexes with free *aza*-NH moiety were developed. These complexes exhibited large Stokes shift, long lifetime, high quantum yield and remarkable luminescence turn-on signals specifically upon binding to G-quadruplexes. It is the first example of using the inorganic light-switch probe for imaging RNA GQ and tracking their un/folding dynamics in live cells.

In Chapter 5, a submicrometer-long G-quadruplex nanowire was constructed by self-assembly of guanine tetranucleotides. It was used to host several metal complexes and further to mediate electron transfer processes in the electrochemical reduction of CO_2 , which was catalyzed by these complexes. Although DNA as a medium for electron transfer has been widely used in photolytic processes, it is seldom applied to the dark reaction of CO_2 reduction by now. Herein, the GQwire modified electrode efficiently enhanced both Faradaic efficiency for CO_2 reduction and total current density, compared with bare metal complex decorated electrode. The high efficiency and selectivity of electrocatalytic CO_2 reduction were attributed to the unique binding of metal complexes on G-quadruplex and electron transfer mediated by GQ nanowire to achieve efficient redox cycling of catalytic centers on the electrode. Therefore, the study provides practical

information on the potential of using conductive biomacromolecules to merge the gap between light and dark reactions in the artificial photosynthesis.

List of acronyms and abbreviations

[Bmim]Tf ₂ N	1-Butyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide
AFM	Atomic force microscopy
bpa	<i>N,N</i> -bis-(1,10-phenanthroline-2-yl)amine
bpy	2,2'-Bipyridine
CD	Circular dichroism
C _{dl}	Double-layer charging
CP	Carbon paper
Cy5-TS	5'-Cy5 tagged (TAACCC) ₆ sequence
dmbpa	<i>N,N</i> -bis-(4,7-dimethyl-1,10-phenanthroline-2-yl)amine
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dppz	Dipyrido[3,2- <i>a</i> :2',3'- <i>c</i>]phenazine
EDTA	Ethylenediaminetetraacetic acid
ESI-MS	Electrospray ionization mass spectrometer
ET	Electron transfer
FBS	Fetal Bovine Serum
FE	Faradaic efficiency

FID	Fluorescent intercalator displacement
FISH	Fluorescence <i>in situ</i> hybridization
FRET	Fluorescence resonance energy transfer
G	Guanine
GC	Gas chromatography
GMP	Guanosine monophosphate
GQ	G-quadruplex
G-tetrad	Guanine quartet
HPLC	High performance liquid chromatography
hTERT	Human telomerase reverse transcriptase
LSV	Linear sweep voltammetry
MLCT	Metal ligand charge transfer
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
Pc	Phthalocyanine
phen	Phenanthroline
ppy	2-Phenylpyridine
TA	Transient absorption
TBE	Tris-borate-EDTA
TEAA	Triethylammonium acetate

TERRA	Telomeric RNA
T_m	Melting temperature
TMPyP4	Tetra- <i>N</i> -methyl-4-pyridyl porphyrin
TMS	Tetramethylsilane
TO	Thiazole orange
TRAP	Telomerase Repeat Amplification Protocol
Tris	Tris(hydroxymethyl)aminomethane
tsGQwire	Tetrastranded GQ nanowire
UV-vis	Ultraviolet-visible

CHAPTER 1

Introduction

1.1 The Nature of G-quadruplex

1.1.1 The General Structures and Topologies of G-quadruplexes

The structure of deoxyribonucleic acid (DNA) has been commonly considered to form a double helix topology, in which two complementary strands non-covalently assembled and coiled around each other.^[1] However, in 1962, a special DNA structure called G-quadruplex (GQ) was hypothesized and reported by Davies and co-workers.^[2] In GQ, four guanine residues were arrayed at each corner of a square and associated nose to tail through Hoogsteen hydrogen bond to construct a stable G-tetrad layer (Figure 1.1). Due to the strong Van der Waals force and π -stacking interaction between each G-tetrad, several G-tetrads are prone to linearly stacked on the top of each other and give rise to a multilayered 3D GQ structure. This unusual structure was later confirmed by Sen and co-workers in 1988.^[3] It was found happened in a certain single-stranded DNA sequence that comprised of tandem guanines (G) bases at physiological salt concentrations. From the crystal structure of GQ, one can see that the length and width of the G-tetrad layer that holds by Hoogsteen hydrogen bonds are much bigger than Watson-Crick base pairs in normal duplex DNA.^[4]

Figure 1.1 Structure of G-quadruplex.

With the subsequent exploring, Sen *et al* further reported and proposed several possible routes for constructing an intermolecular GQ structure from the G-rich single-

stranded DNA.^[5] Some monovalent alkali metal ions and divalent alkaline-earth metal cations can be used to stabilize GQ structure (Figure 1.1). They were believed to be located at the axial channel inside stacked G-tetrad layers. The order of their stabilization ability has been demonstrated as follows: $Mg^{2+} < Ca^{2+} < Ba^{2+} < Sr^{2+}$ and $Li^+ < Na^+ < Rb^+ < K^+$, which is generally associated with the size of these cations.^[6] The stability mainly comes from the interaction between cations and two adjoining G-tetrads, which producing a chelated octahedral structure. Due to its proper size, K^+ is located in the negatively charged central cavity between each pair of tetrads, coordinating with eight carbonyl oxygen atoms of adjacent G-quartets. The distance of formed K-O bond is constant and similar to the K-O bond from potassium and 18-crown-6.^[7] GQ structure obtained in K^+ conditions is much more stable than in other alkali conditions. For example, melting temperature (T_m) of GQ DNA in K^+ containing buffer was more than 20 °C higher compared with the same sequence in Na^+ condition. Different from the position of K^+ in GQ, Na^+ is inferred to have two binding modes,^[8] one is locating within the G-tetrad layer and the other is residing between adjacent G-quartets.

Compared with duplex DNA, these four-stranded GQs are topologically polymorphic and display great structural diversity. normally, the various structures and topologies of GQs are mainly attributed to the nature of compositions in the loop region as well as the solution conditions, such as cations, binders or molecular crowding conditions.^[4] GQs can be divided into intra- or inter- molecular folding structures that are constructed by one (unimolecular), two (bimolecular) or four (tetramolecular) G-rich DNA sequences, respectively.^[9] In general, potential intramolecular GQ (unimolecular) sequences can be simplified as $G_iX_jG_iX_kG_iX_lG_i$, in which i is the number of guanine within every curt G-tract; and X_j , X_k and X_l represent any combination from loop regions. Dimeric quadruplexes are usually consisting of two identical sequences $X_jG_iX_kG_iX_l$; while the building block of tetrameric quadruplexes is described as $X_jG_iX_k$.^[4] In addition,

GQ can be classified as parallel, anti-parallel and (3+1) hybrid topologies according to the strands polarities and the relative arrayed directions.^[10] As shown in Figure 1.2, loop in parallel GQ belongs to propeller conformation; whereas in anti-parallel topologies are lateral or diagonal fashion.^[4] In most cases, residues in the loop region can spontaneously build extra stacking or hydrogen bond to further stabilize GQ DNA structure. In parallel topologies, guanine glycosidic torsion angles can be characterized as *anti*-conformation, while they adopt both *anti* and *syn*-configurations in antiparallel quadruplexes with a given topology.^[4]

Figure 1.2 Loop region in different topologies of G-quadruplex.

Several biophysical methods have been commonly applied in GQ studies, such as circular dichroism (CD), gel electrophoresis, X-ray analysis and NMR (nuclear magnetic resonance) spectroscopy.^[4] X-ray crystal analysis and 2D NMR spectroscopy are used to provide a visualized 3D picture for GQ structure study. CD spectroscopy is a useful and rapid method to discriminate quadruplex topology because it consumes very little sample. Classic parallel and anti-parallel quadruplexes have their characteristic signals in CD spectrum. A positive signal peaked at around 260 nm accompanied with a negative signal peaked around 240 nm represents for parallel structure; while for anti-parallel topology, the typical positive and negative peaks are at ~290 and 260 nm, respectively.^[4] In addition, temperature unfolding (or folding) of GQs can offer several thermodynamic parameters on GQ stability. Melting process of GQ is often determined by ultraviolet (UV) or CD

spectrum. Besides, fluorescence resonance energy transfer (FRET) assay is much convenient for melting temperature (T_m) detection of several sequences simultaneously by employing double-labelled GQ DNA sequences.^[4]

1.1.2 Functionalities of G-quadruplexes

1.1.2.1 Important Biological Roles of G-quadruplexes

Considerable GQ structures have been found in certain regions from human genome, including telomeres,^[11,12] nuclear hypersensitivity element III₁ (NHE III₁),^[13,14] human vascular endothelial growth factor (VEGF)^[15,16] and human *bcl-2* gene.^[17] Recent studies reported telomeric RNA (TERRA), that comes from the transcripts of telomeric DNA (C-rich strand), can adopt GQ structure as well.^[18] The significant biological roles make GQ, as promising target for cancer treatment, attracts a great of attentions.

1.1.2.1.1 G-quadruplexes Structure Inhibit Telomerase Activity

Telomeric DNA is tandem repeats of G-rich DNA segment that located at the end of chromosomes from animals and plants cells.^[19] The sequence in different species showed a bit of difference. In vertebrate cells, it contains repeated d(TTAGGG) tracts that make up of the long (5-8 kilobases) single-stranded overhang of hundreds of bases at 3'-terminal.^[20] Unlike in human somatic cells, telomeric DNA sequences in *Arabidopsis*, *Tetrahymena*, *Oxytricha* and *Bombyx* are d(TTTAGGG)_n, d(TTGGGG)_n, d(TTTTGGGG)_n and d(TTAGG)_n, respectively.^[21-24]

It is worth mentioning that human telomeric DNA can form into several topologies.^[25] In sodium solutions, it exists as a basket GQ structure with TTA loops residing along diagonal and lateral directions, which belongs to typical anti-parallel conformation.^[20] On the contrary, the solid state telomeric structure that crystallized out from potassium solutions exhibits parallel propeller-type topology, in which four guanine columns are aligned toward the same direction.^[26] However, several studies reported the

structure of telomeric GQ determined in K^+ solution are of inconsistent with the solid-state.^[27,28] A hybrid structure in K^+ solution, with both propeller and lateral loops, was determined by NMR method. G-quadruplex exists as an equilibrium between folding and unfolding process in a certain solution condition. Hence, some additional factors can affect the equilibrium and make it prone to form a certain structure.

In human somatic cells, telomere is a protecting unit that capped on the end of chromosomes to endow them survive from genomic instability and breaking that caused by degradation or telomere end-end fusion (Figure 1.3).^[29] Controlling the length of telomere is essential for many biological processes, including cell cycling, cellular immortalization and tumorigenesis.^[30-32] Telomerase is the specialized enzyme that manage to add hundreds of telomeric repeats to the end of chromosomes to maintain the length of telomere and support normal cell proliferation.^[33] Telomerase activity in normal human somatic cells is too low to maintain the constant telomere length. So healthy cells display the conventional circle of growth. However, in cancer cells, more than ninety percent of the population exhibit fierce telomerase activity that induces malignant growth and immortalization. Stabilizing GQ structure will impede the binding of telomerase to telomeres, thus it has been observed with inhibited telomerase activity and shortened telomere under the impaired telomere repeat synthesis process (Figure 1.3).^[29,34]

Figure 1.3 Functions of telomeric DNA at the end of chromosomes.

1.1.2.1.2 Formation of G-quadruplexes Regulate Transcription of Oncogenes

It has been reported that near fifty percent of human genes contain potential GQ forming sequence, such as human and chicken β -globin genes,^[35,36] rat preproinsulin II gene,^[35] retinoblastoma susceptibility genes,^[37] adenovirus serotype 2^[38] and the *c-myc* gene.^[13] These sequences are located close to the promoter regions, which indicated the significant biological functions of GQ on the regulation of gene expression.

The G-rich sequence Pu27 in the NHE III₁ region, which located at the upstream of P1 promoter of *c-myc*, shows the inclination to adopt GQ structure.^[13,14] G-rich region in *c-myc* comprise of more than 4 consecutive G-strands and therefore result in the formation of several parallel GQ isomers that exist in dynamic equilibrium as a mixture.^[10,13] In fact, *c-myc* was the earliest recognized oncogene among Myc gene family. It is related to a great number of human cancers. *c-Myc*, the protein product, was reported with the function to regulate more than 10% of cellular genes and take part in many cellular activities, including cell adhesion, metabolism and apoptosis.^[4,39] This protein also has been demonstrated as a main activator of human telomerase reverse transcriptase (hTERT) catalytic domain from telomerase.^[40] Overexpression of *c-myc* is linked with many tumors, such as carcinoma of colon, cervical carcinoma and mammary carcinoma.^[10,41-42]

It has been reported that about 90% activation of transcription is regulated by NHE III₁ region in *c-myc*.^[43-45] However, the transcription will be repressed once it adopts GQ structure in this region (Figure 1.4).^[29] Studies also found the stabilization of GQ in NHE III₁ region could not only restrain the stimulation of *c-myc* transcription, but also down regulate *c-myc* expression, obstruct cell proliferation and cause delay of leukemia cell apoptosis (Figure 1.4).^[46-48]

In fact, except from the ability to inhibit telomerase activity and oncogene transcription, GQ structure in human somatic cell can regulate DNA replication and suppress translation as well.^[9] These biological functions eventually lead to genome

instability and aborted RNA transcription.

Figure 1.4 G-quadruplex in the promoter region influence on the transcriptional of certain oncogenes.

1.1.2.2 Nanotechnological Functions of G-wires

In 1910, the gelatinous assembly of guanosine monophosphate (GMP) solution have been found with unusual stability.^[49] As revealed by X-ray fiber diffraction studies, the assembly was comprised of tandem of stacked G-tetrads. The properties and the constituent modes of G-quadruplex made it as one of the most potential building units for constructing functional nanostructures. Gilbert and Sen discovered that d(A₄G₄) sequence can self-assembled into a superstructure instead of a tetrastranded GQ-[d(A₄G₄)]₄.^[50] In this DNA superstructure, each G-tetrad layer was arrayed in a head to tail orientation to form a consecutive line, and the poly-A part branched out along G-wire trunk. Henderson and Sheardy groups reported the extraordinarily stable polymers that assembled from d(G₄T₂G₄) sequence (Figure 1.5), with lengths ranged from 10 to 1000 nm under atomic force microscopy.^[51-54] The heights of these wires were around 2 nm and were accordant with G-tetrad diameter. These G-wires are 1D nanowire with a uniform height and width, but few bends/kinks. Erie and co-workers later constructed frayed-wires by using d(A₁₅G₁₅) to process self-assemble (Figure 1.5).^[55] The aggregated structures showed a height of 2.1 nm and lengths ranged from 5 to 200 nm, which are similar to the G-wires formed by d(G₄T₂G₄). After that, Kotlyar and Porath prepared long, monomolecular G-wires by separated out ploy(G) strand from the enzymatically synthesized double stranded

poly(G)-poly(C) DNA.^[56] The presence of divalent metallic cations will promote the formation of G-wire structures. Sr^{2+} was reported to induce the formation of G-wire by G-rich DNA sequences, $\text{d}[(\text{G}_4\text{T}_2)_3\text{G}_4]$ and $\text{d}[(\text{G}_4\text{T}_4)_3\text{G}_4]$.^[57] Ca^{2+} , as well as the molecular crowding conditions, can induce G-wire formation by telomere DNA sequences.^[58,59] The forming G-wires exhibited heat-resistant, mechanically stable properties, as well as the insensitivity to DNase degradation. Unlike double-stranded DNA, GQ structures are stable in more than 50% ethanol and other dehydrating agents. All these properties endow the GQ-based materials as desirable candidates that can exert their functions in biosensor and nanoelectronics development and nanotechnological field.

Figure 1.5 Illustration of higher-order structures constructed by association of G-rich DNA sequences. G-wires were obtained *via* the assembly of $\text{d}(\text{G}_4\text{T}_2\text{G}_4)$; while “frayed wires” are constructed by the assembly of $\text{d}(\text{G}_{15}\text{A}_{15})$ sequence.^[6]

Due to the lowest oxidation potential of guanine among the four deoxynucleotide bases, guanine-rich DNA sequences attracted great interests as functional building blocks in molecular electronics. G-wire structures were found with enough conductivity theoretically,^[60] which indicated that it can be used as nanoscale bioelectronic devices. Recently, the experimental results also showed that the molecular G-wire have the capability to transport electrical current not only on solid phases, but also in aqueous phases.^[61,62] Kotlyar and Porath groups reported reproducible charge transport in GQs, with current ranged from 10 to 100 pA over a distance from 10 to more than 100 nm.^[61] Transport occurs *via* a thermally activated long-range hopping between multi G-tetrad layers. Our group have reported the phenomenon of accelerated charge transport

efficiency over long GQ bridge (more than four G-tetrads layers) with an inverse distance-dependent property.^[62]

The efficient charge transport efficiency along DNA wire attracted great interests in designing such materials for the development of programmable circuits to exert their nanotechnological functions. However, the practical application of GQ in nanotechnological field were rarely reported because of the defect that arise from its self-recognition manner, by which the assembly was uncontrollable. In the case of intermolecular GQ structures or more than four guanine-rich single strands to assembly GQ, a mixture with high complexity will be obtained owing to the various possible combinations among these strands. Phan and co-workers observed the diversity in G-wire surface features from AFM images.^[63] G-wires assembled from d(G₄T₂G₄) mainly exhibited two types, 4.3 or 2.2 nm periodicity, accompanied with two subtypes, left-handed and zigzag subtypes. Thus, using short G-rich DNA sequences to control the assembly of G-wire structures is essential for realizing its nanotechnological functions and more explorations are in demand to achieve the real applications.

1.2 Binders of G-quadruplexes

With the identification of GQ biological roles in human cells, especially their close relationship with a wide range of malignancies, GQ have been widely studied as a significant target for cancer treatment. Numerous organic, inorganic small molecules as well as short peptides and proteins have been developed as GQ binding compounds.^[64] These GQ binders can be generally characterized as GQ cross-linkers and GQ stabilizers. Most of biomolecular and organic binders act as GQ stabilizers by stacking with G-tetrad or hydrophobic/electrostatic interaction with groove regions. Metal complexes can act as both cross-linkers and stabilizers. In general, these ligands bind to GQ mainly through three possible binding modes, which are proposed as the stacking with G-tetrad layers, groove binding and interaction mode with the cation channel at the center of GQ.^[65] Some

of the binders have exhibited effective functions on either *in vivo* GQ detection or anticancer effects *via* blocking telomerase activity or suppressing the corresponding oncogene expression, even further entered clinical trials.

1.2.1 Biomolecules

Balasubramanian's group has screened out a GQ specific antibody, BG4, from a library of 23 billion different single-chain antibody clones. Binding affinity between BG4 and GQ was super high that K_d value could reach sub-nanomolar. The antibody can recognize GQ structure among other nucleic acid structures, such as single stranded DNA, duplex DNA and RNA hairpin. However, it could not discriminate a specific GQ topology, which means that BG4 is a broad antibody for all GQ conformations (parallel, anti-parallel and hybrid GQs).^[66,67]

The Arg-Gly-Gly (RGG) domain located at C-terminal of translocated in liposarcoma (TLS), an oncogenic fusion protein, has also been identified to bind with both GQ human telomere DNA and TERRA GQ.^[68] Further substitution of tyrosine for phenylalanine in the RGG domain (TLRGG3Y) will enhance its selectivity towards TERRA GQ via specifically recognize the 2'-OH of the riboses from the loop region.^[69]

Though these biomolecules exhibited satisfactory binding affinities to GQ, the synthesis and purification processes are complicated and time-consuming. Hence, developing and *in silico* screening of small molecules, including both organic molecules and metal complexes, that is easy to synthesis is much more attractive to researchers.

1.2.2 Organic Compounds

In general, the organic binders of GQ are designed with extensive aromatic rings to achieve the efficient π - π stacking with G-quartet layers. A natural product, telomestatin, as telomerase inhibitor was isolated from the bacteria *Streptomyces anulatus* (Figure 1.6).^[70] It is the first generation of organic GQ binders, which mimic the extensive

aromatic area and macrocyclic ring structure to achieve good stabilization on G-quadruplex. Inspired by it, the porphyrin analog, tetra-*N*-methyl-4-pyridyl porphyrin (TMPyP4) was synthesized for realizing the excellent binding affinity to GQ and efficient restraint on telomerase activity.^[71] The X-ray crystal directly provided the binding between TMPyP4 and the bimolecular human telomere G-quadruplex d(TAGGGTTAGGG), in which porphyrin molecule stacked on GQ structure *via* two modes, stacked either on the external loop of GQ or on the 5'-end G-quartet.^[72] However, rare interaction was observed between GQ and its isomer, TMPyP2 (Figure 1.6).^[71] Although the planar aromatic system enabled TMPyP4 to tightly bind either parallel and anti-parallel GQ structures, the porphyrin can also bind double stranded DNA and compromise the specific targeting on GQ topologies. However, most of these aromatic-ring containing molecules can also bind to duplex DNA, which make them limited for anticancer treatment.

Fluoroquinolones are well known molecules for microbial disease treatment through blocking bacterial DNA gyrase activity.^[4] Several tetracyclic quinolone derivatives that scaffold from it were designed as GQ interactive molecules for cancer treatment, such as QQ58, quarfloxin and FQAs (Figure 1.6).^[73,74] FQAs, with an extension on the phenoxazine ring, showed stronger stacking with G-tetrad layer and enhanced telomeres suppression while maintaining topoisomerase-II poisoning effects. As a first-in-class agent against neuroendocrine tumor, Quarfloxin has entered Phase II clinical trials currently. It exhibits obvious preference for GQ structure, but not other duplex DNA or single stranded sequence.^[4]

Figure 1.6 Structure of some organic compounds that targeted G-quadruplex.

Some bisquinoline derivatives were designed to form an aromatic plane via intramolecular H-bonds. Both shape and size of the aromatic moiety endows them with good selectivity for GQs over duplex DNA and strong inhibitory effects on telomerase activity.^[75] A well-known example is 360A (Figure 1.6), in which two quinolinium groups were linked *via* a 2,6-pyridodicarboxamide unit.^[76] The linker adopts a *syn-syn* conformation that promotes internally H-bond between pyridinyl nitrogen and carbonyl oxygen, and results in a crescent-shaped conformation of the ligand. The ligand hence has optimal size and shape to be locked on top of the external G-tetrad.

In addition, some positively charged groups were further introduced to the organic binders, which not only strengthen its interaction with GQ structures, but also enhanced the water-solubility of the molecules. For example, a series of acridine derivatives have been widely developed due to the important positively charged property that came from the central ring of the chromophore, which may form electrostatic interaction with the

negatively charged cationic channel in GQ.^[77-79] Two well-known acridine analogues, RHPS4 and BRACO19, have been reported with high binding affinity to telomere targets (Figure 1.6). The structure of RHPS4-d(TTAGGGT)₄ adducts was resolved by NMR method.^[80,81] The aromatic rings stacked on the terminal G-tetrad instead of inserting between two G-tetrad layers, with the remaining layer unperturbed. BRACO19, the trisubstituted acridine derivative, was designed to interact with grooves of GQ simultaneously when it stacked onto the terminal G-quartet layer. A crystal structure of the BRACO19-human telomeric GQ complex showed one molecule of BRACO19 sandwich-like stacked between two dimeric quadruplexes.^[82] These reported acridine derivatives showed potent inhibition on telomerase activities and cancer cell proliferations.

Except for the passive binding and stabilizing the already formed GQ structure, the designed molecules can also play a driver-like role in assembly of GQs. Based on perylene skeleton, Fedoroff and co-workers have designed a polycyclic compound-PIPER, with two positive charges (Figure 1.6).^[83] NMR spectrum showed PIPER bound to d(TAGGGTTA)₄ GQ in 1:1, *via* external stacking mode with G-tetrads. It not only exhibited a specific binding preference to GQ rather than single or double stranded DNA, but also promoted the assembly of an intermolecular GQ structure.

1.2.3 Metal Complexes

The various organic binders can be only achieved by changing, adding and reducing functional groups on the initial skeleton of lead compounds. In contrast with organic molecules, the pool metal complex binders can be extended through several other ways, including changing the metal center and modifying the coordination ligands. A plenty of geometries can be obtained in the metal binders due to various spatial arrangements of the electrons in metal center. Compared with organic compounds, metal complexes displayed more advantages as GQ binders.^[65] First of all, the intrinsically electron withdrawing

character in the metal center provides an electron deficiency property of the system by reducing electron density on the coordination ligands. Thus, metal complexes exhibited stronger π - π interactions with G-tetrads. Besides, the positively charged metal center can reside at the cation channel of GQ, which will strength the stabilization through extra electrostatic interaction. In addition, by introducing a good leaving group, metal complexes could not only act as GQ stabilizers, but also as cross-linking agents of GQ, since the electrostatic interaction between metal center and the DNA backbone makes the metal complex easier to access to GQ.

1.2.3.1 Metal Complex Direct Coordinates to GQ

A well-known example of metal complex as cross-linking molecule for DNA is cisplatin, $[\text{Pt}(\text{NH}_3)_2\text{Cl}_2]$, a chemotherapy agent that used for many cancer treatments. The cross-linking predominately between guanine base and cisplatin through the displacement of the chloride ligand from the hydrolysate of $[\text{Pt}(\text{NH}_3)_2\text{Cl}_2]$.

Studies also found that the direct coordination occurred between GQ and metal complex. For example, *trans*- and *cis*- $[\text{Pt}(\text{NH}_3)_2(\text{H}_2\text{O})_2]^{2+}$ compounds were observed cross-linking to two guanines or adenine and guanine in telomeric GQ through platination reaction.^[84] Besides, some polypyridyl Pt complexes have showed site-specific platination on certain adenines of human telomeric GQ structures.^[85]

Based on this, organic molecules have been conjugated with mononuclear platinum (such as cisplatin) to achieve both stabilization and cross-linking interactions with GQ. One example employed a quinacridine aromatic moiety linked to a monofunctional Pt complex through a hydrophilic linker (platinum mono-para-quinacridine, Pt-MPQ) to construct the dual functional GQ binder (Figure 1.7).^[86] After interaction, it showed that the linker stride over G-tetrad layers, with the aromatic organic moiety stacked with one surface G-tetrad; while platination with G2 or G22 on the opposite side of the 22-mer GQ DNA.

Figure 1.7 Chemical structure of Pt-MPQ.

1.2.3.2 Metal Complex Act as GQ Stabilizer

Metal complex with planar geometry was believed as an essential property for GQ stabilizer. Some aromatic planes with appropriate sizes have shown excellent effect on GQ stabilization. Metalloporphyrins were reported as a kind of efficient GQ stabilizers, especially those with positively charged substituents have been proved more beneficial for the targets of stabilization.^[4,65] One example is the pentacationic manganese porphyrin that holds an aromatic core in the center, that is modified with 4 flexible positively charged side-chains (Figure 1.8).^[87] It showed 10000 times higher selectivity towarded GQ structures over double-stranded DNA, with binding constant as high as 10^8 M^{-1} with human telomeric GQ. Another isopropylguanidinium-modified phthalocyanine zinc compound with luminescent property also showed selectivity with GQ (Figure 1.8).^[88,89] Treatment of cells that known to overexpress *c-myc* or KRAS with this zinc complex will induce great diminution in oncogene expression.

Figure 1.8 Structure of some metal complexes that targeted G-quadruplex.

Apart from those binders with macrocyclic ligand, planar metal complexes without the large cyclic coordinated ligands also attracted lots of interests as efficient quadruplex stabilizers recently. This kind of metal binder exploits the intrinsic property of some metal centers to construct the ligands arranging in a planar geometry. Even though the corresponding ligand poorly interacted with GQs due to their so small size or electrical neutrality, formation of a planar metal complex usually exhibited much more enhancement on the binding to GQs. A series of π -delocalized nickel-salphen complexes with positively charged substituents were reported by Neidle and Vilar and their co-workers.^[90] These Ni complexes showed good overlap between their rings and three guanine rings from GQ and exhibited strong stabilization effect on GQ structure. A subsequent exploration of Ni, Cu, Zn and V complexes with the identical salphen ligand demonstrated the importance of metal complex's geometry for interaction with GQs (Figure 1.8).^[91] It is apparently that metal binders in square planar geometry are typically superior to those in square pyramidal conformations.

Several platinum complexes were proposed as good GQ binders due to their square planar geometries. Che's group has synthesized a series of luminescent Pt-dppz complexes (Figure 1.9A), that can bind to quadruplex DNA and inhibited telomerase activities.^[92] The fluorescent intensities of these Pt complexes were enhanced after binding to GQs, which enable them acting as luminescent probes for GQs. The planar geometry of Pt complexes enables them achieve high binding affinity to GQs, however, majority of them exhibited poor selectivity over other types of DNA, even for the discrimination on different GQ topologies. Our group have designed a Pt-Dip complex (Figure 1.9B), in which four phenyl groups extended from the $[\text{Pt}(\text{phen})_2]^{2+}$ core which endowed it with selectivity to parallel *c-myc* GQ by positioning and locking it in the grooves regions.^[93]

Figure 1.9 Structures of (A) $\text{Pt}(\text{dppz-COOH})(\text{N}^{\wedge}\text{C})$; (B) $[\text{Pt}(\text{Dip})_2]^{2+}$ and (C) $[\text{Ru}(\text{bpy})_2(\text{ptpn})]^{2+}$ and $[\text{Ru}(\text{phen})_2(\text{ptpn})]^{2+}$.

or linked to the whole metal complex so that it can intercalate into GQs or stack with G-quartets. Chao and his co-workers have reported two Ru complexes with polypyridyl ligands $[\text{Ru}(\text{N}^{\wedge}\text{N})_2(\text{ptpn})]^{2+}$ ($\text{N}^{\wedge}\text{N}$ is bpy or phen ligand) (Figure 1.9C), which can not only stabilize GQ structures, but also promote the formation of GQ and suppress on HeLa cell proliferations.^[94] Where after, this group reported a series of dinuclear polypyridyl Ru complexes, which are more efficient in inducing and stabilizing telomeric GQ than the mononuclear ones.^[95]

As a summary of the reported studies, metal complexes that no matter if mononuclear, dinuclear or polynuclear metal center, or either square planar, octahedral or other geometries can be exploited as GQ binders after elaborate design and modification. Most can stabilize GQ structures *via* the introduction of an extensive aromatic ring to realize the π -stacking with G-tetrads. However, some can bind to double stranded DNA as well, which will cause the cytotoxicity of normal cells. So far, the metal complexes report can rarely distinguish a specific GQ structures, instead, they showed the ability to stabilize varies GQ topologies. Thus, they are not suitable for achieving disease specificity or therapeutic effects. Hence, to be a successful GQ stabilizer candidate, it not only requires a strong binding affinity and enhanced thermal stability toward G-quadruplex, but also the specific recognition of GQ over duplex DNA structure that is extremely abundant in the genome, and the excellent preference on a special GQ topology. Metal agents are still one of the most potent medicinal candidates for cancer treatment and more research is needed in the field of designing metal drugs with above mentioned capabilities.

1.3 Probes for G-quadruplexes

Though GQ was well-studied *in vitro* and the formation of GQ is indirectly supported by robust biochemical and molecular genetics data, direct evidence for the existence of GQ *in vivo* remains a main problem that need to be solved. The significant

biological roles of GQ *in vivo* triggered extensive interests in signaling the existence of GQ and impelled scientist to make considerable efforts in designing and developing potential molecules for evaluation the abundance of GQ in cells. So far, various GQ-interactive agents were reported to realize GQ-labeling in different analytical ambient, including solution conditions, gel electrophoresis and cell imaging. These agents can be divided into three types based on the performance upon binding to GQ structures: “light-up”, “light-off” and permanent probes. In contrast to the “light-up” probes, luminescence turn-off probes are not suitable for cell imaging studies; while the permanent probes required another recognition moiety to realize the target imaging. By now, various “light-up” probes have been developed, including GQ-specific antibodies, organic and inorganic probes, and a combination of GQ-recognition molecules with luminescent moieties.

1.3.1 GQ-specific Antibody

So far, the most definitive arguments exploited quadruplex-specific antibodies to quantitative visualize cellular GQs. As mentioned above, the engineered and structure-specific BG4 have showed high selectivity and binding affinity to both DNA and RNA GQs. By employing multistep/reagent cascades in BG4-labeling GQ visualization, one can comprehend the modulation of GQ formation during cell cycle progression.^[66,67] However, immune-detection methods are of limited use because of the availability of antibodies and the complex multistep protocols, including sequentially incubated with flag-tagged BG4, anti-flag agents which is either directly linked by a fluorophore moiety, or indirect recognized by the secondary antibody that labeled with a fluorophore. Most importantly, the experimental process need cell fixation and permeation, which is not suitable for live-cell imaging.

1.3.2 Organic Dyes

Cyanine dyes, such as Thiazole orange (TO) and SYBR Green, and ethidium derivatives have been shown to have the ability to stain G-quadruplexes in gel electrophoresis. However, these dyes are not suitable for *in vivo* imaging purpose due to their fluorescence “turn-on” phenomenon upon binding to duplex DNA structures as well, which are widespread exist.

Recently, Monchaud and co-workers reported a common probe-NaphthoTASQ (Figure 1.10), which functioned as a multiphoton light-up luminescent probe for GQ visualization.^[96] The conformation of NaphthoTASQ was transformed from open to the closed state when bound to GQ when assembled into a synthetic G-tetrad layer. This small molecule specifically binds and lights-up GQs rather than any other types of DNA structures. The new biomimetic quadruplex ligand can be directly used for detection of GQ in living cells in only one incubation step, without any treatment for cells, such as fixation and permeation process. Although some organic molecules have showed good GQ imaging capability, two major challenging are need to be solved, one is in discerning excitation wavelength from emission owing to the small Stokes-shift; and the other is their relative short lifetime.

Figure 1.10 Chemical structure of NaphthoTASQ.

1.3.3 Luminescent Metal Complexes

In comparison with these organic fluorophores, luminescent transition metal complexes generally demonstrate large Stokes-shift and display long phosphorescence lifetimes, since the strong spin-orbit coupling in heavy metal nuclei enhances intersystem crossing and allows for triplet emission. One of the most prominent case is Pt-dppz complex, the photoluminescence intensity of which could be enhanced by nearly 300-folds upon binding to GQ (Figure 1.9).^[92] It can also be used as a fluorescent dye to stain GQ DNA on the electrophoretic gel. In addition, Che's group have reported a series of Pt-Schiff base complexes, which could not only stabilize GQ DNA structures and down-regulated the expression of *c-myc* oncogene, but also acted as GQ luminescent probes.^[97] Inspired by this study, Vilar's group reported a redox-activated Pt-salphen complex as GQ DNA binder and luminescent probe.^[98] However, most of luminescent metal complexes did not specifically light-up GQ structures, their emission intensity also enhanced upon addition of other DNA structures, such as double-stranded DNA and poly(dG-dC) structures.^[92,98]

1.3.4 Combination of GQ Recognition Moiety and Luminescent Emission Molecule

As mentioned above, some of the dyes were limited used due to the lack of GQ specificity. To overcome this, a combination strategy was proposed by conjugating a GQ ligand, that showed high binding affinity towards GQ structures, with a fluorescent turn-on probe. The designed TO derivatives, PDC-*M*-TO **2**, exhibited specific selectivity towards G-quadruplexes over duplex DNA structures upon modification of a GQ-targeting moiety (Figure 1.11).^[99] Recently, Huang's group have developed a dual probe for GQ nucleic acids detection, which was comprised of a coumarin-hemicyanine fluorophor and isaindigotone moiety, so that exerting both colorimetric and red-emitting luminescent signals for GQ detection (Figure 1.11).^[100] Except for the organic dyes,

luminescent metal complex was also linked with GQ-recognition part to enhance the probe performance. An Ir(III) complex was reported by conjugating with a GQ groove-binding motif, which resulted in the more efficient GQ probe behavior of the metal probe center (Figure 1.11).^[101]

Figure 1.11 Structures of some G-quadruplex probes.

Another idea was proposed by employing a GQ triggered fluorogenic hybridization probe, in which a GQ-specific fluorescent light-up molecule was linked with a DNA sequence that complementary with the region adjacent to G-rich sequence.^[102] Detection of a particular GQ sequence in living cells can be achieved by changing the nucleotides on the probe. However, this *in situ* visualization means is still limited owing to the small amount of targeted GQ sequence in human cells.

Based on the reported studies, using GQ-specific small molecular binders as luminescence turn-on probes for GQ imaging attracts a broad of interests due to the simple and convenient synthetic process. Thus, luminescent metal complexes, with large Stokes-shifts and long lifetimes, that can selectively bind to GQ structures were considered as potential agents for both therapeutic and live cell imaging targets.

1.4 The Motivation and Significance of the Study

The reported studies provided us with GQ's structural features and functionality. G-quadruplexes played significant roles in disease diagnosis, drug screening, electronic device design and many other aspects. Based on the research, we proposed and focused on GQ related studies including the following parts. Firstly, metal complexes designing and synthesizing of GQ-specific binders were exploited as one of the best choices owing to their own features, structural diversity and easy for synthesis properties. Though a great deal of binding agents exhibited high binding affinity towards G-quadruplexes and the stabilization effects on GQs, they seldom distinguish GQ structures from duplex DNA. Thus, such binders cannot be regarded as anticancer agents due to their potential toxicity to normal human cells. Besides, the recognition of a special GQ topology among the various conformations remains a major challenge for the current binders, which is highly important for the disease specificity on therapeutic effects and as personal drugs. Moreover, though the central channel from GQ structures was believed as a potential binding site, rare studies achieve binding in the ion channel. Hence, we are aiming to designing metal complexes that can not only exclusively bind to GQ structures and stabilize them, but also enhanced the selectivity to a specific GQ topology. In my PhD study, I synthesized a series of noncanonical octahedral Ru(II) complexes, containing a *x-y* planar tetradentate and two *trans-z-axis* ammonia ligands. These compounds efficiently stabilize G-quadruplexes *via* π -stacking between the aromatic planar ligands and external G-tetrad layer. In addition, two ammonia group on z-axis display unique interaction within GQ ion channel, which bestowed both excellent binding against duplex DNA and remarkable selectivity to antiparallel GQ topologies. Furthermore, based on a previous study, we found that the planar configuration is crucial for the stabilization effect of GQ molecular binders. Hence, two planar platinum complexes with an *aza*-bridged *bis*-(1,10-phenanthroline-2-yl)amine (bpa) were synthesized. The two phenanthrolines moieties in

bpa could enter into a flat plane prior binding onto nucleic acids, and therefore endow the two Pt complexes with a remarkable stabilizing ability on DNA and RNA GQs. The introduction of the alkyl tail from *aza* linkage enabled the complexes to distinguish GQ structures in view of the topological folding structures and improved their binding resistance to duplex DNA. These studies paved the way to develop metal complexes as GQ stabilizers for specific folding topology and the applications to disease and/or personalized anticancer medicine/therapy.

Secondly, exploring and developing a series of metal complexes that used for GQ imaging in living cells is another goal in this research. So far, the reported GQ-specific probes, especially those for RNA GQ imaging, have been confronted with many problems, such as a small Stoke shifts, fast photobleaching, non-GQ specific probing and limited in live cell detection. Based on an overall consideration of all issues, a series of platinum complexes, with free *aza*-NH moiety, as GQ probes, for realizing RNA GQ imaging purpose was proposed. These complexes exhibited large Stokes shift, long lifetime, high quantum yield and remarkable luminescence turn-on signals specifically upon binding to G-quadruplexes. It also offered the first example of inorganic light-switch probe for imaging RNA GQ and tracking their un/folding dynamics in live cells.

Thirdly, the consecutive G-rich single stranded DNA sequence which has been shown with potential as a building block in constructing G-wires to exhibit excellent electron transport efficiency was incorporated. As a medium for electron transfer, DNA has been widely applied in photolytic processes but is seldom explored for CO₂ reduction in dark reaction. On the other hand, the binding of metal complexes onto GQ structures provided them with special orientations, which endow them ready for use in catalyzing one step of organic reactions, such as enantioselective Diels–Alder Reaction and sulfoxidation reaction. Hence, in this study, the first to combine the efficient electron transport property of G-wires with the special orientations of metal complexes on GQ has

been proposed and achieved using a novel application of the conductive G-wires based biomolecular materials. The submicrometer-long G-quadruplex nanowire was constructed by self-assembly of guanine tetranucleotides. Upon hosting several metal complexes, GQ nanowire could mediate electron transfer processes in the CO₂ electrochemical reduction, which were catalyzed by the metal centers. As a result of it, the GQwire modified electrode efficiently enhanced both Faradaic efficiency for CO₂ reduction and total current density, compared with bare metal complex decorated electrode. The high efficiency and selectivity of electrocatalytic CO₂ reduction was attributed to the unique binding of metal complexes on G-quadruplex and electron transfer mediated by GQ nanowire to achieve efficient redox cycling of catalytic centers on the electrode. Therefore, the study provides practical information on the potential of using conductive biomacromolecules to merge the gap between light and dark reactions in the artificial photosynthesis.

1.5 References

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CHAPTER 2

Octahedral Ruthenium Complexes that Selectively Stabilize G-quadruplexes

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2.1 Introduction

Nucleic acids with guanine rich sequences have the propensity to fold into a tetra-stranded nucleic acid structures, named as G-quadruplexes (GQ).^[1] The whole structure is usually stabilized by cations such as K^+ and Na^+ , which occupied the central cavity of GQ and neutralized the electrostatic repulsion between O6 of guanines. These G-rich sequences can adopt different topologies with different strand orientations, including parallel, antiparallel and parallel/antiparallel hybrids.^[2-4] The facts that GQ folding sequences exist genome-wide, and play pivotal regulatory roles in biological events, such as telomere elongation and oncogene expression, make GQ topology a high potential drug target, especially in anticancer drug design.^[5-7]

To date, numbers of reported examples have showed that the compounds which contain large planar aromatic rings, including organic molecules and metal complexes with aromatic ligands can achieve large area of π -stacking with G-quartets and hence stabilize GQ DNA (Figure 2.1).^[8-11] However, a successful GQ stabilizer should not only show high binding affinity and elevate thermal stability of GQ structures, but also exhibit excellent selectivity over double-stranded DNA which present overwhelming high abundance in genome. Due to the planar structure of the ligands, the metal complexes as GQ stabilizers are often excellent intercalating agent to the base-pair stacking of duplex DNA.^[12-15] Current successful attempts to enhance selectivity over duplex DNA mainly focus on extending cationic alkyl chains from the peripheral of aromatic ligands to refrain from deep intercalation to the base pair stacking of duplex DNA and to obtain extra stacking and H-bondings with nucleobases and back bonds in the grooves and loops of GQ.^[16,17] Whereas, the interactions between GQ binder and central cation cavity were completely left unharnessed due to the planar tetra-chelation geometry in existing metallo GQ stabilizers. VII/VIII transition metal complexes, such as Ru^{2+} , were well reported as potent DNA binders and exhibited excellent antitumor activities. The octahedral

coordination geometry on Ru^{2+} core can readily construct three dimensional structures to dock Ru complexes onto DNA structures.^[18] However, the canonical Ru complexes with tris-bidentate ligands possess an octahedral structure with C_3 symmetry often well intercalate into base pair stacks with major aromatic ligand, while the ancillary ligands were arranged in right-handed propeller fashion and excellently fit into the major groove of duplex DNA.^[19] Hence none of these tri-ligand Ru complexes often could distinguish G-quadruplex from duplex DNA.^[20,21]

Herein, we designed and prepared a series of novel octahedral Ru complexes (Figure 2.1) with a near C_4 symmetric configuration. In x - y plane, Ru^{2+} was chelated to a planar *N,N*-bis-(1,10-phenanthrolin-2-yl)-amine (bpa) ligand which have nearly the same size of aromatic area as G quartets, but exceeding the width of base pairs in duplex DNA, and would bestow strong π stacking with G quartets to enhance GQ stability. Along z -axis, two axial positions coordinated to small molecular ligands, such as ammonia, could insert and interact with central cation channel to achieve unique extra stabilizing effects on G-quartets. Unlike canonical tri-ligand ruthenium complexes with C_3 -symmetry, complexes in this work possessed a bipyramidal-type octahedral structure (Figure 2.1B). Both the large aromatic ligand in x - y plane and the two *trans*-ligands on z -axis would prevent the complexes from binding/intercalating into duplex DNA, whereas the two interaction modes can efficiently stabilize G-quadruplexes.

Figure 2.1 Structures of G-quartet (A) and Ru complexes (B).

2.2 Results and Discussion

2.2.1 Synthesis of Novel Octahedral Ruthenium Complexes

As shown in Scheme 2.1, to synthesize the precursor **Ru0**, bpa was firstly protonated with trifluoroacetic acid, so that two phenanthrolines (phen) was H-bond to the proton. Hence along with amino linkage, bpa was stabilized into a planar structure. Sequentially, $\text{Ru}(\text{DMSO})_4\text{Cl}_2$ were readily chelated to planar bpa- H^+ and lead one chloride and DMSO into z-axis with a *trans*-coordination to yield **Ru0**.

Scheme 2.1 Synthetic routes for Ru complexes.

As shown in Figure 2.2, UV-vis absorption spectrum of **Ru0** showed a MLCT band around 350 nm that indicated the coordination of equatorial aromatic ligand, bpa. Further introduction of amino ligands on z-axis to yield **Ru1** were achieved by refluxing **Ru0** in the aqueous/MeOH solution of amino ligands. In absorption spectrum of **Ru1**, a new MLCT band rose around 513 nm for the chelation of two z-axial ligands, NH_3 . **Ru2** to **Ru3** were prepared by adjusting either reaction time or amino molecules and were isolated by recrystallization.

Figure 2.2 UV-vis absorption spectrum of **Ru0** (A) and **Ru1** (B).

The crystal structures of **Ru1** and **Ru2** confirmed that the geometric configuration of ruthenium complexes realized our design (Figure 2.3 and Table 2.1). As shown in Table 2.2, the angles among four coordination bonds (N1-Ru1-N2, N2-Ru1-N4, N4-Ru1-N5 and N5-Ru1-N1) was summed to 359.5° , which demonstrated the ruthenium centre adopts a unique equatorial plane structure with four chelating phenanthroline nitrogens. On z-axis, angel of two Ru-NH₃ bonds (N6-Ru1-N7) was 180.0° and bond angle between Ru-NH₃ and equatorial plan (N4-Ru1-N6, N1-Ru1-N6, N4-Ru1-N7 and N1-Ru1-N7) were all approximately 90.0° , which indicated two ammonia molecules on z-axial positions were aligned linearly and perpendicularly to bpa on both sides of the plane. Moreover, the bond lengths of two Ru-NH₃ (Ru1-N6 and Ru1-N7) was 2.1 Å. Once NH₃ in **Ru1** and **Ru2** were aligned with cation channel of G-quadruplex along z-axis, bpa in both complexes would be in appropriate distance to external layer of G quartets to achieve efficient aromatic stacking and hence stabilize and/or bind tightly with G-quadruplex.

Figure 2.3 The crystal structures of **Ru1** and **2** with each atom numbering.

Table 2.1 Crystal data, collection and structure refinement parameters for **Ru1** and **2**.

Chemical formula	C ₂₄ H ₂₁ F ₁₂ N ₇ P ₂ Ru	C ₂₆ H ₂₄ F ₁₂ N ₆ OP ₂ RuS
Formula weight	798.47 g/mol	859.57 g/mol
Crystal system	triclinic	monoclinic
Space group	P -1	P 1 21/c 1
Crystal size (mm ³)	0.040 x 0.100 x 0.300	0.020 x 0.040 x 0.300
Temperature (K)	103(2)	103(2)
<i>a</i> (Å)	8.4109(4)	11.5273(4)
<i>b</i> (Å)	11.4169(6)	12.9572(4)
<i>c</i> (Å)	11.7250(6)	24.0256(7)
<i>α</i> (deg)	74.801(2)	90
<i>β</i> (deg)	76.758(2)	97.6261(17)
<i>γ</i> (deg)	87.087(2)	90
<i>V</i> (Å ³)	1057.59(9)	3556.8(2)
<i>Z</i>	1	4
ρ_{calcd} (g cm ⁻³)	1.618	1.747
μ (mm ⁻¹)	0.547	0.695
F(000)	524	1880
Data / restraints / parameters	5904 / 456 / 428	8994 / 602 / 616
<i>R</i> ₁ [<i>I</i> > 2σ(<i>I</i>)]	0.0365	0.0722
<i>wR</i> ₂ [<i>I</i> > 2σ(<i>I</i>)]	0.0723	0.1928
<i>R</i> ₁ (all data)	0.0506	0.1360
<i>wR</i> ₂ (all data)	0.0796	0.2432
Goodness-of-fit on <i>F</i> ²	1.088	1.084

Table 2.2 Selected Bond Lengths (Å) and Bond Angles (deg) for **Ru1** and **2**.

Ru1		Ru2	
Ru1-N1	2.215(7)	Ru1-N1	2.105(5)
Ru1-N2	1.865(6)	Ru1-N2	2.022(6)
Ru1-N4	1.861(6)	Ru1-N4	2.020(5)
Ru1-N5	2.215(6)	Ru1-N5	2.102(5)
Ru1-N6	2.1237(17)	Ru1-N6	2.130(5)
Ru1-N7	2.1237(17)	Ru1-S1	2.2294(16)
N1-Ru1-N2	80.0(4)	N1-Ru1-N2	80.5(2)
N1-Ru1-N4	179.6(6)	N1-Ru1-N4	171.5(2)
N1-Ru1-N5	99.9(4)	N1-Ru1-N5	107.7(2)
N1-Ru1-N6	89.7(3)	N1-Ru1-N6	88.7(2)
N1-Ru1-N7	90.3(3)	N2-Ru1-N4	91.5(2)
N2-Ru1-N4	100.4(5)	N2-Ru1-N5	169.9(2)
N2-Ru1-N5	178.5(6)	N2-Ru1-N6	87.9(2)
N2-Ru1-N6	90.5(4)	N4-Ru1-N5	79.9(2)
N2-Ru1-N7	89.5(4)	N4-Ru1-N6	88.1(2)
N4-Ru1-N5	79.7(4)	N5-Ru1-N6	86.6(2)
N4-Ru1-N6	90.2(4)	S1-Ru1-N1	87.73(15)
N4-Ru1-N7	89.8(4)	S1-Ru1-N2	89.29(16)
N5-Ru1-N6	91.0(3)	S1-Ru1-N4	95.11(15)
N5-Ru1-N7	89.0(3)	S1-Ru1-N5	96.68(14)
N6-Ru1-N7	180.00(11)	S1-Ru1-N6	175.74(15)

2.2.2 Ru1 Selectively Stabilizes and Binds to GQ Structures

The stabilizing/binding abilities of **Ru1** on GQ and duplex DNA were explored by thermal melting and optical spectra of DNA. The sequences of DNA structures were listed in Table 2.3. Upon addition of one equivalent of **Ru1**, significant enhancement in thermal stability (ΔT_m) were observed for all the G-quadruplexes, where both telomeric GQs received above 20 °C incensement in CD melting temperature. The fact that only 1.9 °C

was observed for ΔT_m of DS26 in the presence of **Ru1**, suggested the weak interactions between **Ru1** and duplex DNA, if there is any.

Table 2.3 Sequences of oligomers (primers) used in this work.

Oligomer	Sequence
<i>c-myc</i>	5'-TGGGGAGGGTGGGGAGGGTGGGGAAGG-3'
c-kit2	5'-CGGGCGGGCGCGAGGGAGGGG-3'
HT21	5'-GGG(TTAGGG) ₃ -3'
HT48	5'-(TTAGGG) ₈ -3'
DS26	5'-CAATCGGATCGAATTCGATCCGATTG-3'

Table 2.4 The parameters that characterize the stabilization (ΔT_m) and binding (K_b and DC_{50}) of **Ru1** with DNA structures.*

DNA	ΔT_m^a (°C)	K_b^b ($\times 10^5$ M ⁻¹)		DC_{50}^c (μM)
		K_b^{Abs}	K_b^θ	
HT21	26 (2) ^d	4.9 (0.2)	2.4 (0.4)	0.40
HT48	23 (2)	4.6 (0.4)	3.4 (0.3)	0.50
<i>c-myc</i>	10 (2)	4.2 (0.2)	3.5 (0.6)	0.25
DS26	1.9 (0.4) ^e	-	-	>2.5

^aMelting temperature incensement of DNA structures upon binding of **Ru1** were determined by the change of CD signal. ^bBinding constants of **Ru1** to DNA structures. ^cFID constants of **Ru1** to DNA structures. ^din Na⁺ buffer ^eMelting temperatures were determined by UV-vis absorption at 260 nm. *Standard deviations were listed in parentheses.

Furthermore, since **Ru1** contains two MLCT bands from equatorial bpa (344~363 nm) and z-axis NH₃ (513 nm), alteration in UV-vis absorption intensity and wavelengths of MLCT bands could indicate the potential interactions between **Ru1** and DNA structures. Both strong intensity decrement and large wavelength redshift were observed for both MLCT bands in the presence of telomeric GQ (Figure 2.4A). As low as 3 μM of GQ HT21 can induce 38% attenuation on bpa- MLCT band, which indicate strong π -stacking of bpa on G-quartet. High binding affinity to all the GQ structures (K_b^{Abs} , $\sim 5 \times 10^5$ M⁻¹) was estimated from UV-vis titration upon fitting into equation 1 (Table 2.4). The obtained binding affinity was on the same order of magnitude as the one that calculated from other reported methods (K_b^θ , $\sim 3 \times 10^5$ M⁻¹). More remarkably, for NH₃-MLCT band,

a large redshift of 18 nm was coincided with 31% hypochromicity upon adding GQ HT21, which indicated that strong interactions with GQ DNA along z -axis significantly weakens the chelation of NH_3 to Ru^{2+} . Remarkably, on the contrary to GQ, negligible change occurred to both MLCT bands upon addition of DS26. Binding affinity to duplex DNA was not able to be obtained by fitting UV hypochromicity (Figure 2.4B). Lack of hypochromicity and bathochromicity of UV-vis spectra of **Ru1** upon addition of duplex DNA, indicated that both aromatic stacking and H-bonding with z -axial ligands were absent and could account for the highly selective stabilization of **Ru1** towards GQ over duplex DNA.

Figure 2.4 UV-vis titration spectra of **Ru1** (25 μM) with increasing concentrations of GQ **HT21** (black to dark cyan: 0 to 8 μM) (A) and **DS26** (black to violet: 0 to 12 μM) (B).

The binding selectivity of **Ru1** was further observed in G4-FID assay and competitive dialysis (Figure 2.5). Thiazole orange (TO) either stacking with G-quartet or binding with groove/loops on GQ DNA were efficiently displaced by submicromolar of **Ru1**, while majority (>70%) of TO remained intercalated in duplex DNA even in the presence of 10 equivalents of **Ru1**. In the competitive dialysis, **Ru1** bound to all the G-quadruplex structures to different extents, rarely any **Ru1** molecules was found binding to duplex DNA. Clearly through both aromatic stacking in x - y plane and interaction with NH_3 along z -axis, **Ru1** can establish unique multi-modal binding to stabilize GQ and hence achieve excellent selectivity over duplex DNA.

Figure 2.5 (A) Plot of TO displacement percentage vs. concentrations of **Ru1** with different DNA sequences. Lines are the exponential fittings of TO displacement curves. (B) Competition dialysis of **Ru1** on various DNA secondary structures.

2.2.3 Ru1 Distinguishes the Topology of G-quadruplexes

Besides the negligible interactions with duplex DNA, **Ru1** can further distinguish the topology of G-quadruplexes. As shown in Table 2.4 and 2.5, ΔT_m of telomeric G-quadruplexes (>20 °C) were 13~16 °C higher than that of *c-myc* GQ (10 °C) and c-kit2 (10 °C). Telomeric G-quadruplex has been reported existed as 3+1 hybrid structure in K^+ buffer, while as basket structure in Na^+ buffer. The thermal stability of both antiparallel HT GQs were elevated more significantly than that of parallel *c-myc* and c-kit2 GQ, especially in Na^+ buffer. Besides, in competitive dialysis, 2- and 5- folds higher in bound concentration of Ru1 is observed on antiparallel HT48 quadruplex than on parallel *c-myc* and c-kit2 GQ.

Table 2.5 The melting temperature incensements (ΔT_m) of different DNA structures in the presence of **Ru1**.

DNA	ΔT_m (°C)
HT21 (in Na^+)	26 (2)
HT21 (in K^+)	20 (1)
HT48 (in K^+)	23 (2)
c-myc	10 (2)
c-kit2	10 (2)
DS26	1.9 (0.4)

The preference of **Ru1** to stabilize antiparallel GQs were further evident by CD spectra (Figure 2.6 and 2.7). In the absence of potassium ion, **Ru1** promoted the emergence of two CD signals, a positive peak at 297 nm and a negative band at 265 nm, which are characteristic to antiparallel G-quadruplex (Figure 2.6). More remarkably, in the presence of K^+ , the major positive band of GQ HT21 at 295 nm showed two unusual alterations upon binding to **Ru1**. A negative peak grew from the band shoulder around 272 nm accompanied by a 3 nm redshift of peak maximum to 295 nm. The spectra variation was consistent with the transition of GQ topology from 3+1 hybrid to basket-type antiparallel GQ,^[22,23] which indicated that **Ru1** can efficiently and specifically induce and stabilize the basket-type GQ, even though hybrid GQ is the dominant topology in potassium buffer. On the contrary, little variation of CD spectra of parallel *c-myc* GQ upon the addition of **Ru1** regardless of the presence of potassium ions. The distinct effects of **Ru1** on the folding topology and thermal stability of telomeric and *c-myc* GQs indicated that antiparallel GQ, especially basket-type folding topology, can accommodate the octahedral Ru1 better than parallel GQ, and consequently achieve higher stability.

Figure 2.6 Circular dichroism spectra of GQ HT21 (A, C) and GQ *c-myc* (B, D) upon titration of **Ru1** in 10 mM Tris buffer (pH 7.4) with (A, B) and without (C, D) 100 mM KCl.

Figure 2.7 HT voltage detected from circular dichroism spectra of GQ HT21 and GQ *c-myc* in the absence and presence of **Ru1**.

2.2.4 The Significance of Two Ammonia Moieties on *z*-Axis

Another two ruthenium complexes (**Ru2** and **Ru3**) were synthesized and the interactions of these two complexes with GQ HT21 were characterized to investigate the origins of selectivity in GQ stabilization for **Ru1**. **Ru2** and **Ru3** contained the same octahedral coordination and aromatic bpa ligands in *x-y* plane, but different *z*-axis ligands were synthesized (Table 2.1 and 2.2, and Figure 2.3). The two *z*-axis ligands, NH_3 in **Ru1** were replaced by one DMSO in **Ru2** or by two methylamine ligands in **Ru3**, respectively. With similar octahedral structure as **Ru1**, **Ru2** and **Ru3** showed negligible T_m elevation on duplex DNA (Table 2.6). While planar platinum bis-phen complexes showed intercalation to duplex DNA,^[24] 3D octahedral coordination with amino ligands blocked at least 4.2 Å of distance along *z*-axis from Ru^{2+} core and prevented the complexes from intercalating between base pairs, which hence accounted for the negligible binding and stabilization of duplex DNA. Whereas for GQ, ΔT_m (HT21) reached 10 °C for **Ru2** and 4.3 °C for **Ru3**. Though planar bpa was remained, the strengths of GQ stabilization were hampered when the number of NH_3 was reduced by replacing the *z*-axis ligand with either larger ligand (DMSO) in **Ru2** or with longer alkyl chains (MeNH_2) in **Ru3**. The bulky DMSO ligand in **Ru2** prevent the complex from approaching G-quartet from DMSO side,

thus diminish its efficiency on stabilization. The extra methyl group on z-axial ligand in **Ru3** increased the distances between bpa ligand and G-tetrad. Hence the π - π interaction between **Ru3** and GQ was significantly weakened and small ΔT_m was yielded.

Table 2.6 The melting temperature incensement (ΔT_m) of DNA structures in the presence of **Ru2** and **Ru3**.

	ΔT_m (<i>c-myc</i>)	ΔT_m (HT21)	ΔT_m (ds26)
Ru2	7.2 (0.5)	9.6 (0.8)	1.2 (0.3)
Ru3	0.8 (0.3)	4.3 (0.3)	1.3 (0.1)

Further investigations on UV-titration of GQ **HT21** showed that significant redshift of NH_3 -MCLT band up to 18 nm occurred to both **Ru1** and **Ru2**, but not **Ru3** (Figure 2.8). The discrepancy indicated the strong interactions between NH_3 ligands and GQ, which was absence in **Ru3**. Since ammonia with similar size as Sr^{2+} can align with cation cavity to stabilize G-quartet, the interaction of NH_3 with external layer of G-quartet would weaken the coordination bond of N-Ru^{2+} and induce large bathochromicity on NH_3 -MLCT band. However, in **Ru3**, methyl group located at the terminals of z-axis. The ammonia group was shielded by methyl group and hence could not interact with the central channel as the case in **Ru1** and **Ru2**.

Figure 2.8 Bathochromicity of Ru-NH₂R (R=H, CH₃) MLCT band upon titration of GQ HT21.

2.2.5 Molecular Docking of Ru1 on Different GQ Structures

Molecular docking of ruthenium complexes on basket and 3+1 hybrid GQ provided further explanations for topological selectivity to basket GQ. **Ru1** showed significantly higher fitting values on both antiparallel telomeric GQs than that on parallel c-myc GQ, which is consistent with the observations that thermal stability and CD spectra of c-myc GQ were much less altered by **Ru1** comparing to telomeric GQs (Figure 2.9). Both **Ru1** and **Ru2** achieved higher fitting values on basket GQ than 3+1 hybrid GQ.

Table 2.7 Fitting values of Ru complexes on various GQ topological structures from molecular docking.

GQ topology (PDB code)	Basket GQ (2mcc)	3+1 hybrid GQ (2mb3)	Parallel c-myc (1xav)
Ru1	94.21	88.06	60.57
Ru2	92.91	82.59	-

In the best docking structure, **Ru1** stacked on top of the external G-quartet in the dual lateral loop side (Figure 2.9A-C). From the top view, bpa showed proper size and location to overlap with the aromatic area of G-quartet. More remarkably, NH₃ lay into the cation channel and three protons were all within the distance of 1.7 Å to O6 of quartet guanines, which indicated strong H-bonds. On the contrary, **Ru1** disturbed the flatness of G-quartet upon stacking on 3+1 hybrid GQ. H-bonds between ammonia protons and guanine oxygens were barely formed with more than 2 Å of distance (Figure 2.9D-F). The discrepancy in the fitting values and docking structures of **Ru1** on two antiparallel GQ structures confirmed the higher stability of basket GQ and better accommodation to **Ru1** via both aromatic stacking and H-bonds in cation cavity, which supported the conversion of CD spectra from 3+1 hybrid GQ to basket GQ upon binding to **Ru1**.

Figure 2.9 Molecular docking of **Ru1** on basket GQ (A-C, PDB: 2MCC) and 3+1 hybrid telomeric GQ (D-F, PDB: 2MB3). (A) side view; (B) top view of the docking structure and (C) H-Bonds between NH_3 and G-quartet. Backbone is represented in blue ribbon and **Ru1** is shown in brown.

2.2.6 Potency Against Telomerase and Cytotoxicity Towards Cancer Cells

Inhibitory activity on telomerase and consequential cytotoxicity of **Ru1** were examined. As shown in Figure 2.10, **Ru1** showed potent efficiency in inhibiting the elongation of telomeric DNA. Since TRAP assay here applied product PCR amplification without removing ruthenium complexes, inhibitory effects of **Ru1** was promisingly limited to telomerase, but not on DNA polymerases, which is a common drawback for other complex-based GQ stabilizers, such as TMPyP4. EC_{50} value of **Ru1** (the concentration which inhibit 50% of telomerase activity) was calculated as 1.9 μM (Table 2.8), which is much lower than the two well-known G-quadruplex binders, TMPyP4 (8.9 μM) and BRACO-19 (6.3 μM).^[25]

Figure 2.10 Inhibition of telomerase activity by different concentrations of **Ru1**. Ladder shows the product of telomerase elongation.

Table 2.8 TRAP assay (EC_{50}) and Cytotoxicity (IC_{50}) of **Ru1** towards various cancerous cell lines.^a

Compound	Cytotoxicity (μM^{-1})					TRAP assay (μM^{-1})
	HT 1080	MDA-MB-231	HeLa	A549	MCF-7	
Ru1	26 (1)	25 (3)	24 (3)	17 (2)	51 (2)	1.9 (0.3)
TMPyP4	38 (6)	41 (5)	21 (4)	43 (1) ^b	90 (3)	8.9 ^c

^a Standard deviations were listed in parentheses.

^b From reference.

^c A modified TRAP assay with eliminating TMPyP4 before PCR step.^[25]

Cytotoxicity of **Ru1** were further examined on variety cancerous cell lines. IC_{50} against HeLa (human cervical epithelioid carcinoma), MDA-MB-231 (human breast carcinoma) and HT 1080 (human fibrosarcoma) were around 25 μM and the values against A549 (Human lung carcinoma) and MCF-7 (human breast adenocarcinoma) were 17 and 50 μM , respectively (Table 2.8). However, the cell viability of NIH 3T3 (mouse embryo fibroblast cells) was still maintained at 75% after treated with 200 μM **Ru1** (Figure 2.11). Thus, cell cytotoxicity indicated **Ru1** were 1.5~2.5 times more potent than

TMPyP4 for different cancer cell lines, while **Ru1** exhibited low cytotoxicity on normal cells.

Figure 2.11 Viability of the normal NIH 3T3 cell (mouse embryo fibroblast cells) in the presence of 3, 6, 12, 25, 50, 100 and 200 μM **Ru1**.

2.3 Conclusion

In this study, three Ru complexes with a unique bipyramidal-type octahedral structure were developed and characterized. The bis-phenanthroline ligands were forced into equatorial plane through an amine linkage and provide aromatic area with proper size and shape to stack with G-quartet and to stabilize G-quadruplexes. The ammonia ligands on the *trans* coordination positions of z-axis bestowed the complexes excellent selectivity against binding duplex DNA via blocking the intercalation of the complexes into the stack of base pairs. In **Ru1** and **Ru2**, NH_3 further formed H-bonds with G-quartet to offer second stabilizing modes on G-quadruplexes. Furthermore, the octahedral Ru complexes showed a topological selectivity for antiparallel G-quadruplex over parallel GQ, particularly for basket-type GQ. Moreover, it exhibited an efficient inhibition on the activity of telomerase, accompanied with a potent cytotoxicity to the cancer cells. Hence, Ru complexes possess the potential therapeutic values for cancer treatment. This study provides a novel strategy to develop octahedral metal complexes as GQ-targeting drug candidates and harness the interactions with cation channel as novel GQ-stabilizing mode.

2.4 Experimental section

All the chemicals were purchased from Sigma-Aldrich. All DNA sequences were purchased from Sangon (Shanghai, China). TRAPeze telomerase detection kit was purchased from Merck Millipore (Darmstadt, Germany). *Taq* DNA polymerase was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Milli-Q water was used in all physical measurement experiments.

Synthesis of Ru^{II} complexes

[Ru(bpa)(DMSO)Cl]Cl₂ (Ru0). The ligand *N,N*-bis-(1,10-phenanthroline-2-yl)-amine (bpa) was prepared as previously reported.^[26,27] bpa was protonated by dissolving in trifluoroacetic acid and precipitated with diethyl ether prior to usage in following synthesis. To prepare **Ru0**, *cis*-[Ru(dmsO)₄Cl₂] (72.0 mg, 0.15 mmol), bpa-H⁺ (72.5 mg, 0.15 mmol) and ethylene glycol (5 mL) were heated at 190 °C for 2 h. The crude were obtained by removing the solvent under vacuum and washed with diethyl ether (yield: 85 %). ¹H NMR (300 MHz, DMSO) δ 10.31 (d, *J* = 4.9 Hz, 2H), 8.89 (d, *J* = 8.0 Hz, 2H), 8.75 (d, *J* = 8.7 Hz, 2H), 8.30 (m, 6H), 8.01 (d, *J* = 8.9 Hz, 2H), 1.68 (s, 6H).

[Ru(bpa)(NH₃)₂](PF₆)₂ (Ru1). **Ru0** (36.9 mg, 0.05 mmol) was suspended in 4 mL MeOH and H₂O mixture (v/v 3:1), followed by addition of 28% ammonium hydroxide solution (1.0 mL). The mixture was refluxed overnight. Methanol was distilled out and the product was precipitated by adding an excessive amount of saturated KPF₆ solution. The solid was filtered and washed with diethyl ether to provide the final product. Crystal of **Ru1** was grown by diffusing diethyl ether into acetone at 4 °C and purple needle crystals were obtained in 40 % yield. ¹H NMR (500 MHz, Acetone-d₆) δ 10.59 (d, *J* = 4.9 Hz, 2H), 8.98 (d, *J* = 8.0 Hz, 2H), 8.83 (d, *J* = 8.5 Hz, 2H), 8.37 (m, 6H), 8.16 (d, *J* = 8.6 Hz, 2H). HR-MS (MeOH) *m/z* (%): 510.0970 (calc 510.0980 for C₂₄H₂₂N₇Ru).

[Ru(bpa)(NH₃)(DMSO)](PF₆)₂ (Ru2). **Ru0** (22.1 mg, 0.03 mmol) was suspended in 4 mL MeOH and H₂O mixture (v/v 3:1), followed by addition of 28% ammonium hydroxide solution (0.5 mL). The mixture was refluxed for 5 h. Methanol was distilled out, and the product was precipitated by adding an excessive amount of saturated KPF₆ solution. The solid was filtered and washed with diethyl ether to provide the final product. Purple needle crystals of **Ru2** were grown by diffusing diethyl ether into acetone at 4 °C (yield: 53 %). ¹H NMR (300 MHz, DMSO) δ 12.83 (s, 1H), 10.18 (d, *J* = 4.7 Hz, 2H), 9.06 (d, *J* = 8.2 Hz, 2H), 8.92 (d, *J* = 8.8 Hz, 2H), 8.39 (m, 6H), 8.10 (d, *J* = 8.9 Hz, 2H), 1.83 (s, 6H). HR-MS (MeOH) *m/z* (%): 571.0848 (calc 571.0854 for C₂₆H₂₅N₆OSRu).

[Ru(bpa)(NH₂CH₃)₂](Cl)₂ (Ru3). **Ru0** (22.1 mg, 0.03 mmol) was suspended in 4 mL MeOH and H₂O mixture (v/v 3:1), followed by addition of 40% aqueous methylamine solution (100 µL). The mixture was refluxed overnight. After distilling the solvent, the obtained solid of **Ru3** was purified and recrystallized by diffusing diethyl ether into methanol at 4 °C (yield: 66 %). ¹H NMR (500 MHz, MeOD) δ 10.41 (d, *J* = 4.8 Hz, 2H), 8.94 (d, *J* = 8.0 Hz, 2H), 8.80 (d, *J* = 8.8 Hz, 2H), 8.36 (m, 6H), 8.10 (d, *J* = 8.8 Hz, 2H), 0.90 (t, *J* = 6.5 Hz, 6H). HR-MS (MeOH) *m/z* (%): 536.1126 (calc 536.1137 for C₂₆H₂₆N₇Ru).

UV-vis absorption titration

Absorption spectra of Ru complexes were recorded on a Cary 100 UV/Vis spectrophotometer. For UV titration experiment, a Tris/KCl buffer (100 mM KCl, 10 mM Tris HCl, pH 7.4) was used and UV/Vis spectra were recorded after each addition of concentrated DNA stock (100 µM) to 25 µM Ru complex solutions in a quartz cuvette (path length = 1 cm) at 25 °C. The approximate value of binding association constant K_b^{Abs} (M⁻¹) was calculated with Eq(2.1),^[28]

$$\frac{D}{\Delta\epsilon_{ap}} = \frac{D}{\Delta\epsilon} + \frac{1}{\Delta\epsilon \times K} \quad \text{Equation 2.1}$$

where D is the concentration of DNA, $\Delta\epsilon_{ap} = |\epsilon_A - \epsilon_F|$, $\epsilon_A = A_{obs}/[Ru]$, $\Delta\epsilon = |\epsilon_B - \epsilon_F|$, ϵ_B and ϵ_F represented the extinction coefficients of the DNA-complex adduct and free complex that is in solution, respectively. The results were got from average of three replicates.

G4-FID assay

FID assay was performed on Varian Cary Eclipse fluorescence spectrophotometer in a 100 mM KCl, 10 mM Tris, pH 7.4 buffer. The concentration of all DNA used was 0.25 μ M. Quadruplex DNA were added with 2 molar equivalents of thiazole orange (TO, 0.5 μ M) and ds26 were added with 3 molar equivalents of TO (0.75 μ M) according to the binding stoichiometry. Metal complexes were titrated to displace TO from DNA by following a 11-steps gradual addition (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0 and 10.0 molar equivalents of DNA). Fluorescence spectra are recorded after each addition and the TO displacement is evaluated by measuring the fluorescence area (FA, from 510 nm to 750 nm, excitation wavelength is 501 nm). The percentage of TO displacement (TOD) is determined using the following equation:

$$TODx = 100 - [(FA_i/FA_1) \times 100] \quad \text{Equation 2.2}$$

with $1 < i < 12$, where FA_1 is the fluorescence area of thiazole orange upon binding to DNA and FA_i are the fluorescence area of TO under various ratios of [**Ru1**]/[TO-DNA]. The percentage of TO displacement ($TODx$) is then plotted as a function of the concentration of **Ru1**.

Competition dialysis Assay

75 μ M of different DNA structures, including duplex and G-quadruplexes (i.e. ds26, HT21, HT48, c-myc and c-kit2) in buffer (10 mM sodium cacodylate with 190 mM NaCl, pH 7.4) were annealed by heating at 95 °C for 5 min and slowly cooling down to room temperature before use. 100 μ L of each oligonucleotide sample was incubated within

Slide-a-Lyzer MINI dialysis unit which contains semipermeable membrane, held by floatation device, in contact with 1 μ M of ruthenium complexes in a beaker. After incubation for 12 hours, 90 μ L of each oligonucleotide solution was taken out, and 10 μ L of 10 % sodium dodecyl sulphate (SDS) was added into each solution to dissociate the complexes from the oligonucleotides. The SDS treated solutions and the free complexes in the dialysis buffer were then analyzed by UV/vis spectroscopy to determine the amount of bound complexes on DNA structures (concentrations of ruthenium complex present inside minus that outside of the dialysis membrane).

CD titration

CD spectra were recorded on a Jasco J-1500 spectropolarimeter using a 1 cm path length cuvette. DNA stock solutions were diluted to 3 μ M with 10 mM Tris (pH 7.4) with or without 100 mM KCl, respectively. CD spectra were recorded from 220-320 nm at a scan rate of 200 nm/min after each titration of complex to DNA solutions. The maximum HT voltage is below 600 V. All CD spectra were baseline-corrected and each curve represented of five averaged scans taken at 25 °C. Final analysis of the data was carried out using Origin 8.0 (OriginLab Corp.). The equilibrium binding constant (K_b^θ) was calculated according to previous report.^[29]

Thermal melting assay

Thermal melting of G-quadruplexes and double stranded DNA were obtained by CD and UV melting, respectively. Due to T_m of duplex DNA cannot be detected by CD melting, we combined UV melting result of duplex DNA as a supplementary with CD melting results of G-quadruplex DNAs, to compare the stabilization effects of Ru complex on these DNAs. 100 μ L of *c-myc* (3 μ M in 10 mM Tris buffer, pH = 7.4, 0.1 mM KCl), *c-kit2* (3 μ M in 10 mM Tris buffer, pH = 7.4, 5 mM KCl), HT21 and HT48 (3 μ M in 10 mM Tris buffer, pH = 7.4, 5 mM KCl), HT21 (3 μ M in 10 mM Tris buffer, pH = 7.4, 5 mM NaCl) and 130 μ L of ds26 (3 μ M in 10 mM Tris buffer, pH = 7.4, 5 mM KCl) were

annealed by heating at 95 °C for 5 min and gradually cooling to room temperature over couple hours. Ru complexes were added to DNA solution to yield a final concentration of 3 µM. For CD melting, ellipticity at 260 nm for *c-myc* and *c-kit2*, and at 295 nm for HT21 and HT48, were monitored upon temperature elevation from 15 to 90 °C with heating ramp of 0.5 °C/min. For UV melting, UV absorption of **ds26** at 260 nm and G-quadruplexes at 295 nm were recorded for the same temperature ramp as CD melting. Melting temperatures were analyzed by Origin 8.0 (OriginLab Corp.). Standard deviation over three repeat experiments were used as error bars.

Telomerase Repeat Amplification Protocol (TRAP) Assay

TRAP assay was performed by using the Millipore TRAPEZE® Telomerase Detection Kit and the protocols were slightly modified to fulfill the requirements in our experiment according to the previously report.^[25] CHAPS Lysis Buffer was used to extract telomerase from HeLa cells. TRAP assay was performed as follows. The “Master Mix” solution (including 5.0 µL 10× TRAP reaction buffer, 1.0 µL 50× dNTP mix, 1.0 µL TS primer, 1.0 µL TRAP primer mix, 2 Units Taq polymerase, 48.0 µL and 1.0 µL cell extract) was prepared in an RNase-free tube. Xx µL of Master mix were combined with XX µL of Ru complexes solution freshly prepared in DEPC-treated water to achieved the final concentration of **Ru1** as 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 µM. The mix was incubated at 30 °C for 30 min for the initial extension of TS by telomerase. To amplify the extension products by PCR, the following procedure was used: 95 °C for 5 min to inactivate the telomerase, followed by 24 cycles at 95 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s. Following PCR, 30 µL of TRAP reaction mixtures (containing 10 µL of 6 × loading dye) were analyzed by 10 % non-denaturing acrylamide gel in 0.5 × TBE (90 min at 400 volts).

Cytotoxicity

MDA-MB-231 (human breast carcinoma cells), HT1080 (human fibrosarcoma cells), HeLa (human cervical cancer cells), A549 (Human lung carcinoma), MCF-7 (human

breast adenocarcinoma) and NIH 3T3 (mouse embryo fibroblast cells) were seeded in a 96-well culture plate (5000 cells per well), and incubated in DMEM medium supplemented with 10% FBS, 1% L-glutamine and 1 % P/S at 37 °C with 5% CO₂ in a humidified atmosphere for 24 hours. The tested compounds were dissolved in bio-grade DMSO, diluted with DMEM medium to the required concentrations (contains <1% v/v DMSO) and added to the wells respectively. The plates were incubated at 37 °C in a 5% CO₂ incubator for 48 h continuously. After incubation, the medium was removed and 200 µL fresh medium contains MTT (20 µL, 5 mg/mL) was added to each well and the plate was incubated at 37 °C in 5% CO₂ for another 4 hours. Then the medium was removed and 150 µL DMSO was added into each well. After the plates were shaken for 10 mins, the optical density of each well was measured on a plate spectrophotometer at a wavelength of 570 nm with background subtraction at 650 nm.

Molecular docking of Pt complexes to GQ topological structures

In docking simulation, GOLD Suite v5.4 (CCDC Software Limited)^[30] is used to dock the crystal structure of two complexes, **Ru1** and **Ru2**, into the basket (PDB ID: 2MCC) and hybrid G-quadruplex structures (PDB ID: 2MB3), respectively. The scoring function was calculated by CHEMPLP. In each simulation, all the torsional angles within 15 Å to the binding site, i.e. the center of the external G-quartet layer, was free to rotate. Subsequently genetic algorithm was employed to find the maximum fitness value among all possible configurations and translational positions of complexes around the binding center on G-quadruplexes. For each simulation, the docking solution with largest fitness value was chosen. The docked structures of ruthenium complexes on GQs were visualized and the H-bond lengths were measured in VMD (NIH Center for Macromolecular Modeling and Bioinformatics, at the Beckman Institute, University of Illinois at Urbana-Champaign).^[31]

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CHAPTER 3

Aza-bridged Bisphenanthrolinyl Pt(II) Complexes: Efficient Stabilization and Topological Selectivity of Telomeric G-quadruplexes

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3.1 Introduction

Nucleic acids with guanine rich sequences have a propensity to adopt tetra-stranded intra- and intermolecular quadruplex structures that are stabilized by a stack of planar aromatic guanine quartets (G-tetrad), in which four guanines are held together by noncanonical Hoogsteen hydrogen bonding.^[1-5] Formation and stability of the whole G-quadruplex structure usually exhibited monovalent cation-dependent manner: $\text{Li}^+ < \text{Na}^+ < \text{K}^+$.^[6-9] These G-rich sequences can adopt different topologies with distinct strand orientation, including parallel, antiparallel and parallel/antiparallel hybrids.^[10-17] G-quadruplexes (GQ) involved in a number of key cellular processes. The well-known example is the human telomeric DNA, which is consists of tandem arrays of simple G-rich (TTAGGG) repeats over several hundred bases.^[18-20] It has been shown that if this single-stranded telomeric DNA folded into a quadruplex, the activity of telomerase will be inhibited and thus impede cell proliferation.^[21,22] Recent studies found that telomeric RNA (*TERRA*), which is transcribed from the C-rich strand of telomeric DNA, was involved in chromatin regulation and remodeling, as well as telomerase function.^[23-25] Besides, many oncogene promoters possess G-quadruplex folding sequences, such as *c-myc*, *bcl-2* and *c-kit*, form rich GQ topology *in vitro* and *in vivo*.^[26] The significant biological roles of G-quadruplex suggested GQ, as a unique family of noncanonical secondary structure, have high potentials as anticancer drug targets for chemotherapeutics.

Up until now, considerable studies focused on the stabilization of G-quadruplexes by using small molecules, such as porphyrin-based complexes and heteroaromatic organic compounds.^[27-32] These compounds with planar aromatic motifs can stack with the multiple layers of G-tetrads at the core of G-quadruplex. At the same time the extensive size and shape of the large aromatic conjugates were also used to distinguish duplex DNA and achieve specific binding to GQs. Besides, sequence and cation dependence of folding polymorphism make it possible to target a specific G-rich sequence by interactions

between binding molecules and unique loop arrangements. Consequentially, a GQ stabilizer with binding preference to a specific G-quadruplex topological structure can reach the goal of disease specific and/or personalized medicines.

Metal complexes have been reported to strongly and selectively interact with quadruplex DNA.^[33-38] As G-quadruplex binders, metal complexes display several advantageous over their organic counterparts. Metal complex with planar heteroaromatic ligand provided strong π stacking with G-tetrad so that efficiently stabilized G-quadruplexes. Besides, both the coordination geometry of metal center, the ligand size and alkyl tails extended from aromatic moieties were easily tuned to obtain topological recognition of GQ targets. Moreover, the electropositive metal center endowed its interaction with cation cavity at the center of G-quadruplexes. Although these metal complexes have shown excellent binding affinity and stabilization on DNA G-quadruplexes, none of them has been demonstrated as RNA G-quadruplex stabilizer until now.

To date, a lot of platinum complexes have been reported as G-quadruplex stabilizers. The square shape of di-ligand coordination on Pt^{2+} provides the necessary size and shape of aromatic area for good π - π stacking with G-quartets.^[39-43] Metal complexes coordinated with phenanthroline and its derivatives have been widely studied as GQ binders and stabilizers due to its excellent size matched with G-quartet. Herein, we designed and synthesized two platinum complexes with bis(1,10-phenanthroline-2-yl)amine (bpa) ligand. Two 1,10-phenanthroline (phen) were connected via an aza-bridge and form a planar configuration under acidic condition. Upon coordination, two $[\text{Pt}(\text{bpa})]^{2+}$ complexes can enter a rigidly flat geometry and bestow strong π stacking with G quartets to enhance GQ stability. Both Pt complexes exhibited significantly higher stabilization effect towards both DNA and RNA G-quadruplexes than duplex DNA.

3.2 Results and Discussion

3.2.1 Synthesis of $[\text{Pt}(\text{bpa})]^{2+}$ Complexes with Planar Structure

The two phenanthrolines in $[\text{Pt}(\text{phen})_2]^{2+}$ provided a near square shape of aromatic area with similar size to that of a G-tetrad.^[39] Previous molecular docking of $[\text{Pt}(\text{dip})_2]^{2+}$, a $[\text{Pt}(\text{phen})_2]^{2+}$ derivative with four corner phenyl groups, showed the two phen ligands underwent a conformation alteration to enter a flatter coordination plane upon stacking with *c-myc* GQ.^[44] This phenomenon suggested a planar geometry of Pt complexes would be essential for the high stabilization effect on G-quadruplex. The X-ray crystal structure of acidified bpa ligand was previously reported to be a near planar structure, since the aza-bridge can facilitate the two phenanthrolines to form an intramolecular H-bond network and to overcome the electron repulsion among the C2 hydrogens of the two phen rings (Scheme 3.1).^[45] Herein, $[\text{Pt}(\text{bpa})]^{2+}$ was expected to have a rigid planar geometry that was similar to the bound $[\text{Pt}(\text{phen})_2]^{2+}$ on GQ. Meanwhile, the aza-bridge offered an opportunity to extend the aromatic ring with alkyl tail via facile synthetic routes. Thus, extra interaction modes with loops region would be achieved by varying the lengths and charges on alkyl tails.

Scheme 3.1 Synthetic procedure of the Platinum complexes.

For the synthesis of two Pt complexes, the bpa ligand was firstly synthesized as previously report.^[45,46] After basified the bpa ligand, alkylation of the amino group as aza-bridge was carried out by refluxing bpa in dimethyl sulfoxide in the presence of KOH and

alkylation agents (iodomethane or 1-(2-chloroethyl)piperidine). The alkylated bpa ligands were then protonated with trifluoroacetic acid so that two phenanthrolyl moieties were stabilized into a planar structure via hydrogen bonds, which make it ready for chelation of Pt(II) (Scheme 3.1). The complex structures were characterized by ^1H and ^{195}Pt NMR and ESI-MS (Figure 3.1 and 3.2).

Figure 3.1 ^1H NMR spectrum of (A) L1 (in DMSO- d_6); (B) 1 (in DMSO- d_6) and (C) ^{195}Pt NMR of 1 (in DMSO- d_6).

Figure 3.2 ^1H NMR spectrum of (A) **L2** (in Methanol- d_4); (B) **2** (in DMSO- d_6).

3.2.2 High Efficiency of Pt Complexes on Stabilizing Both DNA and RNA GQs

The stabilizing effects of Pt complexes on GQs and duplex DNA were first explored by thermal denaturation of DNA. The sequences of DNA structures were listed in Table 3.1.

Table 3.1 Sequences of oligomers (primers) used in this work.

Name	Sequence
ds26	5'-CAATCGGATCGAATTCGATCCGATTG-3'
<i>c-myc</i>	5'-TGGGGAGGGTGGGGAGGGTGGGGAAGG-3'
HT21	5'-GGGTTAGGGTTAGGGTTAGGG-3'
TERRA	5'-uaggguuaggg-3' ^a

^aTERRA is a 12-mer RNA strand.

Upon addition of one equivalent of **1** or **2**, significant enhancement in thermal melting temperatures of G-quadruplexes (ΔT_m) were observed for two DNA G-quadruplexes (Figure 3.3). ΔT_m of telomeric DNA GQs achieved 40 °C, while decent T_m increment up to 16 °C were readily observed for *c-myc* GQ (Table 3.2). The discrepancy

in T_m increments was presumably due to the distinct topology of telomere G-quadruplex. However, for the duplex DNA, only 4.5 and 2.0 °C incensement in melting temperatures was observed in the presence of **1** or **2**, respectively. Such insignificant ΔT_m suggested that the stabilizing abilities of Pt complexes on duplex DNA were much weaker than that on GQs.

Figure 3.3 (A-D) CD Melting of ds26, *c-myc*, HT21 and TERRA in the presence and absence of **1** and **2**. (E-F) The CD spectra of HT21 along with temperature elevation. (E) HT21 alone; (F) HT21 with **1**; (G) HT21 with **2**.

Table 3.2 The increments in melting temperatures (ΔT_m)^a of DNA by **1** and **2**.

DNA	ΔT_m (°C)	
	1	2
HT21	40 (4)	38 (4)
<i>c-myc</i>	16 (3)	16 (4)
TERRA	15 (2)	16 (3)
ds26	4.5 (0.8)	2.0 (0.6)

^a $\Delta T_m = T_m(\text{Pt-DNA}) - T_m(\text{DNA})$, where $T_m(\text{Pt-DNA})$ and $T_m(\text{DNA})$ are CD melting temperatures of DNA structures in the presence and absence of platinum complexes, **1** or **2**, respectively.

Binding affinities of this two Pt complexes to all the DNA structures was abstracted from UV-vis titration (Table 3.3). Although the differences in binding affinities for two Pt complexes towards GQs and duplex DNA were not as high as that in ΔT_m , K_b values still reached nearly 4 - 5 times higher on GQs than that on ds26.

Table 3.3 Binding constants (K_b) of DNA structures with Pt complexes **1** and **2**.

DNA	$K_b (\times 10^5 \text{ M}^{-1})$	
	Pt1	Pt2
ds26	1.8 (0.4)	2.4 (0.1)
<i>c-myc</i>	7.9 (0.8)	9.7 (0.4)
HT21	9.7 (0.4)	11.2 (0.2)

As mentioned above, telomere sequence is transcribed into telomeric repeat-containing RNA, which was found in living cells and regarded as a promising cancer therapeutic target as well [23-25]. So far, some metal complexes have shown excellent binding affinity and stabilization on DNA G-quadruplexes, but none of them has been demonstrated as RNA G-quadruplex stabilizer. Inspired by the such high ΔT_m values on DNA G-quadruplex, the interactions of these two platinum complexes on RNG G-quadruplex were further explored. 12-nt human telomeric RNA sequence (*TERRA*) as the transcript of telomeric DNA, r(UAGGGUUAGGGU) was used in this study due to the known dimeric parallel G-quadruplexes structure characterized by NMR experiment.^[47]

Similar as DNA GQs, K^+ could stabilize the bimolecular *TERRA* RNA GQ by coordinating to guanines from two RNA strands. The thermal stability of *TERRA* GQ in K^+ buffer was characterized by CD melting experiments. Addition of one equivalent of **1** or **2** shifted the melting curve of *TERRA* GQ more than 15 °C towards high temperature (Table 3.2). As expected, the bpa chelated Pt complexes showed the ability to not only stabilize DNA G-quadruplexes, but also the telomeric RNA GQ. The stabilizing ability of both Pt complexes on RNA GQ were as good as the small molecular stabilizer reported up to now, if not better, such as BRACO-19 (in the presence of 2.5 equivalents, ΔT_m for *TERRA* RNA GQ was only up to 11.3 °C).^[48] Since the two Pt complexes showed negligible difference in ΔT_m for RNA GQ, the flat aromatic core of two $[Pt(bpa)]^{2+}$ complexes had the suitable size and shape to offer dominant π -stacking effects to stabilize RNA g-tetrads in *TERRA* GQ.

3.2.3 Stabilizing Preferences of Pt Complexes Toward Antiparallel G-quadruplex Topology

Besides the selectively stabilization effect on GQs over duplex DNA, these two Pt complexes may further distinguish and alter the folding topology of G-quadruplexes. As shown in Table 3.2, ΔT_m of telomeric DNA G-quadruplexes (about 40 °C) were nearly 25 °C higher than that of *c-myc* GQ (16 °C) and *TERRA* RNA GQ (15~16 °C). The thermal stability of the antiparallel HT21 GQs were elevated more significantly than that of parallel DNA and RNA GQs.

The preference of **1** and **2** to stabilize antiparallel GQs could be further evident by CD spectra (Figure 3.4). In the presence of potassium ion, addition of **1** or **2** will promote a new peak at 260 nm with negative ellipticity, accompanied with the exit of the positive peak shoulder around 272 nm. Meanwhile, the major positive band at 295 nm was mildly enhanced. The spectra variation was consistent with the transition of GQ topology from 3+1 hybrid to basket type antiparallel GQ, which indicated that **1** and **2** can efficiently

and specifically induce and stabilize the basket-type GQ, even though hybrid GQ is the dominant topology in potassium buffer.^[9,49,50] On the contrary, the folding structure of both *c-myc* GQ and dimeric *TERRA* RNA GQ have been determined as a parallel topology with propeller-type structure. Little variation in CD spectra of both parallel GQ upon the addition of **1** or **2** was observed, regardless the distinct conformation of backbone pentose (Figure 3.4). The discrepancy that both Pt complexes showed on the folding topology and thermal stability of parallel and anti-parallel GQs, suggested that the planar coordination geometry in **1** and **2** likely possessed a strong inclination to antiparallel GQ, especially basket-type folding topology, and achieve remarkably high stabilizing effects.

Figure 3.4 CD spectra of G-quadruplexes in the presence of **1** and **2**. (A) HT21, (B) *c-myc* and (C) *TERRA*.

3.2.4 Molecular Docking of Pt Complexes on Different GQ Structures

Since topological folding of GQ cannot be exclusively demonstrated by CD spectra,^[51] molecular docking of Pt complexes on three different GQ structures, basket (PDB 2MCC), 3+1 hybrid (2MB3) and parallel GQ (1XAV) were utilized to provide further information for topological selectivity of Pt complexes favoring antiparallel GQ over parallel structure, especially to basket G-quadruplex. Pt complexes showed remarkably higher fitting values on basket and hybrid 3+1 G-quadruplexes topologies (> 82) than the parallel structure (< 67), which is consistent with the observations on the thermal stability and CD spectra of both RNA and DNA GQs. Both **1** and **2** achieved

higher fitting values on basket GQ than 3+1 hybrid GQ (Table 3.4) indicating the inclination to basket folding topology.

Table 3.4 Best fitting values of molecular docking of **1** and **2** on various GQ topological structures.

GQ topology (PDB code)	basket type (2MCC)	3+1 hybrid (2MB3)	parallel <i>c-myc</i> (1XAV)
1	89.6	82.4	57.4
2	103.7	95.3	67.0

In the best docking structure, Pt complexes stacked on top of the external G-quartet in the dual lateral loop side (Figure 3.5A and D). From the top view (Figure 3.5B and E), the bpa ligand showed an appropriate size to overlap exactly right on top of the aromatic area of G-quartet. On the contrary, both **1** and **2** would disturb the flatness of G-quartet upon stacking on 3+1 hybrid GQ (Figure 3.5C and F). The discrepancy in the fitting values and docking structures of Pt complexes on different GQ structures confirmed the better accommodation of both Pt complexes to basket type GQ with a higher stability, which also supported the conversion of CD spectra from 3+1 hybrid GQ to basket GQ upon binding to Pt complexes.

Figure 3.5 Molecular docking of **1** and **2** on basket (PDB: 2MCC, A, D: side view; B, E: top view,) and 3+1 hybrid GQ (PDB: 2MB3, C, F: side view). Backbone and G-quartets in both quadruplexes were represented as cyan ribbon and in stick mode, respectively. **1** (A-C), **2** (D-E) and Pt cation were shown in orange stick and pink ball, respectively.

3.2.5 Effects of the Alkyl Side Chains on Distinguishing DNA Structures

1 and **2** showed 4.5 and 2.0 °C of ΔT_m on duplex DNA, respectively. Stabilizing ability of **2** on duplex was decreased comparing with **1**, which ΔT_m was similar as that of $[\text{Pt}(\text{phen})_2]^{2+}$.^[44] This suggested that longer alkyl chain with a bulky piperidinium terminal in **2** prevent the aromatic ligand in complexes from binding or intercalating into duplex DNA. Remarkably, compared with $[\text{Pt}(\text{phen})_2]^{2+}$, 5 folds higher ΔT_m value on HT21 GQ was achieved by $[\text{Pt}(\text{bpa})]^{2+}$ complexes, while interactions with duplex DNA were comprised. Hence, the pre-planar conformation in $[\text{Pt}(\text{bpa})]^{2+}$ complexes obviously enhanced the binding specificity to G-quadruplexes, while a flexible positive alkyl tail from aza-bridge improved the selectivity over duplex DNA.

Although the melting temperature incensements of GQs upon addition of **1** and **2** were nearly the same, the fitting values of the two complexes on the same GQ structure indicated a difference. Fitting value of **2** to the basket topology was much higher than those of **1**, which was presumably due to the extra hydrogen bonds and/or electrostatic/hydrophobic effects between the loop backbone/nucleobases of GQ and the aza-alkyl tail in **2** (Figure 3.5E). Docking **2** on the basket GQ resulted in the conformation that the alkyl tail resided in the groove next to 3'-terminal. The piperidinium ring was placed parallel to an adenine base in the lateral loop in which several methylene hydrogens were within the distance of hydrogen bonds with purine nitrogens (Figure 3.5E). Whereas in the case of 3+1 hybrid topology, not only these interactions were absence, but accommodation of the alkyl tail into the narrow groove between 3' and 5' terminals altered both the orientation of Pt complex and the flatness of G-tetrads (Figure 3.5F), which would significantly comprise the stability of hybrid GQ and GQ-1/GQ-2 as one biological motif.

In our previous work, the aromatic anchors from two phen ligands in $[\text{Pt}(\text{Dip})_2]^{2+}$ bestow a high binding preference on parallel G-quadruplex, while the current study

showed that $[\text{Pt}(\text{bpa})]^{2+}$ complexes with pre-planar structure exhibited much higher stabilization effects on antiparallel topology. The piperidinium tail showed extra interaction modes with loops region, which assisted the conformation conversion of HT GQ from 3+1 hybrid to basket structure. Hence, by properly tuning the flatness and the peripheral moieties of bis-phen coordination core, Pt complexes were readily to specifically target distinct GQ topological structures, regardless of the backbone composition.

3.2.6 Potential Cytotoxicity Towards Cancer Cells

The aforementioned results demonstrated the two Pt complexes could efficiently stabilize different DNA GQs, as well as telomeric RNA G-quadruplex. These findings encourage us to further investigate the biological activities of the complexes. *In vitro* cytotoxicity of **1** and **2** against HeLa (human cervical epithelioid carcinoma), A549 (Human lung carcinoma) and MCF-7 (human breast adenocarcinoma) cancer cell lines were evaluated by MTT assay. IC_{50} values of **1** and **2** against all the cell lines were from 0.4 to 4 μM , as shown in Table 3.5. While the cell viability of A549, the most sensitive cancer cell line towards Pt complexes, was still more than 50% when 100 μM of the ligands (L1 and L2) or the precursor $(\text{Pt}(\text{DMSO})_2\text{Cl}_2)$ were treated alone (data not shown). This confirmed that the potent cell cytotoxicity were caused by $[\text{Pt}(\text{bpa})]^{2+}$ as a complex, but neither ligand or metal core as individual component. The potency of Pt complexes here was nearly 9 and 6 times more potent than the well-known GQ stabilizer, TMPyP4 and chemotherapy drug, cisplatin, respectively.

Table 3.5 Cytotoxicity (IC₅₀) of **1** and **2** compared with TMPyP4 and cisplatin towards various cancerous cell lines.^a

	IC ₅₀ (μM)			
	TMPyP4	Cisplatin	1	2
Hela	19 (3)	13 (1)	2.2 (0.1)	3.6 (0.1)
A549	43 (1) ^[52]	36 ^[53]	0.351 (0.002)	1.46 (0.01)
MCF-7	90 (3)	15 ^[53]	1.46 (0.04)	3.98 (0.04)

^a Standard deviation were listed in parentheses.

3.3 Conclusion

In this study, two Pt complexes coordinated with aza-bridged *bis*-phenanthroline ligands were designed and synthesized. The aza-bridge enabled the complexes to obtain a flat aromatic structure even prior to the binding of G-quadruplex and achieve remarkably high stabilizing effects on both human telomeric DNA and RNA G-quaruplexes. The alkyl chain extended from aromatic core further decreased the binding and stabilization of the complexes on duplex DNA and hence enhanced the binding specificity towards G-quadruplex. Moreover, both complexes showed topological preferences towards basket-type of antiparallel G-quadruplex evidenced by higher thermal stability and alteration of hybrid to basket GQ folding conformation even in the potassium buffer. The stabilizing effects on human telomeric GQ rendered potent cytotoxicity of the complexes towards a wide range of the cancer cell lines that showed high potentials of the complexes as drug candidates for anticancer therapy.

3.4 Experimental Section

All the chemicals and RNA sequence were purchased from Sigma-Aldrich. All DNA sequences were purchased from Sangon (Shanghai, China). The synthetic route of *N*-methyl-*N*-(1,10-phenanthroline-2-yl)-1,10-phenanthroline-2-amine (**L1**) and *N*-(1,10-phenanthroline-2-yl)-*N*-(2-(piperidin-1-yl)ethyl)-1,10-phenanthroline-2-amine (**L2**) were described in supporting information. Milli-Q water was used in all physical measurement experiments. ¹⁹⁵Pt NMR spectra were recorded on a Bruker AVIII 400MHz NMR

spectrometer. Chemical shift was referenced externally to K_2PtCl_4 in D_2O (δ -1628 ppm) for ^{195}Pt NMR spectra.

Synthesis of two ligands

Synthesis of *N*-methyl-*N*-(1,10-phenanthrolin-2-yl)-1,10-phenanthrolin-2-amine (**L1**). *N,N*-bis-(1,10-phenanthrolin-2-yl)-amine (bpa) was prepared as previously reported.^[45,46] Into DMSO (3 mL) were successively added potassium hydroxide (17 mg) and bpa (20 mg). The resulting deep-orange-colored solution was stirred at r.t. for 30 min. Iodomethane (12.4 μL) was then added and the orange solution was stirred at r.t. for 48 h, and then diluted with water (20 mL), which lead to the immediate deposition of a fine yellow precipitate and was collected by filtration. After washing with water, the yellow solid was dried to yield the compound (80% yield). ^1H NMR (500 MHz, DMSO) δ 9.09 (dd, J = 4.2, 1.5 Hz, H2,2', 2H), 8.45 (dd, J = 8.1, 1.5 Hz, H4,4', 2H), 8.40 (d, J = 8.8 Hz, H7,7', 2H), 7.98 (d, J = 8.8 Hz, H6,6', 2H), 7.96 (d, J = 8.8 Hz, H5,5', 2H), 7.86 (d, J = 8.8 Hz, H8,8', 2H), 7.74 (dd, J = 8.1, 4.2 Hz, H3,3', 2H), 4.09 (s, H1,1',1'', 3H). ESI-MS ($\text{M}+\text{H}$) = 388.41.

Synthesis of *N*-(1,10-phenanthrolin-2-yl)-*N*-(2-(piperidin-1-yl)ethyl)-1,10-phenanthrolin-2-amine (**L2**). Into dimethyl sulfoxide were successively added potassium hydroxide (7.8 mg) and bpa (10 mg). The resulting deep-orange colored solution was stirred at room temperature for 2 h. 1-(2-chloroethyl)piperidine hydrochloride (8.4 mg) was then added and the orange solution was stirred at 110 $^\circ\text{C}$ for 8 h. It was then diluted with water, which lead to the immediate deposition of a yellow precipitate and was collected by filtration. The precipitate was washed with water for several times to yield the compound (40 % yield). ^1H NMR (300 MHz, MeOD) δ 9.01 (dd, J = 4.4, 1.7 Hz, H2,2', 2H), 8.43 (dd, J = 8.2, 1.7 Hz, H4,4', 2H), 8.26 (d, J = 8.8 Hz, H7,7', 2H), 7.88 (d, J = 8.8 Hz, H6,6', 2H), 7.79 (d, J = 8.8 Hz, H5,5', 2H), 7.72 (dd, J = 8.2, 4.4 Hz, H3,3', 2H), 7.71 (d, J = 8.8 Hz, H8,8', 2H), 5.10 (t, J = 6.8 Hz, H1,1', 2H), 2.95 (t, J = 6.8 Hz,

H9,9', 2H), 2.63-2.53 (m, H10,10',10'',10''', 4H), 1.43-1.25 (m, H11,11',11'',11''',12,12', 6H). ESI-MS (M+H) = 485.26.

Synthesis of [Pt(bpa)]Cl₂ complexes

[Pt(L1)]Cl₂ (**1**). Pt(DMSO)₂Cl₂ (11.0 mg) and **L1** (10 mg) were brought to reflux in a mixture of methanol and water with the ratio of 1:1. After 5 hours, the mixture was cool to room temperature and yielded orange precipitates. The crudes were collected by filtration and were washed with methanol for three times to provide the product in 78% yield. ¹H NMR (300 MHz, DMSO-d₆) δ 9.98 (d, *J* = 4.5 Hz, H2,2', 2H), 9.35-9.25 (m, H4,4',7,7', 4H), 8.69 (d, *J* = 9.3 Hz, H8,8', 2H), 8.53-8.33 (m, H3,3',5,5',6,6', 6H), 4.53 (s, 3H). ¹⁹⁵Pt NMR (86 MHz, DMSO-d₆): δ -2272. ESI-MS calcd for [M - Cl]⁺ 617.08, [M - 2Cl]²⁺ 291.06. Found [M - Cl]⁺ 616.96, [M - 2Cl]²⁺ 290.96.

[Pt(L2)]Cl₂ (**2**). Pt(DMSO)₂Cl₂ (11.0 mg) and **L2** (12.5 mg) were reflux in methanol. After 5 hours, the mixture was cool to room temperature and yielded yellow precipitates. The crudes were collected by filtration and were washed with methanol for three times to provide the product in 85% yield. ¹H NMR (500 MHz, DMSO-d₆) δ 10.05 (d, *J* = 4.7 Hz, H2,2', 2H), 9.38 (d, *J* = 9.2 Hz, H7,7', 2H), 9.33 (d, *J* = 8.2 Hz, H4,4', 2H), 8.90 (d, *J* = 9.2 Hz, H8,8', 2H), 8.58 (d, *J* = 8.6 Hz, H6,6', 2H), 8.52-8.45 (m, *J* = 7.6 Hz, H3,3',5,5', 4H), 5.49 (t, *J* = 7.3 Hz, H1,1', 2H), 3.86 (t, *J* = 7.3 Hz, H9,9', 2H), 3.66 (d, *J* = 11.3 Hz, H10,10', 2H), 3.15 (s, H10'',10''', 2H), 1.90 (m, H11'',11''',12,12' 4H), 1.80 (d, *J* = 11.7 Hz, H11, 1H), 1.52-1.37 (m, H11', 1H). ESI-MS calcd for [M - Cl]⁺ 714.17, [M - 2Cl]²⁺ 339.60. Found [M - Cl]⁺ 715.04, [M - 2Cl]²⁺ 339.19.

Thermal melting assay

Thermal melting of G-quadruplexes and double stranded DNA were obtained by CD melting. 100 μL of *c-myc* or HT21 (3 μM in 10 mM lithium cacodylate buffer, pH = 7.4, without KCl), 100 μL of telomeric RNA (6 μM in 10 mM potassium phosphate buffer, pH = 7.0, 15 mM KCl) and 100 μL of ds26 (5 μM in 5 mM potassium phosphate buffer,

pH = 7.4) were annealed by heating at 95 °C for 5 min and gradually cooling to room temperature over couple hours. Pt complexes were added to DNA solution to yield a final concentration of 3 μ M or 5 μ M (only for ds26). Thermal melting was monitored at 260 nm for *c-myc*, 295 nm for HT21, 263 nm for telomeric RNA and 250 nm for ds26, at the heating rate of 0.5 °C/min from 20 to 95 °C. Melting temperatures were analyzed by Origin 8.0 (OriginLab Corp.). Standard deviation over three repeat experiments were used as error bars.

UV-vis absorption titration

For UV titration experiment, 100 μ L of Pt complex solution (20 μ M in 100 mM KCl, 10 mM Tris HCl, pH 7.4) was titrated with 1 μ L of DNA stock (100 μ M in 100 mM KCl, 10 mM Tris HCl, pH 7.4) at 25 °C. Binding association constant K (M^{-1}) was calculated with the literature method.^[54] The results were got from average of three replicates.

CD spectrum and titration

CD spectra were recorded on a Jasco J-1500 spectropolarimeter using a 1 cm path length cuvette. DNA stock solutions were diluted to 3 μ M with 10 mM lithium cacodylate buffer (pH 7.4) with 1 mM KCl for *c-myc* and 10 mM KCl for HT21. 12-nt RNA stock solution was diluted to 6 μ M with 10 mM potassium phosphate buffer (pH = 7.0) with 15 mM KCl, respectively. CD spectra were recorded from 220-320 nm at a scan rate of 200 nm/min after each titration of complex to DNA solutions. The maximum HT voltage is below 600 V. All CD spectra were baseline-corrected and each curve represented of five averaged scans taken at 25 °C. Final analysis of the data was carried out using Origin 8.0 (OriginLab Corp.).

Molecular docking of Pt complexes to GQ topological structures

1 and **2** were first drawn by Gaussian view followed by an optimization step (B3LYP/6-31g* for C, H, N; SDD for Pt) using Gaussian 09 to optimize the coordination structures of two Pt complexes.^[55] In docking simulation, GOLD Suite v5.4 (CCDC

Software Limited) is used to dock the optimized structures of two complexes, **1** and **2**, into the basket (PDB ID: 2MCC) and hybrid G-quadruplex structures (PDB ID: 2MB3), respectively.^[56] Pt cation was set as a dummy atom for docking simulation. The scoring function was calculated by CHEMPLP. In each simulation, all the torsional angles within 15 Å to the binding site, i.e. the center of the external G-quartet layer, was free to rotate. Subsequently genetic algorithm was employed to find the maximum fitness value among all possible configurations and translational positions of complexes around the binding center on G-quadruplexes. For each simulation, the docking solution with largest fitness value was chosen. The docked structures of platinum complexes on GQs were visualized and the H-bond lengths were measured in VMD (NIH Center for Macromolecular Modeling and Bioinformatics, at the Beckman Institute, University of Illinois at Urbana-Champaign).^[57]

Cell cytotoxicity

HeLa (human cervical epithelioid carcinoma), A549 (Human lung carcinoma) and MCF-7 (human breast adenocarcinoma) cancer cell lines were seeded in a 96-well culture plate (5000 cells per well), and incubated in DMEM medium supplemented with 10 % FBS, 1 % L-glutamine and 1 % P/S at 37 °C with 5 % CO₂ in a humidified atmosphere for 24 hours. The tested compounds were dissolved in bio-grade DMSO, diluted with DMEM medium to the required concentrations (contains <1 % v/v DMSO) and added to the wells respectively. The plates were incubated at 37 °C in a 5 % CO₂ incubator for 48 h continuously. After incubation, the medium was removed and 200 µL fresh medium contains MTT (20 µL, 5 mg/mL) was added to each well and the plate was incubated at 37 °C in 5 % CO₂ for another 4 hours. Then the medium was removed and 150 µL DMSO was added into each well. After the plates were shaken for 10 mins, the optical density of each well was measured on a plate spectrophotometer at a wavelength of 570 nm with background subtraction at 650nm.

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CHAPTER 4

[Pt(bpa)]²⁺ Complex: a GQ ‘Light-switch’ for Visualizing and Tracking the Folding Dynamics of RNA GQ in Live Cells

Adapted from manuscript: Lei He, Fangwei Shao

4.1 Introduction

G-quadruplexes (GQ) are a family of non-canonical DNA/RNA structures transiently formed from cellular guanine rich nucleic acids.^[1,2] In the past decade, diverse key roles that DNA GQs play in a plethora of biological processes, such as telomere maintenance, DNA replication and transcription, identified DNA GQs as the promising therapeutic targets for many human diseases.^[2-5] However, the biological functions of RNA GQs, though would be much more intriguing and promising from the aspect of medicine and theranostics comparing to their DNA analogs, are remained rarely known. This is largely due to the paucity and short lifetime of RNA GQ that make it extremely challenging to target RNA GQ and to track their folding dynamics in live cells, especially when currently proper optical probes for RNA GQ is still rare. Early work on GQ-specific antibody limited to *ex vivo* applications and fixed cell imaging due to the complicate multistep or reagents protocols.^[6] Recent development of organic fluorescent probes targeted RNA GQ and progressed to visualize RNA GQ in the live cells.^[7-9] However, the naphthalene^[7] or cyanine^[8,9] scaffold cannot circumvent the common concerns of organic fluorophores, including unideal imaging channel, self-quenching and photo-leaking due to small Stokes shifts, and fast photobleaching. Whereas, metal complexes, as another category of GQ-specific binder,^[10] often exhibit large Stokes shift, long lifetime from triplet excited state emission and tunable metal dependent emission channels. Several salphen complexes and [Ir(ppy)³⁺] (ppy is 2-Phenylpyridine) have shown the promising fluorogenic signals in *ex vivo* protocols.^[11-13] However, since the fluorescence turn-on mechanism is either non-GQ specific^[12,14] or relies on the derivatization of small GQ binders,^[13] the fluorescent selectivity of these inorganic probes against duplex DNA/RNA is often compromised. Herein, inspired by the luminescent-switch manner of [Ru(bpy)₂(dppz)]²⁺ (dppz is dipyrido[3,2-*a*:2',3'-*c*]phenazine) on duplex DNA,^[15] in which H-bonds between aza nitrogen on dppz ligand and solvent rendered fluorescent

quenching in aqueous buffer and fluorescence turn on upon intercalating into duplex DNA, we restored a free aza (-NH-) moiety in our previously reported Pt-bpa complexes (bpa is *N,N*-bis-(1,10-phenanthrolin-2-yl)-amine)^[16] by removing the alkyl chain (Figure 4.1). [Pt(bpa)]²⁺ complexes exhibited high fluorescent quantum yields only on G-quadruplexes and can track the dynamic un/folding of TERRA RNA GQ in live cells.

Figure 4.1 Chemical structures of Pt complexes **1-3**. (b) Crystal structure of **1** (Hydrogen atoms are omitted for clarity).

4.2 Results and discussion

4.2.1 Synthesis and Characterization of [Pt(bpa)]²⁺ Complexes

[Pt(bpa)]²⁺ (**1**) and its derivatives were synthesized *via* chelating of protonated bpa or its derivatives to Pt center from Pt(DMSO)₂Cl₂. Crystal structure of **1** have the additive angles among four coordination bonds (N1–Pt1–N2, N2–Pt1–N4, N4–Pt1–N5 and N5–Pt1–N1) summed up to 360.1° (Figure 4.2 and Table 4.1), and suggested a square planar geometry. With such a flat geometrical structure, [Pt(bpa)]²⁺ and its derivatives bind selectively to DNA and RNA G-quadruplexes.

Figure 4.2 Crystal structure of [Pt(bpa)]²⁺ showing the atom labeling.

Table 4.1a Crystal data, collection and structure refinement parameters for **1**.

Chemical formula	C ₅₁ H ₃₄ F ₁₂ N ₁₀ OP ₂ Pt ₂
Formula weight (g mol ⁻¹)	1483.00
Crystal system	monoclinic
Space group	P 1 21/c 1
Crystal size (mm ³)	0.020 x 0.040 x 0.240
Temperature (K)	103(2)
<i>a</i> (Å)	6.8545(3)
<i>b</i> (Å)	39.3855(16)
<i>c</i> (Å)	17.3529(7) Å
α (deg)	90
β (deg)	98.991(2)
γ (deg)	$\gamma = 90$
<i>V</i> (Å ³)	4627.2(3)
<i>Z</i>	4
ρ_{calcd} (g cm ⁻³)	2.129
μ (mm ⁻¹)	12.723
F(000)	2848
Data / restraints / parameters	8170 / 72 / 705
<i>R</i> ₁ [<i>I</i> > 2 σ (<i>I</i>)]	0.0438
<i>wR</i> ₂ [<i>I</i> > 2 σ (<i>I</i>)]	0.0999
<i>R</i> ₁ (all data)	0.0472
<i>wR</i> ₂ (all data)	0.1014
Goodness-of-fit on <i>F</i> ²	1.220

Table 4.1b Selected Bond Lengths (Å) and Bond Angles (deg) for **1**.

Pt1-N1	2.062(5)
Pt1-N2	1.981(6)
Pt1-N4	1.992(5)
Pt1-N5	2.062(5)
N1-Pt1-N2	81.2(2)
N1-Pt1-N4	172.7(2)
N1-Pt1-N5	106.2(2)
N2-Pt1-N4	91.6(2)
N2-Pt1-N5	172.6(2)
N4-Pt1-N5	81.1(2)

4.2.2 [Pt(bpa)]²⁺ Selectively Stabilize and “Lighted-Up” by GQ Structures

The binding performance of **1** on DNA/RNA G-quadruplexes and double stranded DNA structures were investigated by spectroscopic and thermal melting studies. Nucleic acid sequences we used were listed in Table 4.2.

Table 4.2 Sequences of oligomers (primers) used in this work.

Oligomer	Sequence
TERRA	5'-uaggguuaggg-3'
EBNA1	5'-ggggcaggagcaggagga-3'
<i>c-myc</i>	5'-TGGGGAGGGTGGGGAGGGTGGGGAAGG-3'
HT21	5'-GGG(TTAGGG) ₃ -3'
ds26	5'-CAATCGGATCGAATTCGATCCGATTG-3'
ssDNA	5'-CACACTTCGTGAGAAGTCTCGAGACCCGCACGCTA-3'
ssRNA	5'-gggaaggccagggaaucc-3'
Cy5 probe	5'-Cy5-(TAACCC) ₆ -3'

Thermal melting results showed that the addition of [Pt(bpa)]²⁺ would increase the CD melting temperature (T_m) of distinctive GQ topology (Figure 4.3). Though the enhancement was not so high as alkylated Pt-bpa, **1** can increase modestly T_m of *c-myc* GQ by 7 °C, HT21 GQ by 5 °C and TERRA and EBNA1 GQ by 4 °C, while negligible T_m change was observed on duplex DNA. With peripheral methylation on bpa ligand,^[17] **2** achieved better stabilizing effects on GQ structures (T_m of *c-myc*, HT21, TERRA and EBNA1 was increased by 9, 8, 5 and 9 °C, respectively), presumably due to the interaction of corner methyl group and GQ grooves.

Figure 4.3 Increased melting temperature of different DNA structures (black) with addition of equivalent amount of **1** (3 μ M, red) and **2** (3 μ M, blue).

Circular dichroism (CD) spectra showed no significant alteration while Pt(II) complex is added into GQ solution and indicated that **1** and **2** binding onto GQ structures would not affect their folding structures (Figure 4.4).

Figure 4.4 CD spectrum of different DNA structures (3 μ M) in the absence (black) and presence of **1** (3 μ M, red) and **2** (3 μ M, blue).

1 showed UV-vis spectra with a broad MLCT band peaked at around 425 nm in aqueous buffer. As usual, MLCT band showed hypochromicity upon the addition of duplex DNA. Remarkably, in the presence of GQ structures, both hyperchromicity and

the appearance of a new peak around 447 nm were observed for the MLCT band (Figure 4.5). The higher intensity and bathochromic peak indicated that **1** interacts with GQ in a distinctive mode to that of duplex DNA.

Figure 4.5 UV-vis spectra of **1** (25 μ M) in the absence and presence of different DNA/RNA structures (25 μ M).

The remarkable difference in absorption spectra as well as the obvious selectivity of Pt complexes toward GQ structures over double-stranded DNA was correlated with the distinctive fluorescence signals of **1** on the respective structures. Notably, **1** emits intensively at around 580 nm in the presence of GQ structures (Figure 4.6 and Table 4.3), while the fluorescence of **1** was highly quenched in buffer ($Q=0.0241$). Upon titration, the maximum fluorescent turn-on was achieved in the presence of 10 equivalents GQ. Similar titration experiments that were performed on single stranded or duplex DNA/RNA configurations scarcely trigger any improvement on the luminescence of Pt complexes, even the addition of 50 molar equivalent of nucleic acids (Figure 4.6b). Moreover, the emission intensity of **1** were much higher in the presence of parallel folded RNA and DNA GQs (TERRA and *c-myc*) than anti-parallel GQ (HT21) (Figure 4.6a and Table 4.3).

Figure 4.6 (a) Emission spectra of **1** (1 μM) in the presence of 20 equivalent amounts of different DNA structures ($\lambda_{ex} = 450$ nm). (b) Emission intensities of **1** (1 μM) in the presence of different DNA structures with varies [NA] / [1] ratios.

Table 4.3 Quantum yield of **1** and **2** in the absence and presence of different DNAs.

DNA/RNA	Φ_{em}		
	1	2	3
without DNA	0.0241 (4)	0.0084 (4)	0.0024 (3)
TERRA	0.366 (7)	0.456 (9)	0.103 (6)
EBNA1	0.312 (5)	0.378 (8)	0.078 (5)
<i>c-myc</i>	0.253 (4)	0.465 (9)	0.039 (5)
HT21	0.116 (2)	0.221 (8)	0.026 (3)
ds26	0.015 (1)	0.031 (2)	0.004 (2)
ssDNA	0.040 (2)	0.126 (8)	0.009 (3)
ssRNA	0.063 (4)	0.112 (6)	0.008 (4)

With better binding selectivity and affinity to GQ structures (Figure 4.3), **2** showed higher quantum yield in the presence of *c-myc* GQ (0.465), which is over 55-folds higher compared with compound alone (Figure 4.7b and Table 4.3). The four peripheral methyl groups in **2** would also present some hydrophobic interaction and/or π -stacking with the random coiled single stranded and double helix duplex structures. Though **2** showed higher luminescence on RNA GQ, the selectivity against non-GQ structures is comprised. **3** exhibited the same fluorescence turn-on manner upon binding with GQ structures, however, its quantum yields in the presence of different GQs were much lower than **1** and **2** (Figure 4.7c and Table 4.3).

Figure 4.7 Emission spectra of 1 μ M **2** (a) and **3** (b) in the presence of 20 equivalent amounts of different DNA structures ($\lambda_{ex} = 450$ nm). (c) Compared the emission spectrum of **1**, **2** and **3** in the presence and absence of TERRA GQ and duplex DNA structures.

Furthermore, we found that the fluorescence turn-on manner of **1** was absent by addition of albumin (Figure 4.8), a family of globular protein that exist in blood plasma, which indicated the $[\text{Pt}(\text{bpa})]^{2+}$ complex can be used as a potential GQ DNA/RNA specific probe. These $[\text{Pt}(\text{bpa})]^{2+}$ -type complexes not only exhibited structure-specific “light up” manner towards GQs over duplex and single stranded structures *in vitro*, but also displayed palpable topological selectivity towards parallel GQ.

Figure 4.8 Emission spectra of 1 μ M **1** in the absence (black) and presence of 20 equivalent amounts of c-myc (red) and 1.8 g/mL of albumin (blue) ($\lambda_{ex} = 450$ nm).

4.2.3 Production of a Triplet-state Emission in $[\text{Pt}(\text{bpa})]^{2+}$ upon Binding onto GQ

To illustrate the mechanism of GQ-specific fluorescent turn-on, we firstly study the transient absorption (TA) spectra and the lifetime of the excited states of Pt1 in the presence and absence of GQ. TA difference spectrum of **1** in buffer following 430 nm

excitation, included two negative absorption bands centered at 360 and 440 nm, which can be attributed to the ground state bleaching. Two positive bands around 320 and 400 nm, and a broad and featureless positive band centered between 450 to 690 nm around 550 nm were characteristic of an excited state absorption (Figure 4.9a). Notably, an induced absorption centered around 780 nm was detected upon addition of *c-myc* GQ into **1** (Figure 4.9b). Combined with the lack of stimulated emission signal in the TA spectrum, the low possibility in conformational change of **1** and the notable Stokes shift (>130 nm) we detected in the steady state, this induced signal might be attributed to the absorption of triplet state. Analysis of the transient absorption kinetics at 550 nm revealed a single-exponential decay for **1** alone, which exhibited 2.8 ns lifetime (Figure 4.9c). The lifetime is increased by 6-folds under anaerobic condition. The fluorescent quenching due to energy transfer to triplet oxygen gas further indicated that **1** emits from a triplet excited state. Upon the addition of *c-myc*, nonlinear least-squares analysis unveiled two significantly longer time-components of **1**, 1.7 μ s and 18.6 μ s (Figure 4.9c).

Figure 4.9 (a) Transient absorption decay kinetics of **1** (in buffer, pH=7) in the absence and (b) presence of *c-myc*; (c) Transient absorption decay kinetics of **1** in the absence (black) and presence of *c-myc* (red). (d) Phosphorescent spectra of **1** in the absence (black) and presence of *c-myc* (red).

Phosphorescence spectra from **1** emerged accordingly, which confirmed the triplet state emission (Figure 4.9d). Moreover, after deaeration, the two lifetimes increased to 3.2 μs and 35.8 μs , respectively, and the steady state emission intensity has also doubled (Figure 4.10).

Figure 4.10 (a) Transient absorption decay kinetics. (b) Steady state emission spectrum of **1** in the presence *c-myc* before (black) and after (red) deaeration.

Hence, the long lifetime triplet excited state endows **1** the strong luminescent signal with high quantum yields in the presence of GQ structures. The triplet state emission is stable and intensive that the quantum yields are even higher than the commercial cyanine dyes that widely used for *in vivo* imaging ($\Phi_{\text{Cy3}}=0.04$ and $\Phi_{\text{Cy5}}=0.27$).^[18]

4.2.4 Contribution Factors for the Luminescence Turn-On of $[\text{Pt}(\text{bpa})]^{2+}$ by GQ

To elucidate the effects of GQ binding on the triplet-state luminescence of **1**, we explored three aspects, H-bonds with solvent; thermal vibration; and energy transfer with oxygen gas. Firstly, superior than the phenazine nitrogen atoms from dppz ligand in $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$, the *aza* group in $[\text{Pt}(\text{bpa})]^{2+}$ -type complexes could act as both H donor and acceptor, endowed Pt(II) complexes with dual sensitivity to the subtle changes in the surrounding hydrophobicity. The steady-state absorption and emission spectra of Pt(II) complexes in different solvents were measured. As shown in Figure 4.11, among the three organic solvents, the greatest emission of **1** was observed in dichloromethane

($\Phi_{\text{em}} = 0.0173$), which is neither a proton donor nor acceptor (Table 4.4). In contrast, the quantum yield of **1** in acetonitrile, an aprotic solvent with proton acceptor, declined to 0.0086; while it is even lower in methanol ($\Phi_{\text{em}} = 0.0082$), which acted as both proton donor and acceptor.

Table 4.4 Photophysical data of **1-3** in different solvents at room temperature.

Solvent	Φ_{em}		
	1	2	3
Buffer pH=7	0.0241 (4)	0.0084 (4)	0.0024 (5)
Dichloromethane	0.0173 (8)	0.0244 (9)	0.0008 (4)
Acetonitrile	0.0086 (4)	0.0180 (8)	0.0007 (4)
Methanol	0.0082 (3)	0.013 (1)	0.0005 (2)

Figure 4.11 UV-vis and emission spectra of **1** (a and b), **2** (c and d) and **3** (e and f) in different solvents ($\lambda_{\text{ex}} = 450$ nm).

Moreover, **1** displayed a broader and stronger emission band and was accompanied with

an obvious red-shift in potassium phosphate buffer as compared to other three organic solvents (Figure 4.11b). UV-vis spectrum showed that the MLCT band of **1** peaked at 425 nm in buffer and is blue-shifted 20 nm comparing to that in the three organic solvents, which is indicative to a H-aggregation of **1** (Figure 4.11a). The aggregation may account for the slightly higher quantum yield in the buffer ($\Phi_{\text{em}} = 0.0241$) than that on duplex DNA. The aggregation behavior of **1** was also confirmed by temperature-dependent ^1H NMR spectra. As shown in Figure 4.12, the proton peaks in the aromatic region are broad with inadequate resolution for signal assignment at 298 K, implying the aggregation. The aggregates dissipated when temperature increased, which was denoted by the downfield-shifted performance of the poor signals and their separation into several definite sharp signals. A similar temperature-dependent NMR spectra of highly concentrated solution of **2** was detect as well; while the methylation at *aza* linkage would impede the aggregation, NMR spectra of **3** can readily achieved with high resolution at room temperature.^[16]

Figure 4.12 ^1H NMR spectra of **1** (a) and **2** (b) in DMSO-d_6 with variation of temperatures.

Emission of **2** displayed similar solvent-dependent manner. Formation of hydrogen bond between Pt(II) complexes and solvent would lead to quench in their emission. Both nitrogen and hydrogen atoms from the *aza* linkage forms H-bonds with solvent molecules and contribute simultaneously to the luminescent quenching of $[\text{Pt}(\text{bpa})]^{2+}$ complexes.

[Pt(bpa)]²⁺-type complexes have a length of 12.2 Å and width of 8.6 Å (Figure 4.2), which endow their efficient π -stacking with GQs due to the well-matched size to G-quartet layers.^[1] Our simulation docking showed that **1** can be enwrapped inside c-myc GQ upon stacking on top of the external G-tetrad and the hydrogen bonding between *aza* group and solvent are prohibited (Figure 4.13). However, duplex and single stranded DNA and RNA cannot accommodate **1** to efficiently block the accessibility of *aza*-NH to buffer molecules, due to either the size exclusion and/or the lack of stable interactions. Thus, binding to GQ could protect the *aza* group from the solvent, which would prevent the triplet excited state from thermal dissipation via H-bonding network and render a longer excited-state lifetime and a higher emission intensity.

Figure 4.13 Molecular docking of **1** on *c-myc* GQ (PDB: 2N6C). Backbone is represented as a blue ribbon and **1** is shown in brown.

Secondly, steady state emission results showed that quantum yield of Pt(II) complexes upon binding onto GQ structures far exceeded that in dichloromethane, which indicated other factors may contribute to the luminescence “turn-on” effects as well. By measuring the emission of **1** in methanol/glycerol mixtures with different ratios, we found that the luminescent intensity of [Pt(bpa)]²⁺ was in close connection with solvent viscosities (η) (Figure 4.14). The emission intensity enhanced more than 100-folds higher when the viscosity of the solution increased from 0.6 cP (100% methanol) to 906 cP (100% glycerol). Such enhancement may cause by the vibration or rotation restriction on the

complex. In the case of binding onto GQ, the dynamic motion of the complexes would be high retrained inside GQ structure. With the four peripheral methyl group, **2** can be locked into parallel GQ better than **1** and accordingly achieved higher luminescence turn-on ratio.

Figure 4.14 Plot of the luminescent intensity for **1** versus solvent (a mixture of methanol as glycerol in different ratios) viscosity. Logarithm of luminescent intensity were correlated with logarithm of solvent viscosity.

Thirdly, as a phosphorescent probe, the emission of $[\text{Pt}(\text{bpa})]^{2+}$ and its derivatives are highly sensitive to solvated oxygen in buffer. As shown in Figure 4.15, the emission intensity of **1** has increased around 3 times after deaeration; while the emission intensity of **1** upon binding on *c-myc* GQ was doubled in anaerobic condition comparing with that in air condition (Figure 4.10b). The emission quenching from oxygen is more severe on free $[\text{Pt}(\text{bpa})]^{2+}$ complexes than GQ-bound complexes. Upon binding onto G-quadruplex, one side of the planar Pt(II) complex was protected by GQ and would not be accessible to the free oxygen in the solution. Binding onto GQ could reduce the feasibility of energy transfer from $^3\text{Pt}(\text{II})^*$ to $^3\text{O}_2$ and hence protect the luminescence intensity of the complexes.

Figure 4.15 Steady state emission spectra of **1** in buffer before (black) and after (red) deaeration.

4.2.5 Imaging and Tracking the Un/folding Dynamics of GQ in Live Cells by Using [Pt(bpa)]²⁺

Given the large Stokes shift, long lifetime, high quantum yield as well as the structure-specific “light up” manner, **1** accorded with most of the requirements for luminescent probe that used in bioimaging. The live-cell imaging in Cy3 channel showed strong and distinct luminescence foci in HeLa cells after staining by **1** (Figure 4.16). A majority of the foci were appeared in cytoplasm, whereas a very little amount of the foci was observed in cell nucleus, which indicated **1** can enter the cell nucleus.

Figure 4.16 Live cell imaging of HeLa cells stained with **1** (red). The nucleus were stained with Hoechst (green).

To further elucidate the cellular target of **1**, a colocalization experiment was performed by the introduction of a 5'-Cy5 tagged (TAACCC)₆ sequence (Cy5-TS) that is

complementary to TERRA, a long repeatable RNA G-quadruplex folding sequence. Figure 4.17 showed Cy5 signal distributed in the cytoplasm and indicate the location of single stranded TERRA RNA upon hybridizing to Cy5-TS.

Figure 4.17 (a) Illustration of the colocalization mechanism of **1** and Cy5-(TAACCC)₆. (b) Live-cell imaging of HeLa cells co-stained with **1** (red) and Cy5 (blue). The nucleus was stained with Hoechst 33342 (green).

Figure 4.17b showed that approximately 95 % of Cy5 foci were well colocalized with **1** foci in the cytoplasm (Figure 4.18). Since luminescence of **1** was observed while **1** binds to an RNA GQ, the high colocalization of two probes indicated that majority of TERRA RNA hold both random coil and GQ structure simultaneously.

Figure 4.18 Quantification of the overlapped Cy5 and **1** foci with each other. Approximately 100 cells were measured to show the colocalization of 5'-Cy5 tagged probe and **1**.

To further study the folding dynamics of TERRA GQ, we continuously image the cell in both channels over 1 minute with 10 s interval. The reconstructed video showed that most

of the **1** foci were changing continuously during the observation period, while the Cy5 foci remained largely constant. Movements, merging and splitting of **1** foci were observed from the real-time tracking (Figure 4.19).

Figure 4.19 (a) Tracking the folding dynamics of TERRA *via* co-staining with **1** (red) and Cy5 tagged TERRA probe (blue) in live cells. (b) Time-lapse images of the region denoted by the boxes in a.

Some **1** foci were also found gradually disappeared while new luminescence foci appeared. The distinctive behaviors of the two probes indicated the folding and unfolding of TERRA GQ are highly dynamic comparing to the cellular distribution of the RNA molecules. RNA GQ can dissolve and refold on or faster than the timescale of seconds. Furthermore, around 50 % of **1** foci was outside the fluorescent area of Cy5, which indicate that **1** can also stain other cellular RNA GQ simultaneously (Figure 4.18). These non-TERRA GQ foci also showed dynamic motions which suggests that the folding dynamics could be a universal feature for cellular RNA GQ. Since **1** is rather a GQ binder, instead of a stabilizer, **1** does not prohibit the folding dynamics and showed the potential to be used to real-time track the cellular behaviors of multiple RNA GQs, while induced GQ signal may not be a concern here.

4.3 Conclusion

In summary, we developed a series of novel phosphorescent Pt complexes as light switches for G-quadruplexes. Due to the size, shape and aza NH bridge of the aromatic

ligand, the complexes showed highly sensitive luminescence to the microenvironment, including the accessibility to proton and oxygen molecules, and the constraints on molecular motion. These complexes exhibited large Stokes shift (> 130 nm), long lifetime (> 18 μ s), high quantum yield (> 0.45) and high luminescence turn-on signals specifically upon binding to G-quadruplexes and offered the first inorganic light-switch probe for RNA GQ in live cells. With a dual probe assay, dynamic un/folding of multiple RNA GQ could be simultaneously visualized in live cells. $[\text{Pt}(\text{bpa})]^{2+}$ complexes showed great potentials in live-cell imaging and tracking the un/folding dynamics of RNA GQs.

4.4 Experimental section

RNA sequences and all the chemicals were ordered from Sigma-Aldrich. The DNA sequences were ordered from Sangon (Shanghai, China). The *N,N*-bis-(1,10-phenanthroline-2-yl)-amine (bpa) ligand and Pt complex **3** was synthesized as previously reported.^[16] Milli-Q water was used in all experiments. All DNA and RNA structures were annealed by heating the desire single stranded sequences at 95 °C for 10 min, and cooling down to r.t. gradually over 2 h.

Synthesis of Pt^{II} complexes

Pt complex 1. The bpa ligand was protonated by dissolving in trifluoroacetic acid and precipitated with diethyl ether prior to usage in following synthesis. $\text{Pt}(\text{DMSO})_2\text{Cl}_2$ (20.9 mg, 0.05 mmol) and bpa-H^+ (24.5 mg, 0.05 mmol) were dissolved in ethylene glycol. The solution was heated at 125 °C for 1.5 h. Orange solid was precipitated by adding an excessive amount of saturated KPF_6 solution. The precipitate was wash with water, cold methanol and ethyl ether 3 times for each to provide the product in 76% yield. Crystal of **1** was grown by diffusing acetone into dimethyl sulfoxide at r.t. and orange needle crystals were obtained. ESI-MS (MeOH) m/z : 567.44 (calc 568.10 for $\text{C}_{24}\text{H}_{16}\text{N}_5\text{Pt}$).

Synthesis of **2**

4,7-Dimethyl-1,10-phenanthroline-*N*-oxide (a). To a solution of 4,7-dimethyl-1,10-phenanthroline (480 mg) in acetic acid (8 mL) was added 30% H₂O₂ (580 μ L). The temperature was carefully maintained at 70 °C for 3 h, after which another portion of H₂O₂ (580 μ L) was added, and the solution was continuously heated for an additional 3 h. After cooling to r.t., a third portion of H₂O₂ (360 μ L) was added, followed by leaving the reaction mixture stirring for 6 h. Then, the solution was condensed to around 4 mL under vacuum, followed by addition of water and re-concentration back to a volume of 4 mL. To the resulting dark-brown oil a solid K₂CO₃ was added and the mixture was extracted by CHCl₃ for 3 h. To the CHCl₃ solution a charcoal and MgSO₄ were added. Subsequently, solids were filtered off and the solvent was removed to afford the product (280 mg, 56% yield). ¹H NMR (300 MHz, CDCl₃) δ : 9.09 (d, *J* = 4.1 Hz, 1H), 8.60 (d, *J* = 6.3 Hz, 1H), 7.85 (dd, *J* = 53.4, 9.4 Hz, 2H), 7.44 (d, *J* = 4.0 Hz, 1H), 7.24 (d, *J* = 6.3 Hz, 1H), 2.68 (d, *J* = 24.7 Hz, 6H). ESI-MS (MeOH) *m/z*: 225.15.

2-Chloro-4,7-dimethyl-1,10-phenanthroline (b). To a mixture of the reagent (last step, 226 mg) and NaCl (1.2 g) in DMF (8 mL), a neat POCl₃ (0.3 mL) was added by syringe at 0 °C. Then, the reaction mixture was heated to 100 °C for 6 h. After cooling to r.t., water (5 mL) was added and the mixture was basified with aqueous ammonia and saturated with NaCl. Solids were filtered and solution was extracted with CHCl₃ (3 $\times$ 5 mL). The combined extracts were washed with brine (3 $\times$ 5 mL), dried over MgSO₄ and evaporated. The crude residue was purified by column chromatography (SiO₂; 3:2, EtOAc/hexanes; *R_f* = 0.3) to afford the product as a yellow solid (90 mg, 35% yield). ¹H NMR (300 MHz, CDCl₃) δ : 9.05 (d, *J* = 4.5 Hz, 1H), 7.95 (dd, *J* = 22.9, 9.3 Hz, 2H), 7.44 (d, *J* = 7.3 Hz, 2H), 2.75 (d, *J* = 10.3 Hz, 6H). ESI-MS (MeOH) *m/z*: 243.14.

2-Amino-4,7-dimethyl-1,10-phenanthroline (c). A mixture of **b** (90 mg), acetamide (0.5 g) and K₂CO₃ (355 mg) was heated to 180 °C and stirred for 2 h. After cooling to r.t., water (50 mL) was added and resulting mixture was extracted with CH₂Cl₂ (4 $\times$ 10 mL).

The combined organic extracts were washed with brine (3×10 ml), dried over MgSO_4 and evaporated. The crude residue was purified by flash chromatography (SiO_2 ; 3:7:90, NH_4OH (25%)/ MeOH/EtOAc ; $R_f = 0.3$) to afford **c** as a yellow powder (37 mg, 47% yield). ^1H NMR (300 MHz, CDCl_3) δ : 8.95 (d, $J = 4.4$ Hz, 1H), 7.81 (dd, $J = 39.1, 9.2$ Hz, 2H), 7.37 (d, $J = 4.2$ Hz, 2H), 2.71 (d, $J = 25.4$ Hz, 6H). ESI-MS (MeOH) m/z : 224.21.

***N,N*-bis-(4,7-dimethyl-1,10-phenanthrolin-2-yl)amine (dmbpa, d)**. A mixture of **c** (37 mg) and NaH (40 mg) were dissolved in DMA (2 mL) and heated at 100°C with stirring for 0.5 h. After that, a solution of **d** (40 mg) in DMA was added by dropwise to the mixture. The mixture was stirred for 6 h with refluxing. DMA was distilled out and the crude product residue was purified by flash chromatography (SiO_2 ; 1:2:20, TEA/ MeOH/DCM). The product was acidified with TFA and precipitate by addition of diethyl ether to afford it as a yellow powder (42 mg, 51% yield). ^1H NMR (300 MHz, MeOD) δ 8.89 (d, $J = 4.5$ Hz, 2H), 8.19 (q, $J = 9.3$ Hz, 4H), 7.73 (d, $J = 4.4$ Hz, 2H), 7.45 (s, 2H), 2.88 (d, $J = 5.7$ Hz, 12H). ESI-MS (MeOH) m/z : 430.40.

Pt complex 2. $\text{Pt}(\text{DMSO})_2\text{Cl}_2$ (15.8 mg, 0.04 mmol) and bpa-H^+ (20.3 mg, 0.04 mmol) were dissolved in ethylene glycol and water (in 2:1 ratio). The mixture was heated at 100°C for 2 h. Yellow solid was precipitated by adding an excessive amount of saturated KPF_6 solution. The precipitate was wash with water, cold methanol and ethyl ether 3 times for each to provide the product in 61% yield. ESI-MS (MeOH) m/z : 623.59 (calc 624.16 for $\text{C}_{28}\text{H}_{23}\text{N}_5\text{Pt}$).

Ultraviolet–visible (UV-vis) absorption measurement

Absorption spectra were recorded on a Cary 100 UV/vis spectrophotometer at 25°C . Briefly, the UV-vis spectrum of each Pt complex (10 μM) in different solvents were scanned from 360 to 550 nm.

Luminescent emission measurement

Steady-state emission spectra were recorded on Varian Cary Eclipse fluorescence

spectrophotometer. 10 μ M of each Pt complex in different solvents were excited at 450 nm and emission spectrum were recorded from 510 to 750 nm. The emission quantum yield was measured with Ru(bpy)₃Cl₂ (Φ = 0.055) as reference. For the emission titration experiments, solutions of each Pt complex (1 μ M) were prepared in 10 mM potassium phosphate buffer (pH=7.4). Aliquots of a high concentration of DNA/RNA stock solution were added. The emission spectra were recorded from 520 to 700 nm after each addition of nucleic acids.

Time-resolved transient absorption spectroscopy

Time-resolved nanosecond transient absorption measurements were performed using an Applied Photophysics LKS.60 Nanosecond Laser Flash Photolysis Spectrometer with 430 nm excitation pulses generated from a seed Nd:YAG, Q-switched laser, and the probe was a 150-W Xe lamp aligned normal to the excitation source. Each time-resolved trace was acquired by averaging 20 laser shots at a repetition rate of 1 Hz. The typical laser pump fluence used is $\sim 60 \mu\text{J cm}^{-2}$. The data were collected in transmission geometry with the sample positioned at 90° with respect to both the excitation source and probe light.

Lifetime

A time-resolved transient absorption (pump-probe spectroscopy) was performed in this experiment to measure the lifetime of platinum complex with or without *c-myc* GQ. The platinum complex was excited by a short laser pulse at 430 nm and probed by a delayed pulse to measure the time dependence of the absorption change. The lifetime was obtained by analyzing the TA spectrum at 550 nm.

Circular dichroism (CD) measurement

CD spectra were scanned on a Jasco J-1500 spectropolarimeter. 3 μ M of DNA (or 5 μ M of RNA) solution were prepared in 10 mM potassium phosphate buffer (pH=7.4) with 10 mM KCl. CD spectra of DNA/RNA structures, with or without 1 equiv. amount of Pt(II)

complex, were recorded from 220-320 nm. The maximum HT voltage is below 600 V. For thermal melting assay, ellipticity at 263 nm for *c-myc*, TERRA and EBNA1, 295 nm for HT21 and 245 nm for ds26, were monitored upon temperature elevation from 15 °C to 95 °C with heating ramp of 0.5 °C/min. Final analysis of the data was carried out using Origin 8.0 (OriginLab Corp.). Standard deviation over three repeat experiments were used as error bars.

Live cell imaging experiment

The HeLa cells were grown in DMEM media containing 10% fetal bovine serum at 37 °C, with 5% CO₂ atmosphere. 40000 cells were seeded into an imaging dish (ibidi, μ -dish 35 mm, high) and cultured for 24 h. The cells were stained with 5 μ M of **1** for 20 h. Fluorescence *in situ* hybridization (FISH) was carried out by adding 0.5 μ M Cy5-(TAACCC)₆ in the dishes and incubated for 3 h before the medium was removed. Lastly, 10 μ L of Hoechst dye (10 μ g/mL) was added into the dish and incubated for another 3 h, and then rinsed by FluoroBrite DMEM (Gibco). Digital images were recorded by ZEISS LSM 800 confocal laser scanning microscope with a 100 $\times$ objective lens, and analyzed with Volocity 6.3 software (PerkinElmer Corp.). 100 cells were counted and the s.e.m. was calculated from three replicates.

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CHAPTER 5

G-quadruplex Nanowires Direct the Efficiency and Selectivity of Electrocatalytic CO₂ Reduction

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5.1 Introduction

Electron conductivity of duplex DNA as a redox chemical event over long nanometer distance ignites substantial interests in biochemistry and electric nanodevices.^[1,2] Proteins bearing FeS clusters were proposed to harness electron transfer (ET) through bound duplex DNA for redox signaling.^[3] The similar ET events were also applied to electrochemical sensing of nucleic acids and proteins.^[4] Though the potentials of DNA conductivity for redox biocatalytic reactions have been highly valued, the real applicable example, especially in heterogeneous catalysis, is rarely reported. Recently, a G-quadruplex nanowire showed high efficiency in mediating electron transfer both on solid surface and in aqueous solution.^[5,6] More appealingly, G-quadruplexes (GQ) are well known to offer specific binding topologies and high affinity for the metal complexes with large aromatic ligands.^[7,8] Phthalocyanine (Pc) and its derivatives are one family of the GQ-targeting ligands that can lead the metal-Pc complexes to define binding conformation and orientation on GQ structures.^[9] Metal-Pc complexes, such as Zn(II), Al(III), Ga(III), are widely studied as photosensitizers in photodynamic therapy,^[9-11] while Co(II)-Pc complexes are extensively used as catalytic centers for heterogeneous electrochemical reduction of CO₂.^[12-17] As the main greenhouse gas, CO₂ was considered as one of the largest carbon feedstock for bulk chemicals.^[18] The electrochemical reduction of CO₂ into useful fuels is a promising approach to mimic the dark reaction of photosynthesis and to provide sustainable energy source.^[19,20] Up to date, majority of the studies on CO₂ electroreduction focused on improving redox communications between electrodes and metal catalytic center, and among the metal complexes on the electrode surface.^[19] Many host scaffolds were used to bridge the electron transfer and facilitate the redox reaction between metal catalytic centers and CO₂, such as organic polymer and carbon nanotubes.^[12,13] Though biomolecules, such as nucleic acids and metalloproteins, are vigorously explored as electron transfer medium for the light reaction of artificial

photosynthesis, such biomaterials are rarely used in mediating CO₂ reduction.^[1,21,22]

Herein, we report the first example of using nanoassembly of biomacromolecules, a GQ nanowire, as a surface scaffold to host Co^{II}Pc (Scheme 5.1) on carbon electrodes for efficient and highly selective electro-chemical reduction of CO₂ to CO (Figure 5.1).

Figure 5.1 Illustration of CO₂ electroreduction *via* metal-tsGQwire adducts. (1) tsGQwire self-assembled by annealing dG₄ sequence 10 mM in lithium cacodylate buffer with 10 mM KCl (pH 7.4); (2) metal complexes bind to the formed tsGQwires to introduce the catalytic center; (3) the metal-tsGQwire adducts were applied for the electroreduction of CO₂ molecule.

Scheme 5.1 Chemical structure of (A) Co^{II}Pc; (B) Fe^{II}Pc and (C) [Ru^{II}(bpa)(NH₃)₂]²⁺.

5.2 Results and discussion

5.2.1 Preparation and Characterization of GQ Nanowire

A tetra-guanine sequence, 5'-GGGG-3' (dG₄) was synthesized by solid phase phosphoramidite chemistry and characterized by ESI-MS (Table 5.1 and Figure 5.2). dG₄ can self-assemble into a G-quadruplex nanowire (Figure 5.1). Four strands of dG₄ in theory can fold into G-quadruplexes to form four layers of G-tetrads.^[6] The sequence

contains only guanines to avoid the disruption of G-tetrad stacking and/or blocking the elongation of G-wire from non-guanine bases. Using short oligonucleotides can circumvent the synthetic difficulty in preparing the long polyG strands.^[23,24]

Figure 5.2 ESI-MS spectra of the synthetic DNA sequences. (A) dG₄; (B) dTTG₄ and (C) 5'-FAM-dG₄.

Table 5.1 Sequences of oligonucleotides used in this work.

Oligonucleotide	Sequence
dG ₄	5'-GGGG-3'
FAM-dG ₄	5'-FAM-GGGG-3'
dTTG ₄	5'-TTGGGG-3'
PolyA	5'-A ₅₉ -3'
PolyT	5'-T ₅₉ -3'

To prepare the tetrastranded GQ nanowire (tsGQwire), 1 mM of dG₄ in 10 mM lithium cacodylate buffer with 10 mM KCl (pH 7.4) were thermally annealed. Atomic force microscopy (AFM) image showed annealed dG₄ assemblies stay in rigid linear and unbranched structure (Figure 5.3A). The heights were uniform at ~1.6 nm (Figure 5.3B),

which was consistent with the reported G-wires assembled from polyG.^[23] The length of GQ nanowires were around 220 nm though longer wires up to 400 nm were also observed (Figure 5.3C). Although assembled by short oligonucleotides, nanowires were mainly tetrastranded GQ nanorods and showed no obvious topological polymorphism.

Figure 5.3 (A) AFM image of tsGQwires formed by annealing dG₄ sequence for 24 h. Statistical analyses showing the (B) height and (C) length of tsGQwires by measuring more than 100 distinct wires.

To confirm the assembly of dG₄ to nanowire, we assembled nanowire by annealing dG₄ together with 10% of a fluorescein labeled tetranucleotides of guanine (5'-FAM-dG₄) and studied the gel mobility of the nanowire by native polyacrylamide gel electrophoresis (PAGE) analysis (Figure 5.4). A band in the loading well indicate the nanowire was too large to enter PAGE, while single FAM-dG₄ strands can move downwards on PAGE. Circular dichroism (CD) spectrum of tsGQwires showed a typical tsGQ topology with all of the guanines stay in anti-conformation (Figure 5.5A).^[6]

Figure 5.4 8% non-denaturing polyacrylamide gel electrophoresis (PAGE) analysis of formed tsGQwires. The gel was monitored by FAM fluorescence. Left lane is 5'-FAM-dG₄ as control; right lane is a mixture of 5'-FAM-dG₄ and dG₄ in 1:9 ratio.

Figure 5.5 (A) CD spectra of tsGQwire (10 μ M) and Co^{II}Pc-tsGQwire adduct (with 1:4 ratio) measured in 10 mM lithium cacodylate buffer with 10 mM KCl, pH 7.4. (B) UV-vis spectrum of 4 μ M Co^{II}Pc (black) in the presence and absence of tsGQwire (red). (C) AFM images of tsGQwire that assembled from dG₄ sequence in the presence of Co^{II}Pc. Statistical analyses showing the (D) height and (E) length of tsGQwires by measuring more than 100 distinct wires.

5.2.2 Electroreduction of CO₂ over a Co^{II}Pc-tsGQwire Decorated Electrode

To prepare Co^{II}Pc bound tsGQwire, stock solution of Co^{II}Pc in DMSO were added to pre-annealed tsGQwire in working buffer at the ratio of 1:4 for Co^{II}Pc versus dG₄, since the binding stoichiometry showed four layers of G-tetrad host one Co^{II}Pc complex (Figure 5.6A). Both AFM and CD spectra showed that Co^{II}Pc induced no structure disturbance on tsGQwire (Figure 5.5). The heights and lengths of Co^{II}Pc-tsGQwire were the same as bare tsGQwire, though a larger length distribution was observed (Figure 5.5D and E). Strong π -coupling between Co^{II}Pc and tsGQwire was indicated by the hypochromicity on two Q bands of Co^{II}Pc at 612 and 674 nm, and a 7 nm redshift of the Q bands (Figure 5.5B). Fluorescent intercalator displacement (FID) assay showed a decrease in the emission of thiazole orange (TO) upon addition of Co^{II}Pc (Figure 5.6B), revealed that Co^{II}Pc inserted into the G-tetrad layers and displaced the quondam TO

molecule.

Figure 5.6 (A) Binding stoichiometry between Co^{II}Pc and tsGQwire. (B) Emission intensity of TO decreased upon addition of concentrations of Co^{II}Pc molecules to the tsGQwire/TO solution.

To examine the Co^{II}Pc-tsGQwire catalyzed CO₂ reduction, the working electrode was prepared by simply depositing and drying Co^{II}Pc-tsGQwire solution over a carbon paper. To identify the optimal electrode potential for CO₂ reduction, linear sweep voltammetry (LSV) in 0.5 M of 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide ([Bmim]Tf₂N) in MeCN was applied between -0.4 and -2.5 V vs Ag/Ag⁺ as reference electrode under saturate CO₂ atmosphere. A reduction peak was found around -2.1 V for the working electrodes decorated by either bare Co^{II}Pc or Co^{II}Pc-DNA adducts (Figure 5.7).

Figure 5.7 LSV curves over Co^{II}Pc, Co^{II}Pc-tsGQwire and tsGQwire alone in CO₂-saturated 0.5 M [Bmim]Tf₂N MeCN electrolyte.

The electron coupling between Co^{II}Pc and GQ upon binding barely altered redox

cycle and potential of $\text{Co}^{\text{II}}\text{Pc}$. A higher current density was observed under CO_2 atmosphere than that under N_2 -saturated environment, which indicated the occurrence of CO_2 reduction under this potential (Figure 5.8). tsGQwire alone showed no apparent redox peak within working potential window around -2.1 V (Figure 5.7), which indicated the metal complex remains as the catalytic center for the redox reaction. tsGQwire contributed to the CO_2 reduction via the functions other than being the catalyst.

Figure 5.8 LSV curves over $\text{Co}^{\text{II}}\text{Pc}$ -tsGQwire in N_2 and CO_2 -saturated 0.5 M [Bmim] Tf_2N MeCN electrolyte.

5.2.3 GQ Nanowire Steer the Efficiency and Selectivity of CO_2 Electroreduction

To analyze the electroreduction products, electrolysis of CO_2 was then performed at constant working potentials in a typical H-type cell for 6 h. Under a given working potential within the window of -1.7 V to -2.3 V, the current density on a $\text{Co}^{\text{II}}\text{Pc}$ -tsGQwire electrode was higher than that on bare $\text{Co}^{\text{II}}\text{Pc}$ electrodes (Figure 5.9A, Table 5.2). For both bare $\text{Co}^{\text{II}}\text{Pc}$ and $\text{Co}^{\text{II}}\text{Pc}$ -tsGQwire electrodes, only gas products but no liquid products were observed. Further GC analysis showed CO and H_2 were the main products with a combined Faradaic efficiency (FE) of around 100 %. More remarkably, $\text{Co}^{\text{II}}\text{Pc}$ -tsGQwire showed higher selectivity towards CO against H_2 . FE of CO on $\text{Co}^{\text{II}}\text{Pc}$ -tsGQwire electrode reached 82.4% while the bare $\text{Co}^{\text{II}}\text{Pc}$ electrode showed only 33.2% (Figure 5.9B, Table 5.2).

Figure 5.9 The total current density (A) and Faradaic efficiency of CO (B) at different applied potentials over Co^{II}Pc and Co^{II}Pc-tsGQwire with a 5 h electrolysis.

Table 5.2 The summarized data for the total current density and Faradaic efficiency of CO at -2.1 V over Co^{II}Pc and Co^{II}Pc-DNA electrodes.

	I / mA cm ⁻²	FE / %
Co ^{II} Pc	8.8	33.2
Co ^{II} Pc-tsGQwire	11.5	82.4
Co ^{II} Pc-GQ _{TG}	9.1	36.0
Co ^{II} Pc-dp(AT)	9.5	42.1
Co ^{II} Pc-polyA	8.9	36.7
Co ^{II} Pc-polyT	9.2	35.8

FE maximized at -2.1 V vs Ag/Ag⁺, though higher facilitation effects from tsGQwire were observed at lower working potentials. FE enhancements for Co^{II}Pc-tsGQwire electrodes can reach ~10 folds at -1.7 to -1.9 V. Co^{II}Pc-tsGQwire decorated electrode showed stable CO selectivity over 5 h electrolysis (Figure 5.10).

Figure 5.10 The stability on CO selectivity during 5 h electrolysis over the Co^{II}Pc-tsGQwire decorated electrode at -2.1 V (vs. Ag/Ag⁺).

Table 5.3 The summary of various Co^{II}Pc-based electrodes used in CO₂ electroreduction.

Electrode/ electrocatalysts	Electrode potential / V	Overpotential / V	FE / %	Total current density / mA cm ⁻²	CO partial current density / mA cm ⁻²	Ref
Co ^{II} Pc- tsGQwire	-2.1 V vs Ag/Ag ⁺	0.44	82.4	11.5	9.5	This work
CoPc/CNT (2.5%) ^a	-0.63 V vs RHE	0.52	92	~10	~9.2	[12]
Perfluorinated CoPc	-0.8 V vs RHE	0.69	93	~4.4	~4.1	[13]
CoPc-P4VP	-0.73 V vs RHE	0.63	89	2.0	1.8	[14]
CoPc-PVP	-1.6 V vs SCE	~0.9	84.1	< 1	< 1	[15]
CoPc-graphite	-1.15 V vs SCE	~0.5	60	0.98	~0.6	[16]
CoPc-graphite	-1.75 V vs SCE	--	--	~4	--	[17]

To the best of our knowledge, among the works used Co^{II}Pc-based catalysts, Co^{II}Pc-tsGQwire electrodes showed the highest total (11.5 mA cm⁻²), CO partial current density (9.5 mA cm⁻²) and the lowest overpotential (0.44 V) (Table 5.3). Though the Faradaic efficiency was slightly lower than the best reported Perfluorinated Co^{II}Pc system, it is comparable to many reported systems.

The mechanism of CO₂ reduction to CO over Co^{II}Pc-tsGQwire electrodes was further studied via electro-kinetic analysis. Tafel plot of CO production on Co^{II}Pc-tsGQwire electrodes was obtained by plotting overpotential (η) against the partial current density ($\log(j_{\text{co}})$) in Figure 5.11. By extrapolating the data, we can obtain the equilibrium potential for CO generation at -1.66 V vs. Ag/Ag⁺ (Figure 5.11B). By fitting the linear correlation between η and $\log(j_{\text{co}})$ on Tafel plot from $\eta = 0.12$ V to 0.26 V, we obtained a slope of 105.2 mV dec⁻¹. Thus, the rate determining step of this system is the single electron transfer to CO₂ and the formation of an adsorbed CO₂^{•-} intermediate (CO₂ + e $\rightleftharpoons$ CO₂^{•-}).^[19,20,25-27] A sequential step is kinetically faster and would push the forwarding reaction by depleting CO₂^{•-} with a series of mass transfer reactions.^[19] The electro-kinetic

mechanism accounted for the high performance of Co^{II}Pc-tsGQwire electrode as that occurs to other efficient systems in the literature.^[28-30]

Figure 5.11 (A) Tafel plot for CO production over Co^{II}Pc-tsGQwire electrode; (B) Current densities for CO production under different potentials and the equilibrium potential can be obtained by extrapolation method.

The excellent activity of Co^{II}Pc-tsGQwire electrode may partially result from high electrochemical active surface area. To characterize those of bare Co^{II}Pc and Co^{II}Pc-tsGQwire electrodes, the reduction current density at -2.1 V (vs. Ag/Ag⁺) was plotted against the square root of scan rate (Figure 5.12A). The double-layer capacitances were calculated via the Randles-Sevcik equation. Accordingly, the electrochemical active surface area for Co^{II}Pc-tsGQwire was estimated to be 2.9 times higher than that of bare Co^{II}Pc (Figure 5.12B). It indicated the accessibility of Co redox center to CO₂ is increased, probably due to the fact that the GQ nanowires on the electrode surface confine the Co^{II}Pc molecules into specific binding orientations and surface distributions. Thus, the increase of electrochemical active surface area can be viewed as one of the contributors for the notable enhancement of CO production ability of Co^{II}Pc-tsGQwire.

Figure 5.12 (A) The reduction current density at -2.1 V (vs. Ag/Ag^+) plotted against the square root of scan rate for different electrodes. (B) The summarized data of double-layer capacitance in A.

5.2.4 Electron Conductivity of DNA Structures and Improved Orientation of Metal Complexes Contributed to CO_2 Electroreduction

Upon binding onto tsGQwire, $\text{Co}^{\text{II}}\text{Pc}$ showed significantly enhanced catalytic efficiency and selectivity on CO_2 electrochemical reduction. To elucidate the facilitation factors that tsGQwire played in this catalytic event, we further prepared three $\text{Co}^{\text{II}}\text{Pc}$ -DNA electrodes. Single stranded polyA, polyT, and a duplex dp(AT) annealed by polyA and polyT, were used to construct adducts with $\text{Co}^{\text{II}}\text{Pc}$ complex *via* the same method applied to $\text{Co}^{\text{II}}\text{Pc-tsGQwire}$. In electrochemical reduction of CO_2 , the three $\text{Co}^{\text{II}}\text{Pc}$ -DNA adducts showed ~20% attenuation in current density, and ~60 % reduction in double layer capacitance comparing to those of Pc-tsGQwire electrode (Figure 5.12 and 5.13). As phthalocyanine complex, $\text{Co}^{\text{II}}\text{Pc}$ complex would interact with single-stranded and duplex DNA in a distinctive mode to GQ and show less orderly binding orientation and arrangement,^[9] though docking on single or duplex DNA could probably escort $\text{Co}^{\text{II}}\text{Pc}$ into a better surface orientation for slightly more efficient redox cycling than direct deposition on carbon electrode (Figure 5.12). Whereas binding onto tsGQwire would offer $\text{Co}^{\text{II}}\text{Pc}$ complexes a more optimal spatial orientation and/or distribution on the electrode surface, which made them more accessible to the diffusible CO_2 . This may not

only render a larger active surface area on Co^{II}Pc-tsGQwire electrode than that on either bare Co^{II}Pc or other Co^{II}Pc-DNA adduct electrodes, but also ameliorate the electron communication among Co^{II}Pc molecules that mediated by tsGQwire.^[31]

Figure 5.13 (A) LSV curves over Co^{II}Pc, Co^{II}Pc-tsGQwire, Co^{II}Pc-polyA, Co^{II}Pc-polyT, Co^{II}Pc-dp(AT) and tsGQwire alone in CO₂-saturated 0.5 M [Bmim]Tf₂N MeCN electrolyte. The total current density (B) and Faradaic efficiency of CO (C) at different applied potentials over Co^{II}Pc-tsGQwire, Co^{II}Pc, Co^{II}Pc-polyA, Co^{II}Pc-polyT and Co^{II}Pc-dp(AT) in 0.5 M [Bmim]Tf₂N MeCN solution saturated with 1 atm CO₂ at ambient temperature with a 5 h electrolysis.

Recent report suggested tsGQ nanowire is more efficient in conducting electrons than duplex DNA in aqueous solution and on the surface.^[5,6] To examine whether electron transfer through GQ nanowire would facilitate redox chemistry of Co^{II}Pc, we wrecked the continuity in GQ nanowire by preparing “GQ dot” with DNA sequence, dTTG₄ (Figure 5.2 and Table 5.1). As shown in Figure 5.14, the length of GQ nanowire reduced with the ratio of dTTG₄:dG₄ increasing from 1:9 to 9:1.

Pure dTTG₄ formed a tetrastranded G-quadruplex with four layers of G-tetrad (Figure 5.15) and no high order structures can be observed in AFM image (Figure 5.14F). GQ formed by dTTG₄, **GQ**_{TG}, can still offer the specific binding of Co^{II}Pc due to the quadruplex structure (Figure 5.15B). However, no efficient electron transfer over long nanometer distance could be achieved in Co^{II}Pc-GQ_{TG}. The efficient redox communications between Co^{II}Pc and electrodes, and among Co^{II}Pc centers were no longer available on the surface of Co^{II}Pc-GQ_{TG} electrode.

Figure 5.14 AFM images (A-F) and statistical analyses (a-e) for the lengths of corresponding Co^{II}Pc-tsGQwire in each figure by measuring more than 100 distinct Co^{II}Pc-tsGQwire that self-assembled from a mixture of dG₄ and dTTG₄ at ratios of (A) 9:1; (B) 7:3; (C) 1:1; (D) 3:7 (E) 1:9 and (F) from dTTG₄ only. With two terminal thymines, dTTG₄ can stop the growth of tsGQ nanowire upon adding to dG₄ during thermal annealing.

Figure 5.15 (A) CD spectrum of GQ_{TG} formed by dTTG₄ (10 μM) and Co^{II}Pc-GQ_{TG} adduct (with 1:4 ratio) measured in 10 mM lithium cacodylate buffer with 10 mM KCl, pH 7.4. (B) UV-vis spectrum of 4 μM Co^{II}Pc (black) in the presence and absence of GQ_{TG} (red).

The electrochemical active surface area and catalytic performance of Co^{II}Pc-GQ_{TG} electrode dropped to the similar level as those of Co^{II}Pc-polyA and Co^{II}Pc-polyT, in which single stranded DNA is also incapable of conducting electrons over long distance (Figure 5.12 and 5.16). Thus, upon binding on tsGQwire, the redox cycle of Co⁺/Co²⁺ was amplified significantly. Consequentially, high number of reductive Co center will be accessible to CO₂, which could enhance both reduction efficiency and selectivity of CO₂. Furthermore, the facilitation effects rank from high to low as tsGQwire > dp(AT) > single stranded DNA, short GQ. Notably, Co^{II}Pc on dp(AT) exhibited better performance on CO₂ electroreduction than those on the two single-stranded DNA, though they have similar binding mode with Co^{II}Pc (Figure 5.12 and 5.13). Duplex DNA can mediate an efficient electron transfer from electrodes to bound molecules,^[1,2] and accelerate the charge transfer among metal complexes bound on it.^[32] Hence the electron conductivity of DNA structures contributed to CO₂ reduction as well as the improved relative orientation of DNA bound metal complexes, if not more important.

Figure 5.16 The total current density and Faradaic efficiency of CO at -2.1 V over Co^{II}Pc adducts, in 0.5 M [Bmim]Tf₂N MeCN solution saturated with 1 atm CO₂ at ambient temperature with a 5 h electrolysis.

5.2.5 GQ Nanowire as a Universal Host Scaffold for Various Metal Complexes to Improve Their Performance in CO₂ Electroreduction

Furthermore, the unique features of tsGQwire were not limited to facilitate Co^{II}Pc redox chemistry. We also explored the catalytic performances of another two GQ-targeting complexes, iron(II) phthalocyanine (Fe^{II}Pc)^[6] and [Ru^{II}(bpa)(NH₃)₂](PF₆)₂ (Ru^{II}; bpa is N,N-bis-(1,10-phenanthrolin-2-yl)-amine)^[33] (Scheme 5.1). CD and AFM characterization indicated that the topology and morphology of tsGQwire are not affected upon binding of either Fe^{II}Pc or [Ru^{II}(bpa)(NH₃)₂]²⁺ (Figure 5.17).

Figure 5.17 Structural characterization of metal bound tsGQwire. AFM images of tsGQwires that assembled from dG₄ sequence in the presence of (A) Fe^{II}Pc and (B) [Ru^{II}(bpa)(NH₃)₂]²⁺. (C) CD spectrum of tsGQwire (10 μM, black) and Fe^{II}Pc-tsGQwire (red) and Ru^{II}-tsGQwire (blue) adducts (in 1:4 ratio) measured in 10 mM lithium cacodylate buffer with 10 mM KCl, pH 7.4.

Hypochromic and bathochromic shifts in UV spectra of the two complexes upon

binding to tsGQwire indicate the strong electron coupling between the complexes and GQ nanowire, which could offer the electron propagation pathway for reactivating the reductive complexes (Figure 5.18).

Figure 5.18 UV-vis spectra of (A) 8 μM $\text{Fe}^{\text{II}}\text{Pc}$ and (B) 8 μM $[\text{Ru}^{\text{II}}(\text{bpa})(\text{NH}_3)_2]^{2+}$ (black) in the presence and absence of 40 μM tsGQwire (red).

CO_2 electroreduction on $\text{Fe}^{\text{II}}\text{Pc}$ -tsGQwire and Ru^{II} -tsGQwire electrode showed the same elevations on efficiency and selectivity as that on $\text{Co}^{\text{II}}\text{Pc}$ -tsGQwire electrode (Figure 5.19). On $\text{Fe}^{\text{II}}\text{Pc}$ -tsGQwire electrode, CO_2 reduction yielded only CO and no liquid products. The reduction selectivity over H_2 was significantly enhanced from 30.8% to 76.3% while the total current intensity also increased from 7.5 to 10.7 mA/cm^2 . In the case of Ru^{II} -tsGQwire, similar escalation on current intensity and reduction selectivity were observed *vs* bare metal complex and other Ru^{II} -DNA electrodes, except the sole product turns out to be HCOOH . For both $\text{Fe}^{\text{II}}\text{Pc}$ - and Ru^{II} -DNA adducts, tsGQwire and dp(AT) with the ability to mediate long-range electron transfer showed higher impacts on CO_2 reduction efficiency and product selectivity, while the rest of $\text{Fe}^{\text{II}}\text{Pc}$ - and Ru^{II} -DNA electrodes merely showed any increments. Hence, GQ nanowire showed the same facilitation on various metal redox centers to achieve high reactivity of CO_2 reduction and concurrently to increase the performance of metal catalyst on product specificity. The confinement on catalyst alignment and the amplification on electron communication that GQ nanowire contributes to CO_2 redox chemistry are universal and independent on the metal centers. By proper selection of GQ-targeting metal complexes, tsGQwire electrode

can steer the electrochemical reduction of CO₂ to the desire gas or liquid products with high efficiency and selectivity.

Figure 5.19 The total current density and Faradaic efficiency of (A) CO at -2.1 V over FePc and FePc-DNA adducts, (C) HCOOH at -2.2 V over Ru and Ru-DNA adducts in 0.5 M [Bmim]Tf₂N MeCN solution saturated with 1 atm CO₂ at ambient temperature with a 5 h electrolysis.

5.3 Conclusion

In summary, we developed a self-assembly method to prepare submicron meter long of GQ nanowire from short tetranucleotides. By using tsGQwire as the host scaffold, GQ targeting metal catalysts showed highly effective and unique metal-dependent selectivity on the reduction of CO₂. The efficiency of CO₂ conversion is significantly improved comparing to the bare Co^{II}Pc electrodes and is comparable to the best performance of Co^{II}Pc-catalyst system in the literature. The facilitation effects from GQ nanowire is much better pronounced than other DNA structures and indicate the specific binding on GQ nanowire provide Co^{II}Pc the unique orientation and distribution on the electrode surface. Moreover, the efficient electron propagation *via* GQ nanowire contributes to speeding up the metal redox cycle and leads to the higher efficiency of CO₂ reduction. The work here offers the first example to use biomacromolecules as scaffold to host metal redox center and facilitate electrochemical CO₂ reduction process. As nucleic acids are widely explored as the medium for electron transfer in photolysis, the work here provides the useful information to merge the gap between light and dark reactions in the artificial photosynthesis with conductive biomacromolecules.

5.4 Experimental Section

Chemicals and materials

PolyA and PolyT were purchased from Sangon (Shanghai, China). All phosphoramidites and reagents for DNA (dG₄, 5'-FAM-dG₄ and dTTG₄) synthesis were purchased from Glen Research. The rest of the solvents and reagents for DNA preparation, purification, and reactions were purchased from Sigma-Aldrich. Cobalt(II) Phthalocyanine (Co^{II}Pc, purity > 98%) and iron(II) phthalocyanine (Fe^{II}Pc, purity > 98%) were purchased from Tokyo Chemical Industry. Ru(II) complex ([Ru^{II}(bpa)(NH₃)₂](PF₆)₂) was synthesized according to previous report.^[33] The ionic liquid was purchased from the Centre of Green Chemistry and Catalysis, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences. Working buffer is 10 mM lithium cacodylate buffer with 10 mM KCl, pH 7.4, unless it is stated otherwise.

Oligonucleotide synthesis and annealing

Oligonucleotides (1 mmol) were synthesized by standard protocols of DNA solid-phase synthesis on a MerMade 4 DNA Synthesizer from BioAutomation Inc. The oligonucleotides were cleaved and deprotected by treatment with NH₄OH/MeNH₂ (1:1, v/v) at 65 °C for 10 min and purified by RP-HPLC (MeCN (5–35%) in triethylammonium acetate (TEAA) buffer (0.1 M, pH 7.0) over 30 min.). The oligonucleotides were detritylated with acetic acid (80%) for 15 min and purified by HPLC (5–18% MeCN in TEAA buffer (0.1 M, pH 7.0) over 30 min). The oligonucleotides after purification were confirmed by ESI mass spectrometry and quantified by UV-vis spectrometry (Shimadzu UV-1800).

The oligonucleotides were dissolved in working buffer at a concentration (or combined concentration) of 500 μM as a stock solution. G-quadruplex, tsGQwire and dp(AT) were annealed by heating the desire single stranded samples at 95 °C for 10 min and gradually

cooling down to room temperature over 2 h. The stock solution was diluted to the desired concentration before each experiment.

Native PAGE analysis

A mixture of 450 μM of dG₄ and 50 μM of FAM-dG₄ were anneal in working buffer by heating at 95 °C for 10 min and gradually cooled down to room temperature over 2 h, and were kept at 4 °C for 24 h. 20 pmol of DNA were loaded on an 8% non-denaturing polyacrylamide gel and run at 400 V in 1×TBE buffer (0.089 M tris(hydroxymethyl)aminomethane-borate and 0.002 M EDTA, pH 8.3) with 100 mM KCl for 4 hours at 4 °C. The gels were scanned using Typhoon Trio Variable Mode Imager and the data were processed with ImageQuant TL (version 7.0).

Atomic force microscopy (AFM) characterization

A tsGQwire solutions (5 μL , 3 μM) were dropped on to freshly cleaved mica surface and were left for surface adhesion for 5 min. The sample on mica surface was then washed with Milli-Q water and dried with nitrogen gas. The AFM images were performed *via* Tapping ModeTM on a MultimodeTM AFM (Veeco, Santa Barbara, CA) in connection with a Nanoscope VTM controller, using TESPA-V2 tips (Bruker, Singapore).

Circular dichroism (CD) measurement

CD spectra were recorded on a Jasco J-1500 spectropolarimeter using a 1 cm path length cuvette. CD spectra of tsGQwire (or GQ_{TG}) in working buffer were recorded from 220-320 nm at a scan rate of 200 nm/min in the presence and absence of metal complex (2.5 μM). The maximum HT voltage is below 600 V. All CD spectra were baseline-corrected, and each curve represented of three averaged scans at 25 °C.

Binding stoichiometry measurement

The binding stoichiometry of Co^{II}Pc and tsGQwire were detected as previous report.^[34] Briefly, the total concentration of Co^{II}Pc and tsGQwire were held constant but their mole

fractions relative to one another were varied. Both Co^{II}Pc and tsGQwire were prepared at 10 μM in working buffer. With each addition of tsGQwire or Co^{II}Pc complex, a UV-vis spectrum was recorded. From the obtained absorbance values, a graph of absorbance at 681 nm vs mole fraction was plotted. The mole fraction of Co^{II}Pc was calculated using the equation $X_{CoPc} = V_{CoPc} / (V_{CoPc} + V_{tsGQwire})$, where V represented to the volume. The intersection of the data points defines the binding stoichiometry.

Ultraviolet–visible (UV-vis) absorption measurement

Absorption spectra were recorded on a Cary 100 UV/Vis spectrophotometer at 25 °C. Briefly, the UV-vis spectra of metal complex solution (4 μM of Co^{II}Pc and 8 μM of [Ru^{II}(bpa)(NH₃)₂]²⁺ or Fe^{II}Pc in working buffer) were recorded in the absence and presence of 4 equivalent amounts of DNA.

Fluorescent intercalator displacement (FID) assay

FID assay was performed on Varian Cary Eclipse fluorescence spectrophotometer in the working buffer. The concentration of tsGQwire used was 0.5 μM (2 μM of dG₄) and thiazole orange (TO) was 1 μM. Co^{II}Pc were titrated to displace TO from tsGQwire. Fluorescence spectra (from 520 to 750 nm, excitation wavelength is 503 nm) are recorded after each addition of Co^{II}Pc.

Electrochemical study

To prepare the working electrode, the metal-tsGQwire solution was spread onto carbon paper (CP). All the electrochemical experiments were conducted on the CHI 6081E electrochemical workstation in a three-electrode system at 25 °C. Linear sweep voltammetry (LSV) measurements were carried out in a single compartment cell with three-electrode configuration. These electrodes consisted of the working electrode, a platinum gauze auxiliary electrode, and an Ag/Ag⁺ (0.01 M AgNO₃ in 0.1 M tetrabutylammonium perchlorate-MeCN) reference electrode. The electrolyte was

bubbled with N₂ (or CO₂) for at 30 min to form N₂ (or CO₂)-saturated solution with slight magnetic stirring before our experiment. The LSV measurements were conducted in the potential range of -0.4 V and -2.5 V vs. Ag/Ag⁺ with a sweep rate of 20 mV s⁻¹.

The electrolysis experiments were performed in the H-type cell with a three-electrode system, which consisted of a working electrode, a platinum gauze auxiliary electrode, and an Ag/Ag⁺ (0.01 M AgNO₃ in 0.1 M tetrabutylammonium perchlorate-MeCN) reference electrode. The anode and cathode compartments were separated through a Nafion 117 proton exchange membrane. H⁺ can be transferred from anode compartment to cathode compartment through Nafion 117 proton exchange membrane, which is the proton source. 30 mL of 0.5 M H₂SO₄ aqueous solution was used as anodic electrolyte, and 30 mL MeCN containing 0.5 M 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide ([Bmim]Tf₂N) was used as cathodic electrolyte. Under the continuous stirring, CO₂ was bubbled through the catholyte (2 mL/min) for 30 min before electrolysis. Then, potentiostatic electrochemical reduction of CO₂ was carried out with the continuous gas bubbling (2 mL/min).

The gaseous product was collected by a gas bag and analyzed by gas chromatography (GC, HP 4890D), which was equipped with FID and TCD detectors. The liquid product of electrochemical experiments was analyzed by ¹H NMR (Bruker Avance III 400 HD spectrometer) in DMSO-d₆ with TMS (0.03 %, v/v) as an internal standard.

According to the literature,^[35] CO Faradaic efficiency (FE) can be calculated by

$$FE = nFA\alpha v / j_{total}$$

where n is the number of electrons involved in the electrode reaction, F is the Faraday constant, A is the integral area of CO peak in GC, α is the conversion factors determined from the calibration of the GC with standard samples, v is the CO₂ inflow rate, and j_{total} is the total current density.

The electrochemical activities of the working electrodes were characterized by single-sweep polarography. According to the Randles–Sevcik equation, electrochemical active surface area was determined by measuring the capacitive current associated with double-layer charging (C_{dl}) from the scan-rate dependence of LSVs.^[36] The C_{dl} was estimated by plotting the current density at -2.1 V vs. Ag/Ag⁺ against the scan rate.

The partial current densities for CO under different potentials were measured and the equilibrium potential was obtained *via* extrapolation method. The overpotential was obtained from the difference between the applied potential and the equilibrium potential.^[37] Multiple electrolysis experiments were then performed at each potential to get the average current density (j_{CO}) in the electrolysis cell. The overpotentials were plotted against $\log(j_{CO})$ to obtain the Tafel plots. A linear fitting of the data in Tafel plots is to yield the slope and deduce the mechanism of reduction reaction *via* electrokinetic analysis.

5.5 References

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Summary and Recommendations

In this thesis, several series of metal complexes were developed to function as GQ-targeted binders and stabilizers. The interaction between metal complexes and different GQ structures were studied. It is demonstrated that metal complexes with extensive aromatic ligands binding onto GQs with high specificity could not only play a unique feature in biomedicine, but also biotechnological and nanotechnological fields.

The three noncanonical ruthenium complexes were synthesized and featured in a distinctive double pyramid conformation. With the introduction of an *aza* linkage, two phenanthroline moieties were driving into a horizontal plane and furnishing an extensive π -conjugated system with suitable size and silhouette to bind onto G-tetrads and further stabilize GQs. Two NH_3 molecules on z -axis, in *trans* coordinating spots, provide the Ru compounds with splendid binding preference towards GQ structures against double-stranded DNA through hindering the insertion into their base pair layers. Further comparing the interaction of three Ru complexes with GQs, one can see that the ammonium group in **Ru1** and **Ru2** could form hydrogen bonds with G-tetrads, which will contribute to the stabilization effects on GQs. Moreover, these Ru compounds in octahedral geometry exhibited additional selectivity for antiparallel GQ topologies over parallel ones, especially for the basket topology. Furthermore, the novel Ru complex displayed a remarkable efficiency on depressing telomerase activity, accompanied with an efficient cytotoxicity towards various cancerous cell lines. Therefore, Ru compounds hold promising therapeutic values for cancer treatment. More importantly, the study offers a new strategy for developing GQ-specific metal complexes as potential anti-cancer agents and employ a novel interactive mode, with central channel in GQ, to function the stabilizing effects.

In addition, two Pt compounds that coordinated with bpa ligands were developed to achieve a definite planar configuration before binding onto GQs and therefore realize the

significantly high stabilization outcome on DNA/RNA GQs. The modification of *aza* linker by introducing alkyl chains deduced their binding and stabilizing effects on double-stranded DNA structures and therefore improved the binding preference to GQs. In addition, two Pt compounds displayed topological selectivity for basket (antiparallel) GQ topologies, which is evidenced by the more efficient stabilization on thermal un/folding process and the conformational changes from hybrid to basket topology even within K^+ buffer conditions. The remarkable stabilization on human telomeric G-quadruplex bestowed them with potent cytotoxicity towards various cancer cell lines, revealed the promising potential of these Pt compound as drug candidates for cancer treatment.

Moreover, by removing the alkyl chain in the above two Pt complexes, *aza* NH linkage was restored. Based on the structures, a series of novel phosphorescent Pt complexes as light switches for G-quadruplexes were synthesized. Due to the size, shape and *aza* NH bridge of the aromatic ligand, the complexes showed highly sensitive luminescence to the microenvironment, including the accessibility to proton and oxygen molecules, and the constraints on molecular motion. These complexes exhibited large Stokes shift (>130 nm), long lifetime (>18 μ s), high quantum yield (>0.45) and high luminescence turn-on signals specifically upon binding to G-quadruplexes and offered the first inorganic light-switch probe for RNA GQ in live cells. With a dual probe assay, dynamic un/folding of multiple RNA GQ could be simultaneously visualized in live cells. $[Pt(bpa)]^{2+}$ complexes showed great potentials in live-cell imaging and tracking the un/folding dynamics of RNA GQs.

Furthermore, the self-assembly features of G-rich sequences were employed to build up a sub-micrometer long G-quadruplex nanowire and applied to the electrochemical reduction of CO_2 . By using GQ nanowire to host GQ-targeting metal catalysts, unprecedented high efficiency and selectivity of metal dependent CO_2 reduction was achieved. The metal-GQwire decorated electrode efficiently enhanced both Faradaic

efficiency for CO₂ reduction and total current density, compared with bare metal complex decorated electrode and even comparable to the best performance of CoPc-based catalytic system in previous reports. The improved effects from tsGQwire is obviously efficient than other DNA structures and suggest GQ nanowire provide metal catalyst the unique orientation and distribution on the electrode surface. Moreover, electron transfer mediated by GQ nanowire achieve efficient redox cycling of catalytic centers on the electrode and leads to the higher efficiency of CO₂ reduction. The work here offers the first example to use biomacromolecules as scaffold to host metal redox center and facilitate electrochemical CO₂ reduction process. Therefore, the study provides practical information on the potential of using conductive biomacromolecules to merge the gap between light and dark reactions in the artificial photosynthesis.

Based on these results, further molecular designment and exploration on GQ-targeted theranostics will be conducted to realize the integration of cancer diagnosis and treatment. The GQ-specific binding probes can not only be used as binders to stabilize GQ structures, but also be applied to study and compare the cellular behavior of G-quadruplexes in normal and cancer cells. More information on GQ abundance and its folding dynamics in malignant tumor and normal tissue will be obtained. Hence, for realizing the individual treatment of cancer, the next generation of metal complexes should not only recognize and stabilize a specific GQ topology, but also can be used as a probe for the special GQ type.