

VEGF G-QUADRUPLEX: ITS INTERACTIONS WITH DOXORUBICIN AND
DETECTION WITH A GOLD NANOPARTICLE-AZACYANINE
FLUORESCENCE PROBE

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY



BY
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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
CHEMISTRY

JULY 2020

Approval of the thesis:

**VEGF G-QUADRUPLEX: ITS INTERACTIONS WITH DOXORUBICIN
AND DETECTION WITH A GOLD NANOPARTICLE-AZACYANINE
FLUORESCENCE PROBE**

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

VEGF G-QUADRUPLEX: ITS INTERACTIONS WITH DOXORUBICIN AND DETECTION WITH A GOLD NANOPARTICLE-AZACYANINE FLUORESCENCE PROBE

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Master of Science, Chemistry
Supervisor: Assoc. Prof. Özgül Persil Çetinkol

July 2020, 98 pages

Vascular endothelial growth factor (VEGF) is a signal protein known to be regulating angiogenesis and it is up-regulated in many tumors. It had been reported that VEGF promoter region Pu₂₂ can form a stable parallel G-quadruplex (G4) structure. G4 structures are formed in guanine rich nucleic acid sequences and they have gained considerable importance due to their involvement in vital cellular functions such as transcription. Concurrently, detecting and targeting G4s became an emerging field in drug design research. Within the scope of this thesis, in the first part, the interactions between the anti-cancer agent Doxorubicin (Dox) and the G4 structures formed by the VEGF promoter region Pu₂₂ and Pu₂₂T₁₂T₁₃ were investigated via UV-vis absorption, Circular Dichroism (CD) and Fluorescence spectroscopy experiments. Afterwards, the affinity of Dox towards different nucleic acid structures including several biologically important G4 structures was studied with competition dialysis experiments. Our investigations demonstrated that Dox had significant affinity to the G4 formed by the VEGF promoter region Pu₂₂ and stabilizing the G4 structure. The competition dialysis results revealed that the affinity of Dox to Pu₂₂ was higher than its affinity to other G4 forming sequences as BCL-2, C-myc, Her-2, K-ras and Human telomeric repeat. More importantly, our results

revealed that Dox's affinity to Pu₂₂ was comparable to its affinity to double stranded DNA, which might imply a new plausible action mechanism for Dox *in-vivo*. In the second part, a rapid, facile and sensitive off-on-off gold nanoparticle-Azacyanine5 (AuNPs-Aza5) fluorescence probe for the detection of G4 structures was developed. The proposed AuNPs-Aza5 fluorescence probe was characterized with UV-vis absorption, CD and Fluorescence spectroscopy, as well as Transmittance Electron Microscopy (TEM) and Dynamic Light Scattering (DLS) techniques. This sensing platform selectively responded to G4 structures including VEGF-Pu₂₂, K-ras, C-myc and BCL-2, while exhibiting no similar response to the other secondary structures, such as single, double and triple stranded structures. VEGF promoter region Pu₂₂ was chosen as the model G4 to investigate the analytical merits of the study and the off-on-off AuNPs-Aza5 probe displayed linear response to the model G4 between a concentration range of 0.032-0.347 μ M with a detection limit of 12.66 nM.

Keywords: G-quadruplex, Azacyanine, Doxorubicin, Gold nanoparticles, VEGF

ÖZ

VEGF G-DÖRTLÜ YAPISININ DOKSOROBİSİN İLE ETKİLEŞİMİ VE ALTIN NANOPARÇACIK-AZASIYANİN FLORESANS PROBU İLE TESPİTİ

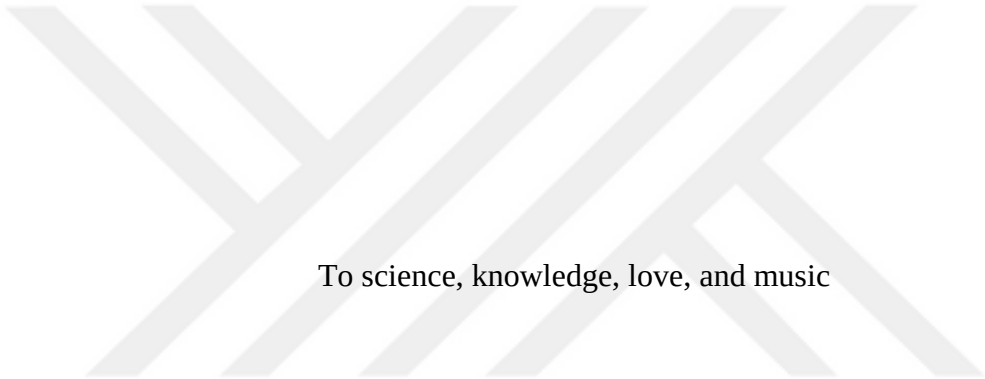
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Temmuz 2020, 98 sayfa

Vasküler Endotel Büyüme Faktörü (VEGF) kan damarlarının oluşumunu sağlayan bir sinyal proteinidir ve tümör hücrelerinde normalden fazla görülmektedir. VEGF promoter Pu₂₂ dizininin paralel yapıda bir G-dörtlü (G4) yapısı oluşturduğu rapor edilmiştir. G4 yapıları nukleik asitlerin guanin bazı bakımından zengin bölgelerinde oluşmaktadır ve bu yapılar, transkripsiyon gibi önemli hücre fonksiyonlarında rol oynadıklarının ortaya çıkmasının ardından, büyük önem kazanmıştır. Aynı zamanda, G4 yapılarının tespiti ve küçük moleküller ile hedeflenmesi ilaç araştırmalarının odak noktalarından biri haline gelmiştir. Bu tez kapsamında, öncelikli olarak, anti kanser ajanı Doksorubisin (Dox) ve G4 yapısı oluşturduğu bilinen VEGF promoter Pu₂₂ ve Pu₂₂T₁₂T₁₃ dizinleri arasındaki bağlanma özellikleri UV-vis, Circular Dichroism (CD) ve Floresans spektroskopisi yöntemleri ile incelenmiştir. Buna ek olarak, Dox'un bir dizi biyolojik olarak önemli G4 yapılarına karşı seçiciliği rekabetçi diyaliz yöntemi ile ortaya konulmuştur. Sonuçlar anti-kanser ilacı Dox ve VEGF promoter Pu₂₂ G4 yapısı arasında güçlü bir etkileşim olduğunu ve Dox'un yapılar üzerinde sağlamlaştırıcı bir etkisinin olduğunu göstermiştir. Gerçekleştirilen rekabetçi diyaliz deneyleri Dox'un, BCL-2, C-myc, Her-2, K-ras ve insan telomerik DNA'sı gibi bilinen diğer G4 yapılarına kıyasla VEGF promoter Pu₂₂ G4 yapısını tercih ettiğini göstermiştir. Daha önemlisi, VEGF Pu₂₂ G4 yapısı ve Dox arasındaki

etkileşimin Dox'un iki sarmal DNA'ya gösterdiği afiniteye yakın olduğu bulunmuştur ve bu sonuç Dox'un etki mekanizmalarına yeni bir yaklaşım kazandırabilecektir. Tezin ikinci kısmında, G4 yapılarını hızlı ve basit bir yöntemle tespiti için bir altın nanoparçacık-azasiyanin (AuNPs-Aza5) floresans probu geliştirilmiştir. Sunulan AuNPs-Aza5 floresans probu, UV-vis, CD ve Floresans spektroskopisi, Transmisyon Elektron Mikroskopisi (TEM) ve Dinamik Işık Saçılımı (DLS) yöntemleri ile karakterize edilmiştir. AuNPs-Aza5 sisteminin seçiciliği incelendiğinde, platformun G4 yapılarından olan VEGF-Pu₂₂, K-ras, C-myc and BCL-2 dizinlerine seçici olarak tepki verdiği, tekli, ikili ve üçlü yapıdaki nükleik asit yapılarına benzer bir tepki vermediği görülmüştür. AuNPs-Aza5 floresans probun analitik ölçülerinin belirlenmesi için model olarak VEGF-Pu₂₂ G4 yapısı seçilmiştir ve probunun 0.032-0.347 μ M Pu₂₂ konsantrasyon aralığında doğrusal bir cevap verdiği gözlenerek saptama sınırı (LOD) 12.66 nM olarak hesaplanmıştır.

Anahtar Kelimeler: G-dörtlüsü, Azasiyanin, Doksorubisin, Altın Nanoparçacık, VEGF



To science, knowledge, love, and music

ACKNOWLEDGMENTS

I would like to thank Assoc. Prof. Dr. Özgül Persil Çetinkol wholeheartedly, for creating the greatest Master's degree experience with her guidance, support and valuable advices.

I gratefully appreciate Scientific and Technological Research Council of Turkey (TUBITAK) for financial support via project number 218Z141.

To my parents, who help me get by the most difficult times and support me to pursue my dreams.

I want to thank my little brother, my best friend, and my source of joy Bartu, for being my inspiration, role model and motivation.

I want to thank my dearest lab mates for keeping me company when I am busy, helping me every way they can and making research the most fun it could be, to my best friends Ayana, Nil, Zeynep, Selin and Ilgın for listening to me and making me laugh whenever I feel frustrated.

I would like to thank to my committee members, Prof. Ceyhan Kayran İşçi, Prof. Yasemin Arslan Udum, Prof. Mesut Muyan and Assit. Prof. Sürayya Özcan Kabasakal for their valuable presence at my thesis defense.

Finally, I thank METU Chemistry Department for giving me the opportunity to do research and educating me to become an enthusiastic researcher.

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LIST OF ABBREVIATIONS

ABBREVIATIONS

AuNPs	Gold nanoparticles
Aza5	Azacyanine5
BCL-2	B-cell lymphoma 2
CD	Circular Dichroism
C-myc	Cellular MYC (Myelocytomatosis)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
dsDNA	Double stranded deoxyribonucleic acid
FRET	Fluorescence Resonance Energy Transfer
Her-2	Human Epidermal Growth Factor Receptor-2
H-telo	Human Telomeric repeat
K _a	Association constant
K-ras	Kirsten rat sarcoma
NMR	Nuclear Magnetic Resonance
RNA	Ribonucleic acid
SPR	Surface plasmon resonance
ssDNA	Single stranded deoxyribonucleic acid
T _c	Cooling temperature
T _m	Melting temperature
UV-vis	Ultraviolet-Visible

CHAPTER 1

INTRODUCTION

1.1 Nucleic Acids

Nucleic acids are long chains of polymers composed of nucleotides. The nucleotide units are made of three main parts which are the sugar, the base and the phosphate group. The nucleic acids are separated into two main groups as Ribonucleic acid (RNA) and Deoxyribonucleic acid (DNA) in means of their structural differences between the building blocks of the nucleotides. In DNA the base is attached to the deoxyribose sugar whereas in RNA the base is attached to ribose sugar. DNA bears adenine (A), cytosine (C), thymine (T) and guanine (G) bases while RNA bears the same bases except it utilizes uracil (U) instead of T. The phosphate groups serve as linkers between successive nucleotides [1]. The nucleic acid structures are one of the most important biological molecules since they possess the hereditary information and are responsible from the survival of living organisms.

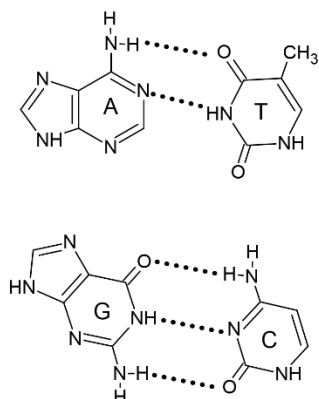
1.2 Structure of DNA

DNA is known as the carrier of genetic information. The structure and function of DNA is responsible for vital processes such as protein synthesis and cell division. The ongoing interest in revealing the aspects of DNA has a potential to enlighten important conflicts in biology such as genetic disorders and cancer [1]. Investigating the DNA structure is crucial in order to gain more information about the storage and expression of the genetic material [2], [3]. It had been revealed that there exists a great structural diversity to DNA which include different conformations and various types of secondary structures [2].

1.2.1 Factors That Govern The Structure of DNA

The structure of DNA is mainly influenced by the variation of bases throughout a strand and its environment. The purine bases, G and A, can associate via hydrogen bonding with the pyrimidine bases, C and T, respectively. There are two main faces of hydrogen bonding in the DNA strand which are called the Watson-Crick and the Hoogsteen faces. In genomic DNA, the interactions between the purine and pyrimidine bases are governed by the Watson-Crick base pairing, where there exist three hydrogen bonds between G:C pairs and two hydrogen bonds between A:T pairs. Hoogsteen hydrogen bonding takes place between purine and pyrimidine base pairs through the sites of purines that are not involved in Watson-Crick base pairing, which are the N7 and O6 atoms of G and N6 and N7 atoms of A bases [4]. The representation of Watson-Crick and Hoogsteen interactions between bases were shown in Figure 1. The interactions between the base pairs can be altered via changes in pH since the presence of Nitrogen atoms in the bases can change protonation states. Additionally, the presence of sugar groups can determine the conformation of DNA structures based on the alterations in the sugar conformation. Unlike the base pairs, sugar groups lack protonation sites, but the Oxygen atoms have the ability to form extensive hydrogen bonding networks to further stabilize the structure of DNA. The phosphate backbone is the most exposed part of the DNA strand and it is susceptible to solvent effects. Accordingly, salt concentrations and hydration levels can change the conformation of DNA. Consecutively, the variation of base pairing can lead to different secondary structures such as double, triple or quadruple DNA via the association of different number of strands in different ways [5].

Watson-Crick base pairing



Hoogsteen base pairing

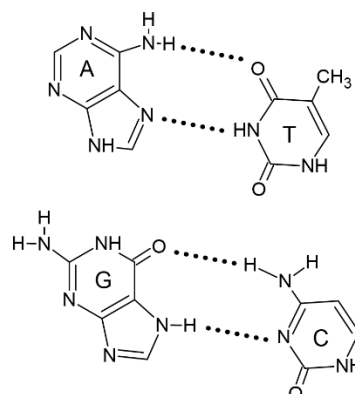


Figure 1. Schematic representations of Watson-Crick and Hoogsteen base pairings.

1.2.2 The DNA Double Helix

Watson and Crick suggested the double helical structure of DNA first in 1953 and received the Nobel Prize in 1962, together with Wilkins, for their discoveries regarding double helix structure of DNA. The suggested structure, known as the B-form DNA, was later on confirmed by X-ray Diffraction studies [6]. Today, it is known that the DNA double helix can also adopt different conformations such as Z and A DNA [7]. The differences in conformations of DNA were found to govern their functions in the cell. B DNA conformation is widely investigated, and it has been found to be the most common DNA double helix conformation [8]. Another example is the Z DNA, which is the energetically more strained conformation of the B-form double helix. Z DNA is observed near important gene sequences since the structural properties of the Z DNA conformation were found to be involved in the functioning of transcription sites and the promoter regions [9], [10]. A-form DNA is the conformation that possess a wider double helix than the B-form DNA. The differences between the conformers of DNA double helix structures are due the variations in the base sequence, temperature and hydration [11].

1.2.3 DNA Secondary Structure

Secondary structures of DNA play an important role in diagnostics and prediction of biological function of gene sequences [12]. One of the examples of DNA secondary structures is triplex DNA which arises from the association of a single strand DNA with a double stranded DNA [13], [14]. Triplex DNA regions are known to be observed especially in long polypurine-polyprymidine stretches and in mirror repeat regions and they interfere with gene expression [15], [16]. DNA triplexes are also seen as powerful tools for genetic manipulation therefore, their structures were characterized with various spectroscopic techniques [17], [18]. Another type of secondary structure is the DNA hairpins which are formed at the single stranded ends of DNA where complementary bases assemble to form pin-like conformations [19], [20]. DNA hairpins were also observed to play a role in cellular processes such as transcription, replication and gene expression [21]. Over past years, G-quadruplexes (G4) have become the most studied DNA secondary structures. These structures are formed in nucleic acids that are rich in G base content and they are found in genomic DNA sequences that hold biologic importance [22]. All these secondary structures of DNA hold great potential to be treated as therapeutic targets since they are involved in gene regulation and they possess structural specificity [12].

1.2.4 The G-quadruplex Structures

G4 DNA structures have gained major attention in the last two decades and have become the most popular secondary structures in nucleic acid research. G-tetrads are formed by the square planar alignment of four G bases through cyclic Hoogsteen hydrogen bonding, coordinated by monovalent cations such as Na^+ and K^+ through the eight oxygen atoms (O6s) of the G-tetrad [5]. The G4s are formed in G rich regions of nucleic acids when G-tetrads associate successively [23]. G4s can adopt different conformations depending on the sequence and the type of cation. Among the cations, K^+ is found to be stabilizing the G4 structures better than the others, due

to the more favorable coordination of the Oxygen atoms involved in the G4 formation [24]. The first G4 structures to have significant biological impact was characterized in the human telomeric overhangs [25], [26]. In humans, 5'-TTAGGG repeats are present at the chromosome ends as single strand overhangs and protect the ends of DNA from damage and shortening. It had been revealed that telomere binding proteins regulate the formation of G4s at the chromosome ends and the presence of G4s serve as recognition elements for replication, transcription and translation [22], [27], [28].

1.2.4.1 The G4 Structures in Human Genome and Their Association with Cancer

The structural features of G4s drew special attention due to their prevalence throughout the human genome [23]. It was anticipated that due to their regulatory roles, G4s may be present in the promoter regions of important genes that are strictly controlled for cell vitality [29]. The proto-oncogene region of Human Epidermal Growth Factor Receptor-2 (Her-2) is one of the first promoter regions that was found to be over-expressed in breast cancer patients [30]. The G4 structure formed by the Her-2 promoter region was studied with Nuclear Magnetic Resonance (NMR) spectroscopy and was characterized to form an intramolecular parallel G4 which held a regulatory role in the transcription of the gene [31]. Additionally, the promoter regions of Kirsten rat sarcoma viral oncogene homolog (K-ras), which shows mutations in 30% of the tumor cells, was characterized to form G4 structures [32], [33]. Another important identification was made-for B-cell CLL/lymphoma 2 (BCL-2) gene product which is responsible from the regulation of controlled cell death [34]. BCL-2 promoter regions are found to be also overexpressed in various cancerous cells and they also form parallel G4s in K⁺ medium [35]–[37]. Another widely studied G4 promoter gene is coding for a protein that is a member of the Master Regulator of Cell Cycle and Proliferative Metabolism (myc) family, C-myc,

and the overexpression of the C-myc is associated with prostatic cancer, breast cancer and leukemia [38], [39].

1.2.4.1.1 The G-quadruplex Formed in the Vascular Endothelial Growth Factor (VEGF) Promoter Region Pu₂₂

Vascular Endothelial Growth Factor (VEGF) plays an important role in development of new cells since it mediates vascular construction and induces cell proliferation. VEGF promotes the formation of new blood vessels by binding to the receptors on the surfaces of endothelial cells, and consequently promoting the nutrient uptake as well as cell growth [40]. A variety of studies reported the upregulation of VEGF transcription in tumor cells and pointed out VEGF as a potential therapeutic target for anti-cancer drugs [41]–[43]. The VEGF promoter region was under intense investigation to understand the molecular aspects of its expression and regulation in the cell. Hurley et. al. studied the G rich proximal promoter region of VEGF and found that an intramolecular G4 structure was adapted by the gene sequence *in vitro* [44]. Furthermore, Yang et. al. successfully characterized the topology of VEGF promoter region Pu₂₂ with NMR spectroscopy and reported that it was forming a parallel G4 structure in presence of K⁺, in solution [45]. The parallel G4 structure adopted by VEGF promoter region Pu₂₂ was shown in **Error! Reference source not found.** The discovery of VEGF G4 topology and its involvement in cancer biology contributed important implications to the correlation between of G4 structures in regulatory genes and tumor cells.

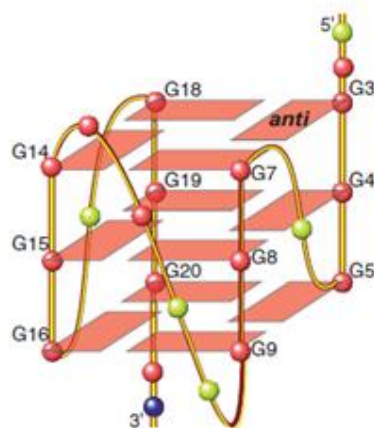


Figure 2. Parallel G4 formed in VEGF promoter region Pu22 [45].*

1.3 Targeting DNA with Small Molecules

Targeting DNA has long been an active area of interest in drug design, especially in discovery of anti-cancer agents [46]. Nucleic acid binders can regulate or suppress gene expression, interfere with transcription and DNA replication. Although biological, chemical, pharmaceutical, and clinical sciences have to work in coordination to come up with new therapeutic strategies, understanding the chemistry behind DNA-small molecule interactions is one of the first essential steps in designing effective drug candidates. Therefore, understanding the thermodynamics, modes of binding, electrostatics, affinity and selectivity features of small molecules are crucial to address nucleic acids and their interactions with drug candidate ligands [47]. The non-covalent DNA binding ligands are generally classified into two groups: intercalators and groove binders.

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1.3.1 Intercalators

Intercalators are mostly composed of an aromatic core that can intercalate in between the bases of DNA and additional functional groups which may facilitate other interactions such as groove binding. The planar aromatic group of an intercalator stacks between the base pairs, affects the DNA structure and thus DNA function [48]. There are two modes of intercalation: classical and threading. In classical intercalation, the aromatic core of the ligand is inserted between the base pairs of duplex DNAs. However, threading intercalation includes simultaneous interaction through minor and major grooves of the DNA and found to associate to the DNA structure more effectively than classical intercalation [49]. Doxorubicin (Dox), which is an effective chemotherapeutic drug that has been in clinical use for over 50 years, is classified as a threading intercalator [50].

1.3.1.1 Doxorubicin

Anthracyclines are composed of a four membered ring system with an anthraquinone chromophore and an aminoglycoside. Dox is an anthracycline derivative anti-cancer agent that has been in clinical use since the 1960s [51]. The structure of the anthracycline derivative Dox is shown in **Error! Reference source not found..** Dox is an intercalator ligand and it binds to duplex DNA by intercalating in between consecutive base pairs [50]. Although the kinetics and modes of action of Dox have been investigated over the past decades, the mechanism of its anti-cancer activity is still not fully understood. Dox induces several effects that result in anti-tumor activity such as inducing DNA damage, repression of gene expression and interference with DNA duplex separation. One of its most studied effect is Topoisomerase II inhibition which results in the disruption of one of the most important mechanisms in cell replication [52]. Along with its anti-cancer activity, Dox causes dose dependent cardiotoxicity due to its redox cycling ability [53]. The underlying mechanisms of the cardio toxic effect of Dox have also been under

intense investigation and several molecules were proposed to prevent the toxicity of Dox [54]–[56]. For instance, Dox and Cisplatin was used in combination to repress VEGF expression in cancer cells as an alternative treatment strategy [57]. Also, interactions between G4 DNA and Dox have been investigated as an additional action mechanism for the drug's anti-tumor effect [58]. Overall, Dox is an anti-cancer drug that has been in clinical use for over 50 years and its mechanism of action still remains controversial.

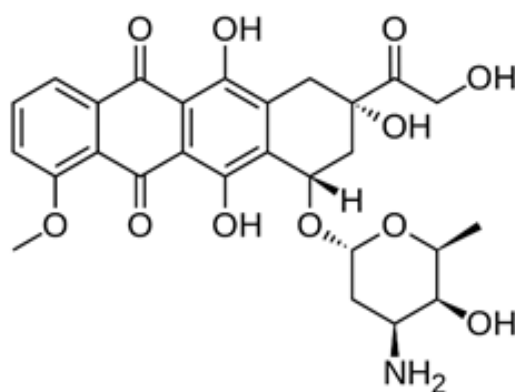


Figure 3. Structure of anthracycline derivative anti-cancer agent Doxorubicin.

1.3.2 Groove Binders

Groove binders can interact with either the major or the minor groove sites of DNA and they are classified according to their site of interaction. Minor groove binders are among the most effective anti-cancer drugs as they block the function of essential enzymes such as polymerases, topoisomerases and helicases through their interaction with DNA [59]. Minor groove binders are known to target A and T rich regions of nucleic acids and new molecules are being added to the literature to increase the sequence and structural selectivity of these ligands [59], [60]. Some widely studied minor groove binders are Netropsin, Berenil, DAPI and Hoechst. On the contrary, the structural insights of the major groove binder ligands have not been studied as extensively as the minor groove binders.

1.4 Targeting G4 Structures with Small Molecules

1.4.1 G4 Structures as Therapeutic Targets

Targeting G4s with small molecules for cancer therapy emerged as a promising approach in clinical research [61]. The G4 targeting therapeutic approach was first studied in 1997 by Hurley and co-workers where they studied the inhibition of telomerase activity via the interactions between 2,6-diamidoanthraquinone and human telomeric G4s [62]. Since then, the interactions between small molecules and G4-forming nucleic acid sequences were studied comprehensively and a diverse selection of new G4-interactive compounds were added to the literature [63]. Neidle et. al. reported G4 interactive Berberine derivatives such as Coralyne and reported that it was able to stabilize G4 forming DNA the most effectively among its other derivatives [64]. The interactions between G4 DNA and Berberine derivatives were studied by also other groups by various spectroscopic methods such as Circular Dichroism (CD), UV-vis for determination of thermal denaturation and Fluorescence spectroscopy for association constant (K_a) calculations [65], [66]. Ragazzon & Chaires used competition dialysis for the discovery of G4 selective ligands and found that methylene blue had selective binding affinity to G4s amongst other nucleic acids which adopt duplex, triplex and single stranded structures [67]. Anthracycline derivatives were also studied as potential G4 selective ligands. Anthracycline drugs Dox and Sabarubicin were investigated for their affinity to human telomeric G4s and the formation of their G4-drug complexes were demonstrated [68]–[70]. The interactions between C-myc promoter Pu₂₂ G4 and Dox and Nemorubicin were investigated with NMR spectroscopy and the complex between C-myc Pu₂₂ G4 and Nemorubicin was found to be the most stable [58]. The research on G4 interactive drugs is progressing with an increasing trend since it provides promising alternatives to overcome the current challenges in cancer therapy [61], [63].

1.4.2 Small Molecule G4 Probes for Cell Imaging and Characterization

Revealing the role of G4s in cellular functioning and their detection have gained significant attention due to their biological relevance mainly in the last two decades. Small molecules for the selective detection of G4s became an active research area since the discovery of such probes can result in alternative methods to gain structural information regarding G4s and consequences of their presence in the human genome [71]. For that purpose, many fluorescent probes were discovered and commonly used fluorescent dyes were improved in terms of their structural selectivity [72]–[74]. One of the selective small molecule probes for G4s was 1,3-imidazolidine-2-thione (IMT), which was used for real time cell imaging of G4 structures [75]. Another fluorescence probe designed for cellular imaging was 6-(2-(anthracen-9-ylmethylene) hydrazinyl)- N2,N4-diphenyl-1,3,5-triazine-2,4-diamine (9CI), which selectively targeted C-myc Pu22 among other G4 forming sequences. Another aspect of using small molecule ligands is to come up with alternative methods for the rapid and facile characterization of G4s since the common techniques for G4 characterization (X-ray Crystallography, NMR and CD spectroscopy) are elaborate and remain challenging. As an instance, Myong et. al. was able to confirm the formation of parallel G4 structures, in both single stranded and duplex DNA, with N-methyl meso-porphyrin (NMM) fluorescence probe [76]. Fluorometric detection of G4 DNA was also achieved by using Cyanine dyes and Carbon Dots (CDs) [74], [77].

1.5 Gold Nanoparticles

Gold nanoparticles (AuNPs) have become an active area of research due to their significant optical properties, unique chemical and physical characteristics such as shape, size and surface properties [78], [79]. AuNPs can be synthesized by the reduction of aqueous HAuCl₄ solution with various reducing agents under different experimental conditions. One of the most common synthesis of AuNPs was reported

by Turkevich. et. al. and the experimental procedure included reduction of heated aqueous HAuCl_4 solution with the addition of sodium citrate solution which induces negative charge on the surface of the nanoparticles [80]. A brief schematic representation of the Turkevich method for the synthesis of negatively charged AuNPs was shown in **Error! Reference source not found.** The resulting deep wine-red colloid after Turkevich synthesis contains citrate-capped, negatively charged AuNPs. The sizes of the synthesized nanoparticles may be manipulated between 2 nm to 100 nm by adjusting the concentration of the precursors and experimental conditions [81].

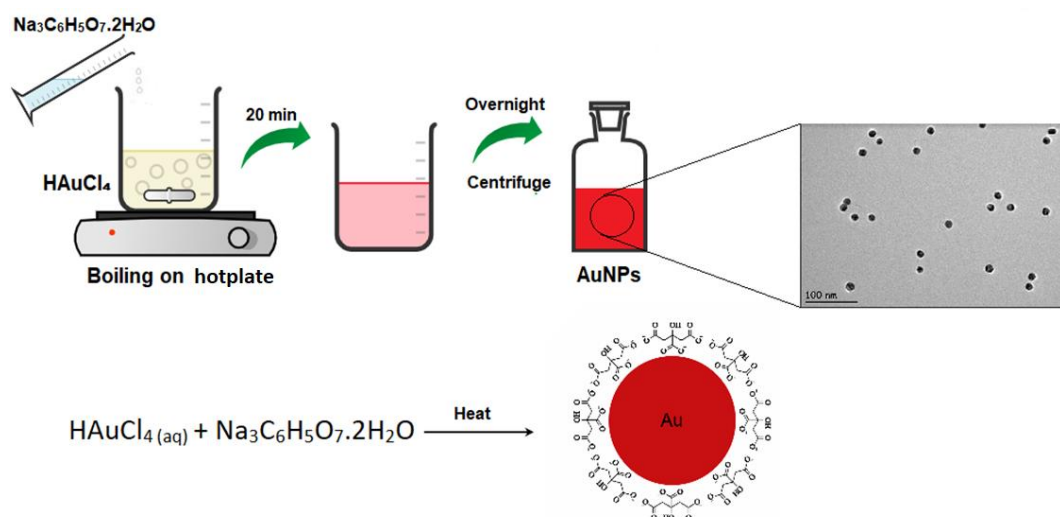


Figure 4. Turkevich method for the synthesis of AuNPs.

The synthesized AuNPs can be characterized by several methods including UV-vis spectroscopy, X-ray analysis and Scanning Electron Microscopy. Most commonly, the presence of the nanoparticles is validated by their characteristic absorption band around 520 nm in UV-vis absorption spectra due to their Surface Plasmon Resonance (SPR) properties. SPR is very useful in AuNPs applications since it depends on the size and shape of the synthesized nanoparticles [81]. Zhong et. al. showed that SPR bands in UV-vis absorption spectra of AuNPs were highly dependent on size since a red-shift was observed in their spectra from 519 nm to 569 nm as the size of the nanoparticles increased from 30 nm to 100 nm [82]. Not only the size effects the spectroscopic behavior of AuNPs, but also their shapes generate significant changes

in their spectroscopic response [83]. The surface properties of AuNPs can be modified by various organic molecules, biomolecules or metals [84]–[86]. In accordance with the tremendous possibilities of application, AuNPs have emerged as key players in many fields of chemistry from catalysis to chemical sensing [87]. Among the wide range of applications, they have become especially important in sensing applications. AuNPs provide cost-effective, sensitive and selective platforms for detecting chemically important species such as toxics, drugs and dangerous ions [79], [84]. Recently, AuNPs have also become extremely useful in biomedical applications such as drug delivery, immunoassays, optical biosensing and detection [79], [88], [89].

1.5.1 Colorimetric Sensing Applications with AuNPs

Colorimetric sensing approaches via AuNPs arise from the dramatic shift in SPR due to the aggregation of dispersed nanoparticles upon addition of an analyte. The red-shift around the absorption band of AuNPs, caused by addition of an analyte can be monitored by UV-vis absorption spectroscopy as well as the observable color change from the deep wine-red to blue [90], [91]. The principle of color change provides simple, sensitive and applicable detection methods for analytes that induce the aggregation effect on the nanoparticles. For instance, AuNPs have been studied for the detection of heavy metals such as Arsenic (As^{3+}), Mercury (Hg^{2+}), Copper (Cu^{2+}), Chromium (Cr^{3+}), Cadmium (Cd^{2+}) and Lead (Pb^{2+}) as well as alkaline earth metals Calcium (Ca^{2+}), Barium (Ba^{2+}) and Strontium (Sr^{2+}) in aqueous solution. AuNPs provide responsive protocols for the determination these ions in the environment which hold great significance since the exposure of living organisms to these metals can be fatal or cause deadly diseases like cancer [92].

1.5.2 Fluorometric Sensing Applications of AuNPs

Fluorometric sensing platforms mostly depend on an analyte that can induce “turn on” or “turn off” effect on the emission intensity. And, in the fluorometric sensing approaches via AuNPs usually the fluorescence of chromophores is quenched by the presence of AuNPs. This phenomena is known as the Forster Resonance Energy Transfer (FRET) [85], [93], [94]. One example of such systems can be the fluorometric detection of Hg^{2+} ions with the “turn on” probe based on the highly fluorescence molecule Rhodamine B and AuNPs [95]. Another reported approach was combining both colorimetric and fluorometric sensing principles of AuNPs, where Carbon Dots (CDs) were used as the fluorescent molecule [96]. The developed “light on” sensor was describing the existence of fluorescence in the presence of SCN^- ions, which otherwise was lost in the presence of AuNPs [96]. The system also took advantage of the highly stable Au-S bonds between the AuNPs and SCN^- ions, which is another widely studied property of AuNPs [97], [98].

1.5.3 The Use of AuNPs for Sensing and Detection of Biomolecules

Fast and cost-effective methods based on the AuNPs are quite advantageous for sensing and detection of biomolecules such as amino acids, enzymes, proteins, hormones and DNA polymers and the research on these sensing platforms increased dramatically over the last decade [90]. Mirkin et. al. developed a highly selective colorimetric DNA-AuNP/ Hg^{2+} platform for detection of Cysteine molecule and reported 100 nm Limit of Detection (LOD) for their system [99]. In addition, Geddes et. al. studied a glucose detection system based on Dextrin modified AuNPs [100]. In that study, when concanavalin A (Con A) molecule with multiple binding sites was added to the Dextrin coated AuNPs system, it caused aggregation of the nanoparticles. When glucose was added to the Dextrin coated AuNPs-Con A mixture, glucose molecules competitively bind to Con A and released the aggregated AuNPs and led to colorimetric sensing of glucose molecules. The FRET mechanism

between AuNPs and fluorophores has also been explored for the detection of biomolecules. A Quantum Dot (QDs) AuNPs sensor was developed for the detection of adenosine and cocaine [101]. AuNPs were also used for the detection of enzymatic activity. As an outstanding example, Song and co-workers developed a platform to detect telomerase activity and their system was governed by the colorimetric response of telomerase primer modified AuNPs (TP-AuNPs) and the presence of exonuclease enzyme Exo1. In their system, whenever there was telomerase activity the primers attached to AuNPs were forming G4 structures and protecting the DNA from exonucleases. When the telomerase activity was absent, the primers attached to AuNPs were not able to form G4s due to lack of telomerase activity, therefore the Exo1 was able to digest the DNA polymers and the AuNPs aggregated which resulted in a colorimetric response [102].

1.6 Thesis Focus

This thesis focuses on revealing the interactions between the famous anti-cancer agent Dox and VEGF Pu22 G4 structure and developing a rapid sensing platform for the detection of G4 nucleic acid structures.

Although Dox is an effective anti-cancer drug, its dose dependent cardio toxic side effects remain an unresolved issue. Therefore, the relationship between Dox and VEGF was previously studied in the scope of combinational therapy investigations. For example, VEGF-B gene therapy was found to inhibit Dox-induced cardiotoxicity [103]. Also, it has been shown that over-expression of VEGF₁₆₅ gene inhibits Dox-induced apoptosis [104]. Recently, several studies suggested the binding of Dox to G4s of human telomeric and C-myc [58]. However, the previous reports had not discussed Dox as an G4 interactive compound and its interactions with G4s were not investigated. Also, none of the studies reported that there might be a possible interaction between the VEGF G4 and Dox even though the combinational therapy approaches demonstrated promising results. Motivated by the above discussions, in the first part of this thesis, we investigated the binding of Dox to VEGF promoter

Pu₂₂ and Pu₂₂T₁₂T₁₃ G4s since VEGF is one of the most important targets in cancer therapy [105]. We also directed our efforts to reveal the affinity of Dox to G4 structures to be able to make a comparison to its affinity reported towards duplex DNAs in the literature. Finally, we wanted to demonstrate the relative affinity of Dox towards VEGF G4s compared to the other biologically important G4 structures such as BCL-2, Her-2, K-ras and C-myc. We believe that the interaction between Dox and VEGF has strong implications towards its anti-cancer activity and demonstrating the selective affinity between Dox and G4 structures may open up new possibilities in cancer research.

There is a definite need for simpler methods to predict the presence of G4 structures in nucleic acids. The common techniques for G4 characterization are CD, NMR, X-ray Crystallography which require expensive instruments, elaborate sample preparation and relatively high amounts of sample [106]. Consequently, less elaborate techniques as the use of fluorescence probes are being explored for monitoring G4 formation and detection. The challenge in developing fluorescence probes is that they need to be sequence selective with strong emission intensity [106]. For the above reasons, we aimed to develop a responsive and facile sensing platform for the detection of G4s. We used the AuNPs platform which is reportedly one of the most effective approaches to detect biomolecules [90]. We developed an off-on-off probe which was governed by the FRET mechanism between the fluorescent Azacyanine 5 molecules and AuNPs. The developed probe was investigated in terms of its size, morphology and selectivity towards G4 structures.

As a whole, this study provides new insights which will contribute to the fundamental knowledge on G4 structures.

CHAPTER 2

EXAMINING THE INTERACTION BETWEEN VEGF PROMOTER REGION AND DOXORUBICIN

2.1 Introduction

It has been thought that angiogenesis and tumor biology are closely related, and the vascular pattern of cancerous cells were widely investigated [107]. It has been shown that VEGF genes are responsible from angiogenesis with in vivo experiments therefore, VEGF became an emerging target for anti-cancer drugs [108]. The structure of VEGF gene sequence was also investigated and recently it has been shown that the gene is forming G4 structures [44], [45]. This immediately, led scientist to question whether VEGF sequences can be targeted with G4-interactive drugs [44].

The anthracycline derivative Dox, was proved to be an effective anti-cancer drug that intercalates to double stranded DNAs (dsDNA) and disrupt the function of vital cellular processes. Dox's relation to G4 structures were also searched since G4 forming gene sequences became important targets in cancer treatment [58], [63]. However, Dox's relation with G4 structures have never been studied comprehensively and the present reports do not discuss any results regarding Dox's affinity towards VEGF G4s [58]. On the other hand, several reports suggested combinational therapy strategies for Dox and VEGF to overcome the dose dependent toxicity of the drug [57], [103]. Evidently, we believe there is a reasonable relationship and a possible interaction between VEGF G4 structures and Dox.

We hypothesized that Dox will show affinity towards the VEGF Pu₂₂ and Pu₂₂T₁₂T₁₃ G4s which were previously characterized by Yang et al. [45]. We also aim to

demonstrate a comprehensive study on Dox's selective affinity towards important G4 structures and suggest that the Dox-G4 interactions are as significant as Dox-dsDNA interactions. Our findings will contribute new aspects to the ongoing studies on the reasoning behind Dox's anti-tumor activity. We will also be demonstrating that Dox can be classified as a G4-interactive ligand which can open up new research topics on increasing Dox's effectiveness as an anti-cancer drug.

2.2 Materials and Methods

2.2.1 Sample Preparation

The Pu₂₂ and Pu₂₂T₁₂T₁₃ G4 DNAs used in the experiments were purchased from Integrated DNA Technologies (IDT) (Coralville, IA, USA). The concentrations of the oligonucleotides were calculated with UV-vis absorption spectroscopy by using the molar extinction coefficients Pu₂₂; ϵ_{260} =205000, Pu₂₂T₁₂T₁₃; ϵ_{260} =200400. The concentration of Dox was calculated with UV-vis absorption spectroscopy using Dox; ϵ_{480} = 115000. Prior to experiments, 3.0 μ M DNA solutions (Pu₂₂ and Pu₂₂T₁₂T₁₃) were prepared in 25mM K-phosphate buffer with 70mM KCl. The preparation of the buffer and salt solutions were described in Appendix A. The DNA solutions were annealed by heating at 92-93°C for 5 minutes in water bath and left overnight for cooling down to ensure the formation of proper G4 structures. Afterwards, needed amounts of Dox for the preparation of 0:1, 1:0, 1:1, 1:2, 1:4, 1:6 and 1:10 (DNA: Dox) equimolar ratio samples were added to the annealed oligonucleotide solutions. The samples were mixed and centrifuged to ensure complete interaction between Dox and DNA. The sample preparation procedure was done separately for Pu₂₂ and Pu₂₂T₁₂T₁₃ G4s, for each DNA versus Dox equimolar ratio sample.

2.2.2 Thermal Denaturation Experiments

Thermal denaturation experiments were performed via UV-vis absorption spectroscopy (Agilent Technologies Cary 8454) and Circular Dichroism (CD) spectroscopy (JASCO J-815 spectropolarimeter). The UV-vis thermal denaturation experiments were performed by varying the temperature between 15-95°C with 2°C/min increments and the CD thermal denaturation experiments were performed by varying the temperature between 15-95°C with 5°C/min increments. Thermal denaturation experiments were performed for all the different equimolar ratio samples described in the previous section.

2.2.3 Determination of Association Constants

Binding (association) constants (K_a) were determined by fluorometric titration experiments using Cary Eclipse Fluorescence spectrophotometer. The DNA solutions (Pu₂₂ and Pu₂₂T₁₂T₁₃) were prepared prior to experiments, by the same annealing procedure and buffer conditions described in the previous section. Briefly, 2.5 mL of 1.0 µM Dox solution was titrated with 50.0 µM oligonucleotide (Pu₂₂ or Pu₂₂T₁₂T₁₃) + 1.0 µM Dox solution with 0.25 µM concentration increments. The parameters for the fluorescence measurements were: Emission spectra collected from 500 to 800 nm, excitation wavelength 485 nm, excitation and emission slits: 5.0 nm and 5.0 nm, operation 800 V, scan rate 600 nm/min. Binding constants were obtained by fitting the Integrated Fluorescence Intensity data between 335 nm and 600 nm as a function of DNA concentration, using the least squares equations:

$$KC_b^2 - C_b(KS_0 + KD_0 + 1) + KS_0D_0 = 0$$

$$F = F_0(C_t - C_b) + F_bC_b$$

where K is the association constant in (M^{-1}), C_b is the concentration of bound ligand, S_0 is the total binding site concentration, C_t is the total concentration of small molecule, D_0 is the total ligand concentration, F is observed fluorescence at each titration point, F_0 is fluorescence intensity of the free small molecule, and F_b is the fluorescence of the bound species [109]. Igor Pro software (Wavemetrics Inc, USA) was used in all the data

analysis. Binding constants were obtained as an average of three replicates for each DNA:Dox titration and reported with standard deviations.

2.2.4 Competition Dialysis Assay

Competition dialysis experiments were accomplished according to the previously reported procedure by Chaires et. al. [67]. Preparation of the buffer solutions used in this experiment were described in Appendix A, the sequences of the nucleic acids were listed in Table 3, Appendix B and the oligonucleotides included in the experimental set up were listed in Table 4, Appendix F. Construction of the calibration curve was also given in Appendix F.

The nucleic acid structures were prepared according to the procedure described by Ren & Chaires [110]. Oligonucleotides including Pu₂₂, Pu₂₂T₁₂T₁₃, BCL-2, C-myc, K-RAS, H-telo, Her-2, dA₃₂ and dT₃₂ were purchased from Integrated DNA Technologies (IDT) (Coralville, IA, USA). Natural DNA of *Micrococcus lysodeikticus* (m-lyso) was purchased from Sigma-Aldrich (St. Louis, MO, USA). M-lyso was sonicated for 30 minutes (5 minutes sonication then, 5 minutes rest) followed by phenol-chloroform extraction before used [111]. Additionally, poly(dA):poly(dT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The concentrations of the oligonucleotides used in this assay were calculated using the extinction coefficient values listed in Table 4, Appendix F. Remaining oligonucleotides were prepared by mixing required amounts nucleic acids as described below:

- Duplex DNA-dA₃₂dT₃₂ was prepared by mixing 1:1 equimolar ratio of dA₃₂ and dT₃₂.
- Triplex DNA-dA₃₂[dT₃₂]₂ was prepared by mixing 1:2 equimolar ratio of dA₃₂ and dT₃₂.
- Duplex RNA-poly(A):poly(U) was prepared by mixing 1:1 equimolar ratio of poly(A) and poly(U).

- Triplex RNA-poly(A):[poly(U)]₂ was prepared by mixing 1:2 equimolar ratio of poly(A) and poly(U).

All the prepared oligonucleotides were annealed according to previously described procedure, in 1xBPES buffer (1.0 mM Na₂EDTA, 6.0 mM Na₂HPO₄, 2.0 mM NaH₂PO₄ and 185 mM NaCl), pH 7.0 (Appendix A).

For each competition dialysis experiment 0.6 mL of 60.0 μM of oligonucleotide, in monomeric units (nucleotide, base pair, triplet and quartet) were put into Pierce (Thermofischer Scientific, USA) 7000 Da molecular cutoff dialysis cassettes. Then, the cassettes were dialyzed against 750 mL of 1.0 μM Doxorubicin solution for 24 hours. The dialysis solution was also prepared in 1xBPES buffer (Appendix A). After the dialysis period, oligonucleotide solutions were taken out from the dialysis cassettes and 1%(w/v) SDS was added to release the bound Dox from the nucleic acids to ensure the Dox molecules were free in solution for measurement purposes. Following that, the Dox concentration in each cassette was determined by Fluorescence spectroscopy. The parameters for the fluorescence measurements were the same with the parameters described in the previous section. The calibration curve for the Dox molecule was constructed using the same instrument and parameters prior to the competition dialysis experiments (Figure 79, Appendix F). Using the linear equation supplied by the calibration curve, the amount of the bound ligand to each oligonucleotide was determined. Corrections were made by considering the dilution effect caused by 1xBPES buffer in each cassette. Two independent dialysis assays were performed for Dox. The ratio of bound ligand (C_b) to free ligand (C_f) was determined by the equation: $C_b / C_f = (C_t / C_f) - 1$ where C_t is the total Doxorubicin concentration in each dialysis cassette after the dialysis period, C_f is the Doxorubicin concentration in the positive control dialysis cassette (which only included 1xBPES buffer).

2.3 Results and Discussion

2.3.1 Circular Dichroism and UV-vis Measurements

CD spectra of samples were measured to observe whether the presence of Dox resulted in any structural alterations in the Pu₂₂ and Pu₂₂T₁₂T₁₃ G4s. CD is a widely used method to characterize secondary structures of proteins and nucleic acids [112]. The G4 structures formed by Pu₂₂ and Pu₂₂T₁₂T₁₃ were previously characterized by Yang et. al. with detailed NMR studies and they are known to form parallel G4s, which display characteristic bands in CD spectra [45]. Parallel G4 structures characteristically display a positive band around 260 nm and a negative band around 240 nm in the CD spectra [5]. Therefore, the formation of parallel G4 structures was validated with CD spectroscopy as shown with the dotted black lines for 3.0 μ M Pu₂₂ (Figure 5) and Pu₂₂T₁₂T₁₃ (Figure 6). Following that, increasing concentrations of Dox was added to G4s to observe the effect of Dox concentration on G4 structures. In Figure 5 and Figure 6 blue, pink, green, yellow and dashed black lines represent 3.0 μ M (1:1), 6.0 μ M (1:2), 12 μ M (1:6), 18 μ M (1:6) and 30 μ M (1:10) Dox additions to 3.0 μ M G4, respectively. In addition to that, solid black lines in Figures 5 and 6 represent the 30.0 μ M Dox in absence of the G4s for comparison. Since Dox is a chiral molecule, it exhibited broad, positive bands around 350 nm and 450 nm, a negative band around 290 nm, another positive band at 234 nm with a shoulder at 250 nm in CD spectra. As can be seen, no significant differences were observed in presence of Dox, meaning that the parallel G4 structure was intact even in the presence of high Dox concentrations. The parallel G4 band at 260 nm decreased upon treatment with Dox which is due to the establishment of interactions between G4s and Dox. Theoretically, the intensity of that band should increase upon addition of Dox as shown in Figure 7 and Figure 8 with the red lines (the composite spectra obtained from the addition of 1:0 and 0:10 DNA:Dox samples) for Pu₂₂ and Pu₂₂T₁₂T₁₃ G4s, respectively. When the spectra of 1:0, 0:10 and 1:10 Pu₂₂:Dox were examined; the ellipticity of 1:0 Pu₂₂:Dox and 0:10 Pu₂₂:Dox samples at 262 nm were

found to be 34.7 mdeg and 2.1 mdeg, respectively. Accordingly, if there was no interaction between Pu₂₂ and Dox, one should expect a spectrum that is equal to the sum of 1:0 Pu₂₂:Dox and 0:10 Pu₂₂:Dox spectra with ellipticity of 36.8 mdeg at 262 nm for 1:10 Pu₂₂:Dox. However, the observed intensity was 23.7 mdeg. In addition, the disappearance of the broad positive bands at 350 nm and 450 nm, also suggested that a different type of chirality is probably induced in Dox structure upon its interaction with Pu₂₂ G4. The same affect was also demonstrated for Pu₂₂T₁₂T₁₃ G4 in Figure 8 where the intensity of the theoretical 1:10 Pu₂₂T₁₂T₁₃:Dox spectra (Figure 8, red line) at 262 was 33.4 mdeg. Similarly, the actual intensity of 1:10 Pu₂₂T₁₂T₁₃:Dox spectra at 262 nm (Figure 8, dashed-black line) was 19.2 mdeg, indicating an interaction between the Pu₂₂T₁₂T₁₃ G4 structure and Dox.

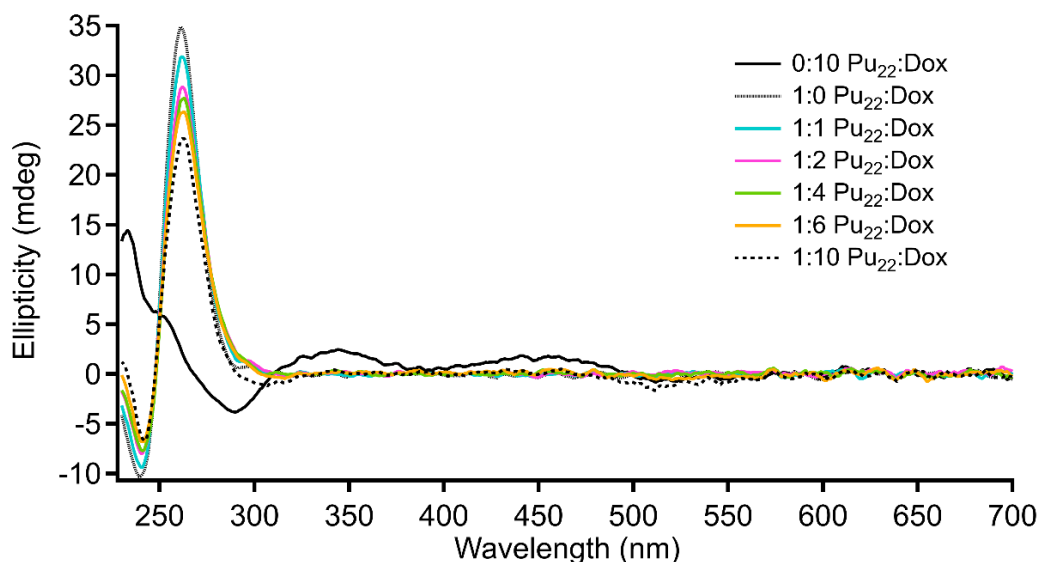


Figure 5. CD spectra of Dox (30.0 μ M) and Pu₂₂ (3.0 μ M) in the presence of increasing Dox concentrations (3.0-30 μ M) at 15°C.

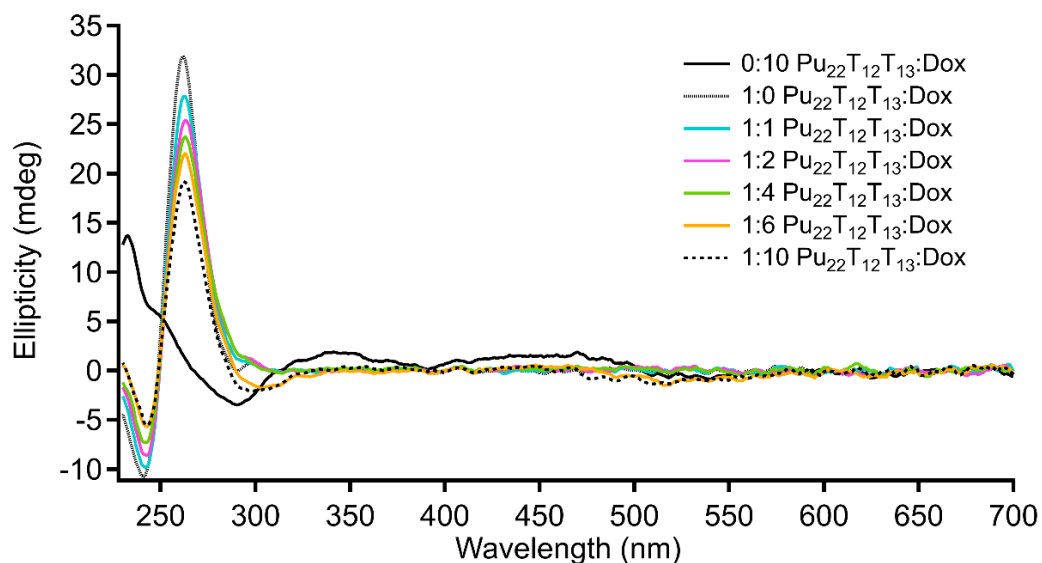


Figure 6. CD spectra of Dox (30.0 μM) and $\text{Pu}_{22}\text{T}_{12}\text{T}_{13}$ (3.0 μM) in the presence of increasing Dox concentrations (3.0-30 μM) at 15°C.

The interactions between Dox and the G4s were also studied with UV-vis spectroscopy. Similarly, UV-vis absorption spectra were generally expected to exhibit changes upon binding of ligands to DNA structures. Figure 9 and Figure 10 displays the UV-vis absorption spectra of Pu_{22} and $\text{Pu}_{22}\text{T}_{12}\text{T}_{13}$ G4s in presence of increasing Dox concentrations, respectively. In Figure 9 and Figure 10 blue, pink, green, yellow and dashed black lines represent 3.0 μM (1:1), 6.0 μM (1:2), 12 μM (1:6), 18 μM (1:6) and 30 μM (1:10) Dox additions to 3.0 μM Pu_{22} and $\text{Pu}_{22}\text{T}_{12}\text{T}_{13}$ G4s, respectively.

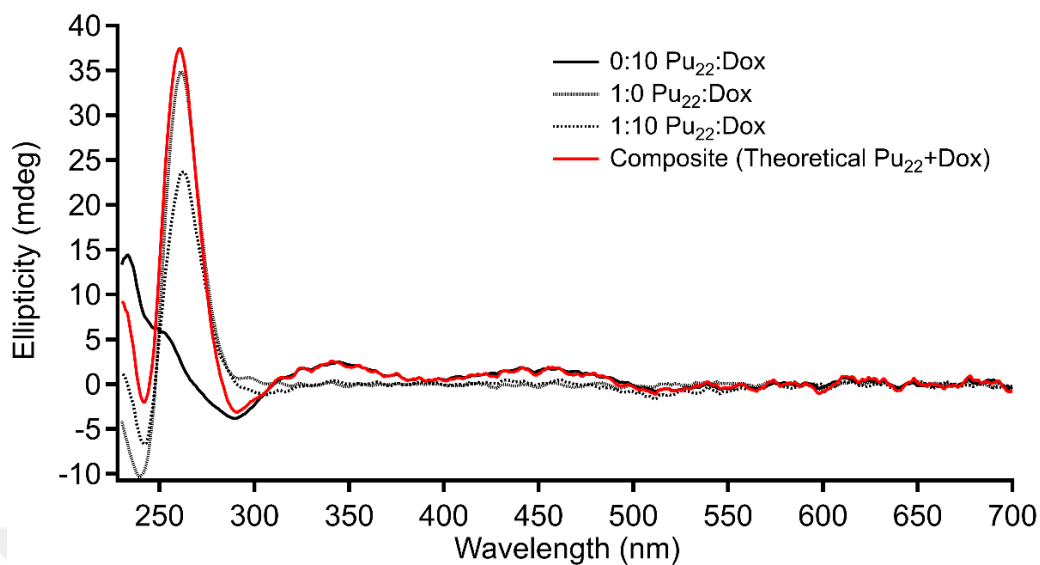


Figure 7. CD spectra of Pu₂₂ G4 (3.0 μM) with increasing Dox concentration (black lines) and theoretical composite of 0:10 and 1:10 Pu₂₂:Dox samples (red line).

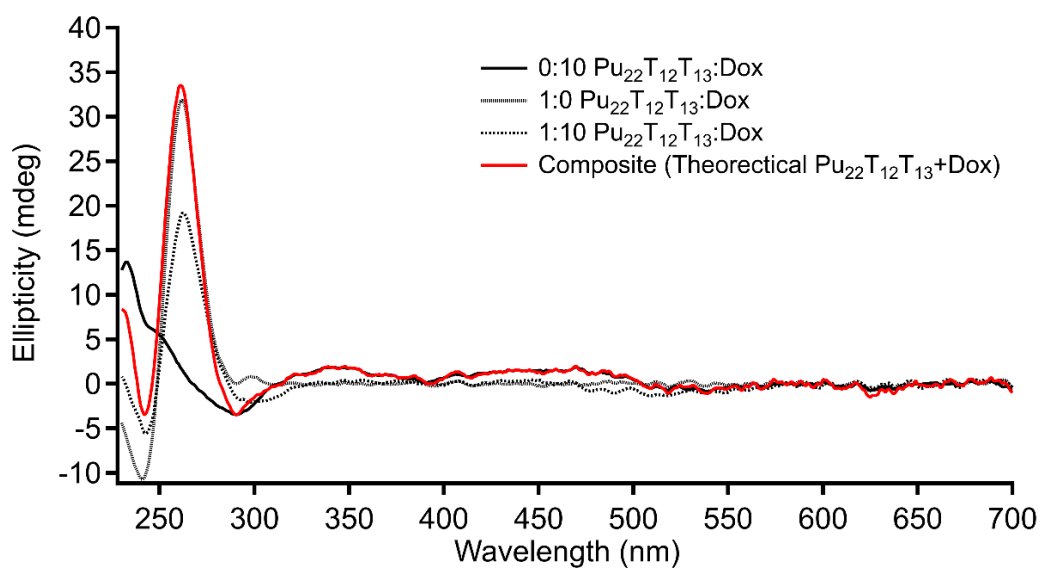


Figure 8. CD spectra of Pu₂₂T₁₂T₁₃ G4 (3.0 μM) with increasing Dox concentration (black lines) theoretical composite of 0:10 and 1:10 Pu₂₂T₁₂T₁₃:Dox samples (red line).

In addition to that, in Figures 9 and 10 solid black lines represent the 30.0 μM Dox in the absence of G4s and dotted black lines represent the 3.0 μM G4s in the absence of Dox (Pu₂₂ and Pu₂₂T₁₂T₁₃ respectively) for comparison. As can be seen from the spectra, Dox exhibited an absorption maximum around 480 nm, which decreased

(hypochromic effect) in presence of both Pu₂₂ and Pu₂₂T₁₂T₁₃ G4s. This result indicated the presence of interactions between Dox and DNAs. If the UV-vis spectra of samples are compared, the decrease in intensity at 480 nm was higher for Pu₂₂. In addition, the presence of G4 structures caused a red shift in the absorbance region of Dox at around 480 nm [47]. The hypochromic effect and the red shift are generally observed due to the stacking of the aromatic moiety of the chromophore in between the DNA base pairs and the magnitude of both the hypochromic effect and the red shift are thought to be correlated with the strength of binding [113], [114]. Here, the magnitude of the hypochromic effect was similar for Pu₂₂-Dox and Pu₂₂T₁₂T₁₃-Dox complexes. As a result, our results clearly displayed that both the hypochromic effect and the red shift were present in the UV-vis spectra of both Pu₂₂-Dox and Pu₂₂T₁₂T₁₃-Dox complexes, conforming the presence of interactions between Pu₂₂ or Pu₂₂T₁₂T₁₃ G4s and Dox.

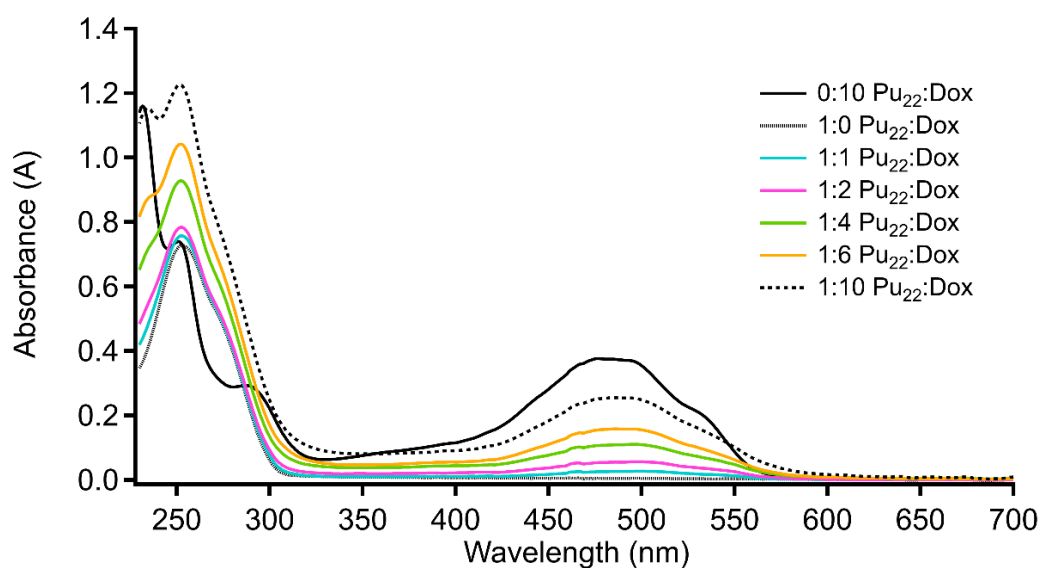


Figure 9. UV-vis absorption spectra of Dox (30.0 μ M) and Pu₂₂ (3.0 μ M) in the absence and presence of increasing Dox concentrations (3.0-30 μ M).

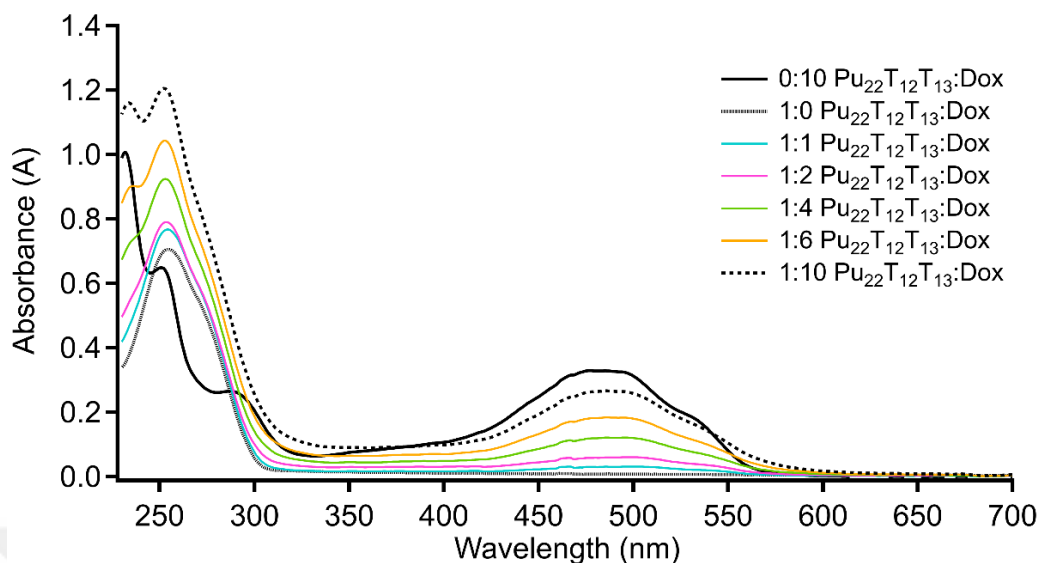


Figure 10. UV-vis absorption spectra of Dox (30.0 μM) and $\text{Pu}_{22}\text{T}_{12}\text{T}_{13}$ (3.0 μM) in the absence and presence of increasing Dox concentrations (3.0-30 μM).

2.3.2 UV-vis Thermal Denaturation Experiments

Thermal denaturation experiments were performed to observe the changes in melting temperatures (T_m) of nucleic acid structures upon their interactions with Dox [115]. An increase in T_m value means that the small molecule inserts a stabilizing effect on the nucleic acid structure and causes it to unfold at higher temperatures. In this study, thermal denaturation (heating) temperatures ($T_h = T_m$) and cooling temperatures (T_c) of samples containing 1:0, 1:1, 1:2, 1:4, 1:6, 1:10 and 0:10 equimolar ratios of DNA (Pu_{22} or $\text{Pu}_{22}\text{T}_{12}\text{T}_{13}$; 3.0 μM):Dox were measured via UV-vis absorption spectroscopy between 15°C and 95°C (the corresponding UV-vis absorption spectra for the thermal denaturation experiments were given in Appendix C, Figures 39-62). The increase in temperature from 15°C to 95°C resulted in unfolding of the G4 structures formed by Pu_{22} and $\text{Pu}_{22}\text{T}_{12}\text{T}_{13}$ sequences. The structural changes in the G4s were monitored by the change in absorbance at 295 nm [115]. Generally a decreasing trend in the UV-vis absorbance at 295 nm was observed as the temperature increased due to the denaturation of G4s [115][116]. However, such a trend wasn't

observed in the thermal denaturation profiles of our G4 samples as given in Appendix C.

The most important outcome of the UV-vis thermal denaturation experiments was that the melting curves obtained at 295 nm (Appendix C) were not revealing the characteristic changes associated with G4s. As just stated above, the melting curves for G4s at 295 nm were generally supposed to display a decreasing trend, while our melting curves were showing mixed trends (Appendix C) [116]. One reason might be the relatively little change observed in absorbance upon unfolding. Another reason might be the interference caused by the presence of Dox since it also absorbs at 295 nm. We concluded UV-vis spectroscopy was not able to resolve the unfolding trends of Pu₂₂ and Pu₂₂T₁₂T₁₃ G4s and therefore, the samples were further investigated with CD spectroscopy.

2.3.3 CD Thermal Denaturation Experiments

CD thermal denaturation experiments were performed to validate the presence of interactions between Pu₂₂ or Pu₂₂T₁₂T₁₃ G4s and Dox since the UV-vis experiment results were controversial. Similarly, CD spectra of the samples containing 1:0, 1:1, 1:2, 1:4, 1:6 and 1:10 equimolar ratios of DNA (3.0 μ M):Dox were measured via CD spectroscopy and the corresponding spectra were given in Appendix D, Figure 63-70. Figure 11 displays the CD spectra of Pu₂₂ G4 (3.0 μ M, in strand) in the absence of Dox, between 15°C and 95°C. The gradual decrease in the positive band around 260 nm was indicating that the unfolding of G4 structure as the temperature increased. Figure 11 displays the CD spectra of Pu₂₂ G4 in presence of 30 μ M Dox (1:10 equimolar ratio sample), between 15°C and 95°C. When the spectra in Figure 11 and Figure 12 were compared, it can be concluded that the gradual decrease was less prompt due to the stabilization effect caused by the presence of Dox.

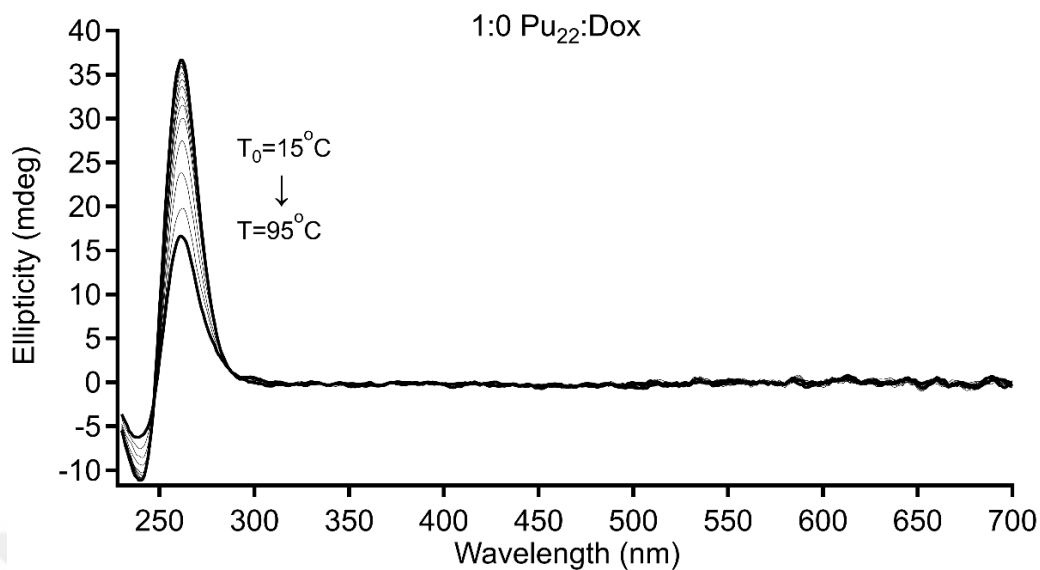


Figure 11. CD thermal denaturation experiment results for 1:0 Pu₂₂ (3.0 μ M):Dox sample. The Ellipticity (mdeg) vs Wavelength (nm) graph shows the CD spectra of 1:0 Pu₂₂:Dox sample between 15°C and 95°C.

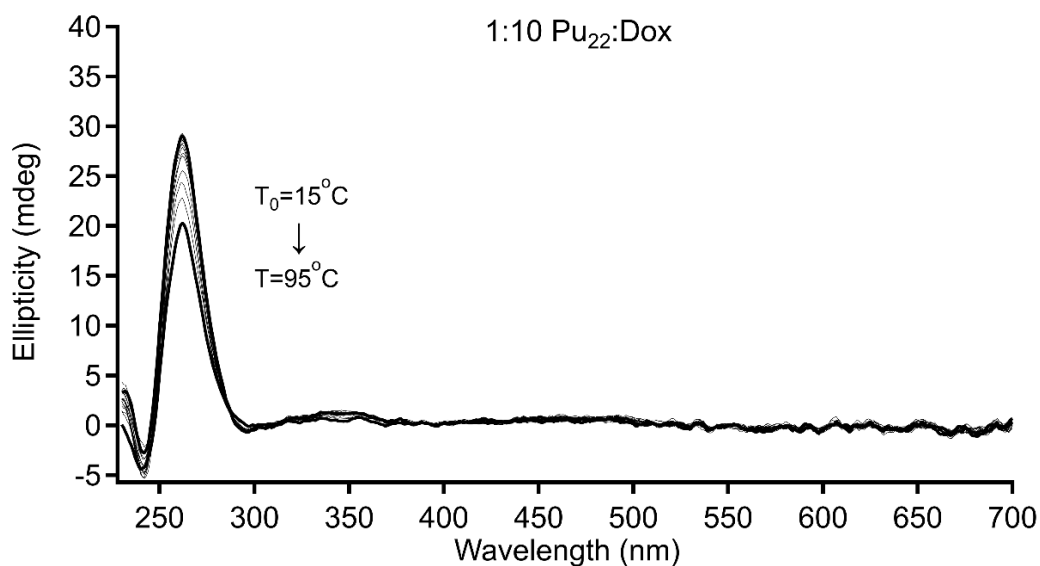


Figure 12. CD thermal denaturation experiment results for 1:10 Pu₂₂ (3.0 μ M):Dox sample. The Ellipticity (mdeg) vs Wavelength (nm) graph shows the CD spectra of 1:10 Pu₂₂:Dox sample between 15°C and 95°C.

The stabilization effect can also be observed if the positive bands at 260 nm and 95°C in Figure 13 for Pu₂₂ in the absence of Dox (black lines) and Pu₂₂ in the presence of Dox (red lines) were compared. The Pu₂₂ at 95°C (Figure 13, dashed black line), G4

positive band exhibited an ellipticity value around 15 mdeg in the absence of Dox, while the positive band was around 20 mdeg in presence of 30 μ M Dox (Figure 13, dotted red line). The change in ellipticity in the spectra between 15°C and 95°C for Pu₂₂ in the absence of Dox was greater than the change in ellipticity for Pu₂₂ in the presence of Dox. The lower value of the ellipticity change indicates that the G4 structure wasn't able to unfold as much as in the absence of Dox as it did in the presence of Dox.

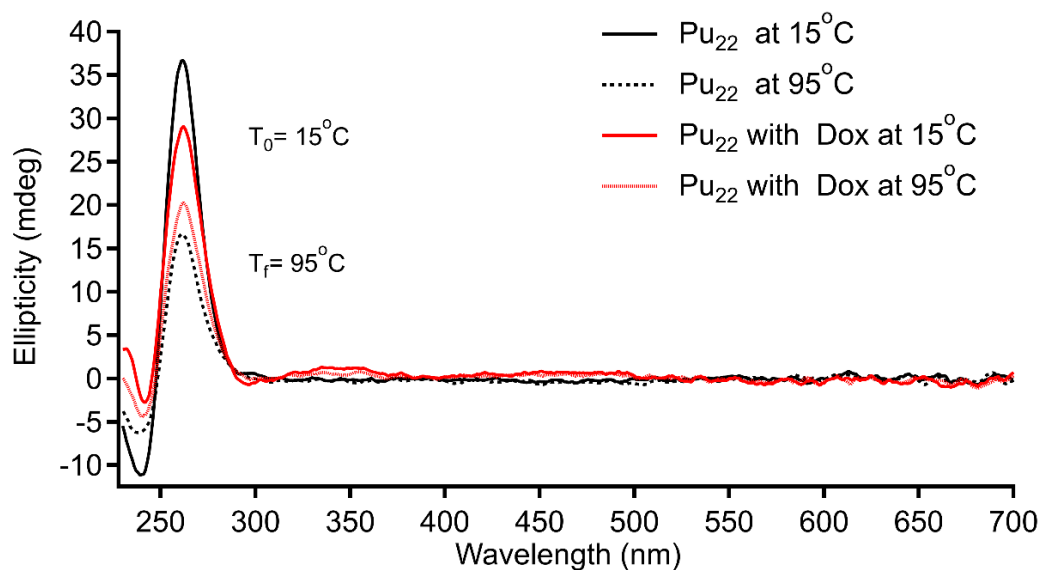


Figure 13. CD spectra of Pu₂₂ (3.0 μ M) and 1:0 Pu₂₂:Dox samples. The Ellipticity (mdeg) vs Wavelength (nm) graph shows the CD spectra of Pu₂₂ (3.0 μ M) in the absence (solid line) and presence of 30.0 μ M Dox (1:10 Pu₂₂:Dox, dashed line) at 15°C and 95°C.

The thermal denaturation experiments were also performed for Pu₂₂T₁₂T₁₃ G4. Figure 14 and Figure 15 shows the CD spectra of Pu₂₂ G4 (3.0 μ M) in the absence and presence of 30.0 μ M Dox (1:10 equimolar ratio sample), between 15°C and 95°C, respectively. Pu₂₂T₁₂T₁₃ G4 exhibited similar results, as the stabilization effect of Dox can be observed from the comparison of the amount of the decrease in the absorption maxima at 260 nm. The decrease in ellipticity at 260 nm observed in Figure 14 was less gradual in Figure 15, where the presence of Dox inserted a stabilization effect on Pu₂₂T₁₂T₁₃ G4.

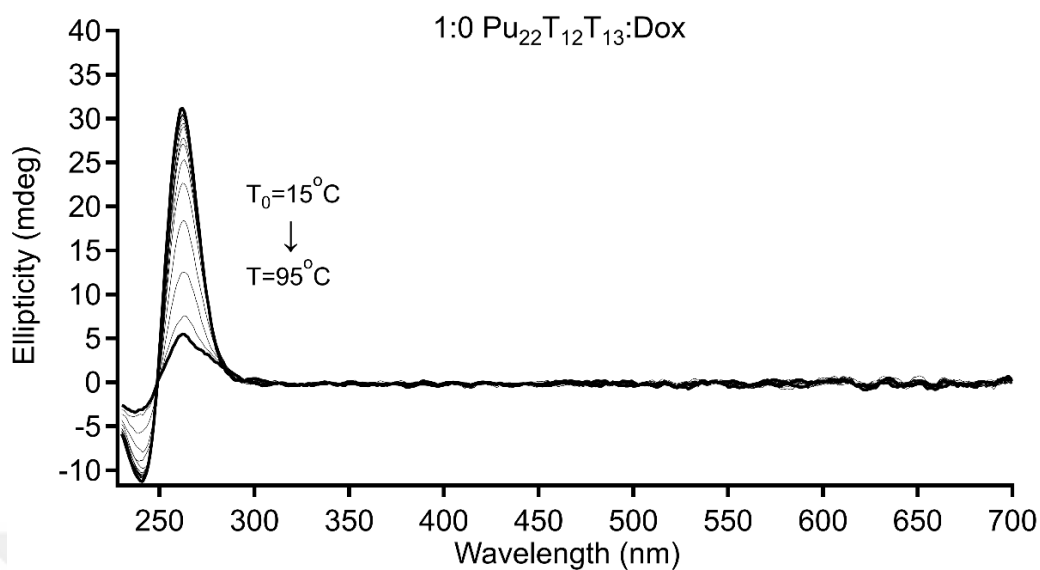


Figure 14. CD thermal denaturation experiment results for 1:0 Pu₂₂T₁₂T₁₃ (3.0 μ M):Dox samples. The Ellipticity (mdeg) vs Wavelength (nm) graph shows the CD spectra of 1:0 Pu₂₂T₁₂T₁₃:Dox sample between 15°C and 95°C.

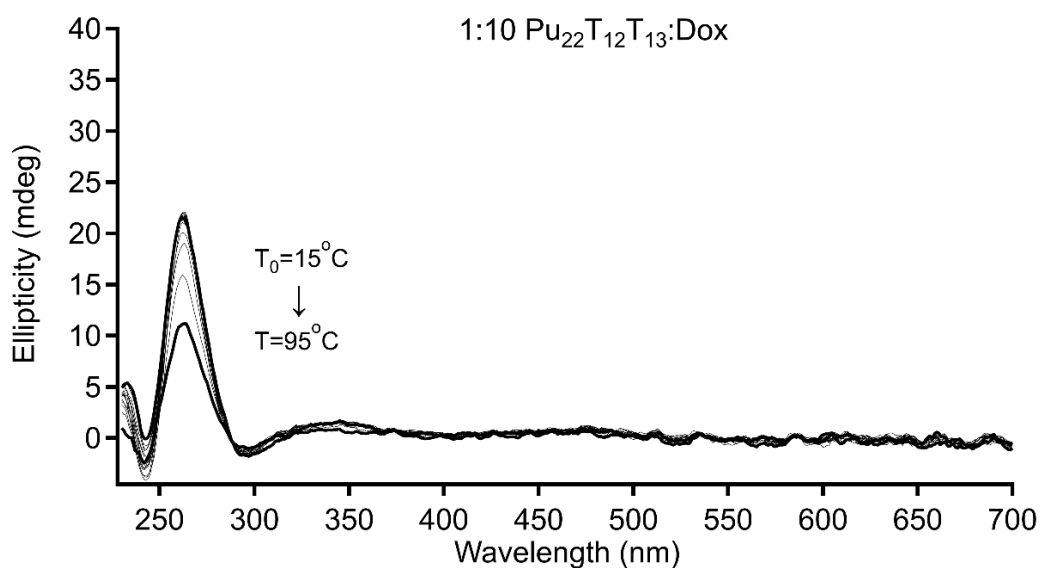


Figure 15. CD thermal denaturation experiment results for 1:10 Pu₂₂T₁₂T₁₃ (3.0 μ M):Dox sample. The Ellipticity (mdeg) vs Wavelength (nm) graph shows the CD spectra of 1:10 Pu₂₂T₁₂T₁₃:Dox sample between 15°C and 95°C.

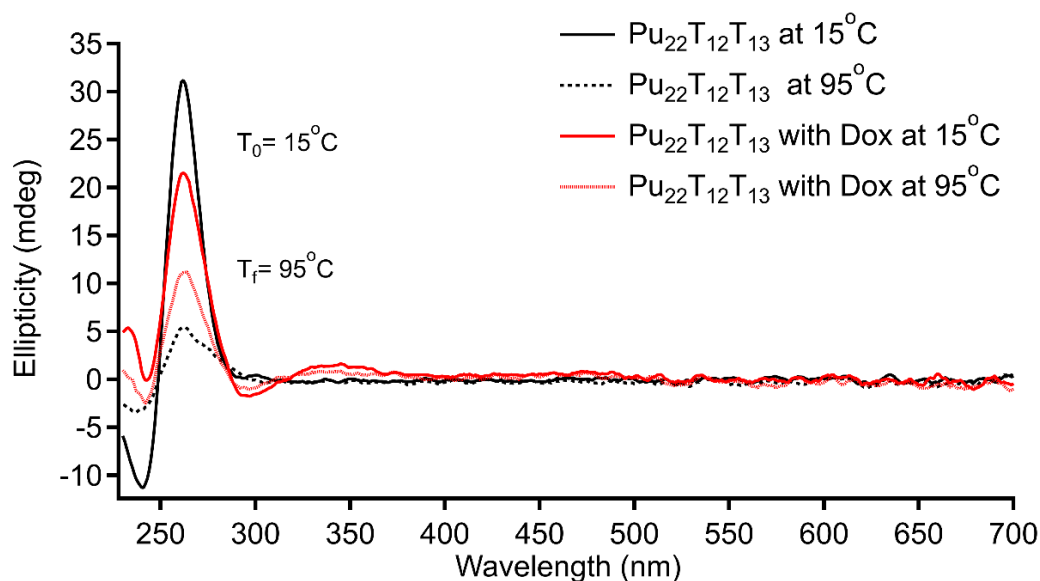


Figure 16. CD spectra of Pu₂₂ (3.0 μ M) and 1:0 Pu₂₂T₁₂T₁₃:Dox samples. The Ellipticity (mdeg) vs Wavelength (nm) graph shows the CD spectra of Pu₂₂T₁₂T₁₃ (3.0 μ M) in the absence (solid line) and presence of 30.0 μ M Dox (1:10 Pu₂₂T₁₂T₁₃:Dox, dashed line) at 15°C and 95°C.

The stabilization effect can also be observed if the intensity of the positive band at 260 nm in Figure 16 for Pu₂₂T₁₂T₁₃ (black lines) and Pu₂₂T₁₂T₁₃ in the presence of Dox (red lines) were compared. At 95°C, The Pu₂₂ G4 positive band exhibited an ellipticity value around 15 mdeg in the absence of Dox (Figure 16, dashed black line), while the positive band was around 20 mdeg in the presence of 30.0 μ M Dox (Figure 16, red dotted line). This indicates that the Pu₂₂T₁₂T₁₃ G4 structure wasn't also able to unfold as much in the absence Dox as it did in the presence of Dox.

The thermal denaturation profiles of the samples were constructed by monitoring the change in ellipticity (mdeg) at 295 nm. Figure 17 shows the denaturation profiles of samples containing different equimolar ratios of Pu₂₂:Dox and Figure 18 shows the melting curves of samples containing different equimolar ratios of Pu₂₂T₁₂T₁₃:Dox. In Figure 17 and Figure 18 blue, pink, green, yellow and dashed black lines represent 3.0 μ M (1:1), 6.0 μ M (1:2), 12 μ M (1:6), 18 μ M (1:6) and 30 μ M (1:10) Dox additions onto 3.0 μ M G4, respectively. In addition to that dotted black lines represent the 3.0 μ M G4 in absence of Dox for comparison. Firstly, the lack of a

plateau region in the denaturation curves given in Figures 17 and 18 indicates that the G4 structures were unable to completely unfold at 95°C which means that the exact T_m values were not obtainable also from the CD thermal denaturation experiments. On the other hand, the right shift of the thermal denaturation profiles for both structures (Pu₂₂ G4, Figure 17; Pu₂₂T₁₂T₁₃ G4, Figure 18) clearly indicated the stabilization effect caused by Dox. If it is assumed that the Pu₂₂ G4 completely unfolded at 95°C, the T_m value of Pu₂₂ was shifted about 5°C in the presence of 30.0 μM Dox, as can be seen in Figure 17. If the same assumption is done for Pu₂₂T₁₂T₁₃ G4, Figure 18 clearly demonstrates the stabilization effect of Dox on the structure as the T_m of G4 was shifted about 10°C in the presence of 30.0 μM Dox. Both Pu₂₂ and Pu₂₂T₁₂T₁₃ G4s in the absence of Dox had melting temperatures around 80°C and the results were in good agreement with previously reported T_m values for these structures [45].

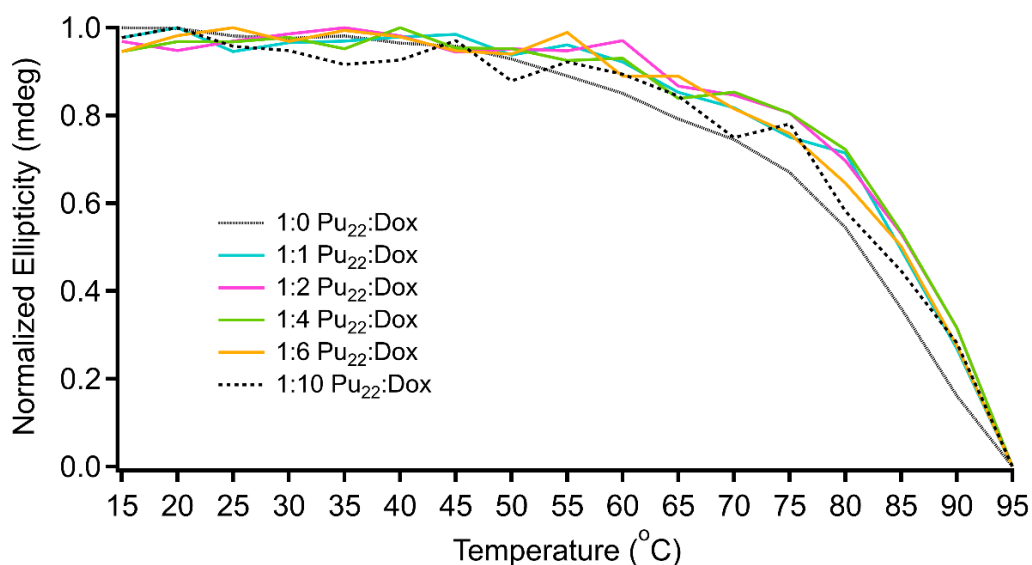


Figure 17. Normalized Ellipticity (mdeg) vs Temperature (°C) graphs of Dox and Pu₂₂ in the absence and presence of increasing Dox concentrations.

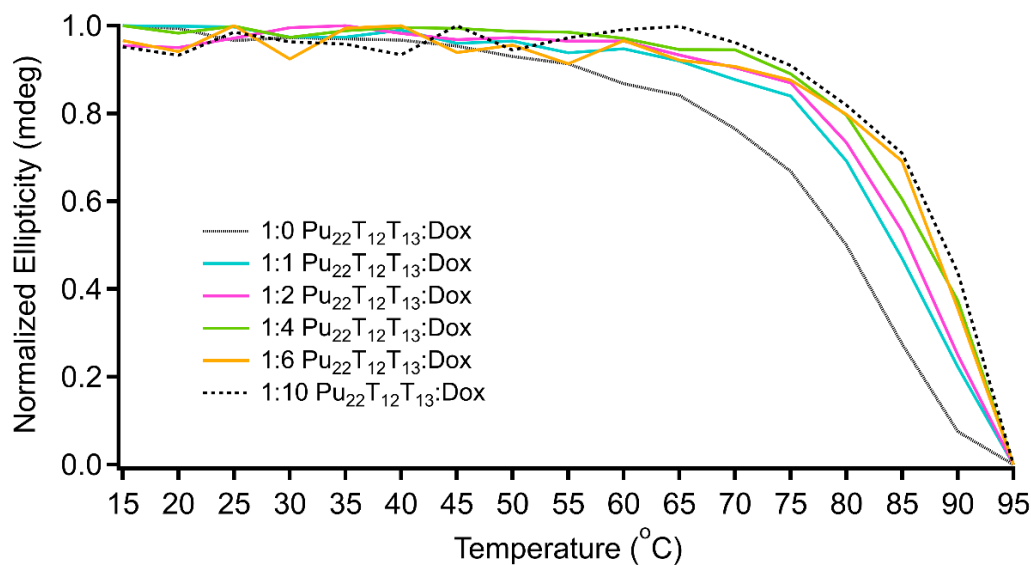


Figure 18. Normalized Ellipticity (mdeg) vs Temperature (°C) graphs of Dox and Pu₂₂T₁₂T₁₃ in the absence and presence of increasing Dox concentrations.

2.3.4 Association Constant Determination

In order to determine K_a values of Pu₂₂-Dox and Pu₂₂T₁₂T₁₃-Dox complexes, Fluorescence titration experiments were performed. Figure 19 represents the titration of Dox with Pu₂₂ and Figure 21 represents the titration of Dox with Pu₂₂T₁₂T₁₃. Fluorescence ability of Dox is quenched when it interacts with the Pu₂₂ or Pu₂₂T₁₂T₁₃ G4s (Figure 19 and Figure 21, respectively). Accordingly, both fluorescence spectra displayed significant decreases in fluorescence intensity upon gradual addition of G4 DNA. For the association constant calculations, the fluorescence titration results are fitted to Integrated Intensity vs Concentration (μM) values and the fittings for Pu₂₂ and Pu₂₂T₁₂T₁₃ were shown in Figure 20 and Figure 22, respectively. The experiments were done in triplicates for the K_a calculations and the 2nd and 3rd replicates were given in Appendix E, Figures 71-78. Thus, the K_a values were reported in Table 1 as average values of the three replicates, with standard deviation. The K_a obtained for Pu₂₂-Dox complex was $7.50 \times 10^6 \pm 0.79 \times 10^6$ and the K_a obtained for Pu₂₂T₁₂T₁₃-Dox complex was $1.36 \times 10^7 \pm 0.29 \times 10^7$. There was a slight increase in the K_a due to the substitution of Gs with T at 12th and 13th positions.

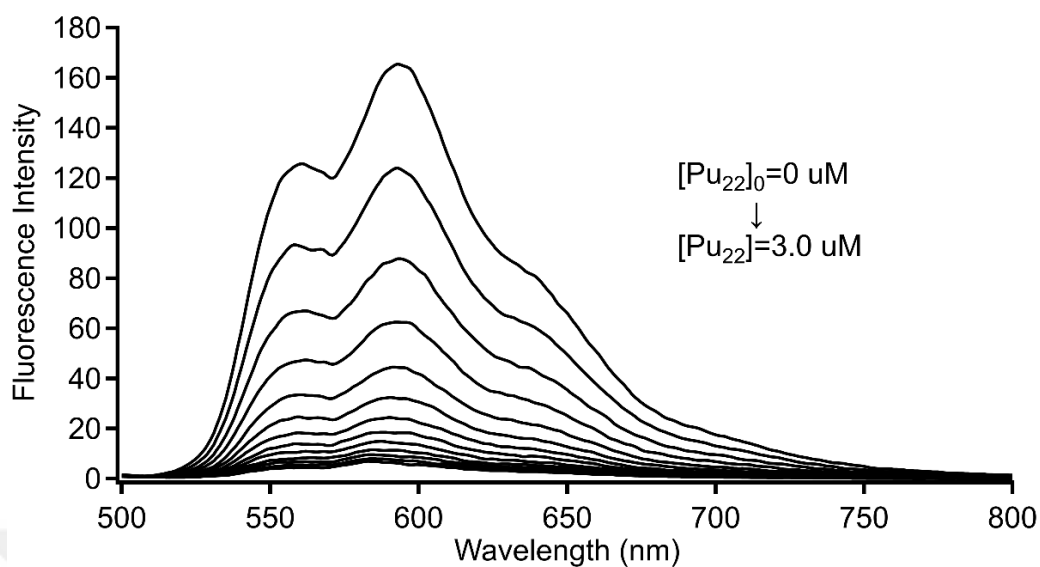


Figure 19. Fluorescence Intensity vs Wavelength (nm) of 1.0 μM Dox titrated with increasing volumes of 50.0 μM Pu_{22} solution with 1.0 μM Dox up to a final 3.0 μM Pu_{22} concentration (1st replicate).

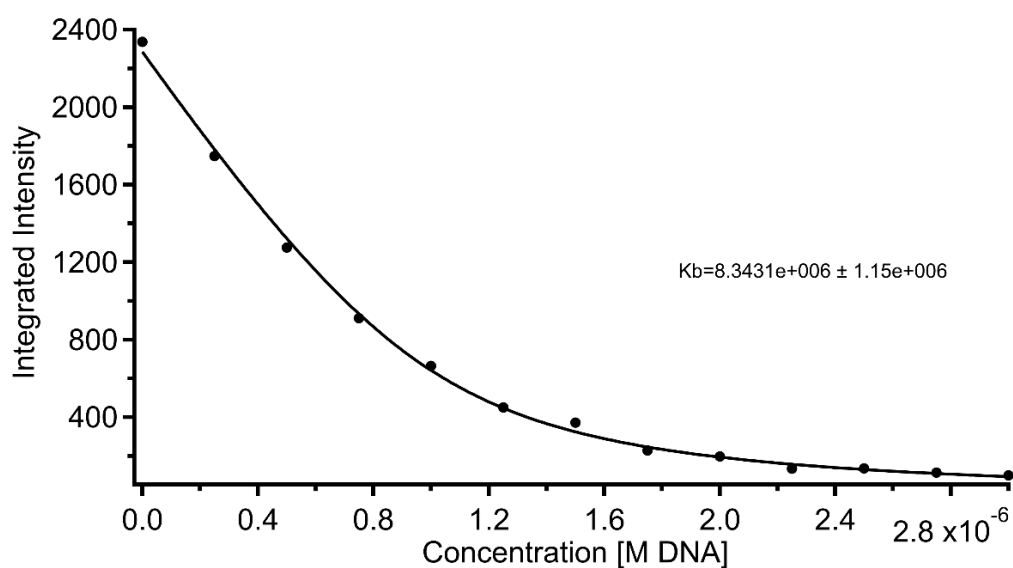


Figure 20. Integrated Intensity vs Concentration (μM in strand) obtained from the titration results in Figure 19.

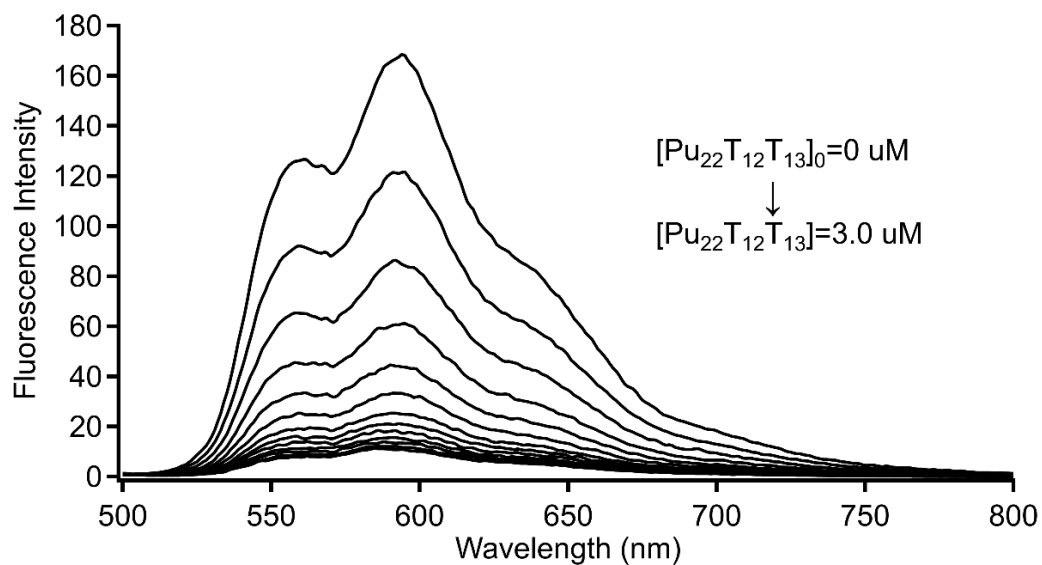


Figure 21. Fluorescence Intensity vs Wavelength (nm) of 1.0 μM Dox titrated with increasing volumes of 50.0 μM $\text{Pu}_{22}\text{T}_{12}\text{T}_{13}$ solution with 1.0 μM Dox up to a final 3.0 μM $\text{Pu}_{22}\text{T}_{12}\text{T}_{13}$ concentration (1st replicate).

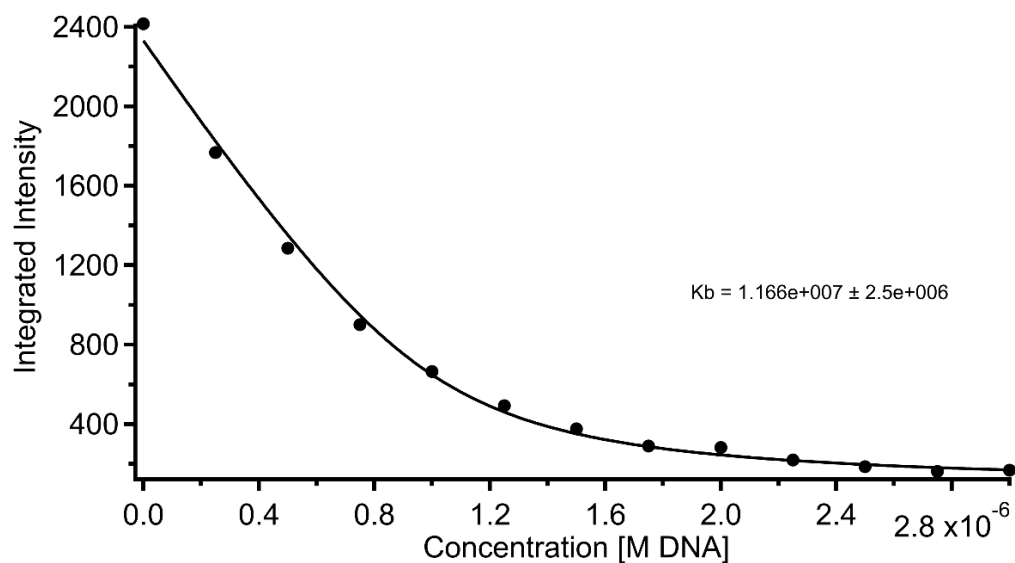


Figure 22. Integrated Intensity vs Concentration (μM in strand) obtained from the titration results in Figure 21.

Table 1. Association constants determined by Fluorescence titration experiments of Dox- Pu₂₂ and Dox-Pu₂₂T₁₂T₁₃.

	Association constants (K _a) for DNA-Dox complexes (M ⁻¹)				
	1 st replicate	2 nd replicate	3 rd replicate	Average	Standard Deviation
Pu₂₂-Dox	8.34x10 ⁶	6.95 x10 ⁶	7.22x10 ⁶	7.50x10 ⁶	0.74x10 ⁶
Pu₂₂T₁₂T₁₃-Dox	1.17x10 ⁷	1.69x10 ⁷	1.23x10 ⁷	1.36x10 ⁷	0.29 x10 ⁷

2.3.5 Competition Dialysis

Competition dialysis experiments were performed in order to reveal the selectivity of Dox towards different nucleic acid structures. Different nucleic acid structures, placed in different dialysis cassettes, were dialyzed against Dox for 24 hours. At the end of the 24 hours long dialysis, the fluorescence intensity in each dialysis cassette was measured and the resulting fluorescence spectra for the 1st and 2nd replicates were shown in Figure 23 and Figure 24, respectively. As can be seen, the highest fluorescence intensity was observed for Pu₂₂T₁₂T₁₃, m-lyso and Pu₂₂; which indicated that these were the structures that had the greatest affinity towards Dox among the other nucleic acid structures. Following that, the C_b/C_f values were calculated for the 1st and 2nd replicates, and the results were listed in Table 2. Figure 25 represents the nucleic acids structures vs C_b/C_f values graph, which also showed that the greatest affinity was reached with Pu₂₂T₁₂T₁₃, m-lyso and Pu₂₂ with C_b/C_f values of 24.07, 21.11 and 20.39, respectively. Our experiments pointed out the well-known affinity between dsDNA and Dox as the binding between genomic duplex DNA m-lyso and Dox was among the highest with C_b/C_f value of 21.11 [117]. It should be noted that the binding of Dox to Pu₂₂ G4 was as great as it was for m-lyso (Figure 25), which suggested that the anti-cancer activity of Dox might be correlated to its binding to VEGF promoter region in addition to its binding to dsDNA. The

results also indicated that the anti-cancer drug Dox was selectively binding to G4s since Dox displayed relatively high affinity towards also BCL-2 ($C_b/C_f = 17.48$) and C-myc ($C_b/C_f = 15.68$)G4s. Additionally, we also observed that Dox had notable affinity to dA₃₂[dT₃₂]₂ triplex DNA, $C_b/C_f = 9.62$. On the contrary, it did not display affinity towards neither the single stranded structure nor the double stranded RNA structures.

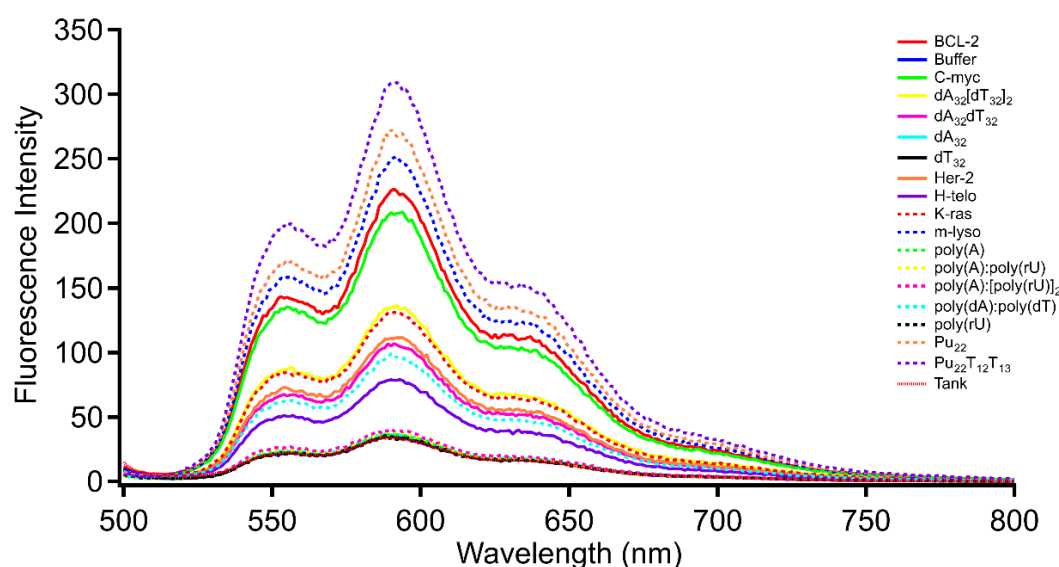


Figure 23. Fluorescence Intensity vs Wavelength (nm) spectra of the samples obtained from the competition dialysis cassettes containing 18 different nucleic acid structures at the end of 24 hours (1st replicate).

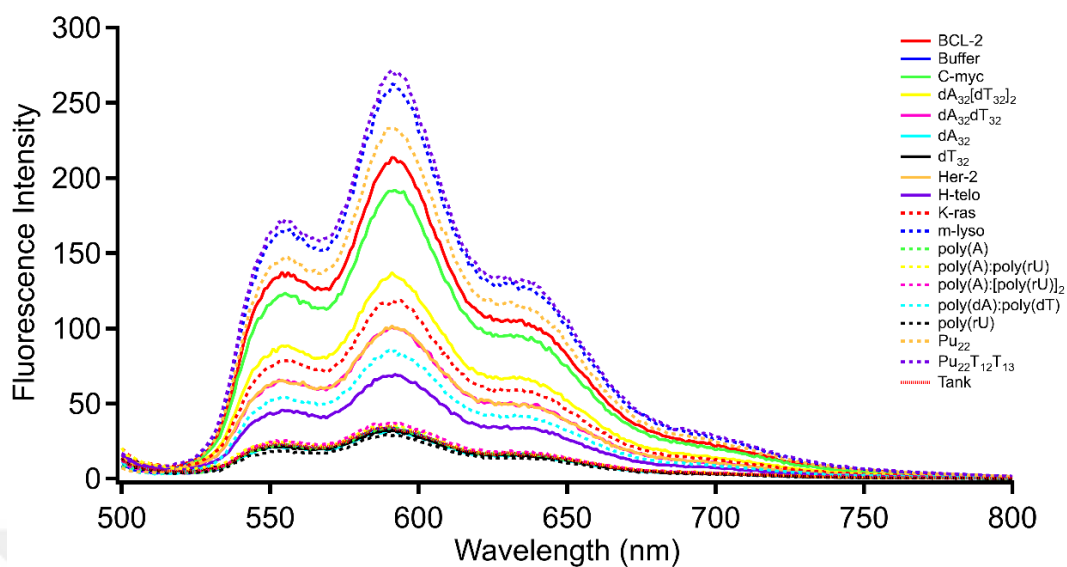


Figure 24. Fluorescence Intensity vs Wavelength (nm) spectra of the samples obtained from the competition dialysis cassettes containing 18 different nucleic acid structures at the end of 24 hours (2nd replicate).

Table 2. Competition Dialysis results of Dox.

Nucleic Structures	Acid	Cb/Cf			
		1 st Replicate	2 nd Replicate	Average	Standard Deviation
Pu ₂₂ T ₁₂ T ₁₃		25.27	22.87	24.07	1.70
Pu ₂₂		21.41	19.37	20.39	1.44
m-lyso		20.00	22.22	21.11	1.57
BCL-2		17.51	17.45	17.48	0.04
C-myc		15.97	15.40	15.68	0.40
K-ras		8.89	8.14	8.51	0.53
Her-2		7.08	6.45	6.77	0.45
H-telo		4.05	3.47	3.76	0.41
dA ₃₂ dT ₃₂		6.58	6.43	6.51	0.10
dA ₃₂ [dT ₃₂] ₂		9.40	9.85	9.62	0.31
poly(dA):poly(dT)		5.71	5.01	5.36	0.50
poly(A):poly(rU)		0.31	0.17	0.24	0.10
poly(A)[poly(rU)] ₂		0.48	0.34	0.41	0.10
dA ₃₂		0.11	-0.09	0.01	0.14
dT ₃₂		0.07	-0.19	-0.06	0.18

poly(A)	0.19	-0.02	0.09	0.15
poly(rU)	-0.16	-0.45	-0.31	0.20
Buffer	0.00	0.00	0.00	0.00
Tank	-0.05	-0.10	-0.08	0.04

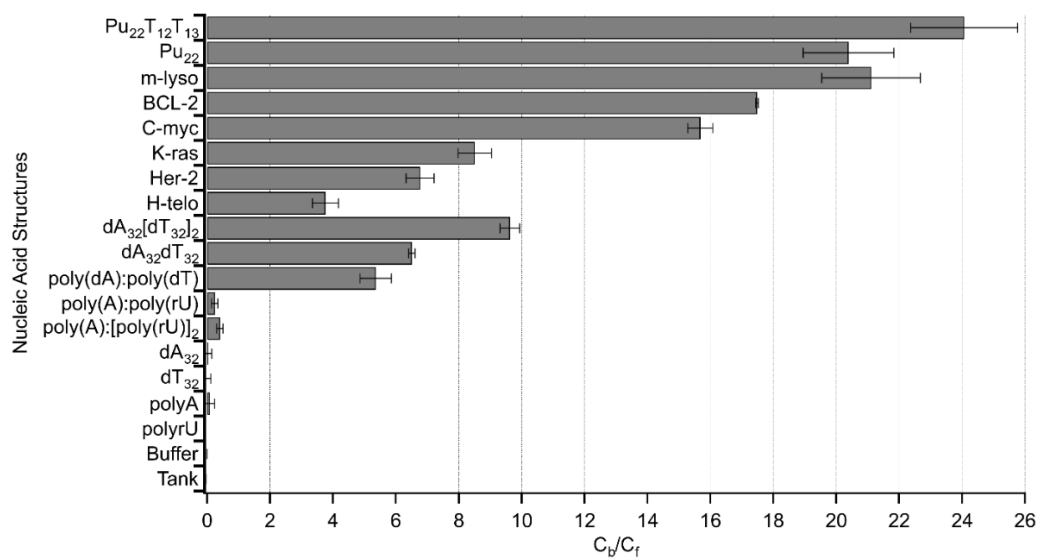


Figure 25. Average C_b/C_f values obtained from the Competition Dialysis experiments.

CHAPTER 3

DEVELOPMENT OF A GOLD NANOPARTICLE-AZACYANINE FLUORESCENCE PROBE FOR THE DETECTION OF G-QUADRUPLEX STRUCTURES

3.1 Introduction

The G4 structures gained major importance in biological events since the discovery of their role in the control of gene expression as well as protein and enzyme binding to DNA strands [22]. Accordingly, revealing their presence in nucleic acids and structural features have become crucial. Therefore, several methods emerged as common characterization techniques for G4 structures. One of those methods is the computational G4 prediction, which employs computer programs that can present whether there is a possible G4 formation in a given nucleic acid sequence [118]. Other methods are NMR, CD spectroscopy and X-ray Crystallization [106]. However, the above-mentioned methods are not applicable in all laboratories. For instance, computer-based prediction methods require experts who have computational background to run the programs efficiently. Also, most of the time, experimental verification is needed. Analysis with NMR spectroscopy needs large sample amounts and high-resolution instruments for appropriate data acquisition. X-ray analysis also require relatively sophisticated instruments, and the crystallization of the structures that can be intricate, slow and tricky. And CD spectroscopy is also not facile enough and only gives accurate information about the topology of the G4s [106].

As an alternative, fluorescence probes emerged as promising platforms for the detection of G4 structures. Highly sensitive and selective fluorescence probes for G4 detection have been reported for both *in vivo* and *in vitro* applications, over the last years. Although the previously proposed detection methods for G4s ease the

characterization process, the probes can still be improved in means of simplicity and ease.

In this study, we aimed to develop a novel detection platform for G4 structures. The developed platform consisted of an off-on-off fluorescence probe which is governed by the FRET mechanism between AuNPs and Azacyanine 5 (Aza5) molecules. We aimed to add an alternative fluorescence probe to the literature, which is accessible, faster to operate and highly selective to G4 structures. This work was published in *Talanta* under the name of “A conjugated gold nanoparticle-azacyanine off-on-off fluorescence probe for sensitive and selective detection of G-quadruplexes” [119].

3.2 Materials and Methods

3.2.1 Synthesis of AuNPs and Preparation of Aza5

The AuNPs were synthesized according to the citrate reduction method reported by Turkevich et.al. with a minor modification [80]. Briefly, 0.0984 g of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ purchased from Sigma-Aldrich (St. Louis, MO, USA), was dissolved in 500 mL Millipore water (0.5 mM HAuCl_4) and the solution was heated until boiling. Then, 50 mL of $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ (Sigma-Aldrich, 38.8 mM) solution was added to the boiling solution of HAuCl_4 , under vigorous stirring. After that, the solution changed color from pale yellow to colorless and then to dark violet. The mixture was boiled under stirring for an additional 20 minutes to ensure the completion of the reaction. Then, a wine-red color appeared indicating the synthesis of citrate capped-AuNPs. Next, the synthesized AuNPs solution was cooled to room temperature and stored in the dark at 4°C overnight. The stock solution of the synthesized and purified AuNPs was diluted with Millipore water prior to the characterization and detection experiments. Dilution was performed as the maximum absorbance of the prepared AuNPs solution was set to 1.17 (at a SPR of 520 nm). The final AuNP concentration in the nanoparticles suspension was estimated to be 3.33 nM using a previously reported molar extinction coefficient (ϵ_{520}) of $2.70 \times 10^8 \text{ M}^{-1}\text{cm}^{-1}$ [120].

The concentration of Aza5 was measured by UV-vis spectroscopy using the molar absorption coefficient ($\epsilon = 25000 \text{ M}^{-1} \text{ cm}^{-1}$) at its maximum absorbance wavelength (407 nm). The prepared stock solution was diluted with DMSO to prepare a final concentration of 2.058 mM solution.

3.2.2 Preparation of AuNPs-Aza5 Fluorescence Probe

The final concentration of Aza5 in the AuNPs-Aza5 probe was 1.21 μM . For the preparation of AuNPs-Aza5 probe, 1.80 μL of Aza5 solution (2.058 mM, in DMSO, as the fluorescence molecule) was added to 3.0 mL of AuNPs suspension and the mixture was vortex-mixed at room temperature. As a result, a displacement reaction between the positively charged Aza5 and negatively charged citrate ions on the surface of AuNPs was likely occurred. Next, 60.0 μL aqueous solution of L-Cysteine (Cys, 1.0 mM) was added to the mixture (which results in the “turn-on” of the fluorescence caused by Aza5 molecules) with vortex mixing and the solution was incubated for 10 minutes at room temperature to allow for adequate reaction time. After that, Aza5 molecules were displaced from the AuNPs surface due the affinity between the sulfur atoms of Cys and AuNPs. As a result, Aza5 regained its fluorescence and the on-state of the fluorescence probe was reached.

3.2.3 Quenching Constant Calculation

Determination of the quenching constant was done by titrating 3 mL of Aza5 (1.21 μM) with AuNPs (3.33 nM) and the titration was performed by 50 μL increments. The parameters for the fluorescence measurements were: Emission spectra collected from 410 to 700 nm, excitation wavelength 407 nm, excitation and emission slits: 5.0 and 5.0, operation 800 V, scan rate 1200 nm/min, respectively. The gradual decrease at 472 nm upon addition of AuNPs was used to calculate the quenching

constant (K_q) and the K_q was determined according to the modified Stern–Volmer equation [121]:

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a K_q [Q]} + \frac{1}{f_a}$$

Where F_0 and F are the fluorescence intensities in the absence and presence of the quencher (AuNPs), respectively, f_a is the fraction of accessible fluorescence, $[Q]$ is the quencher concentration and K_q is the quenching constant.

3.2.4 Characterization of AuNPs-Aza5 Fluorescence Probe by UV-vis Absorption Spectra, TEM, CD, DLS

A double beam JASCO V-730 (Easton, MD, USA) UV-Vis spectrophotometer was used to record the absorption spectra of the samples in the range of 300–900 nm using a 10 mm quartz cell. Transmission Electron Microscope (TEM) micrographs were obtained using a JEOL JEM-2100F (Akishima, Tokyo, Japan) HRTEM instrument operating at an accelerating voltage of 200 kV to characterize the size and morphology of the AuNPs. Before TEM analysis, the samples were carefully placed on commercially available carbon-coated copper grids followed by air drying at room temperature for 24 hours. CD spectra of the samples were recorded using JASCO J-815 spectropolarimeter at wavelength range of 230–700 nm and Dynamic Light Scattering (DLS) experiments were carried out using a Zetasizer Nano-ZS apparatus (Malvern Instruments Ltd, Malvern, UK).

3.2.5 Fluorescence Titration Experiments-Sensitivity Studies

The AuNPs-Aza5 fluorescence probe was titrated with Pu₂₂ G4 structure for the construction of the calibration curve. Briefly, 3060.0 μ L of AuNP-Aza5-Cys probe is titrated with 10.0 μ M of Pu₂₂ with 10.0 μ L increments. The parameters for the fluorescence measurements were: Emission spectra collected from 410 to 700 nm, excitation wavelength 407 nm, excitation and emission slits: 5.0 and 5.0, operation

800 V, scan rate 1200 nm/min, respectively. The titration experiments are repeated 5 times and reported with mean values and standard deviation.

3.2.6 Selectivity Studies

The selectivity of the proposed “off-on-off” fluorescence sensor towards different nucleic acid structures was investigated via recording the emission spectra of the probe in the presence of singlet, duplex, triplex and G4 structures. The parameters for the fluorescence measurements were the same with the parameters described in the previous section. All of the experiments were conducted in triplicate and the mean value of the results was reported with error bars. The oligonucleotides used in this section were described in competition dialysis section and listed in Appendix B. The oligonucleotide solutions were prepared according to the same annealing procedure described in the previous chapter.

3.3 Results and Discussion

3.3.1 Characterization of the Probe by CD, UV-vis Absorption and Fluorescence Spectroscopy

As mentioned in the previous chapter of this thesis, CD spectroscopy is a commonly used technique for the characterization of G4s [112]. The AuNPs-Aza5 fluorescence probe was first characterized by CD, to validate the presence of G4 structure after the addition of all the sensing elements of the off-on-off detection system. In Figure 27, the characteristic bands of the parallel G4 formed by Pu₂₂ was observed in mixture of AuNPs+Aza5+Cys (solid black line), which confirmed that the probe was appropriate to be a G4 sensing platform. The formation of the G4 was further validated by comparing the spectra for samples containing Pu₂₂ in water (Figure 26, dotted line) and Pu₂₂ in AuNPs (Figure 26, dashed line). Presence of AuNPs did not cause any structural changes in Pu₂₂ G4, which also confirmed that AuNPs do not

alter the structure of G4 and can be a compatible platform for G4 detection. In addition, black solid line in Figure 26 showed a slight decrease in the positive band at 260 nm when compared to the positive band shown in Figure 26 dotted line (Pu_{22} in water), which could be correlated with the interaction between Aza5 and Pu_{22} G4.

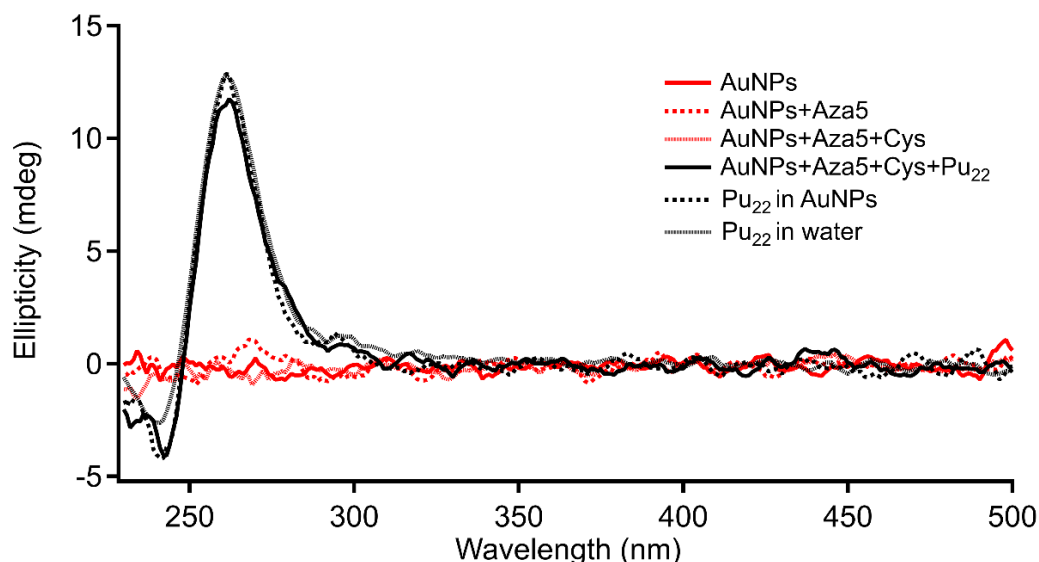


Figure 26. CD spectra of the samples containing AuNPs, AuNPs+Aza5, AuNPs+Aza5+Cys, AuNPs+Aza5+Cys+ Pu_{22} , Pu_{22} in AuNPs and Pu_{22} in Millipore water.

The UV-vis spectroscopy is used to characterize the AuNPs upon addition of the sensing elements. The solid black line in Figure 27 shows the characteristic absorption spectra of the synthesized AuNPs. As can be seen, the synthesized AuNPs showed an absorption maximum around 520 nm, which meant that the synthesis of the nanoparticles was successful and in an agreement with literature [81]. Upon the addition of the optimized concentration of Aza5, the absorption intensity did not undergo a noticeable change (Figure 27, dashed line) indicating that no inter-particle interactions like aggregation and/or a change in their size took place. The treatment of the AuNPs-Aza5 solution with Cys at the optimized conditions also did not result in a significant change in the absorption spectrum of AuNPs suspension (Figure 27, dotted line). The addition of the target G4 structure resulted in a similar LSPR band with a negligible change in its intensity implying the AuNPs-Aza5 did not undergo significant changes during the experiments (Figure 27, dotted-dashed line).

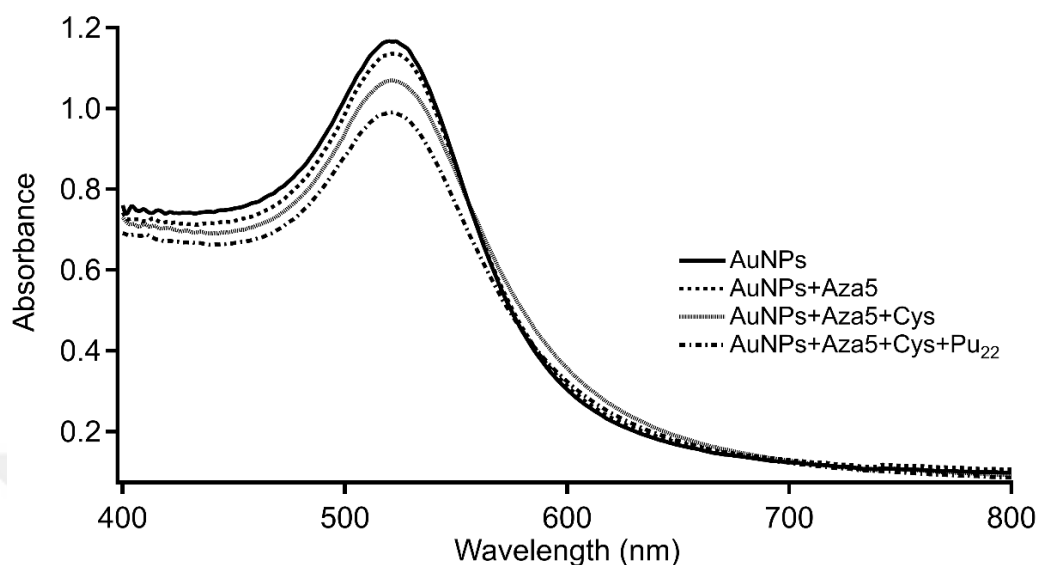


Figure 27. UV-vis absorption spectra of the AuNPs in the absence and presence of Aza5, Aza5+Cys, Aza5+Cys+Pu₂₂.

Figure 28 shows the off-on-off fluorescence response of the probe upon addition of each sensing element and Figure 29 is the photograph of the same samples under UV light. When Aza5 was added to AuNPs the fluorescence was lost and system was in “off” state (Figure 28, dashed line; Figure 29, cuvette A). After the addition of Cys, the fluorescence of Aza5 was restored and the system was in “on” state (Figure 28, dotted line; Figure 29, cuvette B). When the Pu₂₂ G4 structure was added to the AuNPs+Aza5+Cys mixtures fluorescence was quenched and the system was in “off” state (Figure 28, dotted-dashed line; Figure 29, cuvette C). For comparison with a single stranded nucleic acid structure instead of G4, upon addition of dT₃₂, the fluorescence was not quenched as shown in Figure 28 with solid black line and cuvette D in Figure 29. This result demonstrated an initial comparison between the off-on-off AuNPs-Aza5 probe’s fluorescence response towards G4s versus single stranded nucleic acid structures which will be further discussed in the selectivity section.

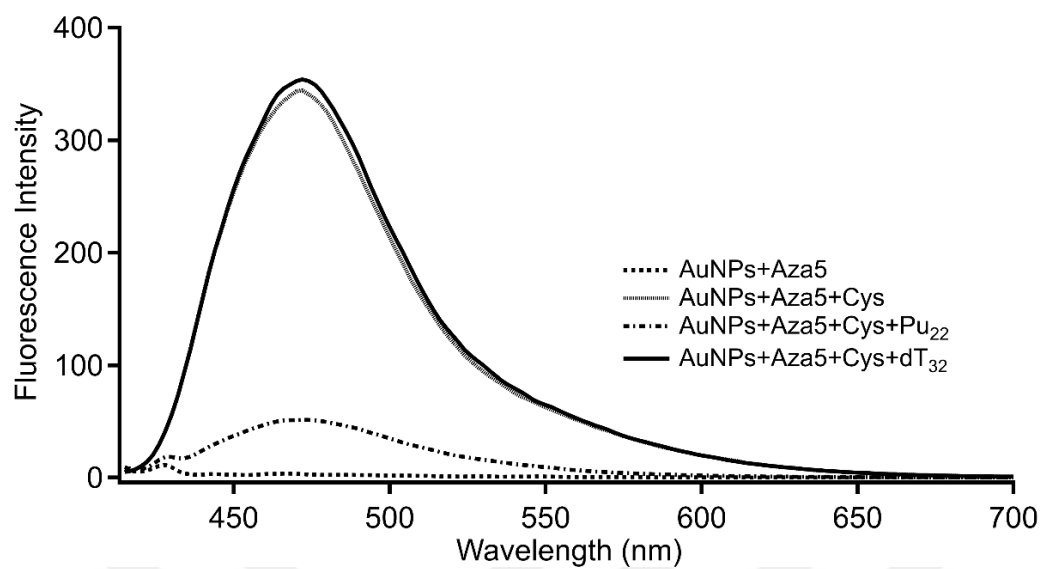


Figure 28. The off-on-off fluorescence response of the probe upon addition of Aza5, Aza-5+Cys, Aza5+Cys+Pu₂₂ and Aza5+Cys+dT₃₂ to the AuNPs.

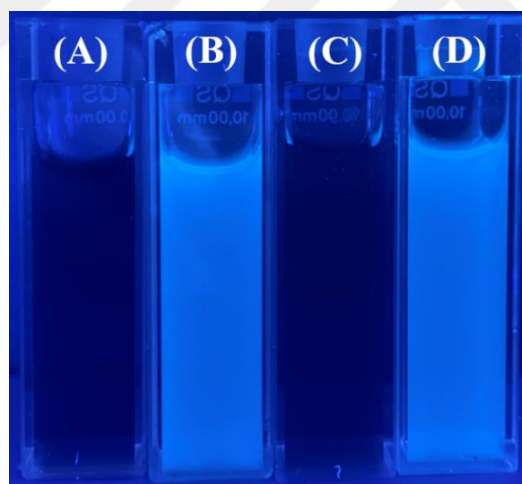


Figure 29. The off-on-off fluorescence response photos of the probe upon addition of (A) Aza5, (B) Aza-5+Cys, (C) Aza5+Cys+Pu₂₂ and (D) Aza5+Cys+dT₃₂ to the AuNPs.

3.3.2 Quenching Constant Calculation

The quenching constant experiment was conducted to demonstrate the quenching ability of AuNPs on Aza5 molecules. In the presence of AuNPs, Aza5 emits almost no fluorescence probably due to the contribution of both FRET between Aza5 molecules and AuNPs and inner filter effect phenomena [79] indicating that Aza5 molecules are adsorbed onto the surface of the AuNPs. The fluorometric titrations of Aza5 with citrate-stabilized AuNPs were carried out and the corresponding modified Stern-Volmer plot was shown in Figure 30. The Stern-Volmer plot in Figure 30 revealed the K_q value as $6.74 \times 10^8 \text{ L mol}^{-1}$ [121].

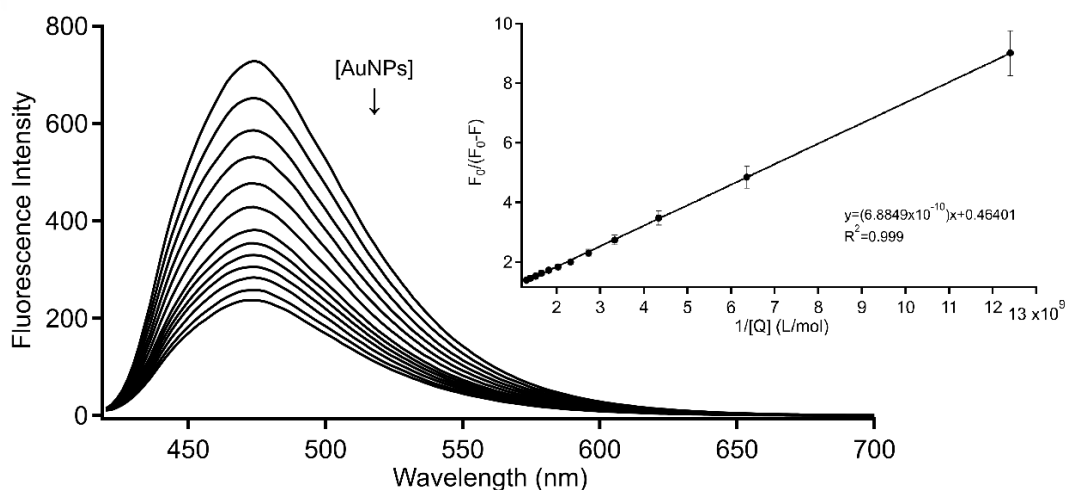


Figure 30. Fluorometric titration of Aza5 with AuNPs for the determination of quenching constant. The Inset represents corresponding modified Stern-Volmer plot of $F_0/(F_0-F)$ versus $1/[Q]$ ($1/[\text{AuNPs}]$). The error bars indicate the SD ($n = 3$).

3.3.3 Characterization of the Probe by DLS and TEM Measurements

DLS measurements were taken to investigate the mean size change of the AuNPs upon addition of each sensing element. Figure 31, from A to B shows the DLS results of AuNPs, AuNPs+Aza5, AuNPs+Aza5+Cys and AuNPs+Aza5+Cys+Pu22 samples, respectively. The DLS measurements revealed that the average diameter of the AuNPs was $12.77 \pm 0.38 \text{ nm}$ (Figure 31 A) and the average size of the AuNPs+Aza5

probe was 11.52 ± 0.98 nm (Figure 31 B). After the addition of Cys to the AuNPs-Aza5 system, average hydrodynamic diameter was determined as 12.08 ± 0.97 nm (Figure 31 C) and finally, upon addition of Pu₂₂ to the AuNPs+Aza5+Cys mixture's average hydrodynamic diameter was determined as 12.14 ± 0.72 nm (Figure 31 D). The DLS results were in good agreement with the UV-vis spectroscopy measurements since both methods revealed that the AuNPs size and distribution properties were not affected after treatment with Aza5, Cys and Pu₂₂ G4.

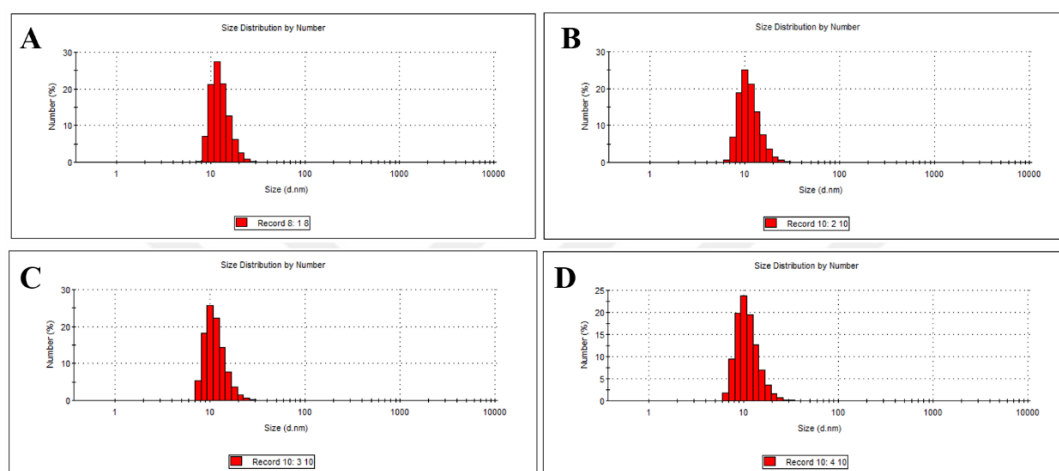


Figure 31. DLS measurements of the sensing probe. From A to D samples contain (A) citrate stabilized AuNPs, (B) AuNPs+Aza5, (C) AuNPs+Aza5+Cys, (D) AuNPs+Aza5+Cys+Pu₂₂.

The TEM images of samples containing AuNPs, AuNPs+Aza5, AuNPs+Aza5+Cys and AuNPs+Aza5+Cys+Pu₂₂ were shown in Figure 32 from A to B, respectively. The TEM micrographs (scale bar: 50 nm) also demonstrated that there were not any significant alterations in the morphology and the size of the AuNPs after addition of Aza5, Cys and the analyte Pu₂₂ G4. TEM images were also in agreement with DLS and UV-vis spectroscopy measurements, which concluded that the AuNPs-Aza5 fluorescence probe was a suitable screening platform for G4 structures.

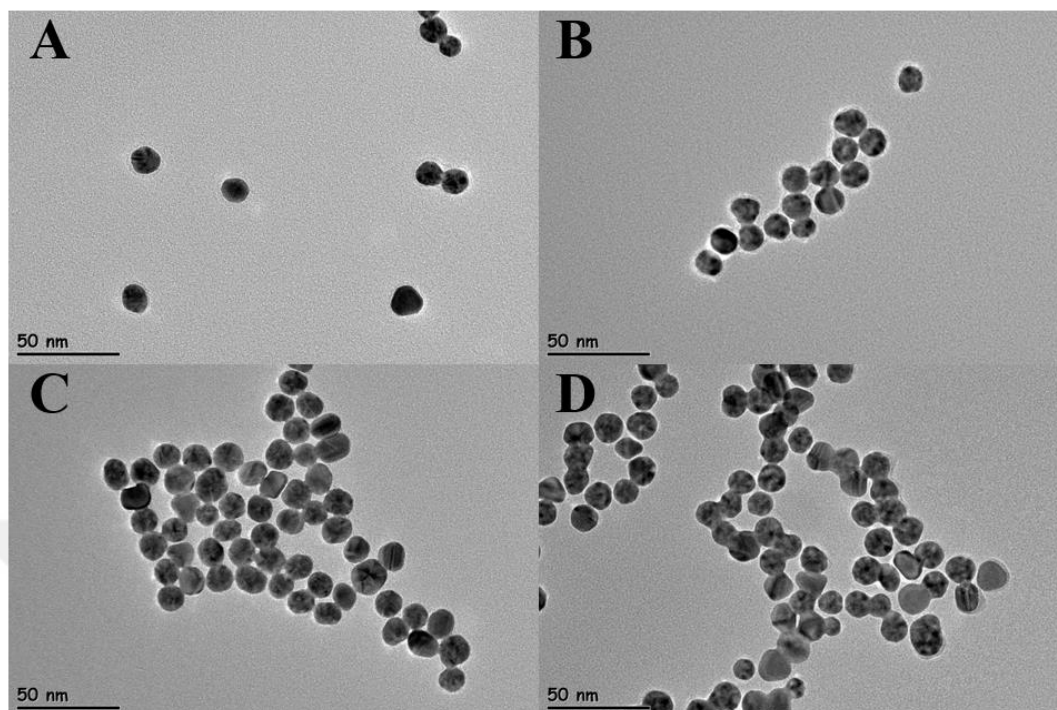


Figure 32. TEM images of the sensing probe. From A to D samples contain (A) citrate stabilized AuNPs, (B) AuNPs+Aza5, (C) AuNPs+Aza5+Cys, (D) AuNPs+Aza5+Cys+Pu₂₂.

3.3.4 Response time and buffer effect

The response time of the AuNPs-Aza5 fluorescence probe for detection of G4 structures was also measured by the duration of the response in 1-hour time interval. Figure 33A shows the Fluorescence Intensity vs Wavelength (nm) graph and Figure 33B is the F_0-F vs Time (min) graph of fluorescence response upon addition of Pu₂₂ G4 to the AuNPs+Aza5+Cys mixture. As can be seen, the system responded to of Pu₂₂ G4 in less than 5 mins and there weren't any alterations in the fluorescence

response afterwards. These results validated that the AuNPs-Aza5 fluorescence probe provides facile platform to detect G4 structures.

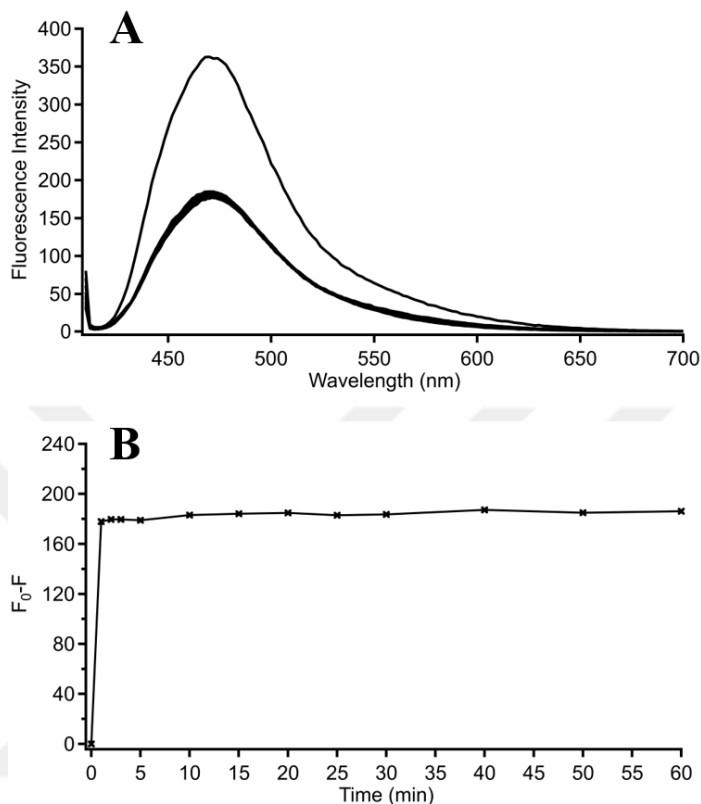


Figure 33. Response time of AuNPs-Aza5 fluorescence probe. (A) Fluorescence Intensity vs Wavelength (nm) graph (B) $F_0 - F$ vs Time (min) graph of fluorescence response upon addition of Pu₂₂ G4 to the AuNPs+Aza5+Cys mixture.

The effect of the buffer on AuNPs-Aza5 fluorescence probe was also investigated to show that the observed selectivity and sensitivity was only caused by the presence of different nucleic acid structures. Each nucleic acid stock solution used in the study was prepared in 25mM K-Phosphate Buffer and 70 mM KCl solution, to assist the formation of secondary structures. Figure 34 shows the Fluorescence Intensity vs Wavelength (nm) graph upon addition of buffer solution to the AuNPs+Aza5+Cys mixture. As can be seen, not only buffer had no reducing effect on the fluorescence response, it caused the fluorescence intensity to increase slightly in time. Therefore, it was concluded that the buffer had no effect on the AuNPs-Aza5 fluorescence probe.

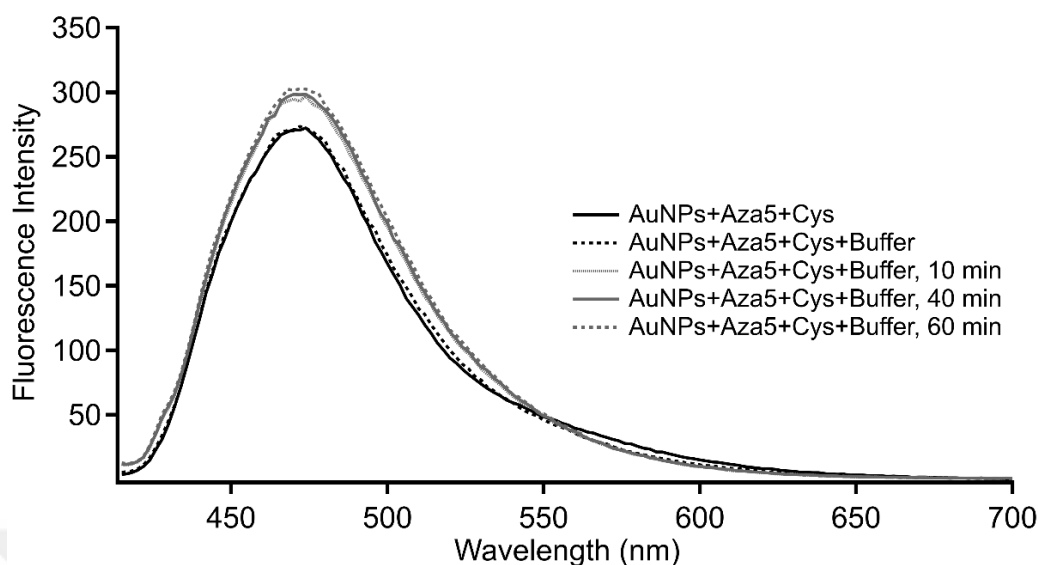


Figure 34. The effect of 25 mM K-Phosphate buffer and 70 mM KCl on the AuNPs-Aza5 fluorescence probe, in 1-hour time interval.

3.3.5 Calibration Titration and Sensitivity Studies

The calibration studies were done by titrating the AuNPs+Aza5+Cys solution with Pu₂₂ G4 structure. Figure 35A shows the decrease in fluorescence upon gradual addition of Pu₂₂ G4, due to the quenching effect caused by the DNA structure. Figure 35B shows the $F_0 - F$ vs [Pu₂₂], where F_0 was the fluorescence intensity in absence of Pu₂₂ G4 at 470 nm (when the system was only containing AuNPs+Aza5+Cys), F is the fluorescence intensity when Pu₂₂ G4 was added to the AuNPs+Aza5+Cys mixture and [Pu₂₂] is the concentration in nM scale. As shown in Figure 35B, the graph started to deviate from linearity after about the addition of 500 nM [Pu₂₂]. Consequently, the calibration plot was constructed between 0.032 to 0.347 μ M Pu₂₂ concentration and it was shown in Figure 36, where the linear fit equation was found to be $\Delta F = 0.6947C + 8.8822$ ($R^2 = 0.9947$) from 0.032 to 0.347 μ M [Pu₂₂] concentration range.

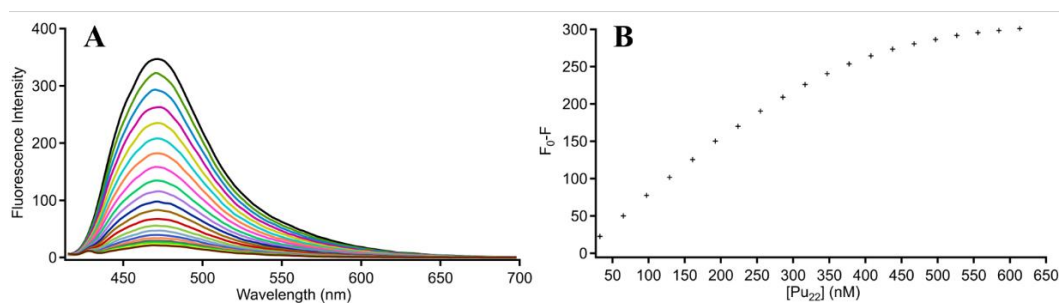


Figure 35. (A) Fluorescence titration profile of the conjugated AuNPs-Aza5 system with increasing concentrations of Pu₂₂ under the optimized conditions, (B) plot of F₀-F at 472 nm vs. Pu₂₂ concentration.

The results demonstrated in Figures 35 and 36 was indicating that the proposed probe can be used for the quantitative analysis of G4 structures. Additionally, LOD of the assay was calculated to be 12.66 nM from the relation $LOD = 3.3 \times (S_b/a)$ where, “S_b” is the standard error of the intercept and “a” is the slope of the calibration curve.

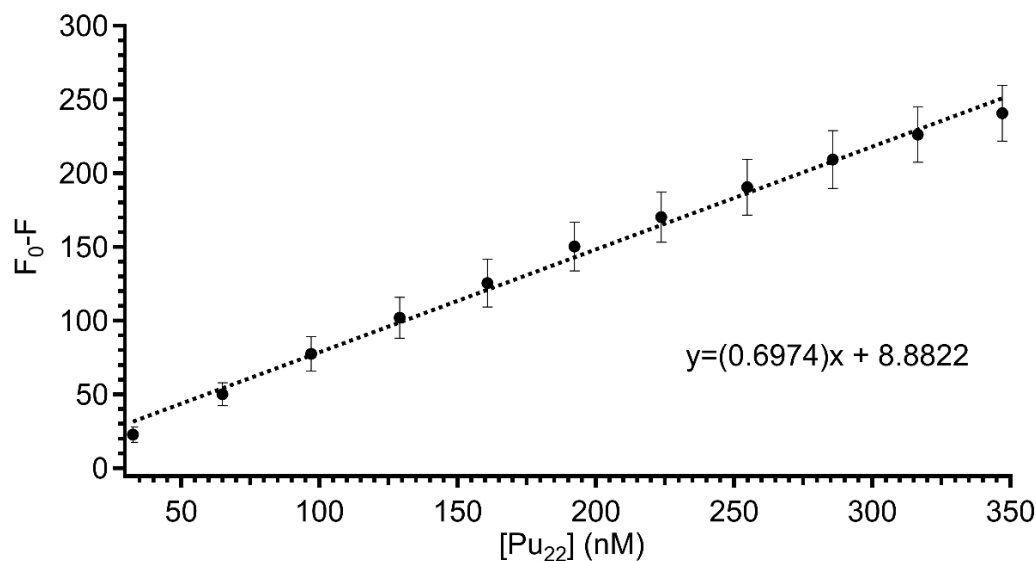


Figure 36. Linear range of the F₀-F at 472 nm vs. [Pu₂₂] (nM) with the linear regression fit equation.

3.3.6 Selectivity Studies

The selectivity studies included single-stranded nucleic acid sequences (dA₃₂, dT₃₂, poly(A) and poly(rU), complementary strands of Pu₂₂comp and Pu₂₂T₁₂T₁₃comp),

double-stranded nucleic acid structures ($\text{dA}_{32}\text{dT}_{32}$, poly(dA)poly(dT)), triple-stranded nucleic acid structures DNA ($\text{dA}_{32}[\text{dT}_{32}]_2$ and $\text{poly(dA)[poly(dT)]}_2$), various G4 structures which were wild-type VEGF-Pu₂₂ (the model G4 structure), Pu₂₂T₁₂T₁₃ (modified Pu₂₂ sequence), another VEGF, BCL-2, C-myc, K-RAS, and H-telo sequences. The selectivity study also included a possible G4 structure formed on a dsDNA (Pu₂₂ds=Pu₂₂:Pu₂₂comp), K-phosphate buffer solution (25 mM, containing 70 mM KCl) and Millipore water as controls groups. In order to screen the selectivity, each nucleic acid structure was added in 350 nM final concentration and the fluorescence response of the AuNPs-Aza5 fluorescence probe to the nucleic acid structures were shown in Figure 37. The decrease in Fluorescence Intensity, $F_0 - F$ vs Nucleic Acids Structures graph is shown in Figure 38.

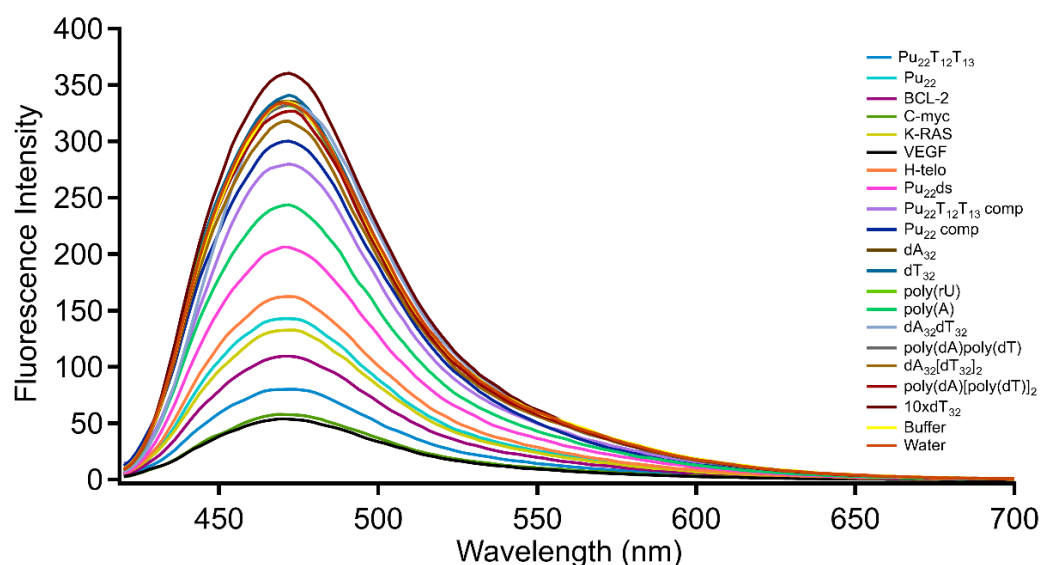


Figure 37. Fluorescence Intensity vs Wavelength (nm) graph of the nucleic acid structures used for the selectivity studies of the off-on-off fluorescence probe upon addition of 350 nM of each structure in monomeric unit.

As can be seen from Figure 38, the fluorescence decrease was not observed for single, double and triple stranded nucleic acid structures. The relatively high $F_0 - F$ value for poly(A) was predicted to be due the affinity between poly(A) and Aza5, which was investigated in previous studies [122]. Moreover, the response for Pu₂₂ds structure was probably due to the formation of G4 in the double strand DNA, which

indicated that the proposed probe was valid to screen G4s formed in dsDNA structures.

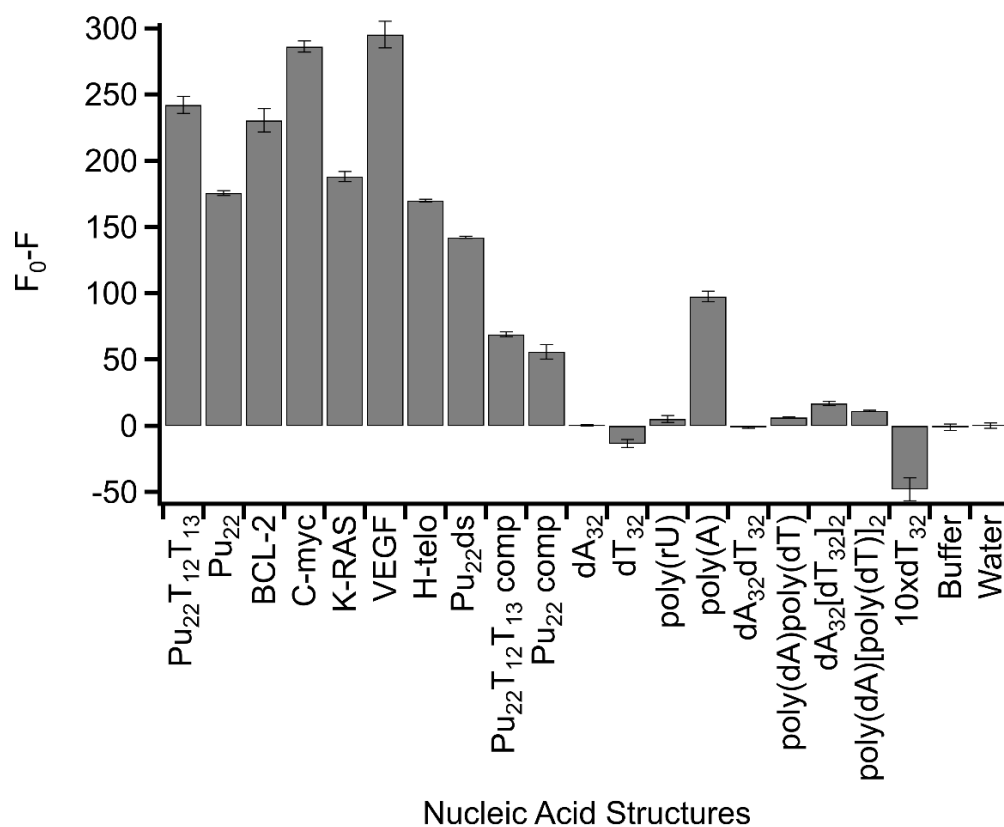


Figure 38. F_0-F values vs Nucleic Acid Structures graph of the selectivity studies. Selectivity studies for the off-on-off fluorescence probe upon addition of 350 nM of each structure in monomeric unit.

CHAPTER 4

THESIS CONCLUSION

4.1 Examining the Interaction Between VEGF Promoter Pu₂₂ and Doxorubicin

This study focused on the interactions between clinically used anti-cancer drug Dox and the G4 structures. The hypochromic effect observed in the UV-vis spectra depicted the presence of interactions between Pu₂₂ G4-Dox and Pu₂₂T₁₂T₁₃ G4-Dox complexes. CD spectroscopy analysis revealed that Dox also had a stabilizing effect on the Pu₂₂ and Pu₂₂T₁₂T₁₃ G4s since the thermal denaturation curves indicated an increase in T_m upon treatment of G4 DNAs with increasing amounts of Dox. Moreover, the calculated K_a values via fluorometric titration experiments implied a strong association between Pu₂₂ G4-Dox and Pu₂₂T₁₂T₁₃ G4-Dox complexes. We also demonstrated a comprehensive study on the affinity of Dox to various biologically important G4 structures. Our competition dialysis assay revealed that the affinity of Dox to promoter G4 structures was comparable to its commonly reported affinity towards double stranded nucleic acids. Overall, the selective affinity of Dox to G4 secondary structures and its association to VEGF promoter region Pu₂₂ and Pu₂₂T₁₂T₁₃ G4s had strong implications on its anti-cancer activity. The relationship between Dox and VEGF genes have been previously investigated. In the context of combinational therapy approaches, it was found that Dox affected VEGF expression and VEGF gene therapy was suggested to reduce the cardiotoxicity of Dox [57], [103]. We are convinced that revealing the interactions between Dox and G4 structures can shed a light on the controversial discussions regarding the action mechanism of the drug and provide solutions to the dose dependent dilemmas. Our findings can contribute the advances in clinical research since revealing the chemical behavior of small molecules may lead to better and promising treatment strategies.

4.2 Development of an AuNPs-Aza5 Fluorescence Probe for Detection of G-quadruplexes

Within the scope of this thesis, a novel and facile turn-off-on-off AuNPs-Aza5 fluorescent probe was developed for the screening and the quantitative detection of the G4 structures. As stated earlier, the present characterization methods for G4 structures in the human genome require complicated methods and large sample amounts. Therefore, fast and straight forward methods have been under investigation to accelerate the research on the issue and we aimed to propose an off-on-off AuNPs-Aza5 for the selective and sensitive detection G4 structures. Spectroscopic methods (CD, UV-vis and Fluorescence spectroscopy), TEM and DLS characterizations concluded that the probe is valid to be used as a detection platform for G4s. The CD analysis revealed that presence of AuNPs, Aza5 and Cys did not distort the secondary structure of G4s. UV-vis absorption, TEM and DLS measurements concluded that the addition of sensing elements to the AuNPs-Aza5 probe did not alter the size and morphology of the nanoparticles. The developed AuNPs-Aza5 probe revealed a high selectivity for G4 structures within the linear range of 0.032-0.347 [Pu₂₂] (μ M) and the LOD was calculated as 12.66 nM. The proposed probe is the first off-on-off fluorescent platform for the detection of biologically important G4 structures that is governed by the FRET mechanism between AuNPs and a cyanine dye. The developed AuNPs-Aza5 probe provides an alternative, simple, and straight forward method to detect the presence of G4s when compared to other techniques such as NMR, CD and X-ray analysis. Additionally, this method can be used as a screening platform for G4s even in absence of spectroscopic instruments since examining the AuNPs-Aza5 platform under a hand-held UV-light source was enough to observe the results of the probe. We believe that, such responsive and accessible detection methods are essential to accelerate research on G4 structures.

REFERENCES

- [1] A. Travers and G. Muskhelishvili, “DNA structure and function,” *FEBS J.*, vol. 282, no. 12, pp. 2279–2295, 2015.
- [2] S. S. Bachurin, M. E. Kletskii, O. N. Burov, and S. V. Kurbatov, “Non-canonical DNA structures: Comparative quantum mechanical study,” *Biophys. Chem.*, vol. 235, no. January, pp. 19–28, 2018.
- [3] T. J. Richmond and C. A. Davey, “The structure of DNA in the nucleosome core,” *Nature*, vol. 423, no. 6936, pp. 145–150, 2003.
- [4] G. Ghosal and K. Muniyappa, “Hoogsteen base-pairing revisited: Resolving a role in normal biological processes and human diseases,” *Biochem. Biophys. Res. Commun.*, vol. 343, no. 1, pp. 1–7, 2006.
- [5] J. B. Chaires and D. Graves, *Quadruplex nucleic acids*. Springer, 2014.
- [6] J. D. Watson and F. H. C. Crick, “Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid,” *Nature*, vol. 171, no. 4356, pp. 737–738, 1953.
- [7] A. Rich and S. Zhang, “Z-DNA: The long road to biological function,” *Nat. Rev. Genet.*, vol. 4, no. 7, pp. 566–572, 2003.
- [8] H. R. Drew *et al.*, “Structure of a B-DNA dodecamer: conformation and dynamics,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 78, no. 4, pp. 2179–2183, 1981.
- [9] D. B. Oh, Y. G. Kim, and A. Rich, “Z-DNA-binding proteins can act as potent effectors of gene expression in vivo,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 99, no. 26, pp. 16666–16671, 2002.
- [10] C. H. Sung, K. Lowenhaupt, A. Rich, Y. G. Kim, and K. K. Kyeong, “Crystal structure of a junction between B-DNA and Z-DNA reveals two extruded

- bases,” *Nature*, vol. 437, no. 7062, pp. 1183–1186, 2005.
- [11] Z. Shakked, G. Guerstein-Guzikevich, M. Eisenstein, F. Frolow, and D. Rabinovich, “The conformation of the DNA double helix in the crystal is dependent on its environment,” *Nature*, vol. 342, no. 6248, pp. 456–460, 1989.
 - [12] C. T. McMurray, “DNA secondary structure: A common and causative factor for expansion in human disease,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 96, no. 5, pp. 1823–1825, 1999.
 - [13] S. Rhee, Z. J. Han, K. Liu, H. T. Miles, and D. R. Davies, “Structure of a triple helical DNA with a triplex-duplex junction,” *Biochemistry*, vol. 38, no. 51, pp. 16810–16815, 1999.
 - [14] I. Radhakrishnan and D. J. Patel, “Solution Structure of an Intramolecular Purine·Purine·Pyrimidine DNA Triplex,” *J. Am. Chem. Soc.*, vol. 115, no. 4, pp. 1615–1617, 1993.
 - [15] R. D. Wells, “DNA triplexes and Friedreich ataxia,” *FASEB J.*, vol. 22, no. 6, pp. 1625–1634, 2008.
 - [16] A. Jain, G. Wang, and K. M. Vasquez, “DNA triple helices: Biological consequences and therapeutic potential,” *Biochimie*, vol. 90, no. 8, pp. 1117–1130, 2008.
 - [17] M. Kamiya, H. Torigoe, H. Shindo, and A. Sarai, “Temperature dependence and sequence specificity of DNA triplex formation: An analysis using isothermal titration calorimetry,” *J. Am. Chem. Soc.*, vol. 118, no. 19, pp. 4532–4538, 1996.
 - [18] P. P. Chan and P. M. Glazer, “Triplex DNA: Fundamentals, advances, and potential applications for gene therapy,” *J. Mol. Med.*, vol. 75, no. 4, pp. 267–282, 1997.
 - [19] M. W. Germann, H. J. Vogel, R. T. Pon, and J. H. van de Sande,

- “Characterization of a Parallel-Stranded DNA Hairpin,” *Biochemistry*, vol. 28, no. 15, pp. 6220–6228, 1989.
- [20] M. L. Wallace, L. Ying, S. Balasubramanian, and D. Klenerman, “FRET fluctuation spectroscopy: Exploring the conformational dynamics of a DNA hairpin loop,” *J. Phys. Chem. B*, vol. 104, no. 48, pp. 11551–11555, 2000.
- [21] J. Chen, N. K. Poddar, L. J. Tauzin, D. Cooper, A. B. Kolomeisky, and C. F. Landes, “Single-molecule FRET studies of HIV TAR-DNA hairpin unfolding dynamics,” *J. Phys. Chem. B*, vol. 118, no. 42, pp. 12130–12139, 2014.
- [22] H. J. Lipps and D. Rhodes, “G-quadruplex structures: in vivo evidence and function,” *Trends Cell Biol.*, vol. 19, no. 8, pp. 414–422, 2009.
- [23] J. L. Huppert and S. Balasubramanian, “G-quadruplexes in promoters throughout the human genome,” *Nucleic Acids Res.*, vol. 35, no. 2, pp. 406–413, 2007.
- [24] S. Burge, G. N. Parkinson, P. Hazel, A. K. Todd, and S. Neidle, “Quadruplex DNA: Sequence, topology and structure,” *Nucleic Acids Res.*, vol. 34, no. 19, pp. 5402–5415, 2006.
- [25] E. H. Blackburn, “Structure and function of telomeres,” *Nature*, vol. 350, no. 6319, pp. 569–573, 1991.
- [26] L. Petraccone *et al.*, “Structure and stability of higher-order human telomeric quadruplexes,” *J. Am. Chem. Soc.*, vol. 133, no. 51, pp. 20951–20961, 2011.
- [27] K. Paeschke, T. Simonsson, J. Postberg, D. Rhodes, and H. J. Lipps, “Telomere end-binding proteins control the formation of G-quadruplex DNA structures in vivo,” *Nat. Struct. Mol. Biol.*, vol. 12, no. 10, pp. 847–854, 2005.
- [28] F. Wang *et al.*, “Telomere- and telomerase-interacting protein that unfolds telomere G-quadruplex and promotes telomere extension in mammalian cells,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 109, no. 50, pp. 20413–20418, 2012.

- [29] A. K. Todd, M. Johnston, and S. Neidle, "Highly prevalent putative quadruplex sequence motifs in human DNA," *Nucleic Acids Res.*, vol. 33, no. 9, pp. 2901–2907, 2005.
- [30] D. J. Slamon, G. M. Clark, S. G. Wong, W. J. Levin, A. Ullrich, and W. L. McGuire, "Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene," *Science (80-.)*, vol. 235, no. 4785, pp. 177 LP – 182, Jan. 1987.
- [31] X. Cui, H. Chen, Q. Zhang, M. Xu, G. Yuan, and J. Zhou, "Exploration of the Structure and Recognition of a G-quadruplex in the her2 Proto-oncogene Promoter and Its Transcriptional Regulation," *Sci. Rep.*, vol. 9, no. 1, pp. 1–12, 2019.
- [32] S. Cogoi and L. E. Xodo, "G-quadruplex formation within the promoter of the KRAS proto-oncogene and its effect on transcription," *Nucleic Acids Res.*, vol. 34, no. 9, pp. 2536–2549, 2006.
- [33] R. K. Morgan, H. Batra, V. C. Gaerig, J. Hockings, and T. A. Brooks, "Identification and characterization of a new G-quadruplex forming region within the kRAS promoter as a transcriptional regulator," *Biochim. Biophys. Acta - Gene Regul. Mech.*, vol. 1859, no. 2, pp. 235–245, 2016.
- [34] P. Agrawal, C. Lin, R. I. Mathad, M. Carver, and D. Yang, "The major G-quadruplex formed in the human BCL-2 proximal promoter adopts a parallel structure with a 13-nt loop in k⁺ solution," *J. Am. Chem. Soc.*, vol. 136, no. 5, pp. 1750–1753, 2014.
- [35] K. W. Yip and J. C. Reed, "Bcl-2 family proteins and cancer," *Oncogene*, vol. 27, no. 50, pp. 6398–6406, 2008.
- [36] J. M. Adams and S. Cory, "The Bcl-2 apoptotic switch in cancer development and therapy," *Oncogene*, vol. 26, no. 9, pp. 1324–1337, 2007.
- [37] J. Dai, D. Chen, R. A. Jones, L. H. Hurley, and D. Yang, "NMR solution

- structure of the major G-quadruplex structure formed in the human BCL2 promoter region,” *Nucleic Acids Res.*, vol. 34, no. 18, pp. 5133–5144, 2006.
- [38] D. M. Miller, S. D. Thomas, A. Islam, D. Muench, and K. Sedoris, “c-Myc and cancer metabolism,” *Clin. Cancer Res.*, vol. 18, no. 20, pp. 5546–5553, 2012.
- [39] N. N. Nupponen, L. Kakkola, P. Koivisto, and T. Visakorpi, “Genetic alterations in hormone-refractory recurrent prostate carcinomas,” *Am. J. Pathol.*, vol. 153, no. 1, pp. 141–148, 1998.
- [40] H. P. Gerber *et al.*, “VEGF is required for growth and survival in neonatal mice,” *Development*, vol. 126, no. 6, pp. 1149–1159, 1999.
- [41] N. Ferrara, “Vascular endothelial growth factor: Basic science and clinical progress,” *Endocr. Rev.*, vol. 25, no. 4, pp. 581–611, 2004.
- [42] M. R. Machein and K. H. Plate, “VEGF in brain tumors,” *J. Neurooncol.*, vol. 50, no. 1–2, pp. 109–120, 2000.
- [43] P. Salven *et al.*, “Vascular endothelial growth factors VEGF-B and VEGF-C are expressed in human tumors,” *Am. J. Pathol.*, vol. 153, no. 1, pp. 103–108, 1998.
- [44] D. Sun *et al.*, “The proximal promoter region of the human vascular endothelial growth factor gene has a G-quadruplex structure that can be targeted by G-quadruplex-interactive agents,” *Mol. Cancer Ther.*, vol. 7, no. 4, pp. 880–889, 2008.
- [45] P. Agrawal, E. Hatzakis, K. Guo, M. Carver, and D. Yang, “Solution structure of the major G-quadruplex formed in the human VEGF promoter in K⁺: Insights into loop interactions of the parallel G-quadruplexes,” *Nucleic Acids Res.*, vol. 41, no. 22, pp. 10584–10592, 2013.
- [46] D. Henderson and L. H. Hurley, “Molecular struggle for transcriptional control,” *Nat. Med.*, vol. 1, no. 6, pp. 525–527, 1995.

- [47] Y. Xie, V. K. Tam, and Y. Tor, "The Interactions of Small Molecules with DNA and RNA," *Chem. Biol. Nucleic Acids*, pp. 115–140, 2010.
- [48] J. Sheng, J. Gan, and Z. Huang, "Structure-Based DNA-Targeting Strategies with Small Molecule Ligands for Drug Discovery," *Med. Res. Rev.*, vol. 33, no. 5, pp. 1119–1173, 2013.
- [49] H. K. Liu and P. J. Sadler, "Metal Complexes as DNA Intercalators," *Acc. Chem. Res.*, vol. 44, no. 5, pp. 349–359, 2011.
- [50] G. Minotti, P. Menna, E. Salvatorelli, G. Cairo, and L. Gianni, "Anthracyclines: Molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity," *Pharmacol. Rev.*, vol. 56, no. 2, pp. 185–229, 2004.
- [51] S. M. Cutts, A. Nudelman, A. Rephaeli, and D. R. Phillips, "The power and potential of doxorubicin-DNA adducts," *IUBMB Life*, vol. 57, no. 2, pp. 73–81, 2005.
- [52] K. M. Tewey, T. C. Rowe, L. Yang, B. D. Halligan, and L. F. Liu, "Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II," *Science (80-.)*, vol. 226, no. 4673, pp. 466–468, Oct. 1984.
- [53] S. Zhang *et al.*, "Identification of the molecular basis of doxorubicin-induced cardiotoxicity," *Nat. Med.*, vol. 18, no. 11, pp. 1639–1642, 2012.
- [54] S. Khiati *et al.*, "Mitochondrial topoisomerase α (Top1mt) is a novel limiting factor of doxorubicin cardiotoxicity," *Clin. Cancer Res.*, vol. 20, no. 18, pp. 4873–4881, 2014.
- [55] L. Li, Q. Pan, W. Han, Z. Liu, L. Li, and X. Hu, "Schisandrin B prevents doxorubicin-induced cardiotoxicity via enhancing glutathione redox cycling," *Clin. Cancer Res.*, vol. 13, no. 22, pp. 6753–6760, 2007.
- [56] L. L. Yi *et al.*, "Topoisomerase II β -mediated DNA double-strand breaks:

- Implications in doxorubicin cardiotoxicity and prevention by dexrazoxane,” *Cancer Res.*, vol. 67, no. 18, pp. 8839–8846, 2007.
- [57] M. C. A. Duyndam, M. P. A. van Berkel, J. C. Dorsman, D. A. P. Rockx, H. M. Pinedo, and E. Boven, “Cisplatin and doxorubicin repress Vascular Endothelial Growth Factor expression and differentially down-regulate Hypoxia-inducible Factor I activity in human ovarian cancer cells,” *Biochem. Pharmacol.*, vol. 74, no. 2, pp. 191–201, 2007.
- [58] L. Scaglioni, R. Mondelli, R. Artali, F. R. Sirtori, and S. Mazzini, “Nemorubicin and doxorubicin bind the G-quadruplex sequences of the human telomeres and of the c-MYC promoter element Pu22,” *Biochim. Biophys. Acta - Gen. Subj.*, vol. 1860, no. 6, pp. 1129–1138, 2016.
- [59] S. M. Nelson, L. R. Ferguson, and W. A. Denny, “Non-covalent ligand/DNA interactions: Minor groove binding agents,” *Mutat. Res. - Fundam. Mol. Mech. Mutagen.*, vol. 623, no. 1–2, pp. 24–40, 2007.
- [60] S. Neidle, “DNA minor-groove recognition by small molecules,” *Nat. Prod. Rep.*, vol. 18, no. 3, pp. 291–309, 2001.
- [61] D. Yang and K. Okamoto, “Structural insights into G-quadruplexes: Towards new anticancer drugs,” *Future Med. Chem.*, vol. 2, no. 4, pp. 619–646, 2010.
- [62] D. Sun *et al.*, “Inhibition of human telomerase by a G-Quadruplex-Interactive compound,” *J. Med. Chem.*, vol. 40, no. 14, pp. 2113–2116, 1997.
- [63] S. A. Ohnmacht and S. Neidle, “Small-molecule quadruplex-targeted drug discovery,” *Bioorganic Med. Chem. Lett.*, vol. 24, no. 12, pp. 2602–2612, 2014.
- [64] M. Franceschin *et al.*, “Natural and synthetic G-quadruplex interactive berberine derivatives,” *Bioorganic Med. Chem. Lett.*, vol. 16, no. 6, pp. 1707–1711, 2006.
- [65] K. Bhadra and G. S. Kumar, “Interaction of berberine, palmatine, coralyne,

- and sanguinarine to quadruplex DNA: A comparative spectroscopic and calorimetric study,” *Biochim. Biophys. Acta - Gen. Subj.*, vol. 1810, no. 4, pp. 485–496, 2011.
- [66] W. J. Zhang *et al.*, “9-Substituted berberine derivatives as G-quadruplex stabilizing ligands in telomeric DNA,” *Bioorganic Med. Chem.*, vol. 15, no. 16, pp. 5493–5501, 2007.
- [67] P. Ragazzon and J. B. Chaires, “Use of competition dialysis in the discovery of G-quadruplex selective ligands,” *Methods*, vol. 43, no. 4, pp. 313–323, 2007.
- [68] I. Manet *et al.*, “Affinity of the anthracycline antitumor drugs Doxorubicin and Sabarubicin for human telomeric G-quadruplex structures,” *Phys. Chem. Chem. Phys.*, vol. 13, no. 2, pp. 540–551, 2011.
- [69] P. Changenet-Barret, T. Gustavsson, D. Markovitsi, and I. Manet, “Ultrafast Electron Transfer in Complexes of Doxorubicin with Human Telomeric G-Quadruplexes and GC Duplexes Probed by Femtosecond Fluorescence Spectroscopy,” *ChemPhysChem*, vol. 17, no. 9, pp. 1264–1272, 2016.
- [70] I. Manet *et al.*, “Complexes of the antitumoral drugs Doxorubicin and Sabarubicin with telomeric G-quadruplex in basket conformation: Ground and excited state properties,” *Photochem. Photobiol. Sci.*, vol. 10, no. 8, pp. 1326–1337, 2011.
- [71] V. Dhamodharan and P. I. Pradeepkumar, “Specific Recognition of Promoter G-Quadruplex DNAs by Small Molecule Ligands and Light-up Probes,” *ACS Chem. Biol.*, vol. 14, pp. 2102–2114, 2019.
- [72] D. L. Ma, Z. Zhang, M. Wang, L. Lu, H. J. Zhong, and C. H. Leung, “Recent Developments in G-Quadruplex Probes,” *Chem. Biol.*, vol. 22, no. 7, pp. 812–828, 2015.
- [73] M. Q. Wang *et al.*, “A benzo(f)quinolinium fused chromophore-based

- fluorescent probe for selective detection of c-myc G-Quadruplex DNA with a red emission and a large Stokes shift,” *Dye. Pigment.*, vol. 168, no. April, pp. 334–340, 2019.
- [74] B. Kumari, R. Kumari, and P. Das, “Visual detection of G-quadruplex with mushroom derived highly fluorescent carbon quantum dots,” *J. Pharm. Biomed. Anal.*, vol. 157, pp. 137–144, 2018.
- [75] S. Zhang *et al.*, “Real-time monitoring of DNA G-quadruplexes in living cells with a small-molecule fluorescent probe,” *Nucleic Acids Res.*, vol. 46, no. 15, pp. 7522–7532, 2018.
- [76] A. Kreig, J. Calvert, J. Sanoica, E. Cullum, R. Tipanna, and S. Myong, “G-quadruplex formation in double strand DNA probed by NMM and CV fluorescence,” *Nucleic Acids Res.*, vol. 43, no. 16, pp. 7961–7970, 2015.
- [77] H. Ihmels, S. Jiang, M. M. A. Mahmoud, H. Schönherr, D. Wesner, and I. Zamrik, “Fluorimetric Detection of G-Quadruplex DNA in Solution and Adsorbed on Surfaces with a Selective Trinuclear Cyanine Dye,” *Langmuir*, vol. 34, no. 39, pp. 11866–11877, 2018.
- [78] M. Das, K. H. Shim, S. S. A. An, and D. K. Yi, “Review on gold nanoparticles and their applications,” *Toxicol. Environ. Health Sci.*, vol. 3, no. 4, pp. 193–205, 2011.
- [79] K. Saha, S. S. Agasti, C. Kim, X. Li, and V. M. Rotello, “Gold nanoparticles in chemical and biological sensing,” *Chem. Rev.*, vol. 112, no. 5, pp. 2739–2779, 2012.
- [80] J. Turkevich, P. C. Stevenson, and J. Hillier, “A study of the nucleation and growth processes in the synthesis of colloidal gold,” *Discuss. Faraday Soc.*, vol. 11, pp. 55–75, 1951.
- [81] J. Kimling, M. Maier, B. Okenve, V. Kotaidis, H. Ballot, and A. Plech, “Turkevich method for gold nanoparticle synthesis revisited,” *J. Phys. Chem.*

B, vol. 110, no. 32, pp. 15700–15707, 2006.

- [82] P. N. Njoki *et al.*, “Size correlation of optical and spectroscopic properties for gold nanoparticles,” *J. Phys. Chem. C*, vol. 111, no. 40, pp. 14664–14669, 2007.
- [83] T. K. Sau and C. J. Murphy, “Room temperature, high-yield synthesis of multiple shapes of gold nanoparticles in aqueous solution,” *J. Am. Chem. Soc.*, vol. 126, no. 28, pp. 8648–8649, 2004.
- [84] H. Haick, “Chemical sensors based on molecularly modified metallic nanoparticles,” *J. Phys. D. Appl. Phys.*, vol. 40, no. 23, pp. 7173–7186, 2007.
- [85] V. K. K. Upadhyayula, “Functionalized gold nanoparticle supported sensory mechanisms applied in detection of chemical and biological threat agents: A review,” *Anal. Chim. Acta*, vol. 715, pp. 1–18, 2012.
- [86] Z. Wang and L. Ma, “Gold nanoparticle probes,” *Coord. Chem. Rev.*, vol. 253, no. 11–12, pp. 1607–1618, 2009.
- [87] L. Qin *et al.*, “‘Gold rush’ in modern science: Fabrication strategies and typical advanced applications of gold nanoparticles in sensing,” *Coord. Chem. Rev.*, vol. 359, pp. 1–31, 2018.
- [88] E. C. Dreaden, A. M. Alkilany, X. Huang, C. J. Murphy, and M. A. El-Sayed, “The golden age: Gold nanoparticles for biomedicine,” *Chem. Soc. Rev.*, vol. 41, no. 7, pp. 2740–2779, 2012.
- [89] P. Ghosh, G. Han, M. De, C. K. Kim, and V. M. Rotello, “Gold nanoparticles in delivery applications,” *Adv. Drug Deliv. Rev.*, vol. 60, no. 11, pp. 1307–1315, 2008.
- [90] M. H. Jazayeri, T. Aghaie, A. Avan, A. Vatankhah, and M. R. S. Ghaffari, “Colorimetric detection based on gold nano particles (GNPs): An easy, fast, inexpensive, low-cost and short time method in detection of analytes (protein, DNA, and ion),” *Sens. Bio-Sensing Res.*, vol. 20, no. March, pp. 1–8, 2018.

- [91] S. C. B. Gopinath, T. Lakshmipriya, and K. Awazu, "Colorimetric detection of controlled assembly and disassembly of aptamers on unmodified gold nanoparticles," *Biosens. Bioelectron.*, vol. 51, pp. 115–123, 2014.
- [92] E. Priyadarshini and N. Pradhan, "Gold nanoparticles as efficient sensors in colorimetric detection of toxic metal ions: A review," *Sensors Actuators, B Chem.*, vol. 238, pp. 888–902, 2017.
- [93] E. Dulkeith *et al.*, "Fluorescence Quenching of Dye Molecules near Gold Nanoparticles: Radiative and Nonradiative Effects," *Phys. Rev. Lett.*, vol. 89, no. 20, pp. 12–15, 2002.
- [94] E. Oliveira *et al.*, "Revisiting the use of gold and silver functionalised nanoparticles as colorimetric and fluorometric chemosensors for metal ions," *Sensors Actuators, B Chem.*, vol. 212, pp. 297–328, 2015.
- [95] C. C. Huang and H. T. Chang, "Selective gold-nanoparticle-based 'turn-on' fluorescent sensors for detection of mercury(II) in aqueous solution," *Anal. Chem.*, vol. 78, no. 24, pp. 8332–8338, 2006.
- [96] D. Zhao, C. Chen, L. Lu, F. Yang, and X. Yang, "A dual-mode colorimetric and fluorometric 'light on' sensor for thiocyanate based on fluorescent carbon dots and unmodified gold nanoparticles," *Analyst*, vol. 140, no. 24, pp. 8157–8164, 2015.
- [97] J. Xu, H. Yu, Y. Hu, M. Chen, and S. Shao, "A gold nanoparticle-based fluorescence sensor for high sensitive and selective detection of thiols in living cells," *Biosens. Bioelectron.*, vol. 75, pp. 1–7, 2016.
- [98] J. Gao, X. Huang, H. Liu, F. Zan, and J. Ren, "Colloidal stability of gold nanoparticles modified with thiol compounds: Bioconjugation and application in cancer cell imaging," *Langmuir*, vol. 28, no. 9, pp. 4464–4471, 2012.
- [99] J. S. Lee, P. A. Ulmann, M. S. Han, and C. A. Mirkin, "A DNA - Gold nanoparticle-based colorimetric competition assay for the detection of

- cysteine,” *Nano Lett.*, vol. 8, no. 2, pp. 529–533, 2008.
- [100] K. Asian, J. R. Lakowicz, and C. D. Geddes, “Nanogold plasmon resonance-based glucose sensing. 2. Wavelength-ratiometric resonance light scattering,” *Anal. Chem.*, vol. 77, no. 7, pp. 2007–2014, 2005.
- [101] J. Liu, H. L. Jung, and Y. Lu, “Quantum dot encoding of aptamer-linked nanostructures for one-pot simultaneous detection of multiple analytes,” *Anal. Chem.*, vol. 79, no. 11, pp. 4120–4125, 2007.
- [102] L. Zhang, S. Zhang, W. Pan, Q. Liang, and X. Song, “Exonuclease I manipulating primer-modified gold nanoparticles for colorimetric telomerase activity assay,” *Biosens. Bioelectron.*, vol. 77, pp. 144–148, 2016.
- [103] M. Räsänen *et al.*, “VEGF-B gene therapy inhibits doxorubicin-induced cardiotoxicity by endothelial protection,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 113, no. 46, pp. 13144–13149, 2016.
- [104] T. Chen *et al.*, “Overexpression of vascular endothelial growth factor 165 (VEGF165) protects Cardiomyocytes against doxorubicin-induced apoptosis,” *J. Chemother.*, vol. 22, no. 6, pp. 402–406, 2010.
- [105] D. R. Senger *et al.*, “Vascular permeability factor (VPF, VEGF) in tumor biology,” *Cancer Metastasis Rev.*, vol. 12, no. 3–4, pp. 303–324, 1993.
- [106] C. K. Kwok and C. J. Merrick, “G-Quadruplexes: Prediction, Characterization, and Biological Application,” *Trends Biotechnol.*, vol. 35, no. 10, pp. 997–1013, 2017.
- [107] J. Folkman, “Angiogenesis in cancer, vascular, rheumatoid and other disease,” *Nat. Med.*, vol. 1, no. 1, pp. 27–30, 1995.
- [108] N. Ferrara, “VEGF and the quest for tumour angiogenesis factors,” *Nat. Rev. Cancer*, vol. 2, no. 10, pp. 795–803, 2002.
- [109] X. Qu and J. B. Chaires, “Analysis of Drug-DNA Binding Data,” *Methods*

Enzymol., vol. 321, pp. 353–369, 2000.

- [110] P. A. Ragazzon, N. C. Garbett, and J. B. Chaires, “Competition dialysis: A method for the study of structural selective nucleic acid binding,” *Methods*, vol. 42, no. 2, pp. 173–182, 2007.
- [111] J. B. Chaires, N. Dattagupta, and D. M. Crothers, “Self-Association of Daunomycin,” *Biochemistry*, vol. 21, no. 17, pp. 3927–3932, 1982.
- [112] S. Paramasivan, I. Rujan, and P. H. Bolton, “Circular dichroism of quadruplex DNAs : Applications to structure , cation effects and ligand binding,” vol. 43, pp. 324–331, 2007.
- [113] M. Sirajuddin, S. Ali, and A. Badshah, “Drug-DNA interactions and their study by UV-Visible, fluorescence spectroscopies and cyclic voltametry,” *J. Photochem. Photobiol. B Biol.*, vol. 124, pp. 1–19, 2013.
- [114] J. Liu *et al.*, “DNA-binding and cleavage studies of macrocyclic copper(II) complexes,” *J. Inorg. Biochem.*, vol. 91, no. 1, pp. 269–276, 2002.
- [115] Y. Zhang, J. Chen, H. Ju, and J. Zhou, “Thermal denaturation profile: A straightforward signature to characterize parallel G-quadruplexes,” *Biochimie*, vol. 157, pp. 22–25, 2019.
- [116] C. M. Olsen, W. H. Gmeiner, and L. A. Marky, “Unfolding of G-quadruplexes: Energetic, and ion and water contributions of G-quartet stacking,” *J. Phys. Chem. B*, vol. 110, no. 13, pp. 6962–6969, 2006.
- [117] V. H. DuVernay, J. A. Pachter, and S. T. Crooke, “Deoxyribonucleic Acid Binding Studies on Several New Anthracycline Antitumor Antibiotics. Sequence Preference and Structure-Activity Relationships of Marcellomycin and Its Analogues as Compared to Adriamycin,” *Biochemistry*, vol. 18, no. 18, pp. 4024–4030, 1979.
- [118] R. del Villar-Guerra, J. O. Trent, and J. B. Chaires, “G-Quadruplex Secondary Structure Obtained from Circular Dichroism Spectroscopy,” *Angew. Chemie*

- *Int. Ed.*, vol. 57, no. 24, pp. 7171–7175, 2018.

- [119] E. Bilgen, M. Forough, and Ö. Persil Çetinkol, “A conjugated gold nanoparticle-azacyanine off-on-off fluorescence probe for sensitive and selective detection of G-quadruplexes,” *Talanta*, vol. 217, p. 121076, 2020.
- [120] M. M. Maye *et al.*, “Gold and alloy nanoparticles in solution and thin film assembly: spectrophotometric determination of molar absorptivity,” *Anal. Chim. Acta*, vol. 496, no. 1–2, pp. 17–27, 2003.
- [121] Y. J. Hu, Y. Liu, L. X. Zhang, R. M. Zhao, and S. S. Qu, “Studies of interaction between colchicine and bovine serum albumin by fluorescence quenching method,” *J. Mol. Struct.*, vol. 750, no. 1–3, pp. 174–178, 2005.
- [122] Ö. P. Çetinkol and N. V Hud, “Molecular recognition of poly (A) by small ligands: an alternative method of analysis reveals nanomolar, cooperative and shape-selective binding,” *Nucleic Acids Res.*, vol. 37, no. 2, pp. 611–621, 2008.

APPENDICES

A. Preparation of Buffer and Stock Solutions

100mM K-Phosphate Buffer Preparation for thermal denaturation and binding constant experiments:

100mM K-Phosphate buffer contains 620 mM KH_2PO_4 and 390 mM K_2HPO_4

100mM K-Phosphate buffer is prepared by using 1M KH_2PO_4 and 1M K_2HPO_4 stock solutions.

1M, 0.5L KH_2PO_4 stock preparation:

$$M = \frac{n}{V} \text{ and } n = \frac{M}{V};$$

$$1M = \frac{n(\text{mol})}{0.5 L}$$

$$n = 0.5 \text{ mol}, \text{KH}_2\text{PO}_4 \text{ Mw: } 136.09 \text{ g/mol and } n (\text{mol}) = \frac{m(\text{g})}{\text{Mw}(\frac{\text{g}}{\text{mol}})}$$

$$0.5 \text{ mol} = \frac{\frac{m}{136.09 \text{ g}}}{\text{mol}} \text{ so; } m(\text{g}) = 68.05 \text{ g}$$

68.05 g KH_2PO_4 is dissolved in 500 mL Millipore water for preparation of 1M KH_2PO_4 solution.

1M, 0.5L K_2HPO_4 stock preparation:

$$M = \frac{n}{V} \text{ and } n = \frac{M}{V};$$

$$1M = \frac{n(\text{mol})}{0.5L}$$

$$n = 0.5 \text{ mol}, \text{K}_2\text{HPO}_4 \text{ Mw: } 174.18 \text{ g/mol and } n (\text{mol}) = \frac{m(\text{g})}{\text{Mw}(\frac{\text{g}}{\text{mol}})}$$

$$0.5 \text{ mol} = \frac{\frac{m}{174.18 \text{ g}}}{\text{mol}} \text{ so; } m(\text{g}) = 87.09 \text{ g}$$

87.09 g K_2HPO_4 is dissolved in 500 mL Millipore water for preparation of 1M K_2HPO_4 solution.

Concentrations are arranged by using the following equation:

$$M_1 \times V_1 = M_2 \times V_2$$

For K_2HPO_4 : $1M \times V_1 = 0.62M \times 1 L$ and $V_1 = 61.50 \text{ mL}$

For KH_2PO_4 : $1M \times V_1 = 0.39M \times 1 L$ and $V_1 = 38.50 \text{ mL}$

The total volume of the above components is equal to 100.0 mL. The buffer solution is completed to 1.0 L with millipore water and the pH of the solution is adjusted to 7.0 with NaOH solution.

500 mM KCl solution preparation for thermal denaturation and binding constant experiments:

$$M = \frac{n}{V} \text{ and } n = \frac{M}{V};$$

$$0.5 M = \frac{n(\text{mol})}{0.1 L}$$

$$n = 0.05 \text{ mol}, \text{KCl Mw:}74.55 \text{ g/mol and } n (\text{mol}) = \frac{m(\text{g})}{\text{Mw}(\frac{\text{g}}{\text{mol}})}$$

$$0.05 \text{ mol} = \frac{\frac{m}{74.55 \text{ g}}}{\text{mol}} \text{ so; } m(\text{g}) = 3.73 \text{ g}$$

3.73 g KCl is dissolved in 100 mL Millipore water for preparation of 500 mM KCl solution.

1X BPES Buffer (pH:7) preparation for competition dialysis experiment:

5X BPES Buffer (pH:7, 0.5L) preparation:

5X BPES buffer contains 925 mM NaCl, 30 mM Na₂HPO₄, 5 mM Na₂EDTA, 10 mM NaH₂PO₄

5X BPES buffer is prepared by using 2 M NaCl, 400 mM Na₂HPO₄, 100 mM Na₂EDTA and 600 mM NaH₂PO₄ stock solutions:

2 M, 0.5 L NaCl stock preparation:

$$M = \frac{n}{V} \text{ and } n = \frac{M}{V};$$

$$2 \text{ M} = \frac{n(\text{mol})}{0.5 \text{ L}}$$

$$n = 1 \text{ mol}, \text{NaCl Mw:}58.44 \text{ g/mol and } n (\text{mol}) = \frac{m(\text{g})}{\text{Mw}(\frac{\text{g}}{\text{mol}})}$$

$$1 \text{ mol} = \frac{\frac{m}{58.44 \text{ g}}}{\text{mol}} \text{ so; } m(\text{g}) = 58.44 \text{ g}$$

400 mM, 0.1 L NaH₂PO₄ stock preparation:

$$M = \frac{n}{V} \text{ and } n = \frac{M}{V};$$

$$0.4 \text{ M} = \frac{n(\text{mol})}{0.1 \text{ L}}$$

$$n = 0.04 \text{ mol}, \text{NaH}_2\text{PO}_4 \text{ Mw:}119.98 \text{ g/mol and } n (\text{mol}) = \frac{m(\text{g})}{\text{Mw}(\frac{\text{g}}{\text{mol}})}$$

$$0.04 \text{ mol} = \frac{\frac{m}{119.98 \text{ g}}}{\text{mol}} \text{ so; } m(\text{g}) = 4.80 \text{ g}$$

100 mM, 0.1 L Na₂EDTA stock preparation:

$$M = \frac{n}{V} \text{ and } n = \frac{M}{V};$$

$$0.1 \text{ M} = \frac{n(\text{mol})}{0.1 \text{ L}}$$

$$n = 0.01 \text{ mol}, \text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O Mw:}372.24 \text{ g/mol and } n (\text{mol}) = \frac{m(\text{g})}{\text{Mw}(\frac{\text{g}}{\text{mol}})}$$

$$0.01 \text{ mol} = \frac{\frac{m}{372.24 \text{ g}}}{\text{mol}} \text{ so; } m(\text{g}) = 3.72 \text{ g}$$

600 mM, 0.1 L Na₂HPO₄ stock preparation:

$$M = \frac{n}{V} \text{ and } n = \frac{M}{V};$$

$$0.6 \text{ M} = \frac{n(\text{mol})}{0.1 \text{ L}}$$

$$n = 0.06 \text{ mol}, \text{Na}_2\text{HPO}_4 \text{ Mw:}141.96 \text{ g/mol and } n (\text{mol}) = \frac{m(\text{g})}{\text{Mw}(\frac{\text{g}}{\text{mol}})}$$

$$0.06 \text{ mol} = \frac{\frac{m}{141.96 \text{ g}}}{\text{mol}} \text{ so; } m(g) = 8.51 \text{ g}$$

Concentrations are arranged by using the following equation:

$$M_1 \times V_1 = M_2 \times V_2$$

For NaCl: $2M \times V_1 = 0.925M \times 0.5L$ and $V_1 = 231.25mL$

For Na₂HPO₄: $0.4M \times V_1 = 0.03M \times 0.5L$ and $V_1 = 25.00mL$

For Na₂EDTA: $0.1M \times V_1 = 0.005M \times 0.5L$ and $V_1 = 25.00mL$

For NaH₂PO₄: $0.6M \times V_1 = 0.01M \times 0.5L$ and $V_1 = 12.50mL$

The total volume of the above components is equal to 293.75mL. The buffer solution is completed to 500 ml with Millipore water and the pH of the solution is adjusted to 7.0 with NaOH solution.

1X BPES Buffer Preparation:

Concentrations are arranged by using the following equation:

$$M_1 \times V_1 = M_2 \times V_2$$

$$5X \times V_1 = 1X \times 1L$$

Therefore, 200mL 5X BPES buffer is diluted to 1.0 L with the addition of 800mL Millipore water and 1X BPES buffer is obtained.

%10 (w/v) SDS solution preparation for competition dialysis experiment:

10.0 g SDS (purchased from Sigma Aldrich 862010) is dissolved in 80 mL Millipore water. The total volume is completed to 100.0 mL with Millipore water.

B. List of Nucleic Acid Sequences Used in This Study

Table 3. List of nucleic acid sequences used in this study.

Abbreviations for the nucleic acids	Sequences
Pu₂₂	5'-CGG GGC GGG CCG GGG GCG GGG T-3'
Pu₂₂T₁₂T₁₃	5'-CGG GGC GGG CCT TGG GCG GGG T-3'
BCL-2	5'-AGG GGC GGG CGC GGG AGG AAG GGG GCG GGA GCG GGG C-3'
K-RAS	5'-GGG AAG AGG GAA GAG GGG GAG G-3'
Her-2	5'- -3'
C-myc	5'-TGG GGA GGG TGG GGA GGG TGG GGA AGG-3'
Human Telomeric repeat (H-telo)	5'-AGG GTT AGG GTT AGG GTT AGG G-3'
dA₃₂	5'-AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AA-3'
dT₃₂	5'-TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TT-3'

C. Uv-vis Thermal Denaturation Experiments and Melt Curves

a. Pu₂₂

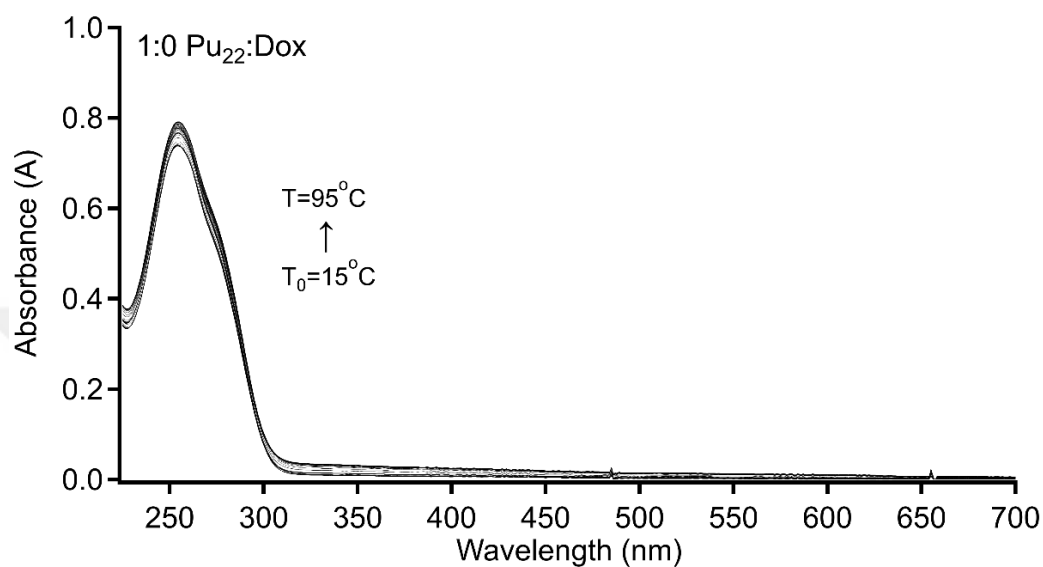


Figure 39. UV-vis absorption spectra of 1:0 Pu₂₂:Dox sample obtained during thermal denaturation experiments.

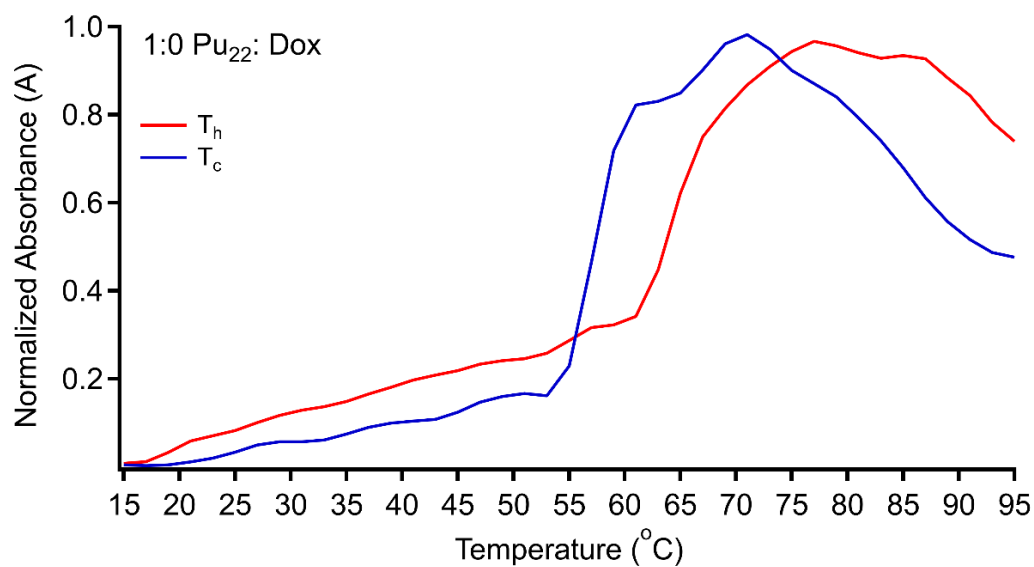


Figure 40. Thermal denaturation curves for 1:0 Pu₂₂:Dox sample obtained by monitoring the UV-vis absorbance at 295 nm. Red curve represents the heating curve (15°C to 95°C) and the blue curve represents the cooling curve (95°C to 15°C).

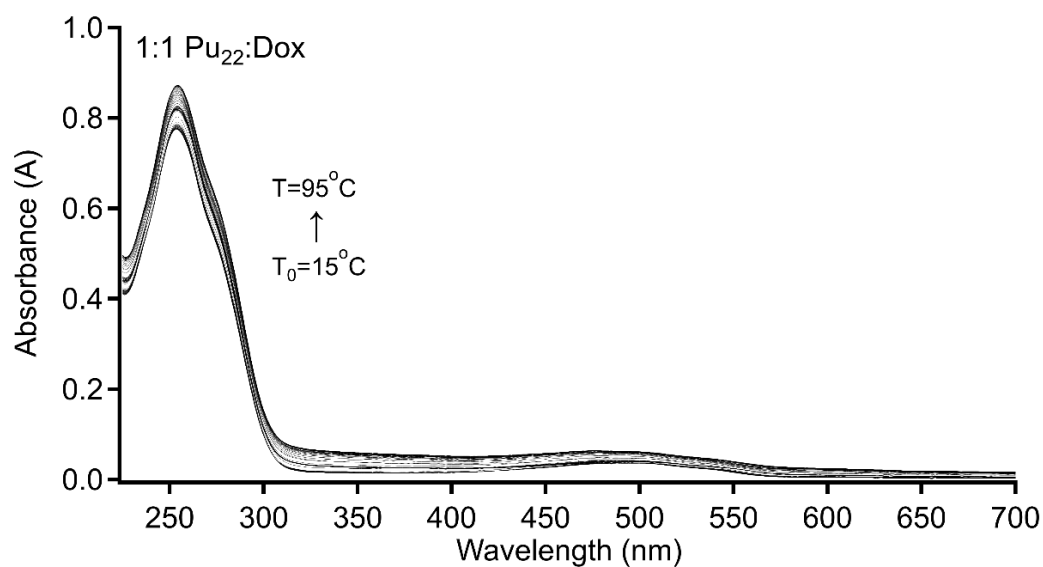


Figure 41. UV-vis absorption spectra of 1:1 Pu₂₂:Dox sample obtained during thermal denaturation experiments.

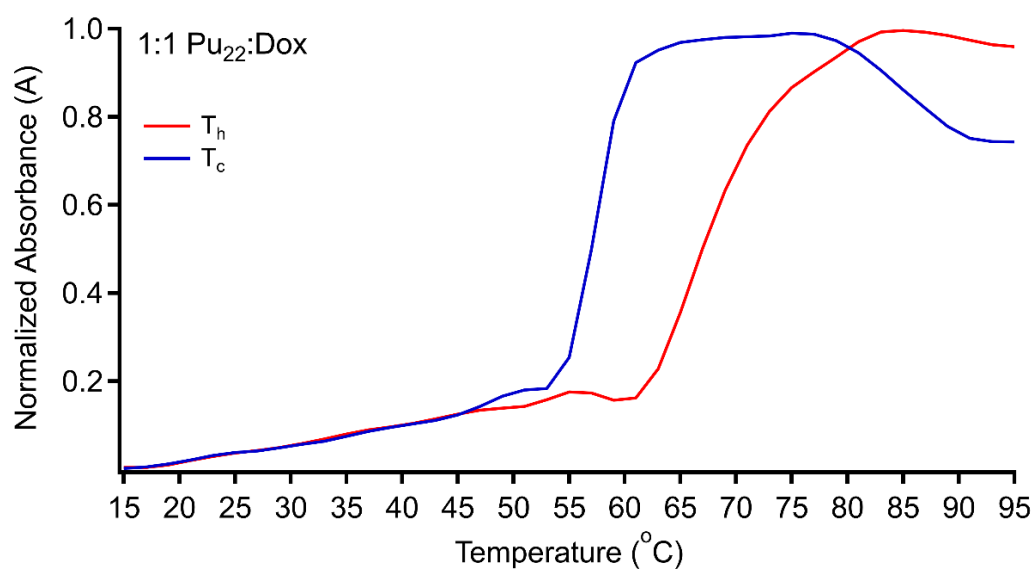


Figure 42. Thermal denaturation curves for 1:1 Pu₂₂:Dox sample obtained by monitoring the UV-vis absorbance at 295 nm. Red curve represents the heating curve (15°C to 95°C) and the blue curve represents the cooling curve (95°C to 15°C).

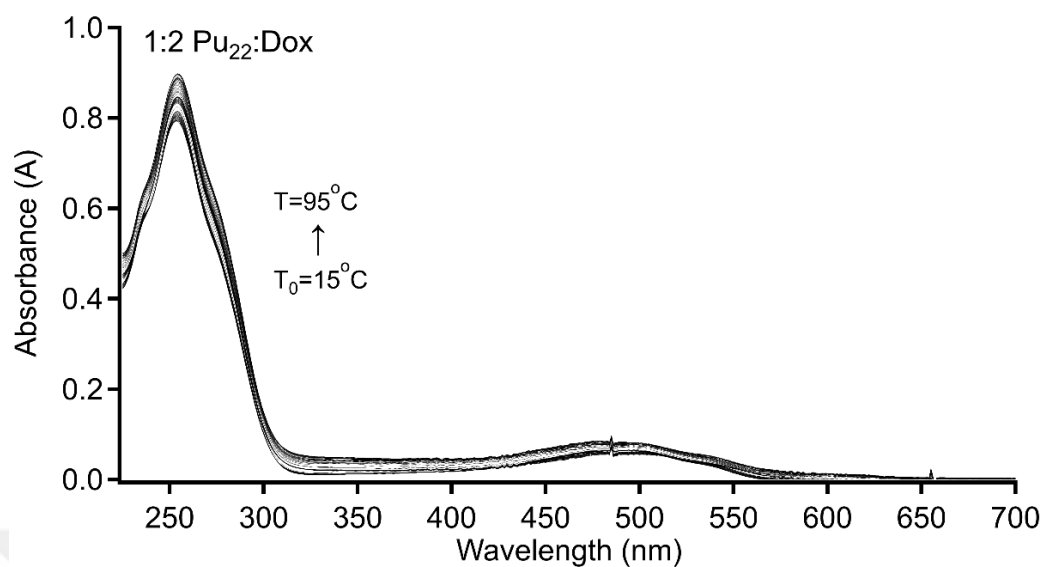


Figure 43. UV-vis absorption spectra of 1:2 Pu₂₂:Dox sample obtained during thermal denaturation experiments.

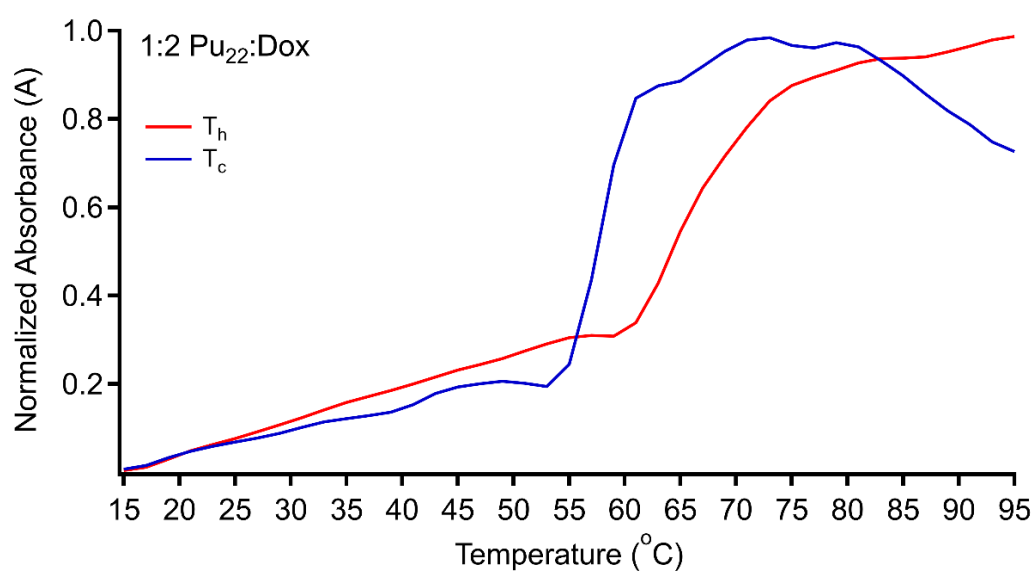


Figure 44. Thermal denaturation curves for 1:2 Pu₂₂:Dox sample obtained by monitoring the UV-vis absorbance at 295 nm. Red curve represents the heating curve (15°C to 95°C) and the blue curve represents the cooling curve (95°C to 15°C).

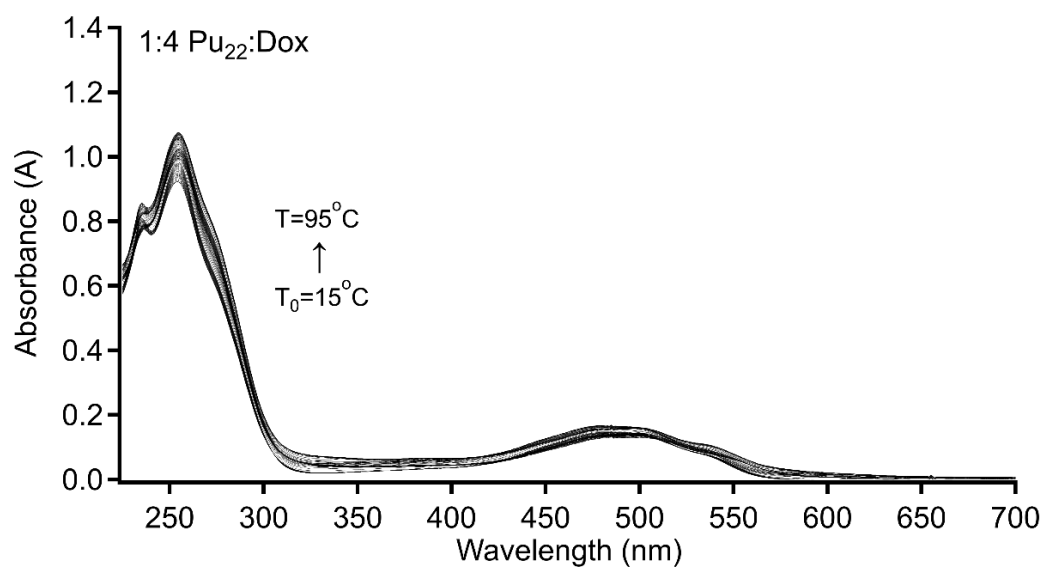


Figure 45. UV-vis absorption spectra of 1:4 Pu₂₂:Dox sample obtained during thermal denaturation experiments.

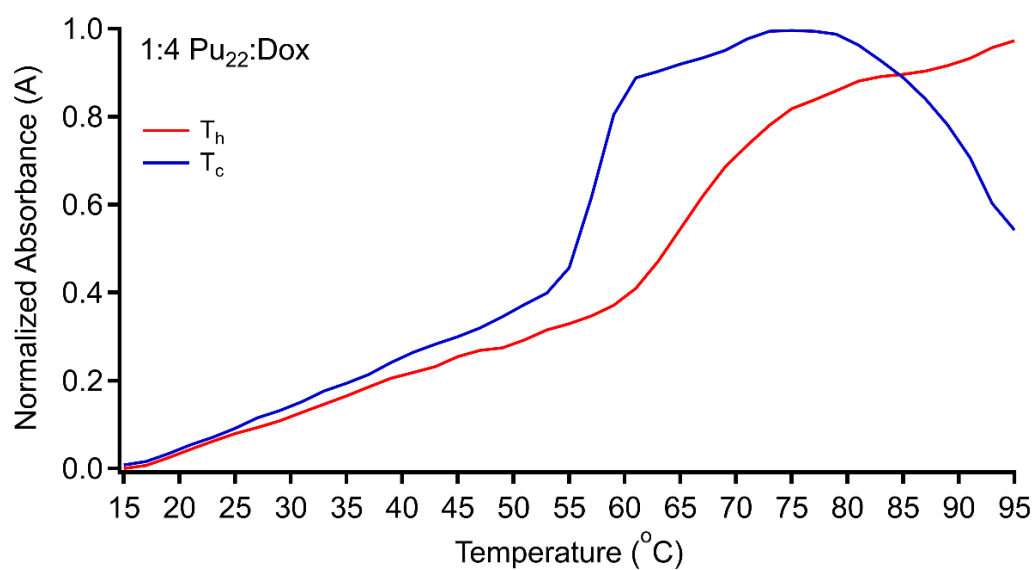


Figure 46. Thermal denaturation curves for 1:4 Pu₂₂:Dox sample obtained by monitoring the UV-vis absorbance at 295 nm. Red curve represents the heating curve (15°C to 95°C) and the blue curve represents the cooling curve (95°C to 15°C).

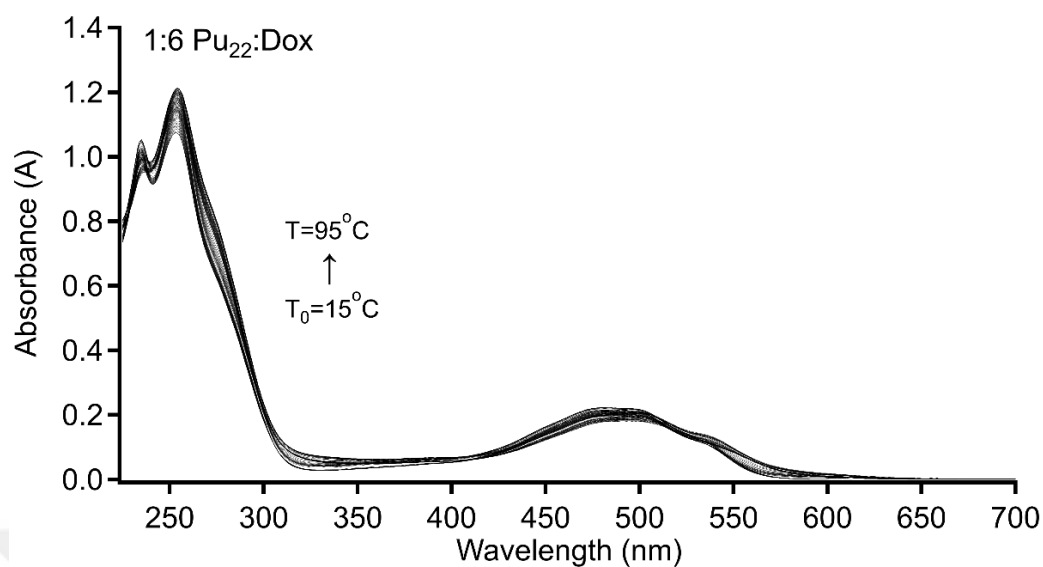


Figure 47. UV-vis absorption spectra of 1:6 Pu₂₂:Dox sample obtained during thermal denaturation experiments.

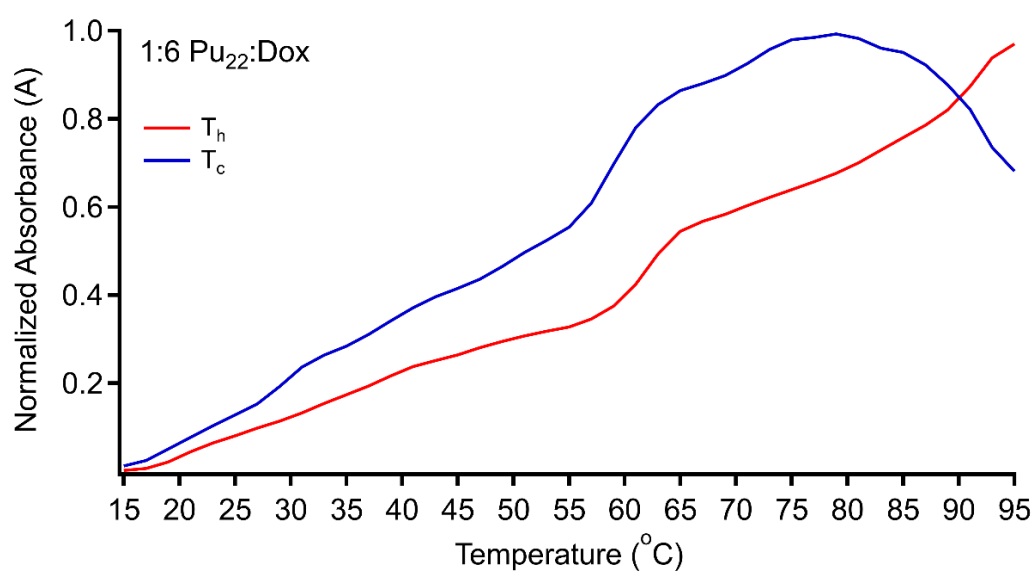


Figure 48. Thermal denaturation curves for 1:6 Pu₂₂:Dox sample obtained by monitoring the UV-vis absorbance at 295 nm. Red curve represents the heating curve (15°C to 95°C) and the blue curve represents the cooling curve (95°C to 15°C).

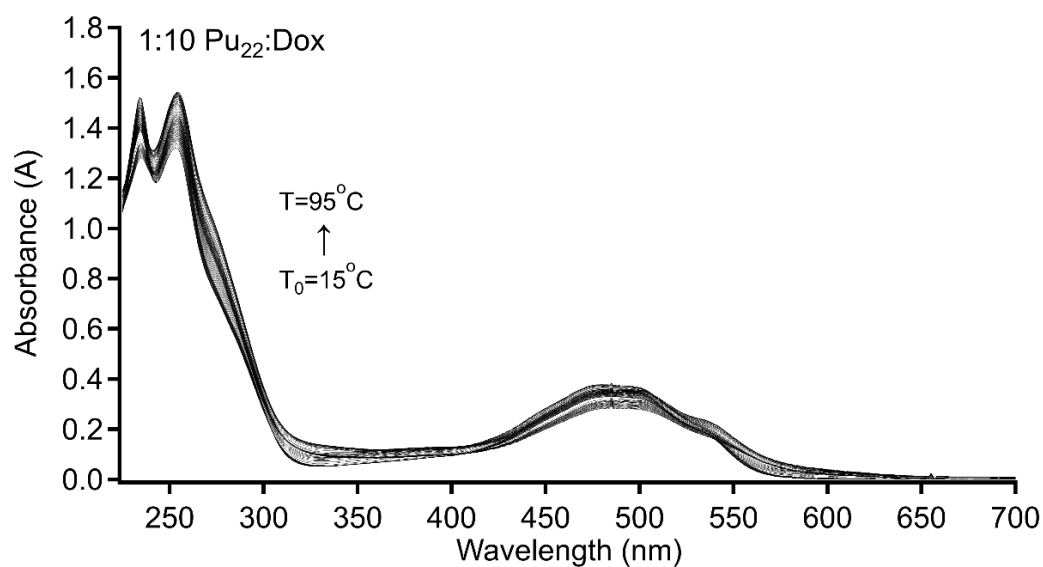


Figure 49. UV-vis absorption spectra of 1:10 Pu₂₂:Dox sample obtained during thermal denaturation experiments.

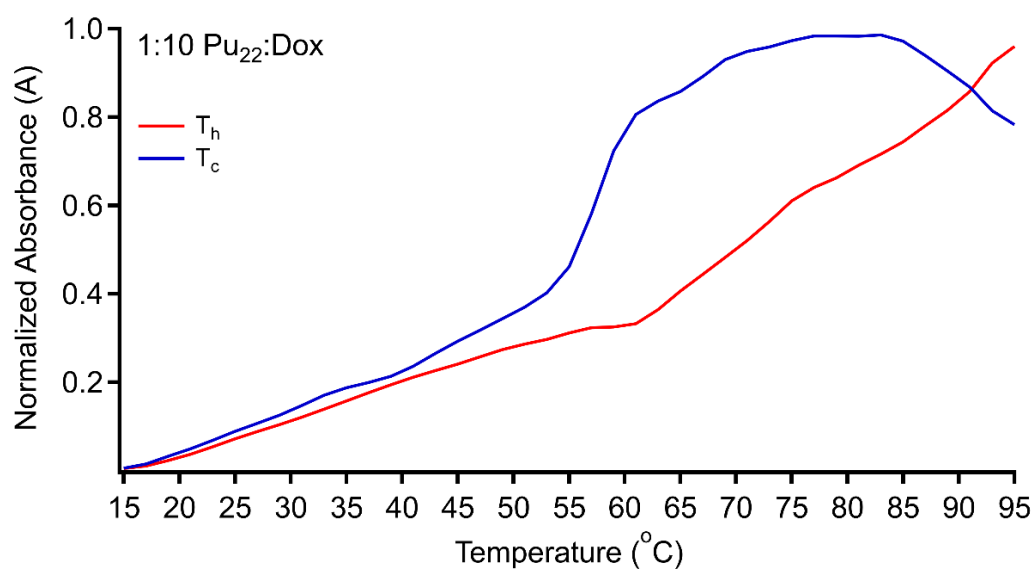


Figure 50. Thermal denaturation curves for 1:10 Pu₂₂:Dox sample obtained by monitoring the UV-vis absorbance at 295 nm. Red curve represents the heating curve (15°C to 95°C) and the blue curve represents the cooling curve (95°C to 15°C).

b. Pu₂₂T₁₂T₁₃

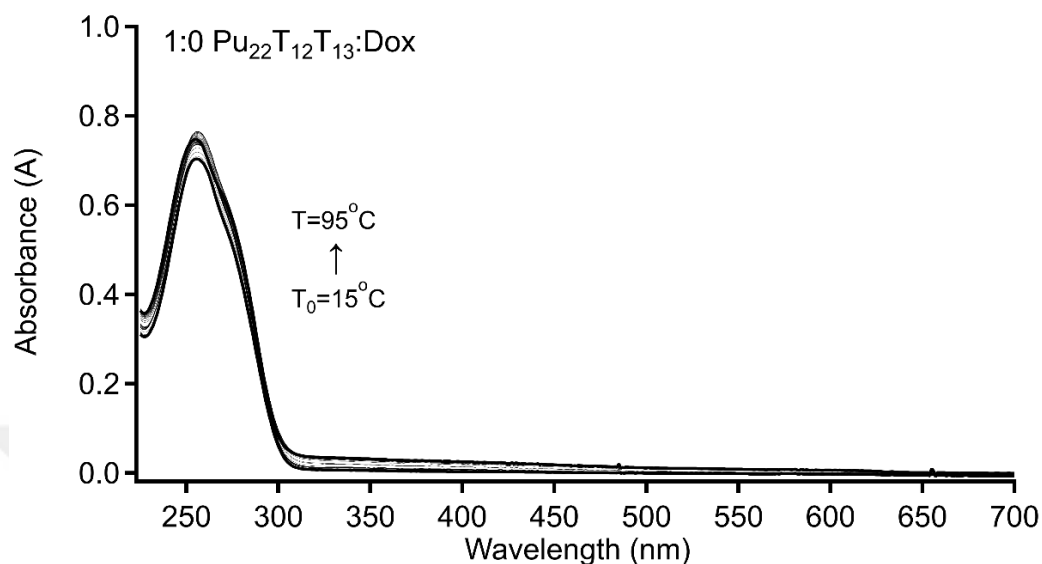


Figure 51. UV-vis absorption spectra of 1:0 Pu₂₂T₁₂T₁₃:Dox sample obtained during thermal denaturation experiments.

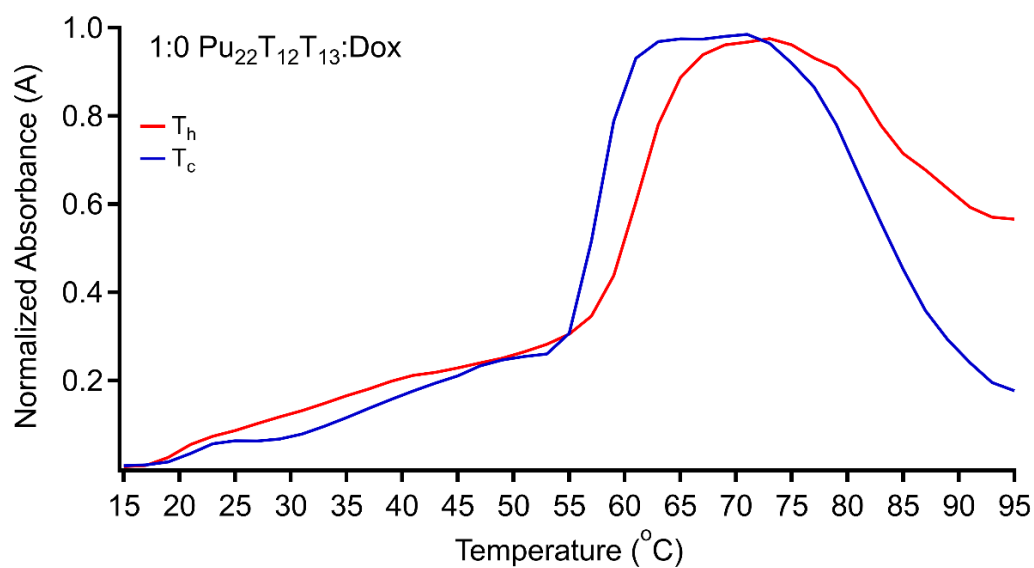


Figure 52. Thermal denaturation curves for 1:0 Pu₂₂T₁₂T₁₃:Dox sample obtained by monitoring the UV-vis absorbance at 295 nm. Red curve represents the heating curve (15°C to 95°C) and the blue curve represents the cooling curve (95°C to 15°C).

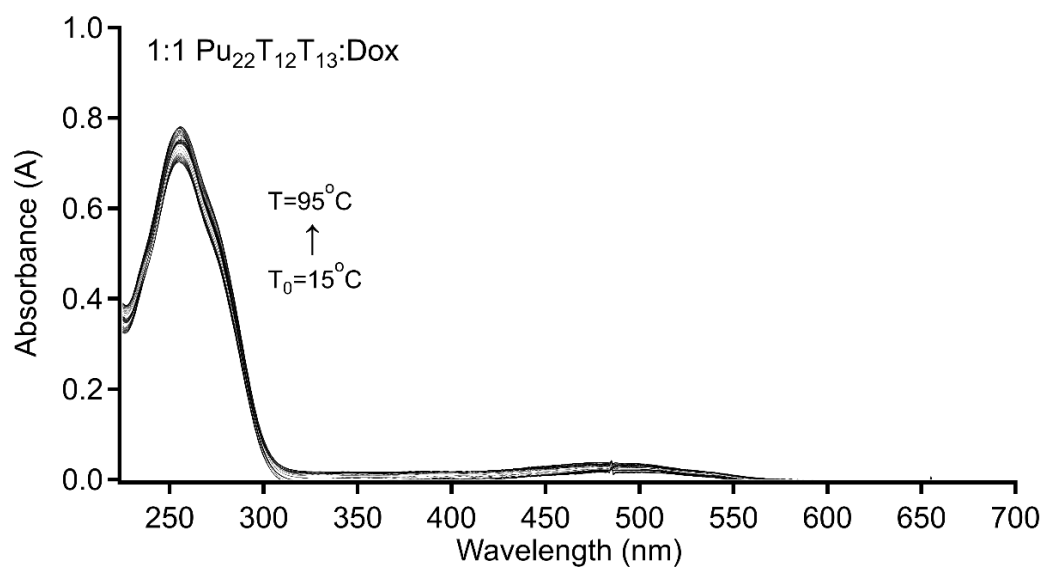


Figure 53. UV-vis absorption spectra of 1:1 Pu₂₂T₁₂T₁₃:Dox sample obtained during thermal denaturation experiments.

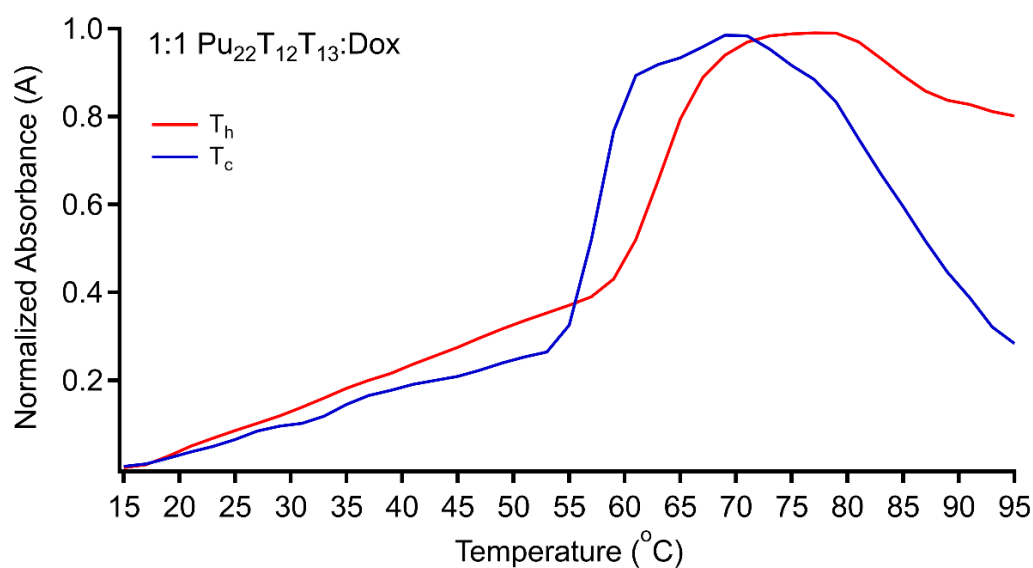


Figure 54. Thermal denaturation curves for 1:1 Pu₂₂T₁₂T₁₃:Dox sample obtained by monitoring the UV-vis absorbance at 295 nm. Red curve represents the heating curve (15°C to 95°C) and the blue curve represents the cooling curve (95°C to 15°C).

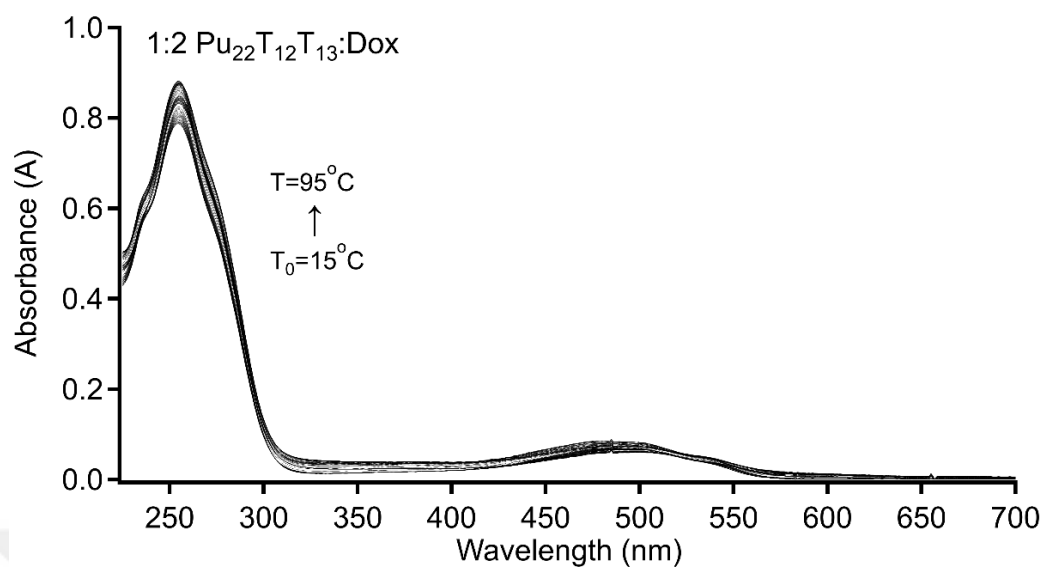


Figure 55. UV-vis absorption spectra of 1:2 Pu₂₂T₁₂T₁₃:Dox sample obtained during thermal denaturation experiments.

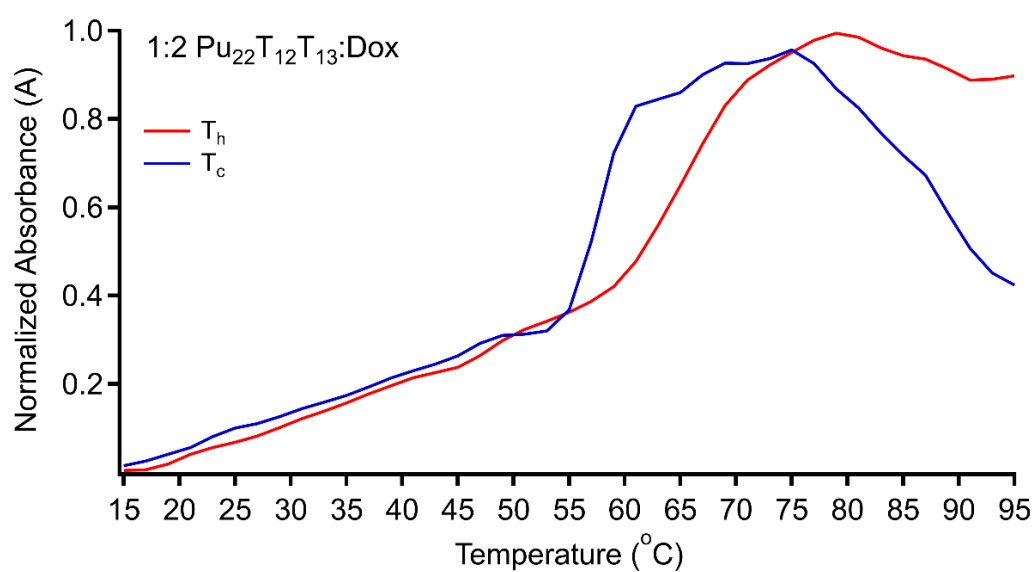


Figure 56. Thermal denaturation curves for 1:2 Pu₂₂T₁₂T₁₃:Dox sample obtained by monitoring the UV-vis absorbance at 295 nm. Red curve represents the heating curve (15°C to 95°C) and the blue curve represents the cooling curve (95°C to 15°C).

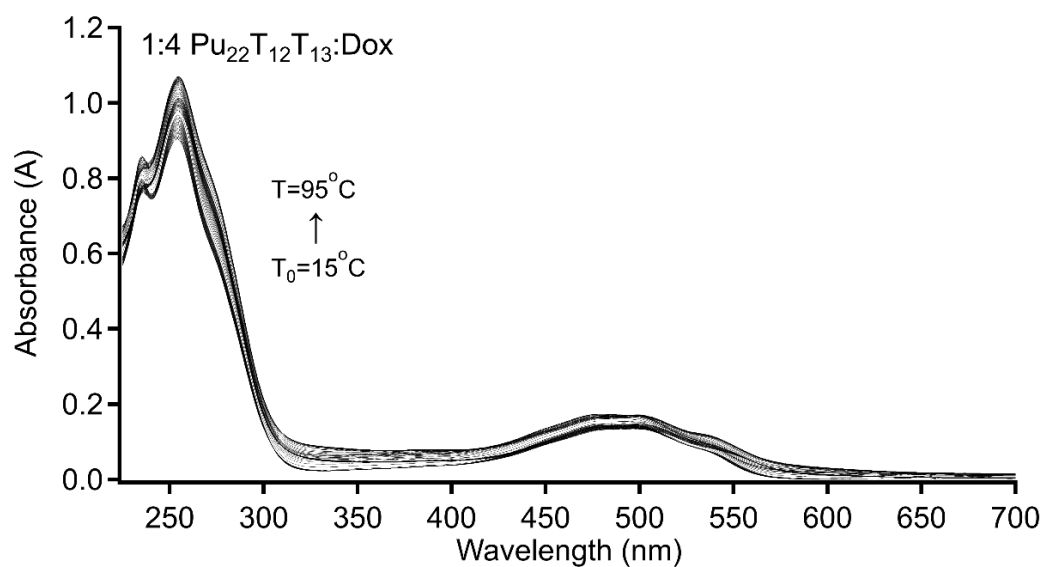


Figure 57. UV-vis absorption spectra of 1:4 Pu₂₂T₁₂T₁₃:Dox sample obtained during thermal denaturation experiments.

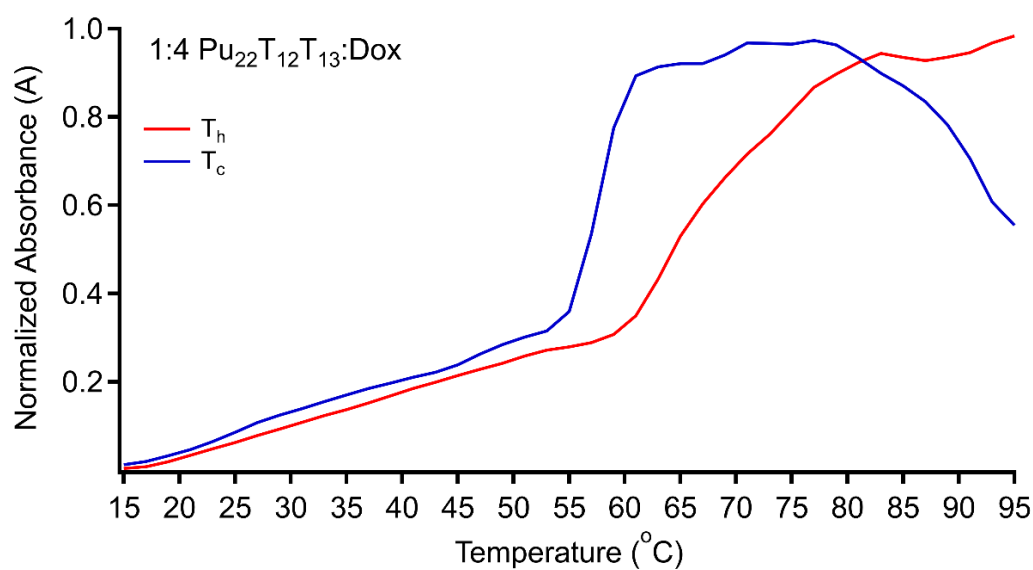


Figure 58. Thermal denaturation curves for 1:4 Pu₂₂T₁₂T₁₃:Dox sample obtained by monitoring the UV-vis absorbance at 295 nm. Red curve represents the heating curve (15°C to 95°C) and the blue curve represents the cooling curve (95°C to 15°C).

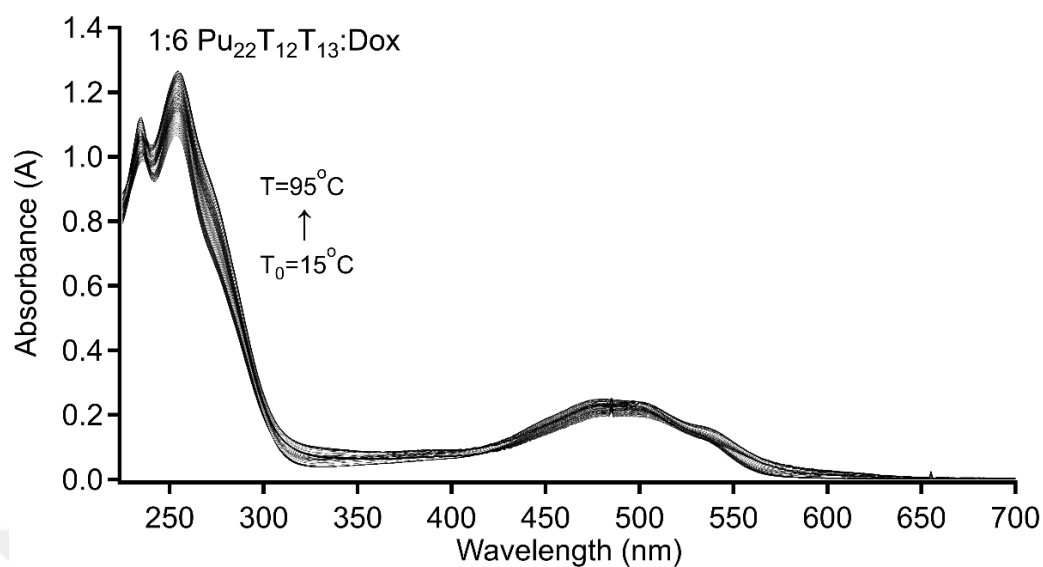


Figure 59. UV-vis absorption spectra of 1:6 $\text{Pu}_{22}\text{T}_{12}\text{T}_{13}$:Dox sample obtained during thermal denaturation experiments.

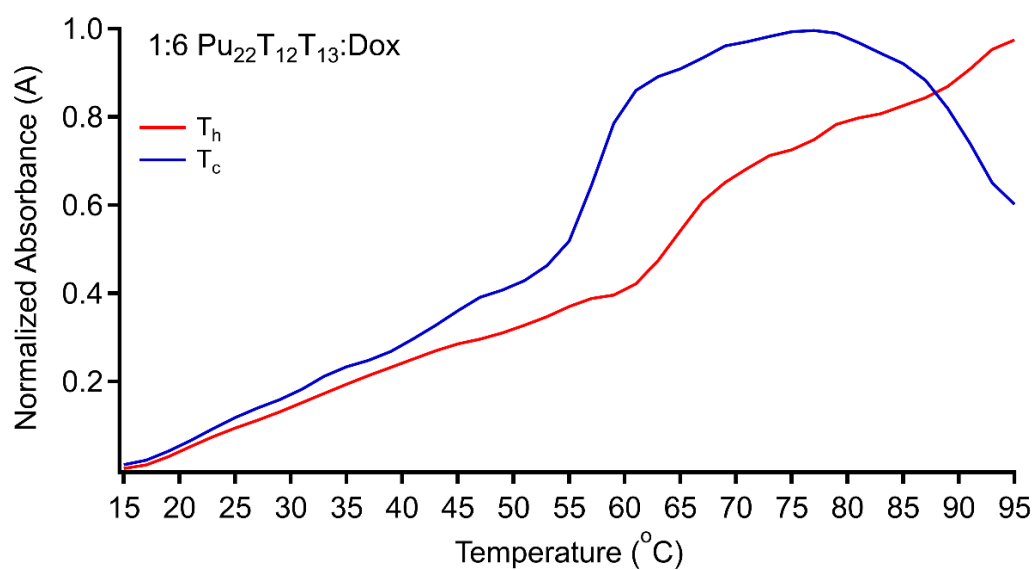


Figure 60. Thermal denaturation curves for 1:6 $\text{Pu}_{22}\text{T}_{12}\text{T}_{13}$:Dox sample obtained by monitoring the UV-vis absorbance at 295 nm. Red curve represents the heating curve (15°C to 95°C) and the blue curve represents the cooling curve (95°C to 15°C).

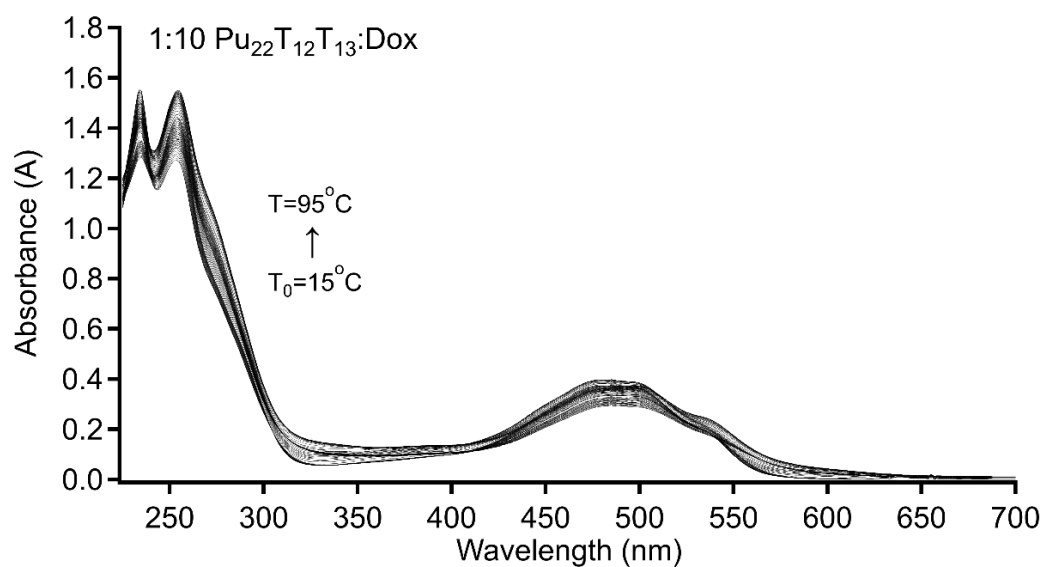


Figure 61. UV-vis absorption spectra of 1:10 Pu₂₂T₁₂T₁₃:Dox sample obtained during thermal denaturation experiments.

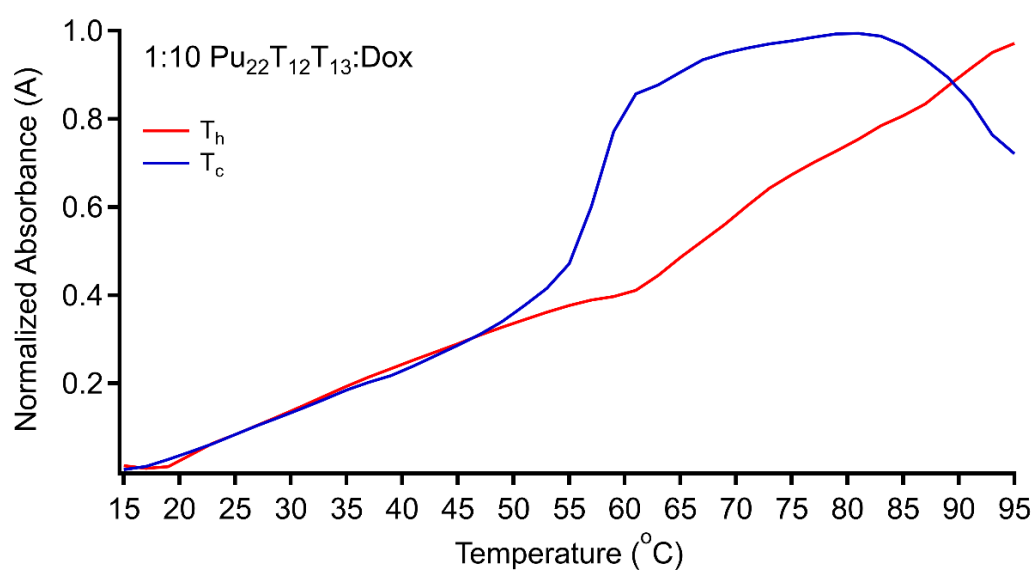


Figure 62. Thermal denaturation curves for 1:10 Pu₂₂T₁₂T₁₃:Dox sample obtained by monitoring the UV-vis absorbance at 295 nm. Red curve represents the heating curve (15°C to 95°C) and the blue curve represents the cooling curve (95°C to 15°C).

D. CD Thermal Denaturation Experiments

a. Pu₂₂

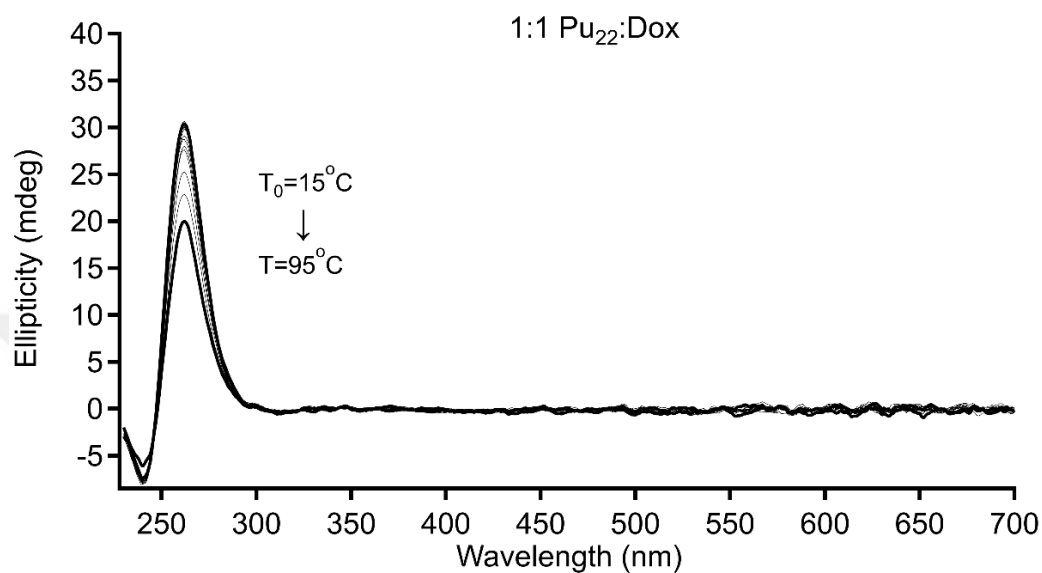


Figure 63. CD thermal denaturation experiment results for 1:1 Pu₂₂:Dox sample. The Ellipticity (mdeg) vs Wavelength (nm) graph shows the CD spectra of 1:1 Pu₂₂:Dox sample between 15°C and 95°C.

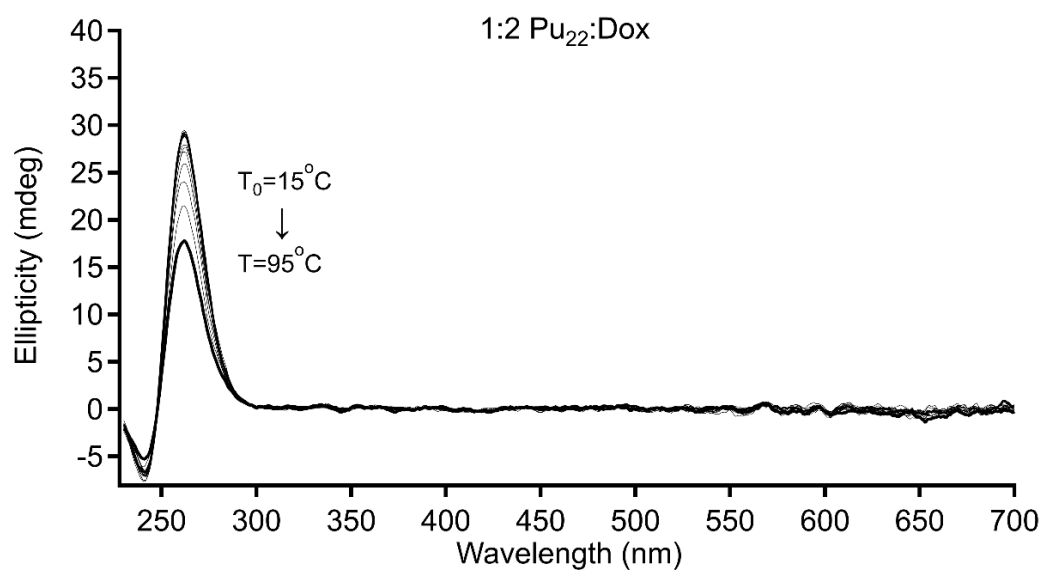


Figure 64. CD thermal denaturation experiment results for 1:2 Pu₂₂:Dox sample. The Ellipticity (mdeg) vs Wavelength (nm) graph shows the CD spectra of 1:2 Pu₂₂:Dox sample between 15°C and 95°C.

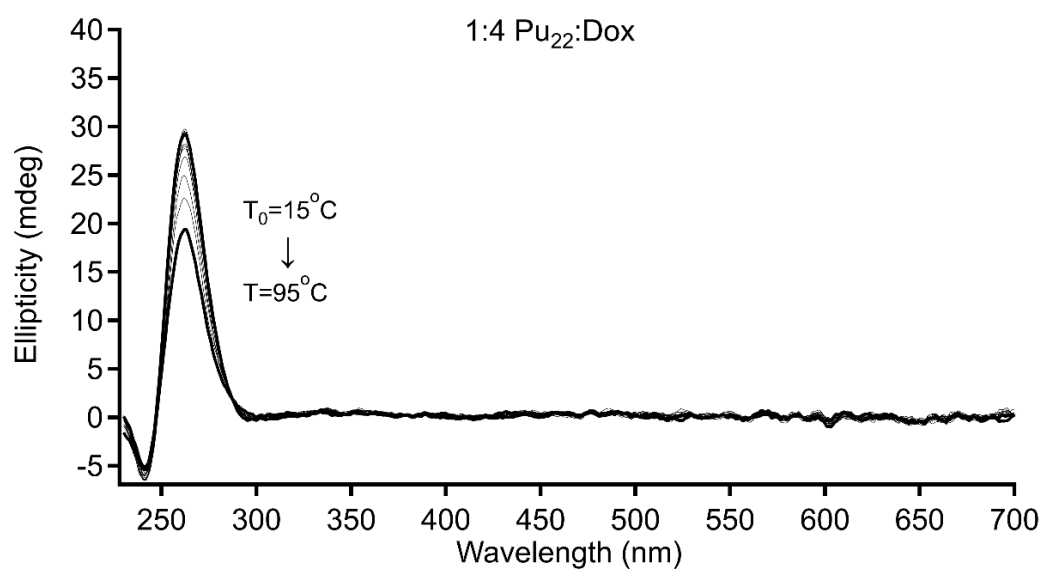


Figure 65. CD thermal denaturation experiment results for 1:4 Pu₂₂:Dox sample. The Ellipticity (mdeg) vs Wavelength (nm) graph shows the CD spectra of 1:4 Pu₂₂:Dox sample between 15°C and 95°C.

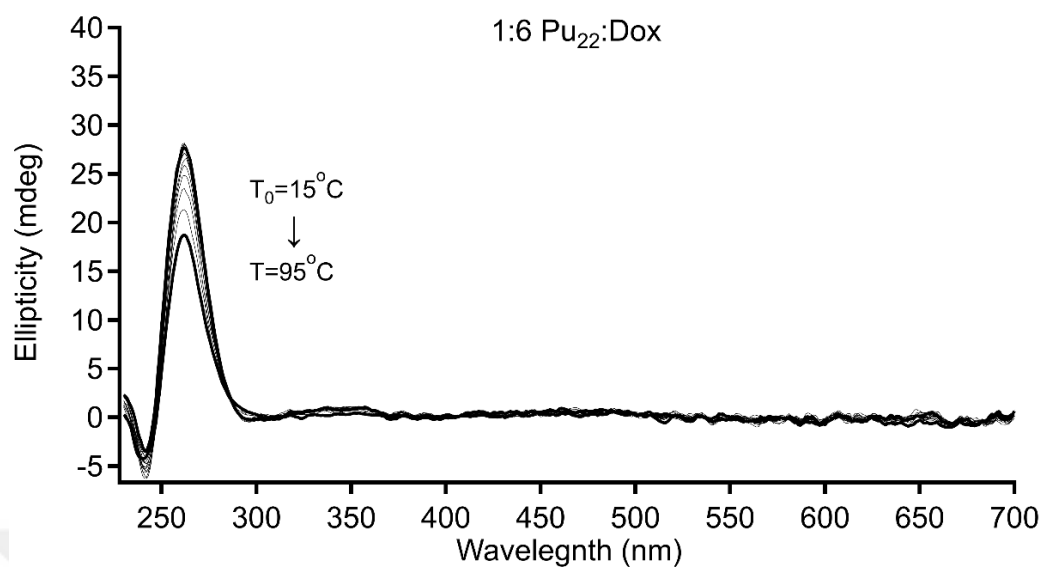


Figure 66. CD thermal denaturation experiment results for 1:6 Pu₂₂:Dox sample. The Ellipticity (mdeg) vs Wavelength (nm) graph shows the CD spectra of 1:6 Pu₂₂:Dox sample between 15°C and 95°C.

b. Pu₂₂T₁₂T₁₃

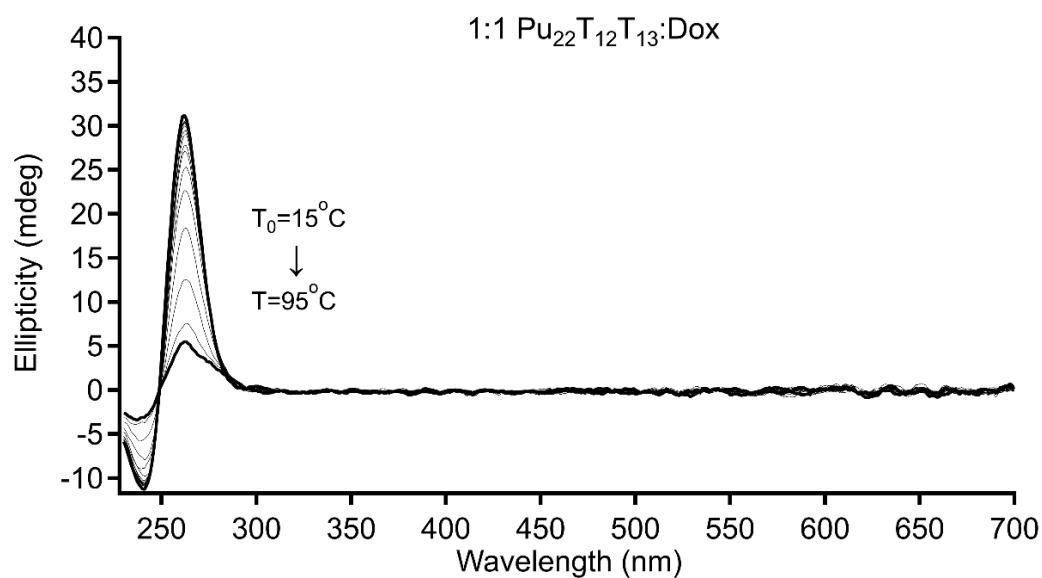


Figure 67. CD thermal denaturation experiment results for 1:1 Pu₂₂T₁₂T₁₃:Dox sample. The Ellipticity (mdeg) vs Wavelength (nm) graph shows the CD spectra of 1:1 Pu₂₂T₁₂T₁₃:Dox sample between 15°C and 95°C.

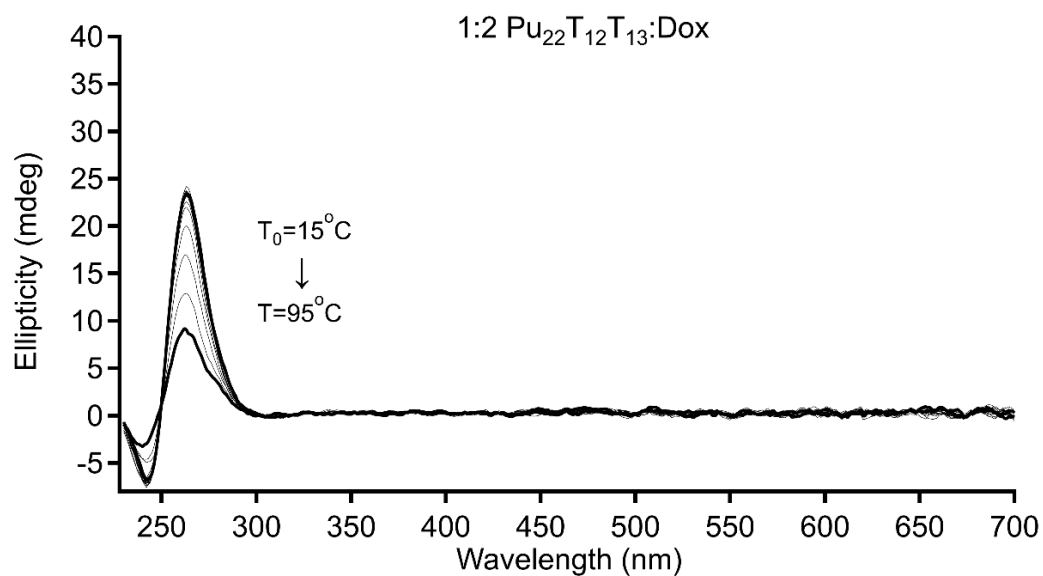


Figure 68. CD thermal denaturation experiment results for 1:2 Pu₂₂T₁₂T₁₃:Dox sample. The Ellipticity (mdeg) vs Wavelength (nm) graph shows the CD spectra of 1:2 Pu₂₂T₁₂T₁₃:Dox sample between 15°C and 95°C.

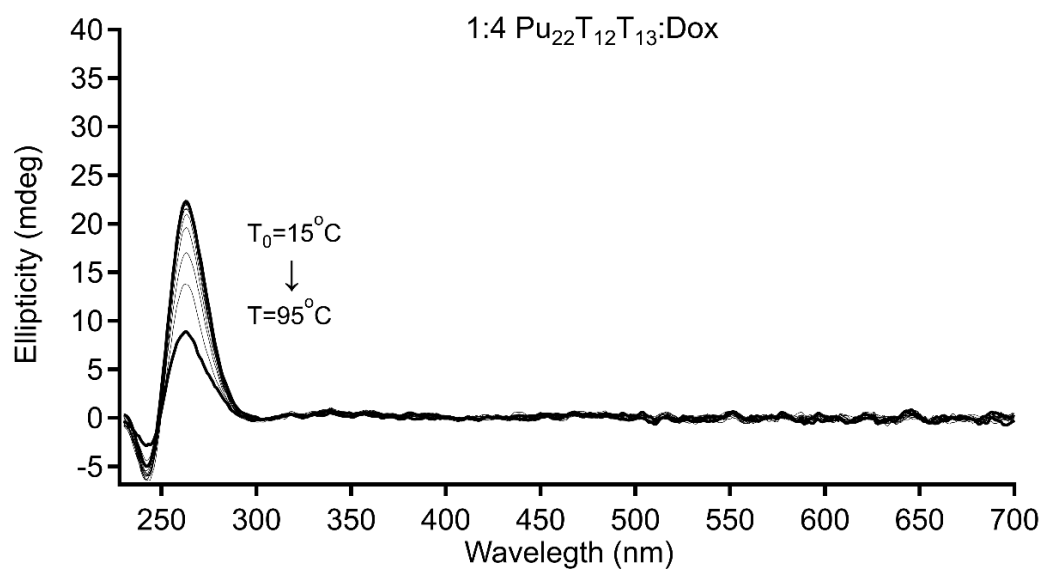


Figure 69. CD thermal denaturation experiment results for 1:4 Pu₂₂T₁₂T₁₃:Dox sample. The Ellipticity (mdeg) vs Wavelength (nm) graph shows the CD spectra of 1:4 Pu₂₂T₁₂T₁₃:Dox sample between 15°C and 95°C.

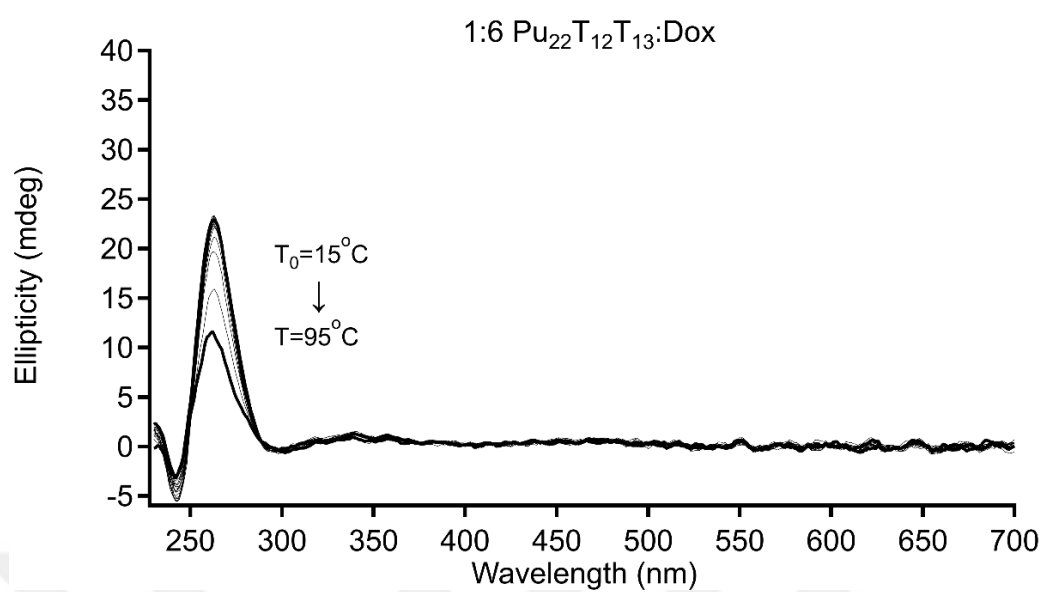


Figure 70. CD thermal denaturation experiment results for 1:6 Pu₂₂T₁₂T₁₃:Dox sample. The Ellipticity (mdeg) vs Wavelength (nm) graph shows the CD spectra of 1:6 Pu₂₂T₁₂T₁₃:Dox sample between 15°C and 95°C.

E. Association Constant Experiments

a. Pu₂₂

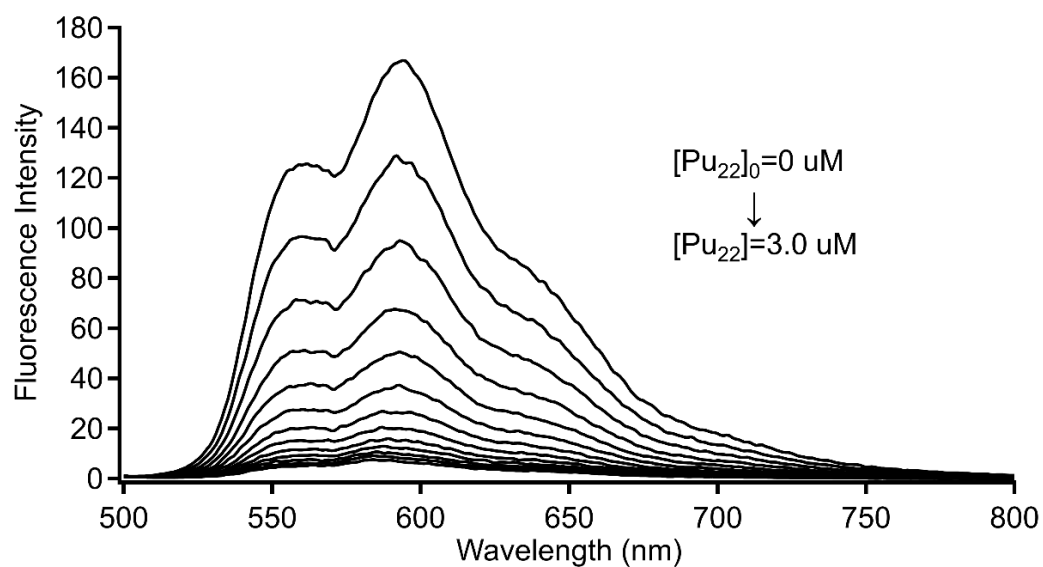


Figure 71. Fluorescence Intensity vs Wavelength (nm) of 1.0 μM Dox titrated with increasing volumes of 50.0 μM Pu₂₂ solution with 1.0 μM Dox up to a final 3.0 μM Pu₂₂ concentration (2nd replicate).

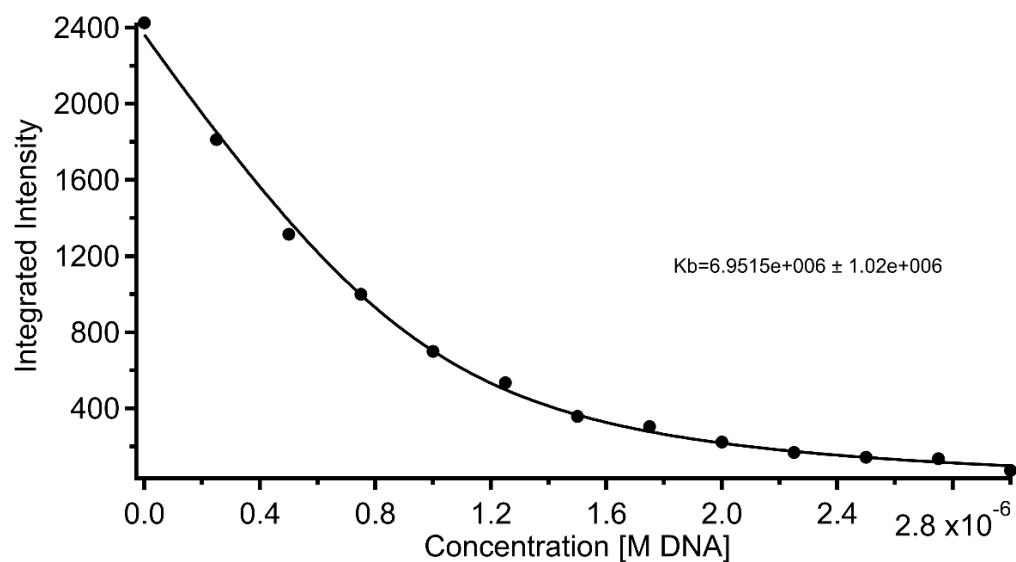


Figure 72. Integrated Intensity vs Concentration (M in strand) obtained from the titration results in Figure 71.

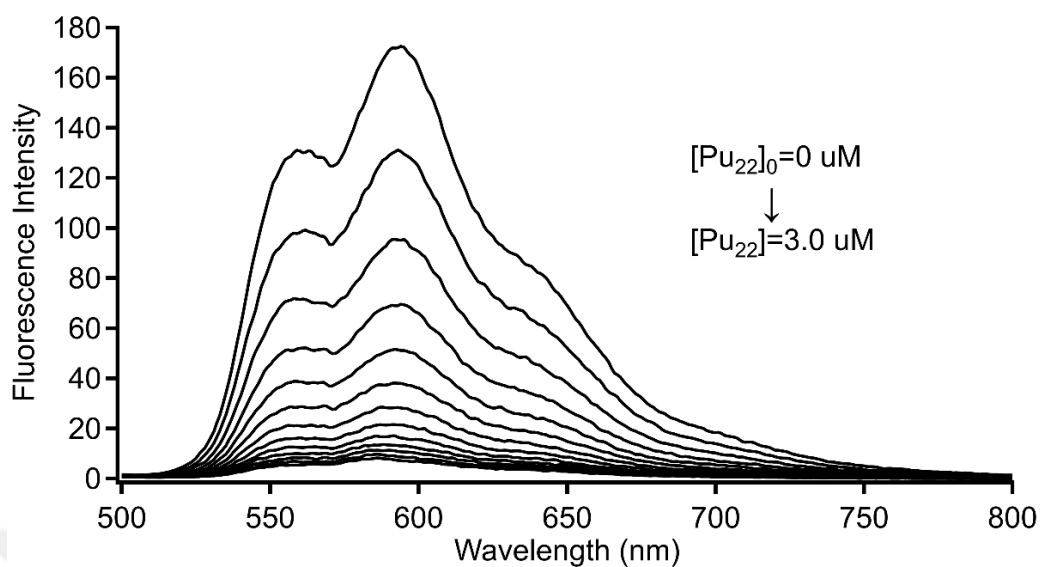


Figure 73. Fluorescence Intensity vs Wavelength (nm) of 1.0 μM Dox titrated with increasing volumes of 50.0 μM Pu₂₂ solution with 1.0 μM Dox up to a final 3.0 μM Pu₂₂ concentration (3rd replicate).

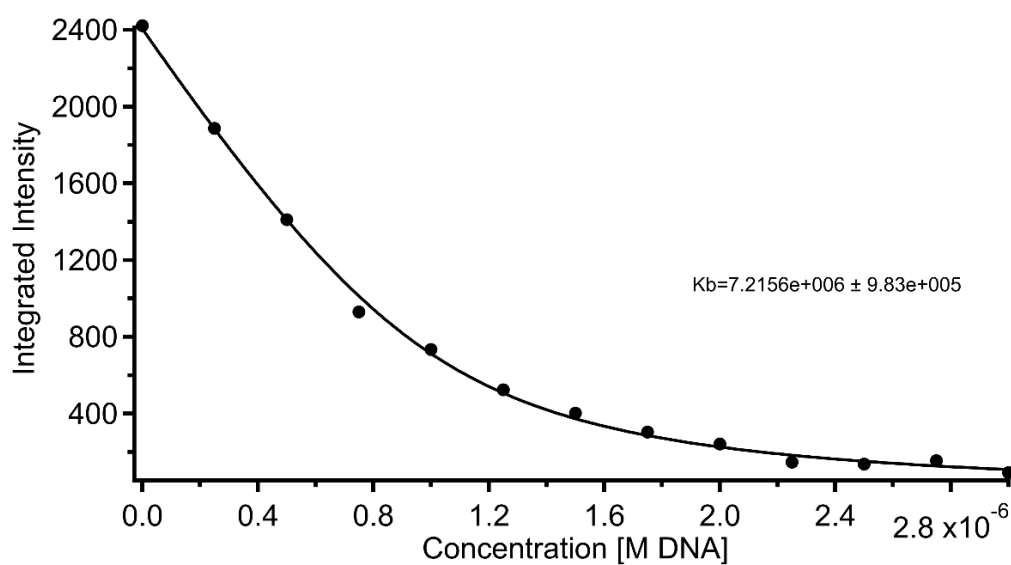


Figure 74. Integrated Intensity vs Concentration (M in strand) obtained from the titration results in Figure 73.

b. Pu₂₂T₁₂T₁₃

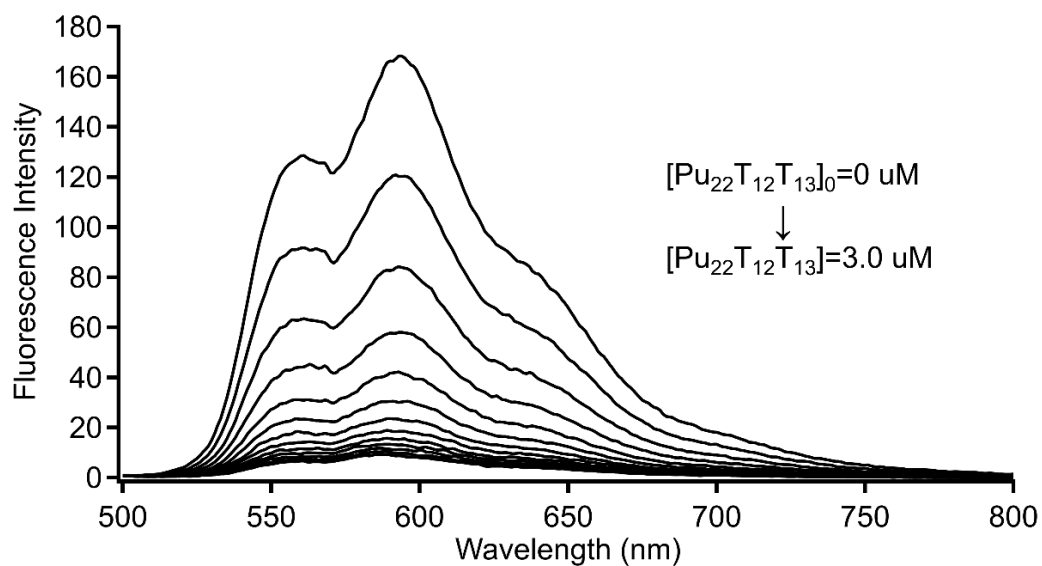


Figure 75. Fluorescence Intensity vs Wavelength (nm) of 1.0 μM Dox titrated with increasing volumes of 50.0 μM Pu₂₂T₁₂T₁₃ solution with 1.0 μM Dox up to a final 3.0 μM Pu₂₂T₁₂T₁₃ concentration (2nd replicate).

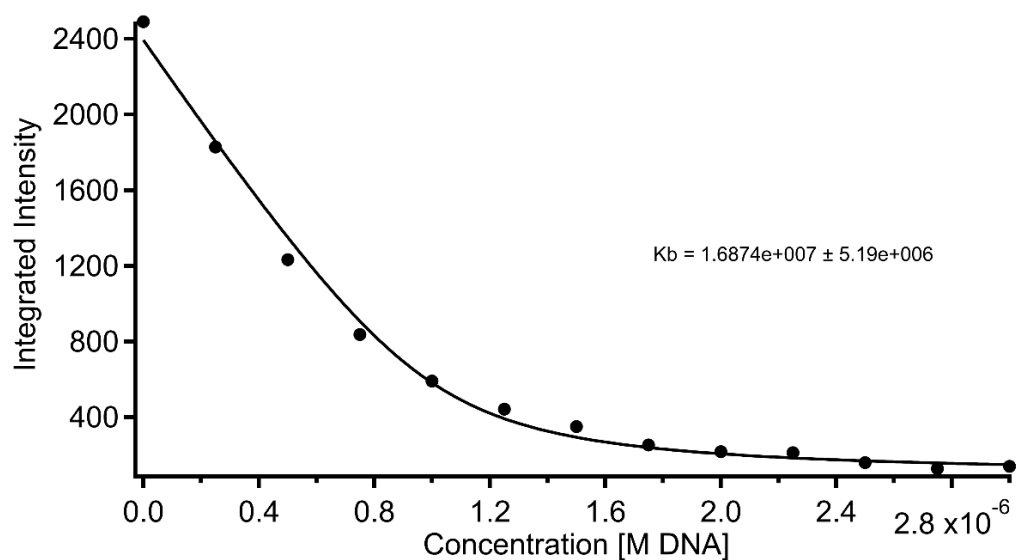


Figure 76. Integrated Intensity vs Concentration (M in strand) obtained from the titration results in Figure 75.

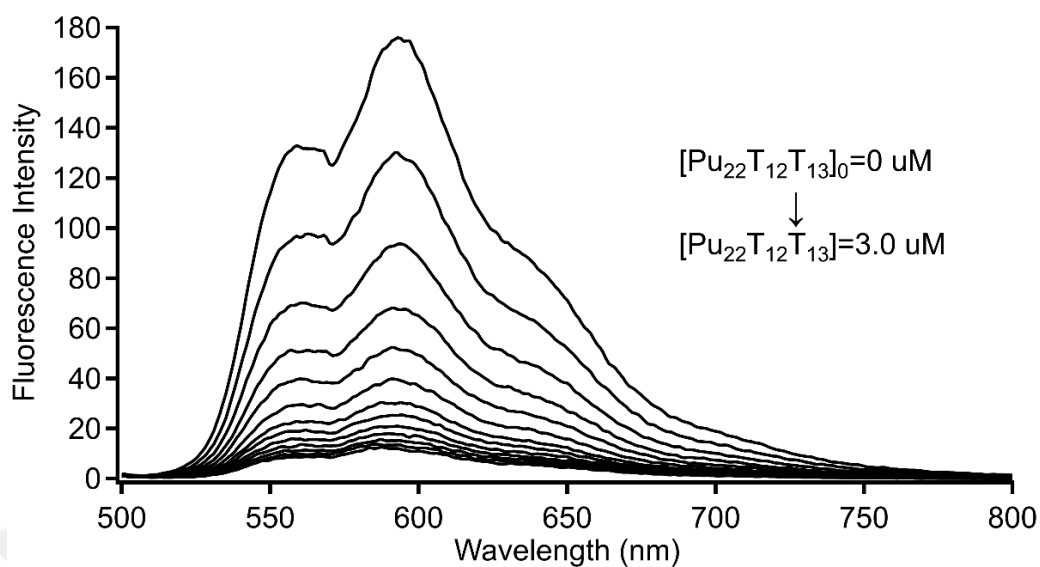


Figure 77. Fluorescence Intensity vs Wavelength (nm) of 1.0 μM Dox titrated with increasing volumes of 50.0 μM $\text{Pu}_{22}\text{T}_{12}\text{T}_{13}$ solution with 1.0 μM Dox up to a final 3.0 μM $\text{Pu}_{22}\text{T}_{12}\text{T}_{13}$ concentration (3rd replicate).

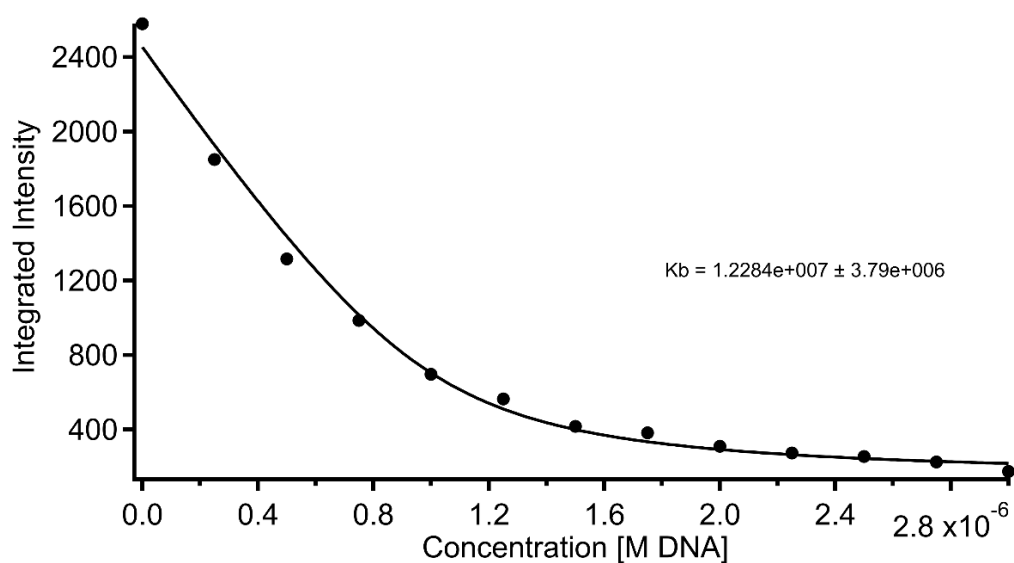


Figure 78. Integrated Intensity vs Concentration (M in strand) obtained from the titration results in Figure 77.

F. Competition Dialysis

Table 4. List of nucleic acid structures used in the competition dialysis assay and their extinction coefficient values for concentration adjustments.

Nucleic Acids	λ (nm)	ϵ ($M^{-1}cm^{-1}$)	Monomeric Unit	Company
Pu₂₂T₁₂T₁₃	260	200400	Strand	IDT
Pu₂₂	260	205000	Strand	IDT
BCL-2	260	41591	Quartet	IDT
C-myc	260	45309	Quartet	IDT
K-ras	260	24646	Quartet	IDT
Her-2	260	42985	Quartet	IDT
H-telo	260	73000	Quartet	IDT
dA₃₂	260	387400	Nucleotide	IDT
dT₃₂	260	259800	Nucleotide	IDT
poly(A)	260	9800	Nucleotide	Sigma Aldrich
poly(rU)	260	9350	Nucleotide	Sigma Aldrich
poly(dA):poly(dT)	260	9800	Base pair	Sigma Aldrich
m-lyso	260	13846	Base pair	Sigma Aldrich

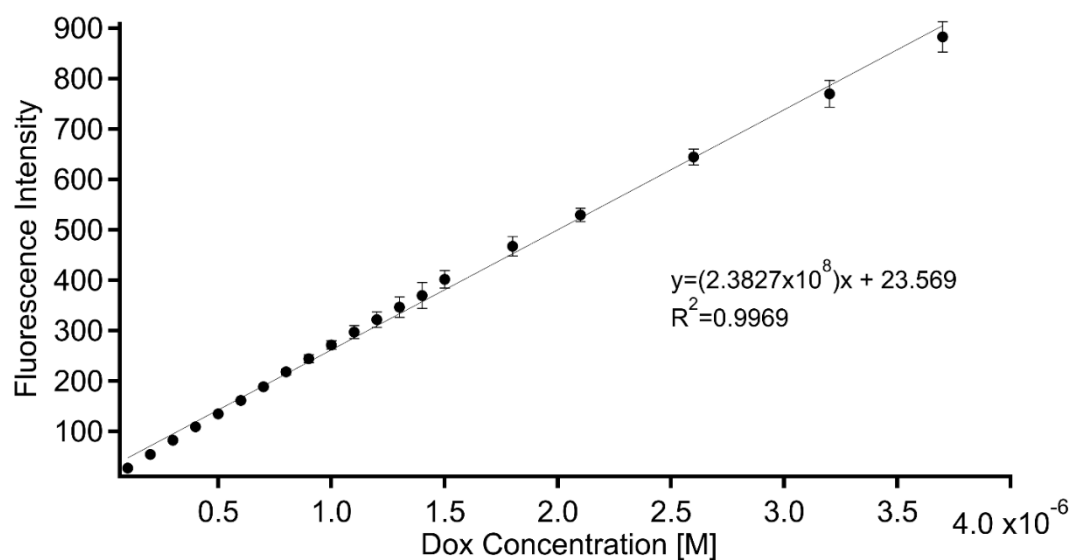


Figure 79. Dox calibration curve constructed for the competition dialysis assay.